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LETTER TO THE EDITOR

A DNA ligase required for active DNA demethylation and genomic imprinting in *Arabidopsis*

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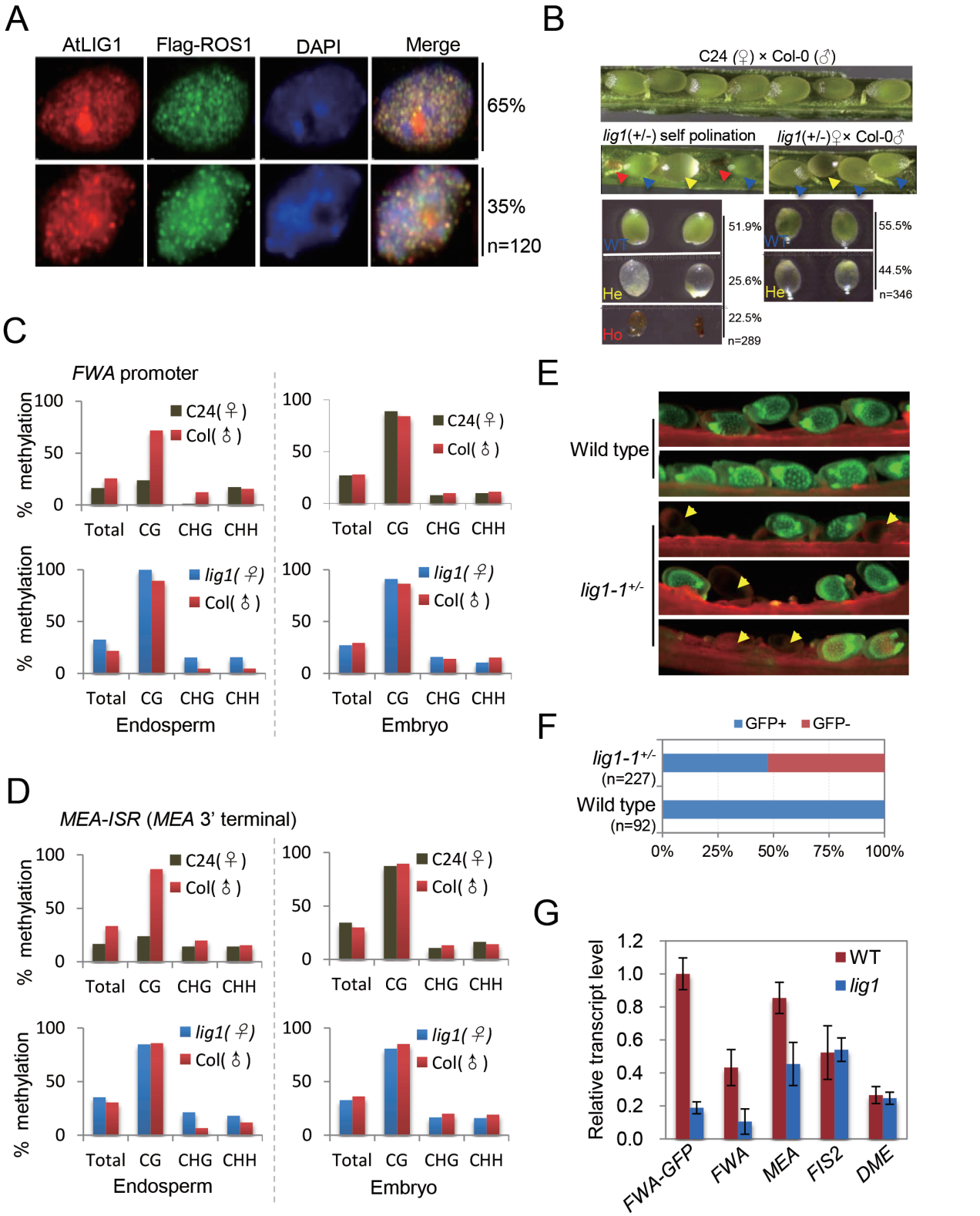
Dear Editor,

Active DNA demethylation plays crucial roles in the regulation of gene expression and gene imprinting. In plants, active DNA demethylation is initiated by the ROS1/DME family of 5-methylcytosine-specific DNA glycosylases via a base excision repair mechanism [1, 2]. ROS1 and DME are bifunctional DNA glycosylases that excise the 5-methylcytosine base and then cleave the DNA backbone at the abasic site, resulting in a gap with a 3′ phosphate or 3′ dRP (3′ α, β-unsaturated aldehyde) terminus. The DNA phosphatase ZDP and the apurinic/apyrimidinic endonuclease APE1L process the 3′ phosphate and 3′ dRP termini, respectively, to generate a 3′ OH group so that downstream polymerases and ligases can fill in the gap with an unmethylated cytosine [3, 4]. Thus far, the DNA polymerases and ligases involved in this active DNA demethylation pathway are unclear. Here we show that *Arabidopsis* DNA LIGASE I (AtLIG1) co-localizes with ROS1, ZDP and APE1L *in vivo*. In addition, we found that AtLIG1 is essential for demethylation and activation of the maternally imprinted genes *FWA* and *MEA* in the endosperm. Our data suggest that AtLIG1 is the major DNA ligase that functions at the last step in active DNA demethylation in *Arabidopsis*.

The *Arabidopsis* genome encodes four DNA ligases: AtLIG1, AtLIG1a, AtLIG4 and AtLIG6 (Supplementary information, Figure S1A) [5]. AtLIG1 carries out the ligation reaction in DNA replication and base excision repair, whereas AtLIG4 is responsible for DNA ligation in the non-homologous end-joining pathway in DNA damage response [6-10]. There are no reported roles for AtLIG1a and AtLIG6. Transcriptome analyses revealed that *AtLIG1a* is probably not expressed, indicating that *AtLIG1* may be the sole source of DNA ligase I activity in *Arabidopsis* [8]. In order to characterize the function of *Arabidopsis* DNA ligases (AtLIGs) in active DNA demethylation, T-DNA insertion lines for these genes were obtained (Supplementary information, Figure S1B). The homozygous mutant of *atlig1* is embryonic lethal and we only obtained heterozygous mutants *atlig1-1* (Col background) and *atlig1-3* (C24 background), which produce small siliques and ~50% aborted seeds (Supplementary information, Figure S2A). We also generated RNAi lines that have a reduced expression of *AtLIG1* (Supplementary information, Figure S2B). Similar to previously reported *AtLIG1* RNAi lines [7], these RNAi lines exhibit a severe dwarf phenotype (Supplementary information, Figure S2C). The *atlig1a-1*, *atlig1a-2*, *atlig4-5* and *atlig6-1* mutants do not exhibit abnormal developmental phenotypes under normal growth conditions. To determine which of the ligases is involved in active DNA demethylation, we designed a chop-PCR assay to measure the DNA methylation level at the *At1g26400* locus. Upon *ROS1* dysfunction, the *At1g26400* locus shows DNA hypermethylation and the DNA becomes resistant to cleavage by the methylation-sensitive restriction enzyme *Hha*I, and thereby can be amplified as a distinct band by PCR. AtLIG1 knockdown by RNAi, but not mutations of other AtLIGs, also led to DNA hypermethylation (Supplementary information, Figure S3A). Moreover, we performed bisulfite sequencing and confirmed that the AtLIG1 RNAi lines show DNA hypemethylation at this locus in the CG context. CHG and CHH methylation levels are also increased, although to a lesser extent (Supplementary information, Figure S3B). To further explore the roles of AtLIG4 and AtLIG6 in active DNA demethylation, we performed whole-genome bisulfite sequencing in the *atlig4atlig6* double mutant but found that the double mutations did not affect DNA methylation levels when compared with *ros1-4* and *rdd* mutants (Supplementary information, Table S1). Collectively, our data suggest that AtLIG1, but not other AtLIGs, is involved in the active DNA demethylation pathway.

Our previous data show that ZDP and APE1L co-localize with ROS1 in subnuclear foci [3, 4]. To test whether AtLIG1 may co-localize with other enzymes in the active DNA demethylation pathway, we performed co-immunolocalization assays. The expression of FLAG-tagged ROS1, driven by its native promoter in *ros1-1* mutant plants, is visualized by an antibody against the FLAG tag. AtLIG1 is stained by a custom-made primary an-

tibody and a fluorescence-tagged secondary antibody. AtLIG1 co-localizes with ROS1 in nucleoplasmic foci, as shown by the strong yellow signals (Figure 1A). In 89% of the cells, AtLIG1 co-localizes with APE1L in the nucleolus and in nucleoplasmic foci, whereas in 11% of the cells, AtLIG1 and APE1L substantially co-localize in



nucleoplasmic foci but not in the nucleolus (Supplementary information, Figure S4A). AtLIG1 and ZDP also co-localize in nucleoplasmic foci in ~87% of the cells (Supplementary information, Figure S4B). Thus, AtLIG1 co-localizes with known components of the active DNA demethylation machinery in distinct subnuclear foci.

The homozygous *atlig1* mutant has been reported to be maternally lethal [11]. Consistent with the previous report, we also observed brown early aborting seeds and white late aborting seeds, which may correspond to homozygous *atlig1* seeds and heterozygous seeds carrying a maternal allele of *atlig1* mutation, respectively (Figure 1B). Maternal lethality phenotypes can be caused by aberrant expression of maternally imprinted genes and defects in the central cell or the endosperm [2, 12, 13]. *FWA* and *MEA* are two well-known maternally imprinted genes, and their maternal expression in the endosperm requires active DNA demethylation initiated by the ROS1 paralog DME [12, 14]. To investigate whether the methylation of *FWA* promoter and *MEA ISR* (intergenic subtelomeric repeat) in endosperm tissues is affected by the *atlig1* mutation, we dissected the seeds of eight DAP (days after pollination) and collected the endosperm tissues from wild-type (WT) and white translucent mutant seeds. Then we carried out bisulfite sequencing analyses of the *FWA* promoter and *MEA ISR* region. Our data show that the maternal CG, CHG and CHH methylation levels of the 5′ direct repeats in the *FWA* promoter in the WT C24 endosperm are 23.81%, 0.8% and 17.14%, respectively (Figure 1C). In the endosperm of late aborting seeds (presumably of the *atlig1-1*$^{+/-}$ genotype with a maternal *atlig1* mutation), the maternal methylation levels of the *FWA* promoter in CG, CHG and CHH contexts are changed to 100%, 15.35% and 15.71%, respectively (Figure 1C). There is a less increase in paternal CG methylation levels in the *FWA* promoter (from 71.85% to 91.11%), and we did not observe any increase in paternal CHG and CHH methylation levels (Figure 1C). The DNA methylation levels in all sequence contexts are similar between WT and late aborting seeds in the embryo (Figure 1C). The maternal CG, CHG and CHH methylation levels of *MEA ISR* in the WT C24 endosperm are 24%, 14.29% and 14.29%, respectively. They are increased to 84.92%, 21.43% and 18.15% in the endosperm of late aborting seeds (Figure 1D). Similarly, the paternal *MEA ISR* methylation levels remain unchanged (Figure 1D). These results suggest that the *FWA* promoter and *MEA ISR* are hypermethylated in the endosperm of mutant seeds harboring a maternal allele of *atlig1*. To examine whether the abnormal seeds in the *atlig1-1*$^{+/-}$ mutant have aberrant expression of *FWA*, we introduced a *pFWA::*Δ*FWA-GFP* reporter into the *atlig1-1*$^{+/-}$ mutant [14]. We found that *atlig1-1*$^{+/-}$ plants produced about 52.4% seeds that were defective in *pFWA::*Δ*FWA-GFP* expression (Figure 1E and 1F). The ratio of GFP-positive and GFP-negative seeds is in accordance with that of normal seeds and aborting seeds. To further measure the mRNA levels of *FWA* and *MEA* in the endosperm of WT and late aborting seeds, we performed real-time PCR and found that the expression levels of *FWA-GFP*, *FWA* and *MEA*, but not *DME* and *FIS2*, are downregulated in the *atlig1* mutant endosperm (Figure 1G). Taken together, our data suggest that the defects in the *atlig1* endosperm are associated with DNA hypermethylation and impaired expression of imprinted genes.

Our findings are consistent with previous data showing that mutations of components in the active DNA demethylation pathway, e.g., DME, SSRP1, ZDP and APE1L, result in defective activation of maternally imprinted genes and cause aberrant seed development [2, 4, 13]. A previous study reported normal expression of *FWA* and a limited, but not significantly altered, expression of *MEA* in the *atlig1* mutant [8]. One possibility for this disparity is that materials of different ecotypes and different developmental stages were used. The previous study used the *atlig1-3* mutant (C24 background) and examined

Figure 1 AtLIG1 is required for genomic imprinting in *Arabidopsis*. **(A)** Dual immunolocalization using anti-AtLIG1 (red) in transgenic lines expressing Flag-ROS1 (green). In all panels the DNA was stained with DAPI (blue). The frequency of nuclei displaying each interphase pattern is shown on the right. **(B)** Phenotype of developing F1 seeds at 7 days after pollination (DAP) in a cross between wild type (WT) and mutant. The percentages of normal, early aborting (homozygous) and late aborting (heterozygous) seeds are shown on the right. **(C**-**D)** Methylation percentage of the 5′ SINE-related repeat of *FWA* **(C)** or *MEA* 3′ terminal **(D)** in the WT embryo and endosperm or mutant embryo and endosperm with maternal *atlig1*. Methylation levels for each fraction were determined by bisulfite sequencing. **(E)** Fluorescence images of *pFWA::*Δ*FWA-GFP* seeds at 4 DAP. *pFWA::*Δ*FWA-GFP* was introduced into *atlig1(+/–)* background by genetic crosses, and homozygous *pFWA::*Δ*FWA-GFP* transgenic plants were selected by genotyping. Yellow arrowheads show seeds with a reduced GFP signal presumably because of their maternal inheritance of *atlig1*. **(F)** The percentages of GFP-positive and GFP-negative seeds in WT and *atlig1* mutant with *pFWA::*Δ*FWA-GFP* reporter. **(G)** Expression levels of imprinted genes in WT and *atlig1* mutant seeds at 3 DAP. *ACT11* was used as an internal control. Two biological replicates were performed, and very similar results were obtained. Standard errors were calculated from three technical repeats (n = 3).

the *FWA-GFP* and *MEA-GUS* signals in the central cell, whereas we used the *atlig1-1* mutant (Col background) and examined the GFP signal in the endosperm.

In conclusion, our study reveals that AtLIG1 is an important component of the active DNA demethylation machinery. This allows us to have a more complete picture of the active DNA demethylation pathway in *Arabidopsis* (Supplementary information, Figure S5).

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)