The atypical histone variant H3.15 promotes callus formation in Arabidopsis thaliana

An Yan¹, Michael Borg², Frédéric Berger² and Zhong Chen¹,*

ABSTRACT

Plants are capable of regenerating new organs after mechanical injury. The regeneration process involves genome-wide reprogramming of transcription, which usually requires dynamic changes in the chromatin landscape. We show that the histone 3 variant HISTONE THREE RELATED 15 (H3.15) plays an important role in cell fate reprogramming during plant regeneration in Arabidopsis. H3.15 expression is rapidly induced upon wounding. Ectopic overexpression of H3.15 promotes cell proliferation to form a larger callus at the wound site, whereas htr15 mutation compromises callus formation. H3.15 is distinguished from other Arabidopsis histones by the absence of the lysine residue 27 that is trimethylated by the POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) in constitutively expressed H3 variants. Overexpression of H3.15 promotes the removal of the transcriptional repressive mark H3K27me3 from chromatin, which results in transcriptional derepression of downstream genes, such as WUSCHEL RELATED HOMEBOX 11 (WOX11). Our results reveal a new mechanism for a release from PRC2-mediated repression through H3.15 deposition into chromatin, which is involved in reprogramming cell fate to produce pluripotent callus cells.

KEY WORDS: Callus formation, H3.15, Regeneration, H3K27me3, Cell fate reprogramming

INTRODUCTION

Plants have a remarkable regenerative capability to heal after injury by regenerating new tissue at sites of wounding. Under appropriate culture conditions, plants can regenerate entire individuals from a piece of tissue or even a single cell (Sugimoto et al., 2011). Wound-induced regeneration is usually initiated with the formation of a proliferating mass of pluripotent cells, termed callus. A callus contains a population of undifferentiated cells with proliferation potential, which are able to re-enter the cell cycle and continue to differentiate into new organs (Ikeuchi et al., 2013). Callus formation can be induced experimentally by wounding or in cell culture on callus-inducing medium (CIM) (Lee and Seo, 2018). Wound-induced callus formation involves the reprogramming of differentiated somatic cells to reacquire totipotency through cell dedifferentiation. This results from the action of transcription factors from the APETALA 2/EThyLENE RESPONSE FACTOR (AP2/ERF) family, including WOUND INDUCED DEDIFFERENTIATION 1 (WIND1), WIND2, WIND3, WIND4 and ENHANCER OF SHOOT REGENERATION 1 (ESR1) (Iwase et al., 2011, 2017). WIND1 promotes wound-induced cell dedifferentiation through the activation of B-Type ARABIDOPSIS RESPONSE REGULATOR (ARR)-dependent cytokinin signaling (Iwase et al., 2011). Auxin-rich CIM-induced callus formation is commonly used as the first step in de novo organogenesis, followed by the incubation of callus on auxin-rich root-inducing medium (RIM) or cytokinin-rich shoot-inducing medium (SIM) to regenerate roots or shoots (Skoog and Miller, 1957). CIM-induced callus formation is initiated from pericycle cells of root explants and pericycle-like cells of aerial organs (Sugimoto et al., 2010). It has been proposed that CIM-induced callus formation resembles lateral root development, regardless of what types of tissue are used as explants (Atta et al., 2009; Sugimoto et al., 2010; Lee and Seo, 2018).

During callus formation, widespread changes in gene expression are required to reprogram the transcriptional state of somatic cells. Such genome-wide changes in transcription coincide with changes in chromatin modifications, including DNA methylation, post-translational modification of histones and exchange of histone variants (Ikeuchi et al., 2015b; Lee and Seo, 2018). The repressive modification H3K27me3 silences leaf-regulatory genes and is essential for leaf-to-callus transition in Arabidopsis (He et al., 2012), and for the prevention of unscheduled reprogramming of differentiated somatic cells (Ikeuchi et al., 2015a). These contrasting roles indicate that dynamic changes in H3K27me3 are probably required for reprogramming during regeneration. The regulation of dynamic changes in chromatin depends on the activities of histone H3 variants (Malik and Henikoff, 2003; Henikoff et al., 2004; Weber and Henikoff, 2014). In multicellular eukaryotes, the histone H3 family comprises three major types of variants: canonical H3.1 and H3.3 variants, and the centromeric variant CenH3 (Malik and Henikoff, 2003; Loyola and Almouzni, 2007; Jiang and Berger, 2017b). In Arabidopsis, H3.1 is essential for the maintenance of H3K27me3 through cell division (Jiang and Berger, 2017a). H3.1 deposition relies on the activity of the Chromatin Assembly Factor 1 (CAF-1) complex (Kaya et al., 2000; Jiang and Berger, 2017a), whereas H3.3 deposition is primarily mediated by a complex containing the chaperone HIRA (Nie et al., 2014; Wang et al., 2018). Regeneration from callus is less efficient in plants deprived of HIRA, whereas CAF-1 mutants are more efficient (Nie et al., 2014). Hence, selective incorporation and dynamic exchange of specific histone 3 variants might be an important mechanism underlying cell fate reprogramming during regeneration.

In addition to the H3 variants present in all multicellular eukaryotes, further H3 variants have been reported in Arabidopsis (Jiang and Berger, 2017b), including H3.10, which is expressed specifically in sperm (Okada et al., 2005; Borg and Berger, 2015), and H3.14, which is expressed in the vegetative pollen cell and in endosperm (Ingouff et al., 2010). With the exception of H3.10 (Borg et al., 2020), the properties and role of other atypical
Arabidopsis histone variants have not yet been elucidated. Here, we report that the histone 3 variant H3.15, which is encoded by the gene HTR15 (Talbert et al., 2012), is involved in cell fate reprogramming during plant regeneration in Arabidopsis. H3.15 rapidly accumulates upon wounding and promotes callus development. H3.15 lacks residue K27 and consequently impacts H3K27me3 dynamics at genes important for regeneration. Our study suggests a new mechanism for the removal of PRC2-mediated gene repression during plant regeneration.

RESULTS

H3.15 is induced at wound sites

To analyze histone 3 variant dynamics during wound-induced regeneration, we analyzed gene expression in response to injury of hypocotyls and roots. We checked all H3 encoding (HTR) genes except HTR7 and HTR11, which might be pseudogenes (Okada et al., 2005; Ingouff et al., 2010), and HTR10, which is specifically expressed in sperm (Okada et al., 2005; Borg and Berger, 2015). Quantitative RT-PCR (qRT-PCR) analysis showed that, among the 12 genes encoding H3 variants, the expression of HTR15 (At5g12910) gradually increased upon wounding, in contrast with other H3 variants coding genes (Fig. 1A,B). We further tested the wound-induced expression of HTR15 using GLUCURONIDASE (GUS) (Fig. S1A) or green fluorescent protein (GFP) reporter lines (Fig. 1C) under the control of the HTR15 promoter. Confocal imaging indicated that GFP expression was detectable at wound sites of roots within 7 h (Fig. 1C) and in hypocotyls within 21 h of wounding (Fig. S1A). HTR15 promoter activity was also detected at wound sites in other tissues, including roots and petioles (Fig. S1B-D). During wound-induced callus formation in roots, pHTR15 first drove expression in the pericycle around the wound site (Fig. 1D-H) and later in proliferating callus cells (Fig. 1C; Fig. S1A). These observations indicate that the expression of HTR15 is induced upon wounding and is sustained during callus formation.

HTR15 is expressed during auxin-induced callus formation

Explants incubated on auxin-rich CIM can form a callus from pericycle cells or pericycle-like cells (Ikeuchi et al., 2013). As HTR15 is expressed in pericycle cells upon wounding, we tested whether HTR15 expression is induced during CIM-induced callus formation. Time-course gene expression analysis using root explants indicated that HTR15 expression was progressively induced during incubation on CIM (Fig. 2A). We further performed confocal imaging and GUS staining using HTR15 reporter lines. pHTR15 activity was strongly induced in the pericycle or pericycle-like cells of root and hypocotyl explants during CIM incubation (Fig. 2B; Fig. S2). These results

Fig. 1. HTR15 expression is induced at wound sites in Arabidopsis. (A) qRT-PCR analysis shows the response of 12 HTRs to wounding. Seedlings (7 days old) were cut at the middle of hypocotyl; the upper parts were removed and the lower parts were incubated on phytohormone-free MS medium. Wounding tissues about 1 mm in length at the cutting site were collected for RNA extraction at indicated timepoints. Data are mean±s.d. (n=3 biological replicates). (B) qRT-PCR analysis of HTR15 expression at the wound site of Arabidopsis hypocotyl. (C) Confocal imaging showing accumulation of pHTR15::3GFP at wound sites of root at different timepoints after wounding. The roots of 7-day-old seedlings were cut at ~2 cm (elongation zone) from the root-hypocotyl junction. The upper ends of seedlings were removed and remaining roots were subjected to confocal imaging. Cell boundaries are stained by propidium iodide. At least 20 seedlings were checked at each timepoint. Arrows indicate callus cells outside the original root-hypocotyl region. (D-H) Confocal sections show HTR15 promoter activity in the pericycle around wound sites 24 h after wounding (D,E,G). The pericycle marker J0121 was used as a reference (F,H). Cross-sections were generated from confocal z stacks. Scale bars: 50 µm.
strongly suggest that HTR15 expression is induced by auxin-rich CIM and sustained during CIM-induced callus formation.

**Auxin is involved in the transcriptional control of HTR15 during callus formation**

Given that the plant hormone auxin is crucial for callus induction (Ikeuchi et al., 2013), we tested the impact of auxin on HTR15 expression during wound-induced callus formation. HTR15 expression was strongly induced by the synthetic auxin 1-naphthaleneacetic acid in lateral root primordium (Fig. S3) and whole seedlings (Fig. S4A), suggesting that HTR15 expression is auxin inducible. Accordingly, the application of the auxin transport inhibitor 1-naphthylphthalamic acid delayed and reduced auxin inducible. Consequently, we found an auxin-responsive element (AuxRE, core sequence TGTCTC) located upstream of the HTR15-coding sequence (Fig. S4C). To test whether this motif controls wound-induced HTR15 expression, we mutated AuxRE in the full-length HTR15 promoter (pHTR15::AuxRE::GUS). GUS staining revealed that this mutation delayed and impaired promoter activity upon wounding (Fig. S4B), suggesting that this AuxRE partially mediated HTR15 induction in response to wounding. In addition, pHTR15::3GFP expression was much higher at wound sites than unwounded sites in root explants incubated on CIM (Fig. S4D). Altogether, these results indicate that HTR15 transcription is mediated both by wounding and auxin during callus formation.

**H3.15 promotes wound and auxin-induced callus formation**

As pericycle cells possess pluripotency to develop into a callus (Atta et al., 2009; Sugimoto et al., 2010), the accumulation of H3.15 within the pericycle suggested that this H3 variant is involved in callus formation. To test the requirement of H3.15 in this process, we created an htr15 knockout mutant using CRISPR/Cas9 technology. The mutated sequence contained a thymine (T) insertion at the 5′ end of the HTR15-coding sequence, creating a frameshift mutation that resulted in multiple premature stop codons (Fig. S5A). The null htr15 allele produced smaller callus than wild type (Fig. 3A,B). This defect was complemented by introducing pHTR15::HTR15 in htr15 (Fig. S5B). To test whether H3.15 could enhance callus formation, we obtained transgenic plants overexpressing HTR15 by fusing full-length genomic DNA to the cauliflower mosaic virus 35S promoter (p35S::HTR15) (Fig. S5C). Overexpression of HTR15 produced significantly larger callus at hypocotyl wound sites compared with wild type (Fig. 3A,B). We further examined whether H3.15 was involved in CIM-induced callus formation. The root or hypocotyl explants were incubated on CIM for 40 days or 28 days, respectively, due to their tissue-specific differences in regeneration. We found that lines overexpressing HTR15 had stronger callus-forming capacity than wild type in both CIM-cultured hypocotyl and root explants, whereas htr15 explants produced significantly smaller callus (Fig. 3C-F). Moreover, complementation of htr15 with pHTR15::HTR15 fully restored callus-forming capacity (Fig. S5D). We therefore conclude that H3.15 plays an important role in promoting wound- and CIM-induced callus formation.

**H3.15 reduces H3K27me3 levels during callus formation**

Because histone H3 variants are variable at some specific amino acid residues, which are crucial for their function, we carefully analyzed the amino acid composition of H3.15. Four amino acid substitutions at positions 31, 41, 87 and 90 discriminate H3.3 from H3.1 in Arabidopsis (Okada et al., 2005; Ingouff and Berger, 2010). Among the four key amino acid residues at positions 31, 41, 87 and 90, only Phe41 (Y41) is conserved in both H3.3 and H3.15, whereas the other three residues vary among H3.1, H3.3 and H3.15 (Fig. 4A). Y41 was shown to be dispensable for the proper deposition of H3.3 (Lu et al., 2018), whereas alanine-31 (A31) in H3.1 is responsible for selective K27 monomethylation by the plant-specific Set domain histone methyltransferases ATXR5/6 (Jacob et al., 2014). The residues at positions 87 and 90 are crucial for interaction with the CAF-1 and HIRA complexes that deposit H3.1 and H3.3, respectively (Shi et al., 2011; Lu et al., 2018). In H3.15, these residues are substituted with residues that are present in neither H3.1 nor H3.3, preventing the prediction of the mechanisms involved in the deposition of H3.15 (Fig. 4A). In addition, HTR15 lacks introns similar to H3.1-encoding genes, whereas H3.3-encoding genes contain introns within the coding sequence. To investigate whether H3.15 deposition is DNA replication-dependent in common with H3.1, we performed EdU...
staining and GUS staining assays to compare the wound-induced expression of HTR15 with DNA replication. As shown in Fig. S6, DNA replication took place 24 h after wounding (Fig. S6A), whereas CYCLIN B1;1 promoter activity was detected 48 h after wounding (Fig. S6B), much later than when HTR15 expression was induced (Fig. 1C), suggesting that H3.15 transcription is activated prior to DNA replication, and H3.15 deposition is possibly not coupled with DNA replication but rather depends on the replication-independent chaperone HIRA, which has also been shown to promote callus formation (Nie et al., 2014). The substitutions in positions 87 and 90 led us to postulate that H3.15 might be deposited less efficiently than H3.3 through interaction with HIRA.

Compared with other Arabidopsis H3 variants, H3.15 shows the lowest degree of homology and lacks K4 (Lys4) and K27 (Lys27) residues (Fig. 4A). K4 methylation is associated with transcriptional activity in eukaryotes, so we tested whether this substitution had an impact on the phenotype observed in htr15 plants. Introducing pHTR15:HTR15-N4K in htr15 mutants complemented the htr15 phenotype (Fig. S5B,D), suggesting that defective methylation at K4 is not related to the impact of H3.15 on callus induction. We identified homologs of H3.15 in close relatives of Arabidopsis thaliana, Arabis lyrata, Arabis halleri and Boechera stricta, and observed that they shared numerous nearly identical substitutions in the region 17-42 of H3.3 and also in the SHAVLAL motif of H3.3 that is involved in the deposition by HIRA (Fig. S7). This contrasted with homologs of the

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**Fig. 3.** H3.15 is required for wound-induced and auxin-induced callus formation. (A) Callus formed at wound site of wild-type, 35S::HTR15 and htr15 hypocotyls 14 days after wounding. (B) Quantitative analysis of callus formation at wound sites of wild-type, 35S::HTR15 and htr15 hypocotyls. After dissection, explants were cultured on phytohormone-free MS medium for 14 days. Box plots represent the distribution of projected callus area (n>28). The horizontal line in the box represents the median, the lower and upper boundaries of the box represent the lower (25%) and upper (75%) quartiles of the data, and the whiskers represent the minimum and maximum values. The statistical significance was determined by a two-tailed unpaired Student’s t-test (**P<0.01). (C,D) CIM-induced callus formation of wild-type, 35S::HTR15 and htr15 hypocotyl explants. The hypocotyl explants were incubated on CIM for 28 days. The length and area of hypocotyl explants were measured with Image-Pro plus 6.0 software. Data are mean±s.d. (n≥14; **P<0.01; two-tailed unpaired Student’s t-test). (E,F) CIM-induced callus formation of wild-type, 35S::HTR15 and htr15 root explants. The root explants were incubated on CIM for 40 days. Data are mean±s.d. (n≥12; **P<0.01; two-tailed unpaired Student’s t-test). Scale bars: 0.5 mm (A); 5 mm (C,E).
other most divergent variant H3.10, which harbors K27 but lacks the proper residues in positions 28-32. We also identified likely homologs of H3.10 and H3.15 in more distantly related species of Brassicaceae, Brassica oleracea, Capsella rubella and Capsella grandiflora, as well as in Medicago truncatula. The degree of homology between the sequences of these proteins supports the idea that H3.15 and H3.10 represent two classes of divergent H3 variants that evolved in dicots. These variants probably play distinct roles because homologs of H3.10 carry the residue K27 and in Arabidopsis this variant is expressed specifically in sperm to reprogram H3K27me3 (Okada et al., 2005; Borg and Berger, 2015; Borg et al., 2020), whereas H3.15 is induced by wounding and callus induction, and is distinguished by the absence of K27.

We thus hypothesized that H3.15 incorporation could result in a loss of H3K27 methylation during callus formation. We performed immunoblotting analysis of 35S::HTR15 and wild type to examine H3K27 methylation levels. 35S::HTR15 showed levels of H3K27me1 and H3K27me3 similar to wild type before CIM induction (Fig. 4B,C). This suggested that ectopically expressed H3.15 is not incorporated in the absence of callus induction by CIM application. However, 40 days after CIM incubation, CIM-induced callus of 35S::HTR15 lines showed a dramatic reduction in both H3K27me1 and H3K27me3 levels compared with wild type (Fig. 4B,C). Hence, specifically during CIM-induced callus formation, overexpression of H3.15 globally reduces H3K27me1 and H3K27me3 levels compared with wild type.

To further test whether H3.15 function is correlated with the modification of K27, we introduced K27 in place of H3.15 H27 (His27) (Fig. 5A) and overexpressed this mutant version in transgenic plants. Interestingly, H27K substitution did not block the effect of H3.15 on callus formation, as 35S::HTR15-H27K still showed higher callus-forming capacity than wild type (Fig. 5B,C). We hypothesised that this was because of several other amino acid residues being substituted around K27 in H3.15 compared with wild type (Fig. 5A). A31 (Ala31) is crucial for selective monomethylation of H3 at K27 (Jacob et al., 2014; Jiang and Berger, 2017a), whereas residues 28 and 29 are crucial for trimethylation by PRC2 (Moritz and Trievel, 2018). We thus made further substitutions and inserted residues between 22 and 35 to restore an amino acid sequence identical to that of H3.1 (Fig. 5A). A31 (Ala31) is crucial for selective monomethylation of H3 at K27 (Jacob et al., 2014; Jiang and Berger, 2017a), whereas residues 28 and 29 are crucial for trimethylation by PRC2 (Moritz and Trievel, 2018). We thus made further substitutions and inserted residues between 22 and 35 to restore an amino acid sequence identical to that of H3.1 (Fig. 5A). Transgenic plants overexpressing this mutated form of HTR15 (35S::HTR15-KA) showed a similar phenotype to wild type (Fig. 5A). A31 (Ala31) is crucial for selective monomethylation of H3 at K27 (Jacob et al., 2014; Jiang and Berger, 2017a), whereas residues 28 and 29 are crucial for trimethylation by PRC2 (Moritz and Trievel, 2018). We thus made further substitutions and inserted residues between 22 and 35 to restore an amino acid sequence identical to that of H3.1 (Fig. 5A). Transgenic plants overexpressing this mutated form of HTR15 (35S::HTR15-KA) showed a similar phenotype to wild type (Fig. 5B,C).
Fig. 5. Amino acid substitution analysis of H3.15. (A) Amino acid substitution of H3.15. (B) CIM-induced callus formation in wild type, 35S::HTR15, 35S::HTR15-H27K and 35S::HTR15-KA. The hypocotyl explants were incubated on CIM for 28 days. Scale bar: 5 mm. (C) Quantitative analysis of callus formation in wild-type, 35S::HTR15, 35S::HTR15-H27K and 35S::HTR15-KA hypocotyl explants. Data are mean±s.d. (n≥10; **P<0.01; two-tailed unpaired Student’s t-test). (D) Western blot assay of H3K27me1 and H3K27me3 levels in CIM-induced callus. Wild-type, 35S::HTR15, 35S::HTR15-H27K and 35S:: HTR15-KA hypocotyl explants were incubated on CIM for 30 days. H3 served as a loading control. (E) Quantification of H3K27me1 and H3K27me3 western blot signals in CIM-induced callus. y-axis values are fold changes of signals in transgenic lines compared with wild type. Data are mean±s.d. (n=3 biological replicates). *P<0.05; two-tailed unpaired Student’s t-test.

(Fig. 5D,E), indicating that the amino acid residues surrounding K27 affected H3K27me3 accumulation. Altogether, these results demonstrate that the function of H3.15 is strongly linked with the dynamics of histone methylation at K27.

H3.15 acts upstream of WOX11 and LBD18 to promote CIM-induced callus formation

To further understand how H3.15 regulates cell dedifferentiation and proliferation during callus formation, we examined the expression of key callus-forming regulators, including WOX11, LATERAL ORGAN BOUNDARIES-DOMAIN (LBDs) and ETHYLENE RESPONSE FACTOR 115 (ERF115) in CIM-cultured hypocotyl explants of wild type and 35S::HTR15. WOX11, LBD16, LBD18, LBD29 and ERF115 expression was enhanced in 35S::HTR15 compared with wild type (Fig. 6A), suggesting that overexpression of H3.15 promotes transcription of these regulators during callus induction.

We further tested whether H3.15 was deposited in chromatin at the WOX11 and LBD18 loci. We produced plants overexpressing the fusion of H3.15 with 3×FLAG (Fig. S8). We performed ChIP assays with transgenic 35S::HTR15-3×FLAG hypocotyl explants cultured on CIM for 30 days. The relative enrichment of H3.15 was
enrichment and H3K27me3 levels during callus formation. If H3.15 was directly involved in reducing H3K27me3 during callus formation, we would expect that overexpression of HTR15 would reduce H3K27me3 enrichment at the WOX11 locus further than in wild type in response to callus formation. We performed ChIP-qPCR analysis of H3K27me3 levels at the WOX11 locus in wild type and 35S::HTR15. Hypocotyl explants cultured on CIM for 30 days were harvested. The relative enrichment of H3K27me3 on the WOX11 chromatin was calculated by normalizing the amount of immunoprecipitated fragment to input DNA and then by normalizing the value for the 30 days CIM sample against that for day 0 explants. qPCR analysis showed that H3.15:3×FLAG was enriched on WOX11 and LBD18 chromatin both at the promoter and within the gene body (Fig. 6B,C). This indicated that H3.15 was incorporated in chromatin of WOX11 and LBD18 when overexpressed, which in turn likely impacted the levels of H3K27me3 at these loci.

A previous study profiled H3K27me3 dynamics during the leaf-to-callus transition in Arabidopsis (He et al., 2012). Using these data, we found that H3K27me3 levels at the WOX11 and LBD18 loci are reduced in the CIM-induced callus compared with non-callus leaf tissue (Fig. S9). Using ChIP-qPCR, we confirmed that enrichment of H3K27me3 at the WOX11 locus was reduced during callus formation in the wild-type background (Fig. 6D). In plants expressing H3.15:3×FLAG under the control of the 35S promoter, H3.15 was not deposed at the WOX11 locus in the absence of callus induction (Fig. 6E). However, enrichment of H3.15:3×FLAG was increased in the callus when compared with non-induced explants (Fig. 6E, day 0), suggesting that there is a negative correlation between H3.15 enrichment and H3K27me3 levels during callus formation. If H3.15 was directly involved in reducing H3K27me3 during callus formation, we would expect that overexpression of HTR15 would reduce H3K27me3 enrichment at the WOX11 locus further than in wild type in response to callus formation. We performed ChIP-qPCR for H3K27me3 at the WOX11 locus in wild type and 35S::HTR15 explants after CIM incubation, and found that H3K27me3 levels were indeed lower in 35S::HTR15 than in wild type (Fig. 6F). Therefore, these results supported the notion that the deposition of H3.15 at the WOX11 locus is triggered by callus formation, facilitates the removal of H3K27me3 at the WOX11 locus and promotes expression of WOX11 during callus formation. Consistently, a pWOX11::GUS reporter confirmed that WOX11 promoter activity is higher in 35S::HTR15 than wild type during CIM incubation (Fig. S10). Previous studies showed that WOX11 promotes callus formation on leaf explants incubated on CIM (Liu et al., 2014), whereas ectopic expression of LBD16, LBD17, LBD18 and LBD29 resulted in spontaneous callus formation without exogenous phytohormone in Arabidopsis (Fan et al., 2012). Our results suggest that H3.15 promotes callus formation via the WOX11 and LBD-regulated
pathway by directly causing the removal of H3K27me3 and activating the expression of WOX11 and LBD genes.

**DISCUSSION**

H3K27me3 is typically associated with gene repression (Kouzarides, 2007; Zhang et al., 2009; Roudier et al., 2011; Ikeuchi et al., 2015b). Under normal growth conditions, PRC2-mediated H3K27me3 maintains the differentiated states of mature somatic cells and prevents unscheduled cell dedifferentiation by repressing cell fate reprogramming regulators in *Arabidopsis* (Ikeuchi et al., 2015a). During plant regeneration, repression of cell dedifferentiation regulators by H3K27me3 is removed through H3K27 demethylation or other mechanisms that facilitate the activation of cell fate reprogramming and subsequent plant regeneration (Liu et al., 2014; Zhang et al., 2017). Hence, dynamic reprogramming of H3K27me3 is crucial for cell fate transition during plant regeneration. In this study, we reveal how the histone variant H3.15 promotes wound- and auxin-induced callus formation. Overexpression of *HTR15* enhanced the capacity to form callus, whereas its depletion compromised this response. Reporter analyses suggest that H3.15 accumulates in the root pericycle or pericycle-like cells of aerial organs, in which callus formation is initiated (Che et al., 2007; Atta et al., 2009; Sugimoto et al., 2010). H3.15 lacks the K27 residue that carries the repressive mark H3K27me3, and we have shown how H3.15 deposition correlates with a decrease of H3K27me3 in *HTR15* overexpression lines. Further studies are required to profile the global deposition of H3.15 and the mechanism that drives H3.15 replacement. Therefore, based on the data derived from *HTR15* overexpression lines, we propose that H3.15 deposition leads to the replacement of nucleosomes carrying H3K27me1 or H3K27me3 at specific loci by unmarked nucleosomes, resulting in the removal of these modifications and the reprogramming of downstream transcription.

Our study identified WOX11 and LBD18 as direct downstream targets of H3.15. WOX11 is required in the first step of cell fate transition from procambium cells to root founder cells during de novo root organogenesis. Overexpression of *WOX11* results in rapid callus formation on CIM (Liu et al., 2014). WOX11 functions in cooperation with LBD16 to promote cell fate reprogramming of callus formation regulators to promote cell fate reprogramming required for callus formation (Fig. S11). As we have gathered evidence for H3.15 orthologs among dicots, similar mechanisms might work to mediate plant regeneration in other plant species; thus, our findings will probably extend to other species of flowering plants. The model reveals a mechanism whereby plant cells rapidly de-repress key meristem regulators and reprogram cell fate to reacquire pluripotency. In the moss *Physcomitrella*, regeneration involves H3K27me3-dependent trans-de-differentiation of differentiated cells into stem cells without the generation of callus (Ishikawa et al., 2019). Bryophytes encode only CenH3, H3.1 and H3.3 variants (Bowman et al., 2017), and H3.15 probably evolved in flowering plants to facilitate the reprogramming of differentiated cells to initiate callus formation, which is required to regenerate new tissues after partial loss of tissue through injury. It will be of interest to investigate whether H3.15-like variants also contribute to developmentally programmed regeneration and whether H3.15 variants could be harnessed to improve plant cloning for biotechnological purposes.

**MATERIALS AND METHODS**

### Plant materials

All *Arabidopsis* plants used in this study were in the Col-0 background. The reporter line J0121 was described previously (Laplaze et al., 2005). *Arabidopsis* seeds were sterilized with 75% ethanol and 15% bleach, and germinated on half-strength Murashige and Skoog (MS) plates [2.21 g/l MS basal medium with vitamin powder, 0.5 g/l 2-(N-morpholino)ethanesulfonic acid (MES), 10 g/l sucrose and 8 g/l agar (pH 5.7)]. Seven-day-old seedlings were transferred to soil and grown in a growth chamber at 22°C with a 16 h light and 8 h dark photoperiod.

### Plasmid construction

For construction of p35S::*HTR15*, the *HTR15*-coding region was amplified from genomic DNA. After digestion, the fragment was inserted into pGIIK-p35S-LIC-NOS (De Rybel et al., 2011). For generation of the *htr15* mutant, CRISPR/Cas9 technology was used as described previously (Wang et al., 2015). A pair of sgRNA targets (DT1, AGACAGCTGGTAAGGCAAC; DT2, AGATCAACTATCGTCT) in *HTR15* were selected and cloned into the pHHE401E vector. Then the CRISPR construct was transformed into the *Arabidopsis* Col-0 background via Agrobacterium strain GV3101. The homozygous *htr15* mutant was identified by DNA sequencing. For the complementation of *htr15*, a genomic fragment of *HTR15*, which included the promoter region and stop codon, was cloned into pGIIK-LIC-NOS to obtain pHTR15::*HTR15*. This construct was transformed into the homozygous *htr15* mutant. To generate *p35S::HTR15-3×FLAG* construct, a genomic DNA fragment containing the full length of *HTR15* (without stop codon) was cloned into pGIIK-LIC-35S-3FLAG-NOS. The *HTR15-H27K*, *HTR15-N4K* and *HTR15-27K31A* mutations were created by site-directed mutagenesis following a protocol described previously (Liu and Naismith, 2008). All binary vector constructs were introduced into *Agrobacterium* strain GV3101, containing the pGreen helper plasmid pSOUP, and transformed into *Arabidopsis* Col-0 using the floral dip method (Clough and Bent, 1998). Transgenic seedlings were first selected on MS agar plates with appropriate antibiotics and further confirmed by qRT-PCR, histochemical GUS assays or confocal imaging. Primers for plasmid construction are listed in Table S1.

### Callus formation assay

After 3 days at 4°C in the dark, *Arabidopsis* seeds were germinated on half-strength MS plates [2.21 g/l MS basal medium with vitamin powder, 0.5 g/l MES, 10 g/l sucrose, and 8 g/l agar (pH 5.7)]. To induce callus formation by wounding, 7-day-old seedlings were dissected with microscissors at the middle of the hypocotyl (the upper end of hypocotyls and cotyledon were removed). Remaining seedlings were incubated on half-strength MS plates. To induce callus from root or hypocotyl explants, plant roots or etiolated hypocotyls were excised and transferred to auxin-rich CIM (3.21 g/l Gamborg B5 medium with vitamin powder, 20 g/l sucrose, 0.5 g/l MES, 8 g/l phytoagar and 2.2 μM 2,4-D, 0.2 μM kinetin) and incubated at 22°C under long-day conditions. Three independent experiments were performed.

### Gene expression analysis

Total RNA was isolated from *Arabidopsis* hypocotyls at wound sites or from callus, induced by CIM, using the cDNA synthesis Kit (Thermo Scientific, K1639) from 2 μg of total
RNA in a 20 µl reaction. qRT-PCR was performed on an ABI StepOnePlus Real-Time PCR System (Life Technologies) using SYBR green (KAPA SYBR Fast qPCR Kit) according to the manufacturer’s instructions. Relative expression levels were calculated using the ΔΔCt (cycle threshold) method, and PP2AA3 was used as an endogenous reference gene. Three biological replicates with two technical replicates were performed. Primers for qRT-PCR are listed in Table S1.

**GUS staining**

Tissues were prefixed in 90% acetone on ice for 20 min and incubated in GUS staining buffer [50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronicide, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide, and 0.1% (v/v) Triton X-100] at 37°C for 2 h. Stained samples were then cleared of chlorophyll in an ethanol series (35%, 50%, 75% and 90%) followed by clearing in chloral hydrate for several hours and then photographed using a light microscope.

**Confocal imaging**

Samples were first stained with 5 µg/ml of propidium iodide and then rinsed in water to remove excess propidium iodide before slide mounting. All images were taken with a Nikon A1R Laser Scanning Confocal Microscope. For Gfp detection, a 488 nm laser was used for excitation and emission light wavelength was 510 to 550 nm; for propidium iodide detection, excitation light wavelength was 561 nm and emission was 631 to 690 nm.

**EdU staining**

After cutting, the roots of 7-day-old Cot-0 seedlings were transferred onto half-strength MS medium supplemented with 10 µM EdU (Click-iT EdU Imaging Kit, Invitrogen). The roots were then fixed at different time points in 3.7% (v/v) formaldehyde with 1% (v/v) Triton-X 100 in 1×PBS solution for 30 min. EdU staining was performed with the Click-iT EdU Alexa Fluor 647 Imaging Kit following the manufacturer’s instructions. Nuclei were labeled with Hoechst 33342 stain. The samples were imaged using a Nikon A1R Laser Scanning Confocal Microscope. For the detection of EdU staining, the excitation wavelength of 380 nm was used and emission light wavelength was 490 nm. To collect the fluorescence of Hoechst 333 staining, a 405 nm laser was used for excitation and emission light wavelength between 425 and 475 nm was collected.

**Western blot**

The extraction and purification of histones from plants has been described previously (Yan et al., 2007; Mahrez et al., 2016). Briefly, ~100 mg of root explants were homogenized in histone extraction buffer [0.25 M sucrose, 1 mM CaCl2, 15 mM NaCl, 60 mM KCl, 5 mM MgCl2, 15 mM PIPES (pH 7.0), 0.5% Triton X-100, 10 mM sodium butyrate and a protease inhibitor cocktail]. After centrifugation for 20 min at 10,000 g, pellets were resuspended with 0.2 M H2SO4 and incubated overnight at 4°C. After centrifugation for 10 min at 17,000 g, total histones from the supernatant were precipitated with concentrated trichloroacetic acid to a final concentration of 33%. The histone pellet was washed twice with ice-cold acetone and air-dried for 20 min at room temperature. Pellets were then dissolved in double distilled water. All steps were carried out at 4°C or on ice, unless specified. Protein immunoblotting was performed with anti-H3 (Abcam, ab1791, 1:1000), anti-H3K27me3 (Millipore, 07-449, 1:800) or anti-H3K27me1 antibody (Millipore, 07-449, 1:800) for 2 h or overnight at 4°C with mixing. The Protein A/G magnetic beads were then added to the histone solution and collected using a magnetic stand after incubation for 2 h at 4°C with mixing. The beads were then collected and washed three times with IP Wash Buffer 1 and once with IP Wash Buffer 2. The washed beads were then incubated with the elution buffer for 40 min at 65°C. The eluted chromatin and the 10% input control were added with 5 M NaCl and 20 mg/ml Proteinase K, and incubated at 65°C for 2-6 h for reverse crosslinking. DNA was then recovered using a DNA clean-up column and reagents (Pierce Magnetic ChIP Kit, Thermo Scientific), and eluted in 50 µl of elution buffer. Purified DNA (1 µl) was subjected to qRT-PCR. Primer pairs used for the ChIP assays are listed in Table S1.

**ChIP**

ChIP assays were performed as described previously with modifications (Yamaguchi et al., 2014). Hypocotyl explants (300-500 mg) were fixed with 10 ml 1% formaldehyde solution under vacuum infiltration conditions. After adding glycine to a concentration of 125 mM to quench the crosslinker, the fixed samples were washed twice with ice-cold PBS solution and ground to a fine powder in liquid nitrogen. The resultant powder was resuspended in 2.5 ml of ice-cold PBS containing 25 µl of the Halt Cocktail and filtrated through Miracloth (Calbiochem). Labeled by centrifugation the protein bands were quantified by ImageJ and normalized to the loading control. The nuclei mixture was digested with micrococcal nuclease (Pierce Magnetic ChIP Kit, Thermo Scientific) and sonicated with an ultrasonic cell disruptor to break the nuclear membrane. An aliquot of solubilized chromatin (10%) was saved as an input control, and the remainder was incubated with anti-flag antibody (Abcam, ab1162, 1:80) or anti-H3K27me3 antibody (Millipore, 07-449, 1:80) for 2 h or overnight at 4°C with mixing. The Protein A/G magnetic beads were then added to the chromatin solution and collected using a magnetic stand after incubation for 2 h at 4°C with mixing. The beads were then collected and washed three times with IP Wash Buffer 1 and once with IP Wash Buffer 2. The washed beads were then incubated with the elution buffer for 40 min at 65°C. The eluted chromatin and the 10% input control were added with 5 M NaCl and 20 mg/ml Proteinase K, and incubated at 65°C for 2-6 h for reverse crosslinking. DNA was then recovered using a DNA clean-up column and reagents (Pierce Magnetic ChIP Kit, Thermo Scientific), and eluted in 50 µl of elution buffer. Purified DNA (1 µl) was subjected to qRT-PCR. Primer pairs used for the ChIP assays are listed in Table S1.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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