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EARLY FLOWERING IN SHORT DAYS (EFS) regulates the seed size in *Arabidopsis*

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Post-transcriptional modifications, including histone modifications and DNA methylation, alter the chromatin landscape to regulate gene expression, thus control various cellular processes in plants. EARLY FLOWERING IN SHORT DAYS (EFS) is the major contributor for H3K36 methylation in *Arabidopsis* and is important for plant development. Here, we find that *EFS* is expressed in different stages of embryo morphogenesis, and the *efs* mutant produces larger embryo that results in enlarged seeds. Further analysis reveals that an imprinted gene *MOP9.5* is hypomethylated at the promoter region and its expression is derepressed in *efs* mutant. *MOP9.5* promoter is marked by various epigenetic modifications, and we find that following the increase of H3K36me3, H3K27me3 and H3K9me2 levels are reduced in *efs* mutant. This data indicates an antagonistic regulation between H3K36me3 and DNA methylation, and/or H3K27me3 at *MOP9.5*. Our results further show that both maternal and paternal EFS alleles are responsible for the seed size regulation, which unraveled a novel function of EFS in plant development.

DNA methylation, EFS, histone modifications, seed size, gene imprinting

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INTRODUCTION

Chromatin architecture regulates the gene expression in response to environmental and developmental cues, thus control various physiological and cellular processes, including flowering, pathogen defense, gene transcription, DNA replication and repair and so on (reviewed in (Berr et al., 2011; He et al., 2011)). To achieve the desired chromatin landscape, plant is under numerous post-transcriptional modifications, including histone modifications and DNA methylation throughout development. DNA methylation is associated with transcriptional silencing of genes involved in various biological processes, including flowering time, imprinting, floral organ identity, fertility and leaf morphogenesis (Gehring, 2013; Rodrigues and Zilberman, 2015; Zhu et al., 2016). In *Arabidopsis*, DNA methylation occurs in three different contexts, CG, CHG and CHH, and different methyltransferases are required to maintain the specific methylation context (Law and Jacobsen, 2010). For example, *MET1*, a homolog of mammalian *Dnmt1*, is required to maintain the CG methylation (Kankel et al., 2003), plant specific *CMT3* is required for CHG (Lindroth et al., 2001), and *de novo* DNA methyltransferase *DRM2*, the homolog of *Dnmt3* of mammals, is required for establishing all the methylation contexts, especially CHH methylation (Cao and Jacobsen, 2002). DNA methylation and histone modifications control the gene imprinting, a biased gene expression phenomenon depends on whether the allele is inherited from the male or female parent (García-Aguilar and Gillmor, 2015; Gehring, 2013; Köhler et al., 2012). DNA hypo-

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methylation mutants *met1* and *ddm1* cause parent-of-origin effect to regulate the seed size (FitzGerald et al., 2008; Xiao et al., 2006a; Xiao et al., 2006b). In *Arabidopsis*, many imprinted genes have been identified to be either maternally or paternally expressed (Köhler et al., 2012), and results have shown that they are implicated in different pathways, including hormone signaling, ubiquitin protein degradation pathway, histone and DNA methylation regulation, small RNA biogenesis and so on (Gehring, 2013; Köhler et al., 2012).

Histone modifications are either associated with transcription activation or repression, depending on the position and state. For example, H3K36 and H3K4 are linked with activation, while H3K9 and H3K27 are associated with repression. Interplay between DNA methylation and H3K9me2 has been reported in *Arabidopsis* and is well established (Bernatavichute et al., 2008; Du et al., 2012; Du et al., 2015). Along with H3K9me2, chromatin regions that enriched with another repressive mark H3K27me3 are found hypomethylated in *Arabidopsis* (Zhang et al., 2007), indicating the interplay between these repressive marks and DNA methylation to regulate the gene expression. Interestingly, the interplay between active histone marks and DNA methylation has also been reported in *Arabidopsis* and rice. Loss of function *JMJ14*, a histone demethylase of H3K4, displays an increase in H3K4 methylation and DNA hypomethylation at non CG-sites (Lu et al., 2010). In rice, H3K4me3 levels are increased at LINE element Karma in *jmj703* mutant, while DNA methylation is dramatically reduced (Cui et al., 2013). These studies suggest the antagonistic correlation between H3K4 and DNA methylation.

In mammals, additional domains present in DNA methyltransferases play a role in mediating the crosstalk between DNA methylation and histone modifications. For example, presence of the PWWP domain, the H3K36 methylation reader, in DNA methyltransferase DNMT3B had been suggested a role of H3K36me3 in recruiting the DNA methylation to the target regions (Dhayalan et al., 2010; Vermeulen et al., 2010). Further study indeed revealed the important role of H3K36me3 recognition by PWWP for the DNMT3B activity in gene transcription (Baubec et al., 2015).

In *Arabidopsis*, deposition of H3K36me3 is mainly through SET DOMAIN GROUP 8 (SDG8) (also named as EARLY FLOWERING IN SHORT DAYS (EFS)/CCR1/ASHH2 (He et al., 2011; Zhao et al., 2005)), the major H3K36 methyltransferase. Total levels of H3K36me3/me2 are greatly reduced in *efs-3* mutant, thus cause various defects in biological processes, including early flowering, shoot branching, carotenoid biosynthesis, plant pathogen response, weakened innate immunity, seed gene repression, and brassinosteroid-regulated gene expression (Berr et al., 2010; Cazzonelli et al., 2009; Cazzonelli et al., 2014; Dong et al., 2008; Grini et al., 2009; Palma et al., 2010; Tang et al., 2012; Wang et al., 2014; Xu et al., 2008; Yang et al., 2014). However, whether H3K36 methylation coordinates with DNA methylation to control the gene expression in *Arabidopsis* has not yet been studied.

Here, from the analysis of the *efs* mutant, we report that *efs-3* produces significantly enlarged seeds due to the increase in embryo and central vacuole during the embryogenesis, indicating that *EFS* plays a role to ensure the proper seed size and weight in *Arabidopsis*. We further demonstrated that DNA methylation levels are significantly reduced at the promoter of an imprinted gene *MOP9.5*. In addition to DNA hypomethylation, H3K36me3 level is increased while H3K27me3 and H3K9me2 are decreased in *efs-3* mutant at *MOP9.5* promoter, indicating an antagonistic crosstalk between active (H3K36me3) and repressive marks (DNA methylation and H3K27me3) at *MOP9.5* promoter. In brief, our results revealed a new function of EFS in plant development.

RESULTS

efs produces significantly enlarged seeds in *Arabidopsis*

From the analysis of *efs-3* mutant, we observed that the significant enlarged seeds are present in *efs-3* siliques compared to that in Col-0 (Figure 1A). The enlarged seeds can also be found in other three *EFS* allelic mutants (*ccr1-1*, *SALK_065480C/ashh2-1* and *WiscDsLox432B04*; data not shown), and the seed size phenotype can be fully complemented (Figure 1A), indicating that EFS is involved in seed development in *Arabidopsis*.

To quantify the enlarged seed size phenotype in *efs-3* mutant, we measured the seed dimensions and found that *efs-3* mutant showed an increase in seed area compared with Col-0 (Figure 1A and B). We also found that *efs-3* mutant showed 23% increase in seed length compared with Col-0, with an average seed length of 536 and 436 μm, respectively (Figure 1C). Similarly, *efs-3* mutant showed 28% increase in seed width compared with Col-0, with an average seed width of 300 and 234 μm, respectively (Figure 1C). To investigate whether the increase in seed size changes the seed weight, we calculated the 1,000 seeds weight. Indeed, *efs-3* mutant has almost double seed weight compared with Col-0 (Figure 1D), and the increases in seed length, width and weight are fully complemented in the *EFS* complementation line (Figure 1A–D). Together, these results showed that *efs-3* mutant produces heavier and larger seeds compared with Col-0.

To further dissect the enlarged seed size phenotype in *efs-3* mutant, we visualized the different stages of embryo morphogenesis (Figure 1E). In early stages of embryo morphogenesis, *efs-3* mutant did not show any obvious difference in embryo size compared with Col-0, while central vacuole is

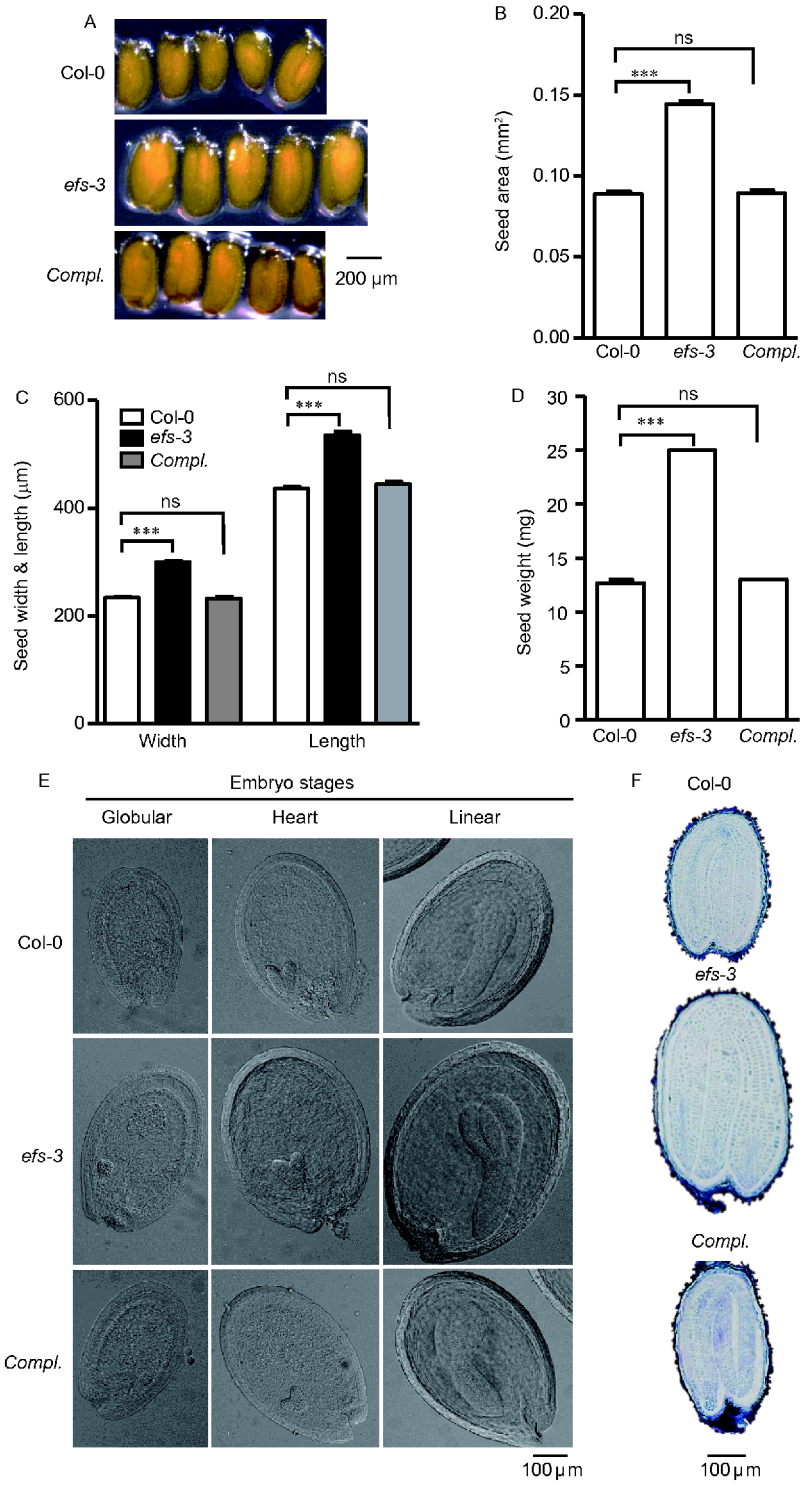

Figure 1 (Color online) *efs-3* produces enlarged seeds. A, Dry seeds of wild-type (Col-0), *efs-3* and the complemented plants (*Compl.*). Bar=200 μm. B, Seed area of Col-0, *efs-3* and *Compl.* Seeds were measured by cellSens (OLYMPUS, Japan). The presented value is the average of 50 seeds. C, Bar chart representing the average seed length and width of *efs-3*, Col-0 and *Compl.*, *n*=50. D, 1,000 seeds weight of *efs-3*, Col-0 and *Compl.* The data presented is the average of three biological replicates. E, Seeds clearing of developing siliques of *efs-3*, Col-0 and *Compl.*, Bar=100 μm. F, Seeds section of dried seeds from Col-0, *efs-3* and *Compl.*, Bar=100 μm. ***, significant difference (P<0.001); ns, no significant difference.

spacious in *efs-3* mutant. However, at linear stage of embryo morphogenesis, *efs-3* mutant showed relatively larger embryo size compared with Col-0. This larger embryo phenotype of *efs-3* mutant is further confirmed in mature seeds (Figure 1F, bottom). Furthermore, we also observed that cells in the mature embryo are more spacious in *efs-3* mutant compared with Col-0 (Figure 1F). The enlarged embryo at linear stage and increased cell spacing phenotypes observed

in *efs* mutant are fully complemented in the *EFS* complementation line (Figure 1E and F). These results indicate that EFS is important for embryo development, and larger embryo is likely to increase the seed size and weight in *efs-3* mutant.

MOP9.5, a maternal expressed gene is hypomethylated in *efs*

Maternal and paternal genomes discretely regulate the seed size through the reconciliation of DNA methylation and chromatin modifications (García-Aguilar and Gillmor, 2015; Gehring, 2013; Köhler et al., 2012). DNA hypomethylation mutants *met1* and *ddm1* cause parent-of-origin effect to regulate the seed size (FitzGerald et al., 2008; Xiao et al., 2006a; Xiao et al., 2006b). The results from these studies and the observation of the larger seed size in *efs-3* mutant led us to hypothesize that DNA methylation levels are impaired at imprinted genes in *efs-3* mutant. Therefore, we employed the Chop-PCR to investigate whether the DNA methylation levels in the known imprinted genes (Hsieh et al., 2011; Köhler et al., 2012; Vu et al., 2013; Wolff et al., 2015) are altered in *efs-3* mutant (Figures 2A and S1 in Supporting Information). From all the tested imprinted genes, *MOP9.5* showed an obvious decrease in DNA methylation levels in different tissues of *efs-3* mutant compared to Col-0 (Figures 2A and S1 in Supporting Information), while DNA methylation levels are moderately altered on *MEA*, another imprinted gene that encodes the key component of Polycomb repressive complex 2 (PRC2) (Baroux et al., 2006; Gehring et al., 2006) (Figure S1 in Supporting Information). *MOP9.5*, also called *AtPI4Kγ3*, a type II phosphoinositide 4-kinase, is a maternally expressed imprinted gene (Vu et al., 2013), and is reported to play a role in abiotic stress response and floral transition (Akhter et al., 2016). We also observed the DNA hypomethylation at *MOP9.5* promoter in *met1* heterozygous plants (Figure 2A). Data from genome-wide DNA methylation sequencing and locus specific bisulfite sequencing revealed that the promoter of *MOP9.5* is mainly methylated at CG sites compared with CHH or CHG sites (Figures 2B and S2A in Supporting Information). Furthermore, CG and CHG types DNA methylation levels at *MOP9.5* promoter are dramatically decreased in *efs-3* mutants compared with Col-0, which suggests that *EFS* may help to establish the DNA methylation at this imprinted gene.

MOP9.5 expression is derepressed in *efs*

As DNA methylation is involved in the regulation of imprinting, as well as transcriptional repression in *Arabidopsis* (Calarco and Martienssen, 2012; Gehring, 2013), we then investigated whether the DNA hypomethylation at *MOP9.5* promoter lead to de-repression of *MOP9.5* in *efs-3* mutant. Indeed, qRT-PCR result showed that the expression of *MOP9.5* is increased in flowers, buds and seedlings in *efs-3* mutant compared with Col-0 (Figure 2C). Moreover, *MOP9.5* expression is found higher in flowers compared with other tested tissues, and dramatically increased in *efs-3* flowers (Figure 2C). Similar to the *efs-3* mutant, *met1* heterozygous also showed an increase in *MOP9.5* expression compared with Col-0 (Figure 2D), which is even higher than in *efs-3*. Because the main methylation type that impaired at *MOP9.5* promoter in *efs-3* mutant was CG methylation, and DNA hypomethylation mutants *MET1* and *DDM1* cause parent-of-origin effect to regulate the seed size (Xiao et al., 2006a), we speculated that DNA hypomethylation at *MOP9.5* promoter in *efs-3* mutant is likely through altering the expression of *MET1* and/or *DDM1*, thus regulates the seed size. Contrary to our expectations, *MET1* and *DDM1* expression did not show obvious differences in *efs-3* mutants compared with Col-0 (Figure 2D). These results suggest the involvement of other pathways and/or DNA methyltransferases in parallel to *MET1* and *DDM1* to regulate the DNA methylation levels at *MOP9.5* in *efs-3* mutant.

Maternal and paternal alleles of *EFS* regulate the seeds size

Because *efs* produces enlarged seeds and DNA methylation levels are impaired at the imprinted gene *MOP9.5*, we speculated whether EFS has parent-of-origin effect to regulate the gene imprinting. To test this, we made the reciprocal crosses between *efs-3* mutant and Col-0. We found that F_1 population of seed size is enlarged in either ways of crosses (Figure 3A), suggesting that EFS may regulate the imprinted genes in both maternal and paternal directions. We further quantified the seed width, length and area of the F_1 populations obtained from the reciprocal crosses, and the significant enlarged seed size phenotype is confirmed from F_1 populations in comparison with Col-0 (Figure 3B and C). However, the enlarged seed size observed in F_1 populations is lesser than the *efs* mutant. Furthermore, the seeds of F_1 populations were found to be slightly bigger in case of *EFS* maternal allele compared with paternal allele (Figure 3A–C), suggesting that maternal allele of *EFS* contributes more to the seed size regulation. To further confirm the potential involvement of *MOP9.5* in regulation of seed size phenotype observed in *efs* mutant, we quantified the *MOP9.5* expression in seedlings, endosperm and pollen of F_1 populations obtained from the reciprocal crosses of *efs* and Col-0 (Figure 3D–F). The expression of *MOP9.5* in seedlings and endosperm correlates with the seed size phenotype observed in F_1 populations. However, *MOP9.5* expression is repressed in pollen of *efs*, indicating that maternal allele of *EFS* de-repressed the expression of *MOP9.5*, thus control the seed size phenotype. Therefore, we also checked the seed size of loss-

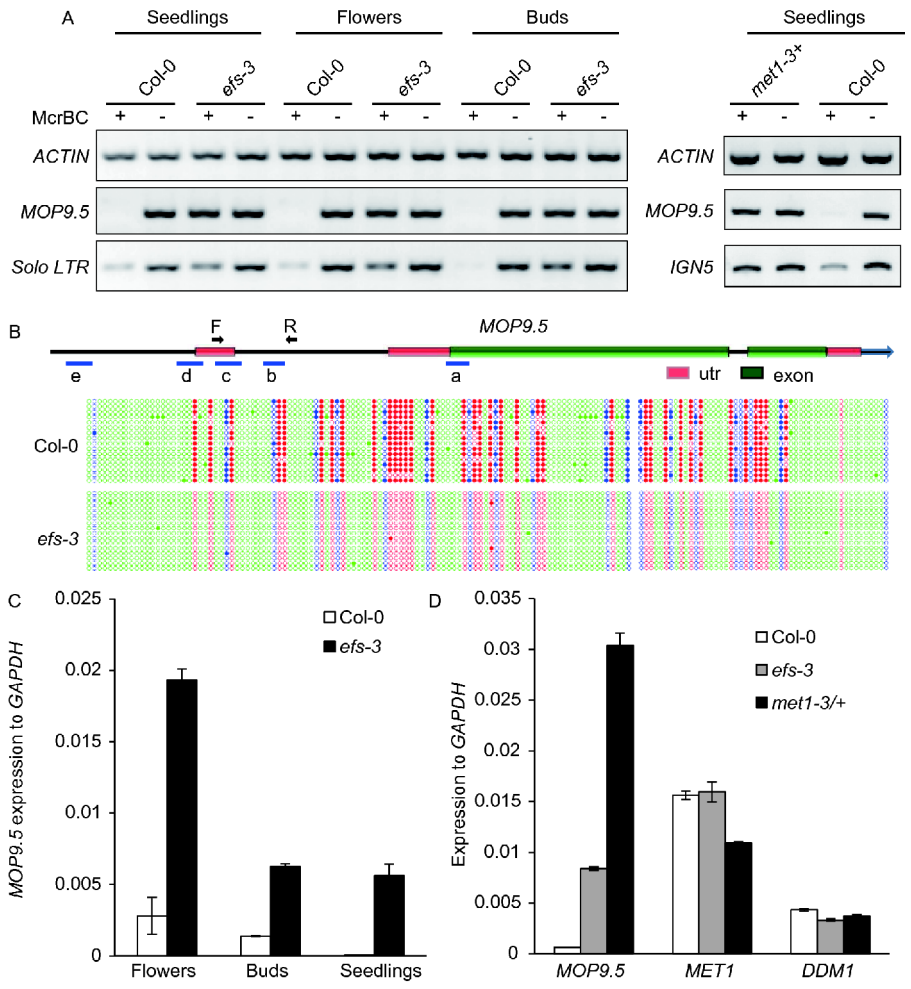

Figure 2 DNA methylation at *MOP9.5* promoter is decreased, resulting in the increased expression of *MOP9.5* in *efs-3*. A, Chop-PCR of *MOP9.5* in different tissues of Col-0, *efs-3* and *met1-3* (heterozygous). *IGN5* and *solo LTR* are positive control while *ACTIN* is the internal control. McrBC is a methylation-sensitive enzyme that specifically digests methylated DNA, and the bands with McrBC treatment represent the non-methylation levels. B, Bisulfate sequencing of *MOP9.5* promoter. Red, CG methylation; blue, CHG methylation; green, CHH methylation. The data presented is from the 16 individual clones. Gene structure of *MOP9.5* is presented and primers used for bisulfate sequencing are labeled as F and R. red, utr; blue, exon C, The mRNA expression of *MOP9.5* in the different tissues of Col-0 and *efs-3*. D, The mRNA expression of *MOP9.5*, *MET1* and *DDM1* in the seedlings of Col-0, *efs-3* and *met1-3* (heterozygous). *GAPDH* is used as an internal control. The data presented is the average of three biological replicates. Bar, SD.

of-function *MOP9.5* (*mop9.5-1* and *mop9.5-2*), and found that the seed size was changed, but not significantly different to Col-0 (Figure S2B–D in Supporting Information). Furthermore, over expression of *MOP9.5* in Col-0 did not show the obvious seed size difference (data not shown), suggesting the involvement of other imprinted genes in addition to *MOP9.5* to regulate the seed size phenotype that observed in *efs*.

Histone modifications are altered at *MOP9.5* promoter in *efs*

It has been shown that *de novo* gene body DNA methylation requires the H3K36 methyltransferase activity and the proper reading of H3K36me3 mark in mammalian cells (Baubec et al., 2015). As we observed DNA hypomethylation at *MOP9.5* promoter in *efs-3* mutant (Figure 2), and the fact that EFS is a major contributor for H3K36 methylation in *Arabidopsis* (Zhao et al., 2005), we anticipated an interplay between H3K36me3 and DNA methylation at *MOP9.5* promoter in *Arabidopsis*. Conversely to our anticipation, H3K36me3 level is increased at *MOP9.5* promoter in *efs-3* mutant compared with Col-0 (Figure 4A), indicating that DNA hypomethylation at *MOP9.5* promoter promotes H3K36me3 level, and thus increasing the expression of *MOP9.5* in *efs-3* mutant. As *EFS* encodes a major H3K36 methyltransferase in *Arabidopsis*, this result suggests that *EFS* regulates the *MOP9.5* expression independent of its methyltransferase activity. Because *EFS* mutant has been reported to display an increased H3K27me3 level at flowering genes following the decreased in H3K36me3 level (Shafiq et al., 2014; Yang et al., 2014), and H3K27me3 also controls the expression of imprinted genes (Köhler et al., 2012; Ohnishi et al., 2014), suggesting the possible in-

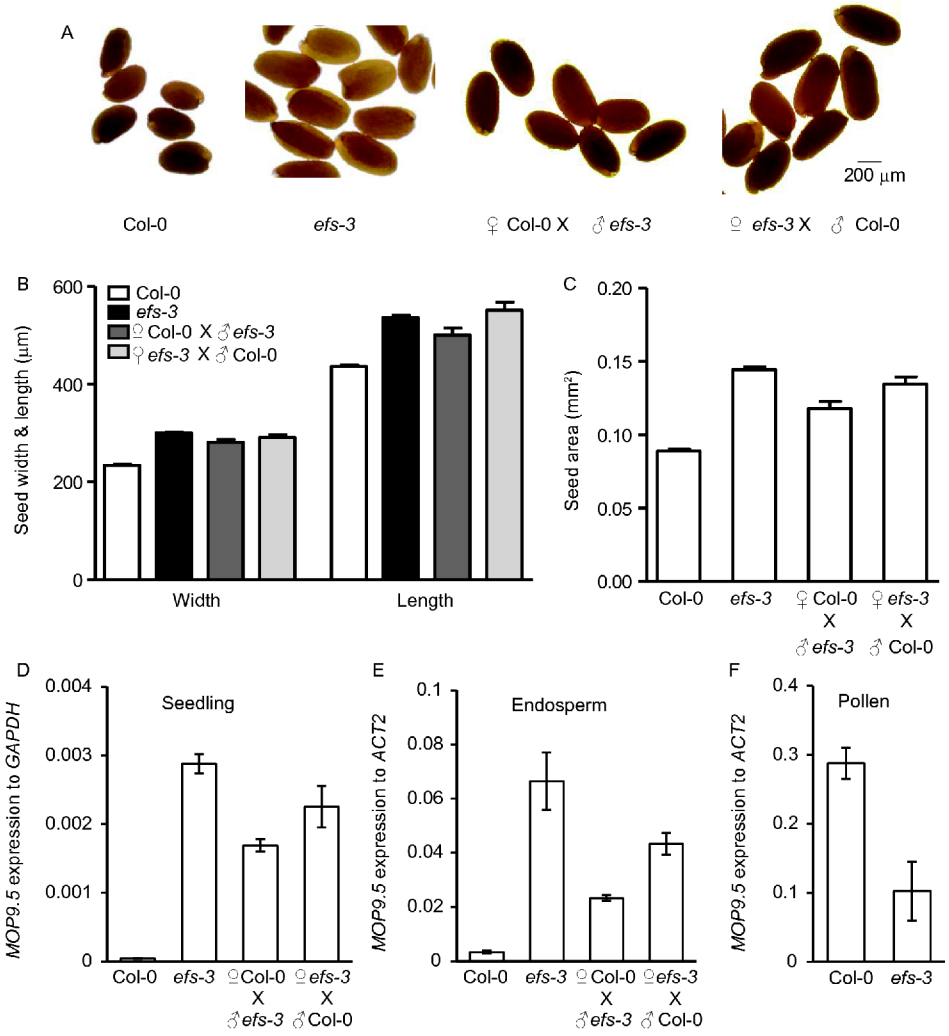

Figure 3 Maternal and paternal alleles of EFS regulate the seeds size. A, Dry seeds of Col-0, *efs-3* and F_1 population obtained from the reciprocal crosses between *efs-3* and Col-0. Bar, 200 μm. B, The average of seed width & length. C, Seed area of Col-0, efs-3 and F_1 population from the reciprocal crosses. The mRNA expression of *MOP9.5* in the seedlings of F_1 plants (D), in the endosperm (E), in the pollen (F). *GAPDH* and *ACT2* are used as internal controls. The data presented is the average of three biological replicates. Bar, SD.

volvement of H3K27me3 at *MOP9.5* promoter. Therefore, we investigated whether the H3K27me3 level is changed at *MOP9.5* promoter in *efs-3* mutant. Indeed, H3K27me3 level is decreased at *MOP9.5* promoter in *efs-3* mutant compared with Col-0 (Figure 4B), further reinforcing the antagonistic correlation between these two histone marks. As DNA methylation has been reported to have a self-reinforcing loop mechanism with H3K9me2, we also investigated the H3K9me2 at *MOP9.5* promoter. H3K9me2 level is slightly decreased at *MOP9.5* promoter in *efs-3* mutant compared with Col-0 (Figure 4C). Along with these histone modifications, we also observed that histone H3 level is decreased at *MOP9.5* promoter in *efs-3* mutant (Figure 4D), indicating that nucleosome density is changed at *MOP9.5* promoter in *efs-3* mutant compared with Col-0. Together, these results indicate that expression of *MOP9.5* is under the control of various epigenetic modifications that likely coordinate to regulate the seed size, and mutation of *EFS* will disrupt the balance among these epigenetic modifications.

EFS is expressed in pollen, ovules and developing seeds

To further dissect the role of *EFS* in regulating seed size, we examined the expression pattern of *EFS* in pollen, ovules and different stages of embryo morphogenesis with p*EFS::GUS* reporter (Cazzonelli et al., 2010). We found that *EFS* is highly expressed in pollen (Figure 5A) and ovules (Figure 5B), suggesting the important roles of EFS in pollen and ovule. Furthermore, we also investigated whether the *EFS* is expressed during the different stages of embryo morphogenesis, and our data indeed revealed that *EFS* is expressed in different stages during the embryo morphogenesis, including globular (Figure 5C), heart (Figure 5D) and linear stages (Figure 5E) of embryogenesis. Interestingly, *EFS*

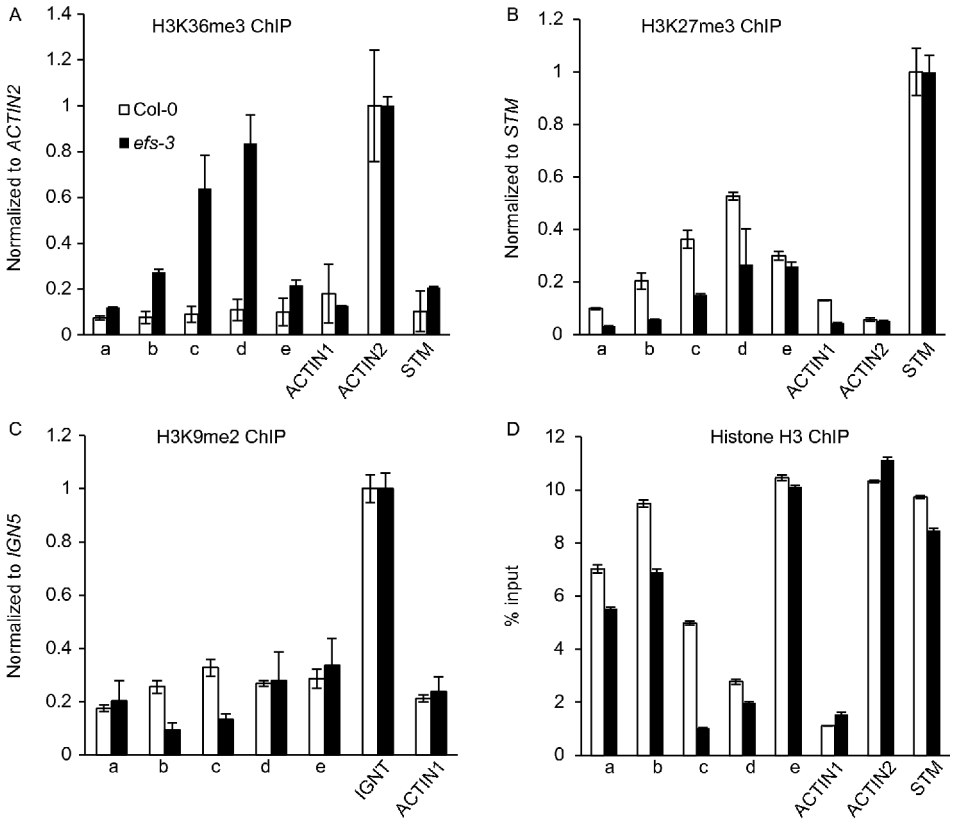

Figure 4 Histone modifications are altered at *MOP9.5* promoter in *efs-3*. The regions for ChIP-qPCR primers are labeled as a–e in Figure 2B. *ACTIN2* is the positive control for H3K36me3, *STM* is the positive controls for H3K27me3, and *IGN5* is the positive control for H3K9me2. % of input is calculated. H3K36me3 is normalized to *ACTIN2*, H3K27me3 is normalized to *STM*, and H3K9me2 is normalized to *IGN5*. The data presented is the average of three biological replicates. Bar, SD.

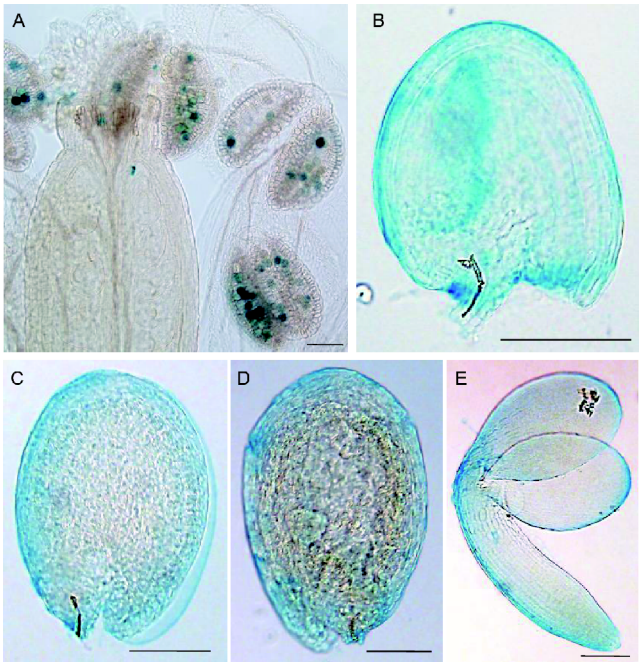

Figure 5 (Color online) *EFS* is expressed in pollen (A), ovule (B) and developing seeds (C–E). *pEFS::GUS* lines were used to stain the *GUS* in developing siliques. Bar, 100 μm.

expressed mainly in the seed coat during the early stages of embryo development. Together, *EFS* is expressed in pollen, ovules and different stages of embryo morphogenesis, which is in line with its effects on embryo development and seed size regulation in both maternal and paternal directions (Figures 1 and 2C).

DISCUSSION

Post-transcriptional modifications, alone or in combination, decide the chromatin landscape and are altered in response to different environmental and developmental cues, thus regulate the gene expression to control the biological processes in plants. Interplay between histone modifications (H3K9, H3K4 and H3K27) and DNA methylation has been reported in *Arabidopsis* (Bernatavichute et al., 2008; Lu et al., 2010; Zhang et al., 2007) and the biological functions of these modifications may vary among different species (Zhao et al., 2017). However, interplay between DNA methylation and H3K36me3 has not yet been tested in *Arabidopsis*. In this paper, we investigated the interplay between DNA methylation and H3K36me3 in *efs-3* mutant by studying the im-

printed genes that were reported to be under the regulation of DNA methylation. Among the tested imprinted genes, DNA hypomethylation in CG and CHG, especially CG context was observed at the imprinted *MOP9.5* promoter in *efs-3* mutant (Figure 2A), thus showed an increase in *MOP9.5* expression (Figure 2C). Although *MOP9.5* promoter is mainly hypomethylated in CG context in *efs-3* mutant, we found that the expression of *MET1*, which encodes the *de novo* CG methyltransferase, is not altered in *efs-3* mutant (Figure 2D). Furthermore, *DDM1* expression also did not change in *efs-3* mutant (Figure 2D). This suggests that the recruitment of either MET1 or DDM1 to *MOP9.5* is impaired in *efs*, or the involvement of other genes and/or pathways in parallel to *MET1* and *DDM1*. In addition, we looked into the RNA-seq data of *efs-3* (Li et al., 2015), and found that DNA demethylases, *ROS1* (repressor of silencing 1) and *DML2* (demeter like-2) are downregulated in *efs-3* mutant, further eliminating the possible direct role of DNA demethylases at *MOP9.5*. RNA-directed DNA methylation (RdDM) pathway is required for *de novo* DNA methylation of cytosine in all sequence contexts (Matzke and Mosher, 2014; Wendte and Pikaard, 2017). *Arabidopsis* has evolved two additional plant specific RNA polymerases, RNA Pol IV and RNA Pol V. RNA Pol IV and V transcribed long non-coding RNAs that are required for the gene silencing through RdDM pathway (Du et al., 2015; Matzke and Mosher, 2014; Wendte and Pikaard, 2017). Therefore, with fact that RNA Pol V binding to *MOP9.5* promoter and the presence of siRNA in Col-0 (Figure S2E in Supporting Information), it is possible that RNA Pol V mediated long coding is being transcribed at *MOP9.5* promoter that recruits AGO4-siRNA complex and *de novo* DNA methyltransferase *DRM2* to establish the DNA methylation, thus results in gene silencing. Therefore, we hypothesize that H3K36me3 collaborates with RdDM pathway to regulate the DNA methylation and gene expression of *MOP9.5*. However, whether EFS collaborates with RdDM pathway is yet to be explored.

Along with DNA methylation, we also found that H3K36me3 is increased at *MOP9.5* promoter (Figure 4A), while H3K9me2 and H3K27me3 are decreased in *efs-3* mutant (Figure 4B and C). These results indicate that the increase of H3K36me3 may be due to the other H3K36 methyltransferases that somehow participate to control the seed phenotype in the absence of *EFS*. Although genome-wide levels of H3K36me3 are not changed in another H3K36 methyltransferase SDG26 loss of function mutant, H3K36me3 levels are reduced at individual loci (Berr et al., 2015). Similarly, SDG4 could be another candidate for H3K36me3 (Cartagena et al., 2008). However, whether SDG26 and/or SDG4 are responsible for H3K36me3 at *MOP9.5* promoter in the absence of *EFS* is yet to be investigated. It has been shown that H3K36me3 modification is responsible for DNA methylation in mammalian cells (Baubec et al., 2015), denoting the interplay between H3K36me3 and DNA methylation. Here, we observed an increase in H3K36me3 and decrease in DNA methylation at *MOP9.5* promoter, indicating an antagonistic correlation between these two marks. However, whether this antagonistic correlation between DNA methylation and H3K36me3 exists throughout the genome in *Arabidopsis* is yet to be investigated. In addition to the antagonistic role of H3K36me3 with DNA methylation, we observed that H3K27me3 level is decreased in *efs-3* mutant at *MOP9.5* promoter (Figure 4B). This antagonistic relationship between H3K36me3 and H3K27me3 has already been reported at flowering genes (Shafiq et al., 2014; Yang et al., 2014). Notably, in *Arabidopsis*, the H3K27me3 marked chromatin regions are significantly hypomethylated (Zhang et al., 2007). Our data showed the decrease of H3K27me3 and H3K36me3 levels at *MOP9.5* promoter. This indicates that decrease in H3K27me3 at *MOP9.5* promoter is likely due to the increase in H3K36me3. In brief, our results showed that different post-transcriptional modifications coordinate with each other to regulate the gene expression of *MOP9.5*.

Our reciprocal crosses between *efs-3* mutant and Col-0 showed the enlarged seed size in F_1 population in both maternal and paternal directions (Figure 3A–C), and as *EFS* is expressed in pollen and anther (Figure 5A and B), this data suggested that *EFS* regulates the gene imprinting in both maternal and paternal directions. However, it requires further analysis to determine the parent-of-origin dosage effect of *EFS* in regulating the seed size. Furthermore, overexpression of *MOP9.5* in Col-0 (data not shown), and *mop9.5* mutants did not show significant change of seed size (Figure S2B–D in Supporting Information), indicating that only *MOP9.5* is not responsible for the increased seed size observed in *efs-3* mutant and pointing towards the involvement of other genes that could also participate to regulate the seed size. Together, these observations suggested that there might be other pathways and/or genes that regulate the seed size in *efs-3* mutant. Besides gene imprinting, seed size is regulated through the IKU pathway, ubiquitin–proteasome pathway, G-protein signaling, mitogen-activated protein kinase signaling pathway, phytohormones and transcriptional regulatory factors (reviewed in (Li and Li, 2016)). Among the phytohormones, *EFS* is involved in brassinosteroid regulated gene expression in *Arabidopsis*, and expression of a few genes, such as *CKX5*, *CKX1* (Orozco-Arroyo et al., 2015), *ABA1*, *ABA4*, and *ARF2* (Li and Li, 2016; Li et al., 2015) are altered in *efs-3* mutant that may contribute to the enlarged seed size observed, and our results did not rule out the possibility that *EFS* may regulate the seed size through the phytohormones.

In summary, *EFS* is expressed in different stages of embryo morphogenesis to ensure the proper seed size and weight of *Arabidopsis*, and both maternal and paternal alleles

of *EFS* are participated in gene imprinting to regulate the seed size (Figure 3A–C). Furthermore, our work highlighted the interplay between DNA methylation and H3K36me3/H3K27me3 at the imprinted *MOP9.5* gene locus, and raised the question that whether and how these post-transcriptional modifications coordinate with each other to regulate the gene imprinting genome-widely in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials

All *Arabidopsis thaliana* plants are Columbia ecotype (Col-0). Loss-of-function *EFS* mutants used in this study are *efs-3*, *ccr1-1*, *ashh2-1* (SALK_065480C), and WiscDsLox432B04. *pEFS:EFS* in *efs* had been reported in (Ko et al., 2010). *efs-3* mutant is previously reported in (Kim et al., 2005), while *met1-3* (CS16394), *mop9.5-1* (SALK_048798) and *mop9.5-2* (GK-171F04) were ordered from The European *Arabidopsis* Stock Centre (NASC). Primers for genotyping are listed in Table S1 in Supporting Information. Surface-sterilized seeds were placed on MS medium (Murashige and Skoog salt base; Sigma, USA) containing phytagel (0.25%, w/v) supplemented with 1% (w/v) sucrose and placed at 4°C for 2 days for stratification. Plants were grown under long-day conditions at 22°C with a 16 h of light/8 h of dark cycle.

Chop-PCR

C hop-PCR was performed as previously described by (Zhang et al., 2014). Briefly, genomic DNA was extracted from 2 weeks seedlings, flowers and buds with CTAB, followed by the digestion of methylation-sensitive restriction enzyme (McrBc, USA). Equal amount of digested and undigested DNA were used as the template for 28 cycles of PCR amplification, followed by agarose gel electrophoresis and ethidium bromide staining. Chop-PCR primers are listed in Table S1 in Supporting Information.

DNA methylation analysis

For bisulfite sequencing, genomic DNA was extracted from 2-week-old seedlings with CTAB. Bisulfite treatment was performed using the EpiTect Plus DNA Bisulfite Kit (Qiagen 59124, USA). To analyze the DNA methylation at *MOP9.5* promoter, bisulfite-treated DNA was amplified by nested PCR primers (Table S1 in Supporting Information). The PCR fragment was cloned into pGEM-T Easy (Promega, USA), and the ligation product was transformed into DH5α cells. At least 20 single colonies from the transformation were sequenced for each PCR product, and the results presented are from 16 colonies for each genotype.

RNA extraction and RT-qPCR

For qRT-PCR, total RNA was extracted using the Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Followed by DNase treatment with Turbo DNase (Ambion, USA), RT-PCR was performed using Superscript III Reverse Transcriptase (Invitrogen). Primers used for qRT-PCR are listed in Table S1 in Supporting Information.

Microscopy

Seeds clearing was performed following the protocol described by Roszak and Köhler (Roszak and Köhler, 2011). In brief, different stages of developing siliques were fixed in ethanol: acetic acid (9:1) and washed by 70% ethanol. Finally, the seeds were isolated and mounted in clearing solution (glycerol/chloral hydrate/water in a ratio of 1:8:3) on slides, and observed with Olympus BX51 microscopy.

ChIP-qPCR

Nuclei were extracted from 3 g of crosslinked plant material by using Honda buffer as described previously by (Sun et al., 2013). In all histone ChIP reactions, sonication, immunoprecipitation, DNA recovery and purification were performed as previously described by (Sun et al., 2013). The antibodies used were: anti-H3 (Abcam, ab1791, USA), anti-H3K27me3 (Millipore, 07-449, USA), anti-H3K36me3 (Abcam, ab9050), anti-H3K9me2 (Millipore, 07-441). All ChIP experiments were quantified by quantitative PCR (qPCR) in triplicates with appropriate primers listed in Table S1 in Supporting Information.

GUS staining

GUS staining was performed following the protocol published by (He et al., 2017). Flowers and developing siliques were cut longitudinally and fixed in ice-cold 90% acetone for 1 h at −20°C. After washing three times with PBS, the tissue was immersed in staining solution, including X-gluc and vacuum-infiltrated for 20 min. After staining for 2 days at 37°C, the samples were mounted in clearing solution and observed with Olympus BX51 microscopy.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 DNA methylation screening of imprinted genes by Chop-PCR in flowers and buds of Col-0 and *efs-3*. McrBC is a methylation-sensitive enzyme that specifically digests methylated DNA, and the bands with McrBC treatment represent the non-methylation levels.

Figure S2 *mop9.5* has a normal seed size, while *MOP9.5* is methylated and is the target of RNA Pol V.

Table S1 Primers information

The supporting information is available online at http://life.scichina.com and https://link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.