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ORTH/VIM proteins that regulate DNA methylation are functional ubiquitin E3 ligases

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Summary

Appropriate methylation of genomes is essential for gene regulation. Here, we describe the six-member *ORTHRUS* (*ORTH*) gene family of *Arabidopsis thaliana* that plays a role in DNA methylation *in vivo*. *ORTH1*–*ORTH5* are predicted to encode proteins that contain one plant homeodomain (PHD), two really interesting new gene (RING) domains, and one set ring associated (SRA) domain, whereas *ORTHlike-1* encodes a protein with only one RING and SRA domain. cDNAs for ORTH1, ORTH2, ORTH5 and ORTHlike-1 were isolated, and when expressed as glutathione-*S*-transferase (GST) fusion proteins, were capable of promoting ubiquitylation *in vitro* with the E2 AtUBC11. ORTH1 promotes ubiquitylation when paired with additional AtUBC8 family members. ORTH1 proteins with substitutions in metal–ligand binding residues in each ORTH1 RING domain individually, and ORTH1 truncation derivatives lacking one or both RING domains, were tested for their ability to catalyze ubiquitylation *in vitro*. In these assays, either ORTH1 RING domain is capable of promoting ubiquitylation. The PHD alone is not active as an E3 ligase, nor is it required for ligase activity. GFP-ORTH1 and GFP-ORTH2 are nuclear-localized in transgenic Arabidopsis plants. Overexpression of ORTH1 or ORTH2 in Arabidopsis leads to an altered flowering time. Inspection of DNA methylation at *FWA* and *Cen180* repeats revealed hypomethylation when ORTH proteins were overexpressed. Once initiated, a late-flowering phenotype persisted in the absence of the ORTH transgene, consistent with epigenetic effects at *FWA*. We conclude that ORTH proteins are E3 ligases mediating DNA methylation status *in vivo*.

Keywords: RING, E3 ligase, DNA methylation, ubiquitylation, ORTH/VIM, SRA.

Introduction

Cytosine methylation of genomic DNA is an important element in the epigenetic regulation of gene expression (for recent reviews see Gehring and Henikoff, 2007; Henderson and Jacobsen, 2007). In plants, cytosine methylation can occur in CG, CHG or CHH sequence contexts (where H = A, T or C). In Arabidopsis, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) is responsible for *de novo* methylation at all sites, whereas maintenance methylation requires multiple activities. The DNA methyltransferase MET1 is responsible primarily for maintenance of pre-existing CG methylation (Finnegan and Dennis, 1993; Ronemus *et al.*, 1996), whereas maintenance of CHG methylation requires a plant-specific DNA methyltransferase, CHROMOMETHYLASE 3 (CMT3), and also a histone H3 lysine 9 methyltransferase KRYPTONITE/SUVH4 (KYP) (Henderson and Jacobsen, 2007). Small RNAs play a key role in targeting DNA methylation, especially in plants, in a process that is not yet completely understood (Henderson and Jacobsen, 2007; Huettel *et al.*, 2007; Matzke *et al.*, 2007; Wassenegger *et al.*, 1994). Asymmetric methylation of CHH is maintained in a small RNA-dependent *de novo* methylation pathway performed by DRM2 (Henderson and Jacobsen, 2007).

Plants defective in either *de novo* methylation or maintenance methylation exhibit a wide variety of phenotypic alterations. In *met1* mutants, dramatic hypomethylation takes place genome-wide (Zhang *et al.*, 2006). This leads to stochastic phenotypes caused by the aberrant expression of genes silenced by methylation, including a late-flowering phenotype derived from the ectopic expression of *FWA*, altered flower development and decreased fertility (Finnegan *et al.*, 1996; Kankel *et al.*, 2003; Soppe *et al.*, 2000).

Another family of proteins implicated in regulating DNA methylation in Arabidopsis, which is much less understood, is the ORTHRUS/VARIANT IN METHYLATION (ORTH/VIM) protein family (Johnson *et al.*, 2007; Liu *et al.*, 2007; Woo *et al.*, 2007). They share domains with and are likely to be orthologous to the mammalian UHRF (Ubiquitin-like containing, PHD, Ring Finger) proteins (Bronner *et al.*, 2007), which are important for the maintenance of DNA methylation (Bostick *et al.*, 2007; Sharif *et al.*, 2007). ORTH/VIM/UHRF proteins are characterized by the presence of an SRA domain, (SET [Su(var), Enhancer of Zeste, Trithorax] and RING [Really Interesting New Gene] Associated), a PHD and a RING domain. The SRA domain, also known as the YDG domain (for three highly conserved amino acids), as the name suggests, usually occurs in conjunction with two different types of proteins: SET domain proteins, or the UHRF/ORTH/VIM proteins with one or two RING domains. The SET domain generally performs the catalytic histone methyltransferase activity of the Arabidopsis SRA-SET proteins, such as KYP (Baumbusch *et al.*, 2001). The SRA-SET proteins are unique to the plant kingdom, whereas the SRA-RING proteins, UHRF/ORTH/VIM, are found in many species with cytosine methylation, such as mammals, zebrafish, honeybees and plants.

The SRA domain has been reported as a methylated DNA binding domain (Johnson *et al.*, 2007; Unoki *et al.*, 2004; Woo *et al.*, 2007). Additionally, the UHRF1 SRA domain has a strong preference for hemimethylated CG (Bostick *et al.*, 2007). However, histone binding has also been ascribed to the SRA domain. The SRA domain of the murine UHRF1 binds H1, H2B and H3, and ubiquitylates histones *in vitro* (Citterio *et al.*, 2004). The human UHRF1 selectively binds to methylated H3K9 peptides, and this activity is dependent on both the PHD and SRA domains (Karagianni *et al.*, 2008). ORTH2/VIM1 has also been shown to associate with histones H2B, H3 and H4, and the centromere H3 histone variant HTR12, *in vitro* (Woo *et al.*, 2007), although the domain responsible is not known.

The RING domain is an approximately 40–60 amino acid region with an octet of histidine/cysteine residues spaced to chelate two zinc atoms (Borden and Freemont, 1996). This structure binds the E2 carrying activated ubiquitin and facilitates ubiquitin transfer to substrate proteins (Lorick *et al.*, 1999). Curiously, only a few, out of the more than 400 predicted, Arabidopsis RING proteins, in addition to the ORTH/VIM proteins, contain multiple RING domains (Stone *et al.*, 2005). PRT1 (PROTEOLYSIS1) has two N-terminal RING domains, and participates in the proteolysis of a subset of ubiquitin pathway substrates bearing specific N-terminal amino acids (Stary *et al.*, 2003). It remains to be determined whether the two RING domains can function independently in supporting ubiquitylation. Multiple members of the Arabidopsis ARIADNE family of RING proteins, like their animal counterparts, contain two RING or RING-like domains separated by a conserved region, designated IBR for 'in between RING' (Mladek *et al.*, 2003). Mutations in either RING domain results in lethality in Drosophila (Aguilera *et al.*, 2000); however, no clear evidence exists to suggest the second RING domain is independently active in ubiquitylation.

ORTH/VIM proteins also contain a PHD, which consists of an octet of histidine/cysteine residues, reminiscent of the RING domain (Aasland *et al.*, 1995; Borden and Freemont, 1996; Schindler *et al.*, 1993). Searches for physiological substrates for the PHD have demonstrated binding to substrates as diverse as phosphoinositols (Gozani *et al.*, 2003) and the trimethyl lysine 4 of histone H3 (Li *et al.*, 2006; Pena *et al.*, 2006; Shi *et al.*, 2006; Wysocka *et al.*, 2006). It has also been suggested that PHD can confer E3 ligase activity to proteins, although those proteins were either determined to be RING domains or the assays were not reproducible (Goto *et al.*, 2003; Lu *et al.*, 2002; Yonashiro *et al.*, 2006).

Previous studies have yielded some insight into the biological function of ORTH/VIM proteins. A screen for natural variation in cytosine methylation in Arabidopsis revealed a role for *ORTH2/VIM1* in centromeric repeat methylation (Liu *et al.*, 2007; Woo *et al.*, 2007). Additionally, the amino terminus of ORTH2/VIM1 containing the PHD binds to NtSET1 in a yeast two hybrid experiment (Liu *et al.*, 2007). Overexpression of GFP-tagged VIM1 also led to a late-flowering phenotype with increased levels of *FLC* (Liu *et al.*, 2007).

Substantial work on the role of the vertebrate ORTH/VIM homolog, UHRF1, has revealed roles in cell-cycle regulation (Bonapace *et al.*, 2002; Jeanblanc *et al.*, 2005), cellular transformation (Jenkins *et al.*, 2005; Mousli *et al.*, 2003), DNA damage repair (Muto *et al.*, 2002), replication of pericentromeric heterochromatin (Papait *et al.*, 2007), liver regeneration (Sadler *et al.*, 2007) and recruiting DNMT1 to replication foci (Bostick *et al.*, 2007; Sharif *et al.*, 2007). The mechanism of many of these processes has not been thoroughly investigated, and further studies of the domains of UHRF1 and its Arabidopsis counterparts are essential.

Herein we report on additional characterization of the Arabidopsis ORTH/VIM family of proteins. We demonstrate that ORTH proteins possess E3 ligase activity, requiring either, but not both, RING domains for E3 activity with AtUBC8 family members *in vitro*. The PHD did not

contribute to *in vitro* E3 ligase activity. As reported previously (Liu *et al.*, 2007), ORTH1/2 overexpressing lines flower later. We further explored the molecular mechanism for this alteration, and demonstrate decreased methylation at *FWA* as the likely cause for delayed flowering. *ORTH1/2* overexpression also reduces methylation at *Cen180* repeats. These data support the role of the ORTH family as E3 ligases that play a role in controlling epigenetic mechanisms at both heterochromatic and euchromatic loci in plants.

Results

ORTH domains and phylogenetic analyses

Inspection of the Arabidopsis predicted proteome for multiple RING domain-containing proteins identified five members of the ORTHRUS/VIM family and a related protein ORTH-LIKE1 (ORL1/VIM6). ORTH proteins contain an N-terminal PHD and two RING domains flanking an SRA domain (Figure 1a, upper). ORL1/VIM6 is missing the N-terminal PHD and C-terminal RING domain, but contains the SRA and N-terminal RING domains. Proteins with both SRA and RING domains are referred to here as SRA-RING proteins. Mammalian SRA-RING proteins have an altered domain organization compared with plant ORTH proteins, with an N-terminal ubiquitin-like domain and an SRA domain that is flanked N-terminally by a PHD and C-terminally by a RING domain (Figure 1a, lower).

Additional Arabidopsis proteins contain an SRA domain without a PHD or RING domain. These include ones with a SET domain (referred to here as SRA-SET proteins), as well as those without any other described protein–protein interaction domain (SRA-only). The SRA domains of all Arabidopsis SRA-RING family members were compared with selected SRA-RING proteins from other species, and with the other Arabidopsis SRA-SET and SRA-only proteins (Figure 1b). Phylogenetic analysis using the entire protein results in the same conclusions (Figure S1 and data not shown).

Within the AtORTH family, ORTH1, ORTH4 and ORTH5 form a clade (Figure 1a). ORTH1 has 95% similarity to ORTH4 and 91% amino acid identity to ORTH5 across the entire protein, and ORTH4 and ORTH5 have overall 99% amino acid and 99% nucleotide identity. In contrast, ORTH1 is 70% and 62% identical to ORTH2 and ORTH3, respectively, and ORTH2 and ORTH3 have 73% amino acid identity. Interestingly, ORTH2 and ORTH3 (At1g57820 and At1g57800, respectively) are separated by only one predicted pseudogene, and ORTH4 and ORTH5 (At1g66040 and At1g66050, respectively) are similarly separated one predicted open reading frame apart, suggesting that duplication events gave rise to the ORTH2/ORTH3 and ORTH4/ ORTH5 pairs.

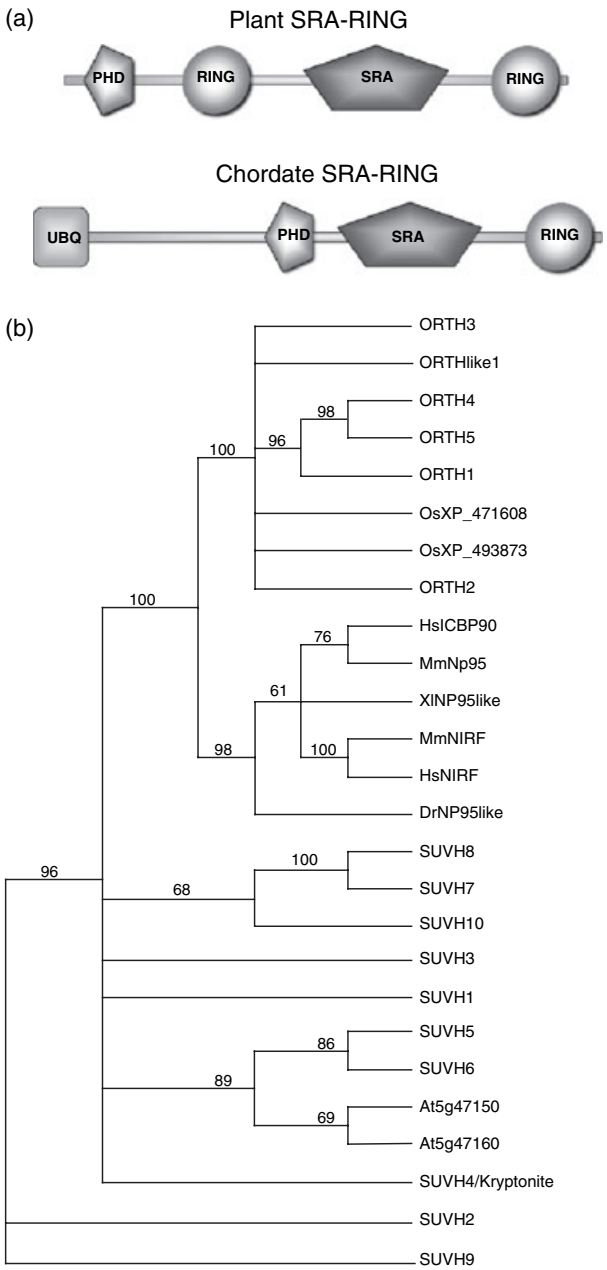

Figure 1. ORTH domains and phylogenetic analysis.
(a) Domain organization of plant SRA-RING (top) and chordate SRA-RING (bottom) proteins.
(b) Phylogenetic tree of the SRA domain-containing proteins. The tree is based on the SRA domain only. Included in the analysis are all SRA domain-containing proteins of Arabidopsis, and select SRA-RING proteins of other organisms. Designations are ORTH1/VIM3 (At5g39550), ORTH2/VIM1 (At1g57820), ORTH3/VIM5 (At1g57800), ORTH4/VIM4 (At1g66040), ORTH5/VIM2 (At1g66050) and ORL/VIM6 (At4g08590). Arabidopsis SRA-domain-only proteins are listed by their AGI code (At5g47150, At5g47160). Arabidopsis SRA-SET domain-containing proteins are listed as previously designated with the abbreviation SUVH (Baumbusch *et al.*, 2001). Rice SRA-RINGs are listed by NCBI locus code. NCBI locus codes for other organisms are as follows: HsICBP90 (AAF28469), MmNP95 (AAK55743), XlNP95like (AAH72079), MmNIRF (AAH60241), HsNIRF(NP_690856) and DrNP95like (AAT68031). Abbreviations: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*).

Comparisons of the SRA domains reveal that the SRA-RING proteins of Arabidopsis have greater similarity to SRA-RING proteins from chordates, than they do to Arabidopsis SRA-SET proteins. This implies that SRA-RING proteins were present in the common ancestor of plants and animals. In contrast, the plant-specific Arabidopsis SRA-SET proteins are present in a clade with the SRA-only proteins, suggesting that they are more closely related to each other than to the SRA-RING proteins.

To determine which genes are expressed, effort was expended to isolated cDNAs for all six members. cDNAs were isolated for *ORTH1*, *ORTH2*, *ORTH5* and *ORL1* using RT-PCR. Attempts to isolate cDNAs for *ORTH3* and *ORTH4* failed using TAIR (http://www.arabidopsis.org) annotations for their predicted open reading frames. Thus, the expression of *ORTH3* and *ORTH4* is uncertain. The 99% nucleotide identity between *ORTH4* and *ORTH5* coding regions makes expressed sequence tag (EST)/cDNA gene assignments challenging. Two *ORTH4* ESTs/cDNAs are listed on TAIR (1/08), but their sequences (GenBank EL177826 and EL265513) are 100% identical to both *ORTH4* and *ORTH5*, and the clones are not available for further sequencing, so their assignment to a single locus cannot be made. The cDNA sequences that are ascribed to *ORTH5* have few *ORTH5*-specific nucleotides, indicating that these are annotated correctly, and that there is no *ORTH4* cDNA in that set. Only a single EST is listed for *ORTH3*, and this assignment appears to be correct. Thus, there is no evidence for expression of *ORTH4*, and there is scant evidence for *ORTH3* expression. These data suggest that only a subset of ORTHs significantly contribute to ORTH activity *in vivo*.

ORTH family members are active as E3 Ubiquitin ligases

The RING domain is associated with the ability to function in ubiquitin transfer (Borden, 2000; Kraft *et al.*, 2005; Lorick *et al.*, 1999; Stone *et al.*, 2005), although not all RING proteins demonstrate *in vitro* E3 ligase activity. The murine SRA-RING domain-containing protein mUHRF1 is active in *in vitro* ubiquitylation assays with the E2 UBCH5B (Citterio *et al.*, 2004). To determine if ORTH proteins function as E3 ligases, full-length ORTH1, ORTH2, ORTH5 and ORL proteins were expressed in *Escherichia coli* as glutathione-*S*-transferase (GST) fusions, purified on glutathione beads and tested for the ability to promote ubiquitylation *in vitro*, as previously described (Kraft *et al.*, 2005; Stone *et al.*, 2005). All three ORTH proteins were active with the Arabidopsis E2 UBC11 (Figure 2a) and as expected, omission of E1, E2 or ORTH protein eliminates the attachment of ubiquitin to higher molecular weight proteins. Similarly, ORL1, which lacks the C-terminal RING and N-terminal PHD, was also active (Figure 2a).

We asked whether ORTH proteins require a specific E2 or E2s for activity. UBC11 is a member of the UBC8 family of Arabidopsis E2s (Kraft *et al.*, 2005). ORTH1 was tested with additional E2s of the UBC8 family (UBC8, UBC10, UBC28, UBC29 and UBC30), as well as with members of three other E2 subgroups (UBC27, UBC32, UBC34 and UBC36) (Kraft *et al.*, 2005). ORTH1 shows robust activity with UBC8 family members UBC8, UBC10 and UBC11 (Figure 2b). ORTH1 also shows activity, albeit diminished, with other more divergent UBC8 family members UBC28 and UBC29 (Figure 2b). ORTH1 was not active with the other E2s tested; UBC34 (Figure 2b) and UBC27, UBC30,

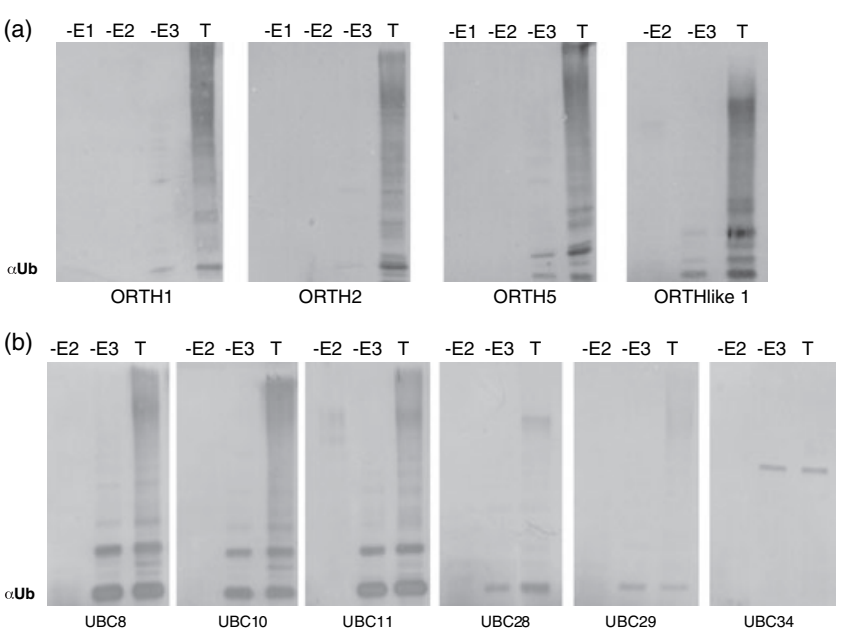

Figure 2. ORTH family members possess E3 ligase activity.
(a–b) *In vitro* ubiquitylation assays. The complete reaction (T) contains E1, 6HIS-E2 and GST-tagged E3, ubiqutin and ATP. The reaction was fractionated by SDS-PAGE, the proteins transferred to a membrane and the products visualized with anti-ubiquitin antibodies. Free ubiquitin has migrated off of the bottom of the gel. Anti-ubiquitin bands visualized in the –E3 lane correspond to E2-Ub adducts. Activity was determined by comparing –E3 reactions to complete reactions for the presence of high molecular weight proteins immunoreactive with anti-ubiquitin antibody (Kraft *et al.*, 2005; Stone *et al.*, 2005). The experiments were repeated at least twice with similar results.
(a) GST-ORTH proteins were tested with the E2 UBC11.
(b) GST-ORTH1 was tested with different E2s to determine E2–E3 specificity. The E2 used is listed below its corresponding western blot.

UBC32 and UBC36 (data not shown). Thus, recombinant GST-ORTH1 is most active *in vitro* with E2s from the UBC8 subfamily.

A single RING domain is sufficient for robust in vitro *ubiquitylation activity*

To determine the domain associated with the E3 ligase activity of ORTH1, codons for conserved metal–ligand residues in the PHD and each RING domain were individually targeted for mutagenesis. These residues have been shown for many RING proteins to be essential for ligase activity (Lorick *et al.*, 1999; Stone *et al.*, 2005). Codons for the PHD metal–ligand binding positions 3–6, for the N-terminal RING metal–ligand binding positions 3–6 or for the C-terminal RING metal-ligand binding positions 4 and 5 were mutagenized to encode alanine, creating proteins called mPD for mutant PHD, mNR for mutant N-terminal RING and mCR for mutant C-terminal RING (Figure 3a). When these GST-ORTH1 fusion proteins were tested for ubiquitylation activity with UBC8, all three retained robust activity in end-point assays (Figure 3b). Time course experiments to detect differences in ubiquitylation rates showed that activity of each mutant protein was largely unaffected relative to wild-type GST-ORTH1 (Figure 3c), indicating that alteration of one RING domain or the PHD does not dramatically interfere with the *in vitro* ubiquitylation activity of ORTH1.

To further characterize the RING domain responsible for E3 ligase activity of ORTH1, the open reading frame was truncated to express GST-ORTH1 proteins lacking one or more domains; lacking either the C-terminal RING only (C-Trunc; Figure 4b), the C-terminal RING and SRA domains (P/NR; Figure 4c) or the PHD and N-terminal RING domains (SRA/CR; Figure 4d). These truncations were assayed with UBC11 in *in vitro* ubiquitylation assays, and were compared with the activity of the full-length protein (Figure 4a). All truncated proteins exhibited E3 ligase activity with UBC11, demonstrating that either RING domain can provide E3 ligase activity *in vitro*. The autoubiquitylation of these proteins can be seen in the anti-GST blots, visualized as slowed migration in the complete reactions only, resulting from covalent addition of ubiquitin to the GST-ORTH fusion protein (Figure 4, below each activity blot).

The contribution of the PHD to ubiquitylation activity was additionally tested by comparing the reactions of a truncated ORTH1 protein with only the PHD to that of the identical protein, except with substitutions in four of the eight metal–ligand binding residues in the PHD (mPHD; Figure 4e). In these reactions, there were faint higher molecular weight anti-ubiquitin immunoreactive bands, but the profile was identical between reactions with or without the ORTH-PHD (–E3 lanes) and reactions containing either ORTH-PHD or

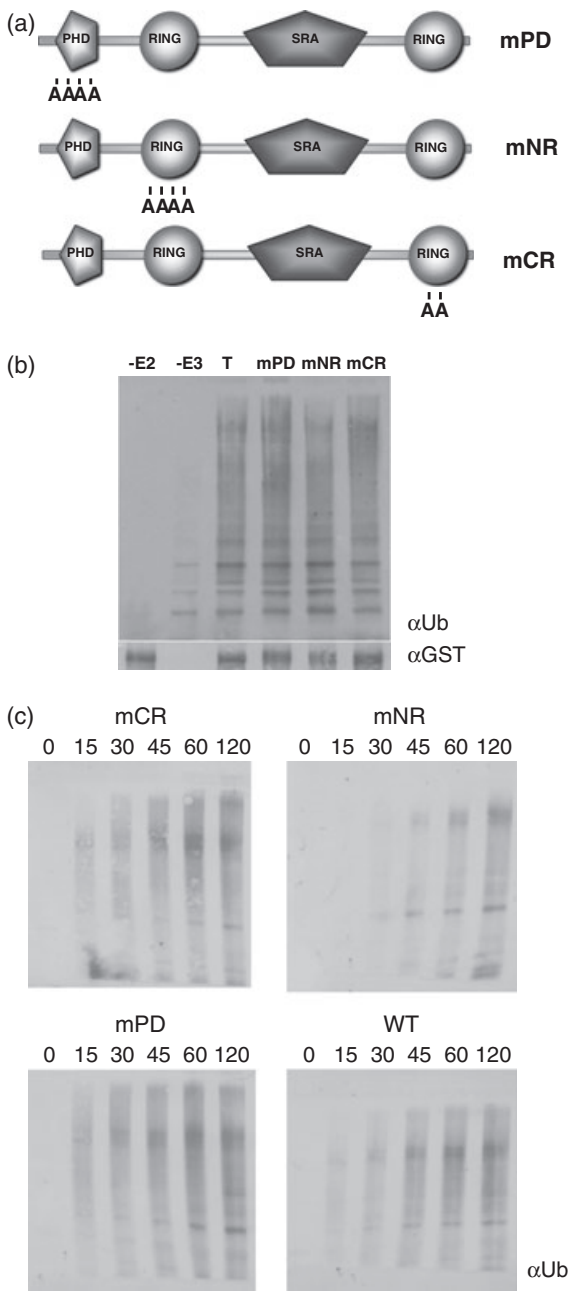

Figure 3. Substitutions in a single zinc-chelating domain do not affect ubiquitylation activity of full-length ORTH1 *in vitro*.
(a) Schematic of the ORTH1 protein and the amino acid changes introduced. Codons for the internal CHCC of the ORTH1 proteins mPD or mNR, or the internal HC of mCR, were altered to encode for alanine.
(b) Single-domain mutations have no effect on ubiquitylation in end-point assays. Glutathione-*S*-transferase (GST)-tagged ORTH1 and mutant proteins were tested for ubiquitylation activity, as described in Figure 1, to determine the contribution of each domain. Ubiquitylated proteins were visualized by anti-ubiquitin western blots. Anti-GST western blots (below) illustrate the equal input of the E3 ORTH1 at time zero.
(c) Time course of ubiquitylation activity of wild-type ORTH1 (wild type, WT, bottom left) and mutant ORTH1 proteins (designated as in panel a). Aliquots of assays were removed at the times indicated (in min) and were stopped with SDS.

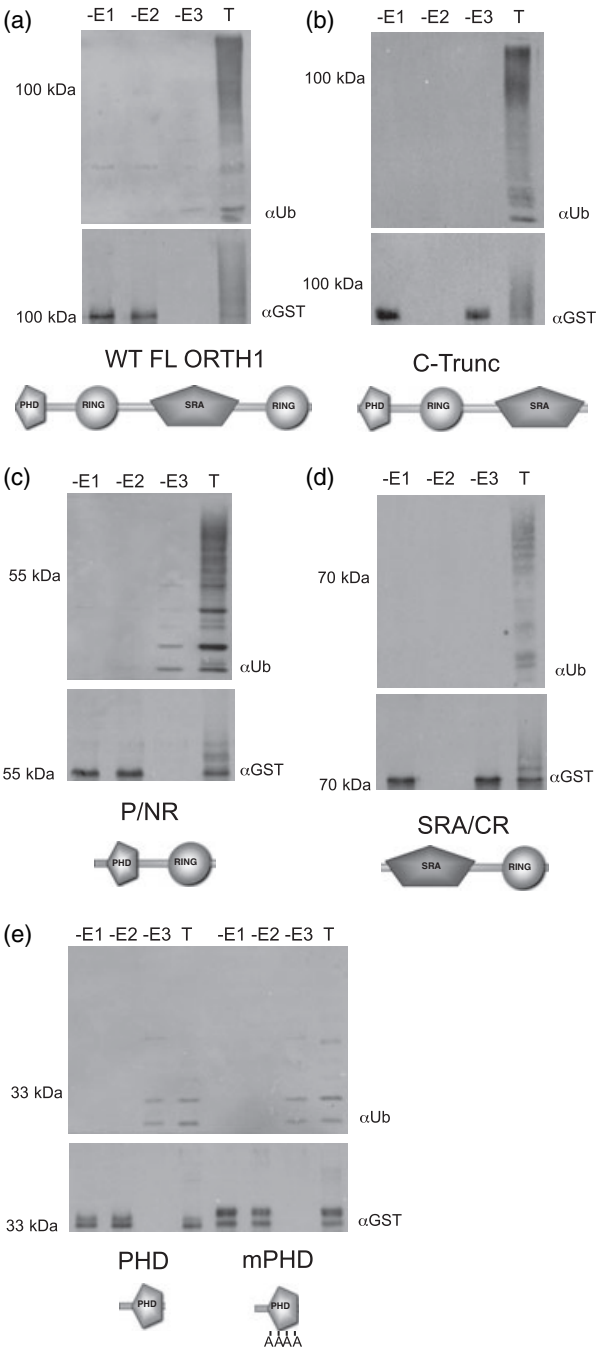

Figure 4. ORTH1 proteins with either RING domain, but not the PHD, have E3 ligase activity.
Truncations of ORTH1 (name and schematic form under their respective western blots) were expressed as glutathione-*S*-transferase (GST) fusions, and were tested for *in vitro* ubiquitylation activity.
(a–e) Top, anti-ubiquitin immunoblot to detect ubiquitylated products; bottom, anti-GST to visualize E3 ligase. Reactions lacking either E1, E2 or E3 (–E1, –E2, –E3, respectively) are compared with comparable reactions with all components (T).
(e) mPHD has the CCHC of the core metal ligands, positions 3–6, all changed to alanine. Full-length ORTH1 assays (a) were included for comparison.

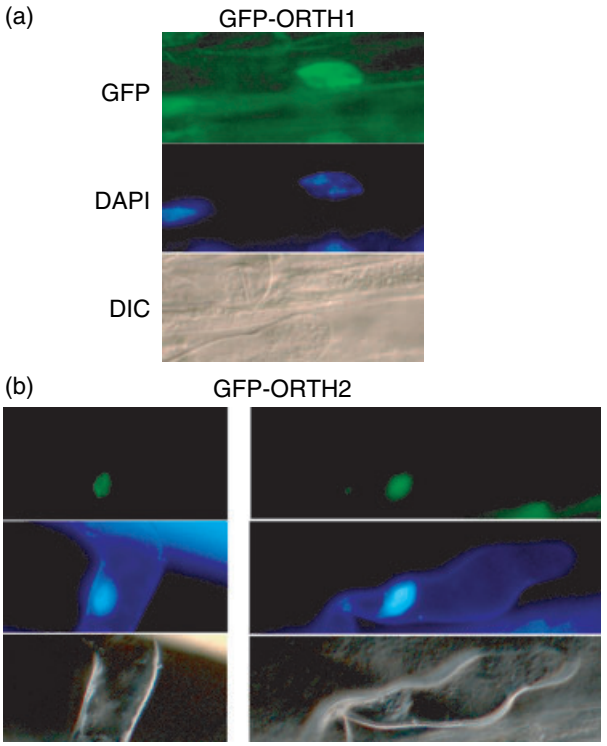

Figure 5. ORTH1 and ORTH2 are localized to the nucleus.
Confocal analysis shows GFP-ORTH1 (a) and GFP-ORTH2 (b) are localized to nuclei (top panels). Corresponding 4′,6-diamidino-2-phenylindole (DAPI; middle panels) and differential interference contrast (DIC) images (bottom panels) are included to show the position of nuclei and the cellular organization/outline.

ORTH-mPHD (T lanes). There does not appear to be any ubiquitylation activity associated with the closely related PHD, at least in these *in vitro* assays.

GFP-ORTH1 and GFP-ORTH2 are localized to the nucleus

To determine the intracellular localization of ORTH proteins, constructs for expression of ORTH1 and ORTH2 in fusion with GFP were made and stably transformed into Arabidopsis. GFP was visualized by fluorescence microscopy and compared with 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining. Both GFP-ORTH2 and GFP-ORTH1 co-localized with DAPI-staining nuclei (Figure 5a,b, respectively). Thus, ORTH1 and ORTH2 are located primarily in nuclei.

Overexpression of ORTH1 *or* ORTH2 *affects DNA methylation-mediated gene silencing*

While producing plants for intracellular localization studies, we observed that GFP-ORTH1 and GFP-ORTH2 expressing transgenic plants exhibited delayed flowering, whereas GFP-ORL plants did not (data not shown and Figure S2). Similarly, Liu *et al.* (2007) recently reported that expression

of GFP-ORTH2/VIM1 under the control of a constitutive promoter leads to a mild to moderate late flowering phenotype, whereas expression of GFP-ORL/VIM6 does not. To further explore the molecular basis of this effect, multiple T_1 plants were analyzed for *FWA* expression. *FWA* is a homeodomain transcription factor completely silenced by DNA methylation in all cells of wild-type plants, except in the developing endosperm. Hypomethylation of the *FWA* promoter leads to ectopic expression, causing a late-flowering phenotype (Soppe *et al.*, 2000). Using RNA extracted from leaf tissue, no expression of *FWA* is observed in wild-type plants, whereas varying levels of expression are seen in almost all independent T_1s transformed with either *GFP-ORTH1* or *GFP-ORTH2* by real-time PCR of cDNA relative to *ACTIN* (Figure 6a). Further analysis by Southern blotting confirmed hypomethylation at the *FWA* promoter. Increased digestion of genomic DNA by the methylation-sensitive enzyme *CfoI* was present in all GFP-ORTH1- and GFP-ORTH2-expressing lines, compared with the negative control, untransformed Col ecotype (Figure 6b, upper panel).

A correlation is observed between *FWA* expression levels and *FWA* methylation levels throughout the independent T_1s for both GFP-ORTH1 and GFP-ORTH2. Higher *FWA* expression is generally coincident with decreased methylation (GFP-ORTH2 #16), and lower *FWA* expression is coincident with a more wild-type level of methylation (GFP-ORTH2 #10). Noticeable late-flowering phenotypes are only observed in lines with the highest expression of *FWA* (GFP-ORTH2, #12, #15 and #16, data not shown).

To test if the late-flowering phenotype was the function of a dominant negative form of ORTH protein caused by the GFP tag, an expression construct for untagged-ORTH1 was also introduced into Col under the control of the *35S* promoter. Flowering time, expressed as the leaves present prior to transition to flowering, was measured in segregating T_2 seedlings derived from independent T_1s, and five of the 10 lines had an average leaf number higher than wild type (Figure S3a). This late-flowering phenotype was also associated with decreased methylation at the *FWA* locus (Figure S3b, top panel). Therefore, plants overexpressing either ORTH1/VIM3 or ORTH2/VIM1 exhibit a late-flowering phenotype that is likely to be caused by the ectopic expression of a hypomethylated *FWA* locus.

Loss of *ORTH2/VIM1* leads to decreased methylation at the heterochromatic *Cen180* repeats (Woo *et al.*, 2007). To test if overexpression of ORTH family members affects methylation at the *Cen180* repeats, in addition to the *FWA* locus, *Cen180* Southern blots were performed on similarly digested genomic DNA from wild-type Columbia and transgenic lines. Decreased *Cen180* DNA methylation was observed in GFP-ORTH1, GFP-ORTH2 and untagged-ORTH1 lines (Figure 6b and Figure S3b; lower panels).

Finally, to test if *FWA* hypomethylation was stable in subsequent generations, T_2 plants from two GFP-ORTH1 (#2, #3) and GFP-ORTH2 (#8, #16) lines were planted. The flowering time was measured, and plants were genotyped for the presence of the ORTH transgene. As expected, the average flowering time of all T_2 lines was delayed relative to wild-type Columbia plants (Figure 6c). In addition, the presence or absence of the GFP-ORTH expressing transgene

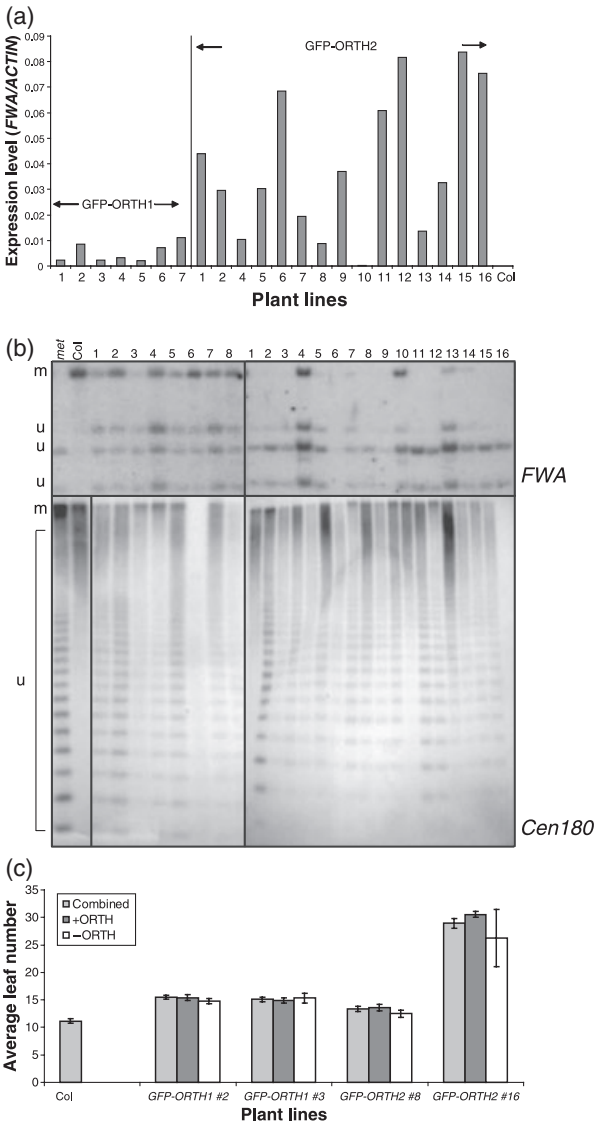

Figure 6. The late-flowering phenotype of *GFP-ORTH1* and *GFP-ORTH2* plants is caused by decreased DNA methylation.
(a) Relative *FWA* levels from seven *GFP-ORTH1* and sixteen *GFP-ORTH2* independent T_1 plants, compared with Col plants.
(b) Southern blots for *FWA* (upper) and *Cen180* bp repeats (lower) with DNA from eight *GFP-ORTH1* and fifteen *GFP-ORTH2* independent T_1 plants. Col DNA is fully methylated (m), whereas *met1-3* (*met*) DNA is severely unmethylated (u).
(c) Flowering time analysis for the T_2 generation of two independent GFP-ORTH1 (#2, #3) and GFP-ORTH2 (#8, #16) lines. The number of leaves produced before the transition from vegetative tissue was counted in at least 26 plants from each line. The plants were then genotyped for the presence (+ORTH) or absence (–ORTH) of the transgene.

did not affect the flowering time (Figure 6c). This shows that ORTH overexpression in the T_1 generation leads to a stable and heritable late-flowering phenotype.

Discussion

The Arabidopsis ORTH/VIM family contains six family members; however, ORL1/VIM6 lacks the N-terminal PHD and C-terminal RING domain present in the other five family members. In addition, ORL1 overexpression does not have the same effect as overexpression of ORTH1 or ORTH2, suggesting that ORL1 may have a unique function (this work and Liu *et al.*, 2007). Of the ORTH1–ORTH5 proteins, only three may contribute significant activity *in vivo*, because *ORTH3/VIM5* and *ORTH4/VIM4* appear to have very low or limited expression. Information on their expression cannot be determined from available microarray studies. *ORTH3/VIM5* sequences are not on the most commonly used expression array (ATH1), and *ORTH4* and *ORTH5* are 99% identical in the coding region, and cannot be distinguished on the current arrays. Among the expressed ORTH/VIM proteins, there are differences in expression patterns. Using *in situ* hybridization, Liu *et al.* (2007) detected RNA in maturing or mature embryos from *ORTH2/VIM1* and *ORL1/VIM6*, but not from *ORTH5/VIM2*. Thus, only a subset of the ORTH proteins is likely to be present at significant levels *in vivo* at any given time.

Previous work in many laboratories has demonstrated that the presence of a RING domain confers E3 ligase activity (Kraft *et al.*, 2005; Lorick *et al.*, 1999; Stone *et al.*, 2005). Consistent with these reports, we show here that ORTH1, ORTH2, ORTH5 and ORL1 show a clear ability to function as E3 ligases *in vitro.* The related mammalian SRA-RING proteins, mouse UHRF1/Np95 and human UHRF2/NIRF, were active *in vitro* with the human E2s, UbcH5b (Citterio *et al.*, 2004) and UbcH5a, respectively, and UHRF2 was not active with UbcH7 (Mori *et al.*, 2004). The UBC8 family of Arabidopsis is more similar to the human UbcH5 E2s than any other Arabidopsis E2 family (Kraft *et al.*, 2005), and thus the E2–E3 pairing of UHRFs and AtORTH family members appears to be shared.

ORTH1–ORTH5 are distinguished from most other Arabidopsis RING proteins, and from the chordate SRA-RING proteins, in containing two RING domains. Activity assays with truncated ORTH1 proteins lacking one or more domains, or with full-length ORTH1 proteins with amino acid substitutions in one RING domain, showed that either RING domain alone is functional in the transfer of ubiquitin. However, it is still possible that both RING domains function synergistically in the transfer of ubiquitin to the endogenous target(s), or that one RING domain has a novel function *in vivo*.

mUHRF1/Np95 interacts with and ubiquitylates core histones *in vitro* and *ex vivo*, with a preference for H3 (Citterio *et al.*, 2004). UHRF2/NIRF interacts with and ubiquitylates PCNP (PEST-containing nuclear protein), a protein of unknown function, *in vitro*, and enhances its ubiquitylation *in vivo* (Mori *et al.*, 2004). Arabidopsis ORTH2/VIM1 expressed in plants interacted with the recombinant histones H2B, H3 and H4, and a centromere-localized H3, HTR12 (Woo *et al.*, 2007), although a direct interaction was not demonstrated, as another protein in complex with ORTH2/VIM1 could be binding to the histones. In our hands, GST-ORTH1 was able to ubiquitylate histone H3 *in vitro*; however, another Arabidopsis non-ORTH RING E3 also shares this ability (data not shown). Thus, we could not determine whether ORTH proteins specifically ubiquitylate histone H3 in our assays. ORTH2/VIM1 interacts with tobacco SET1, but whether SET1 is an ORTH substrate was not tested (Liu *et al.*, 2007). Clearly, further analysis to determine additional interacting proteins or characterize ORTH-containing complexes will allow testing for ORTH-mediated ubiquitylation.

The SRA domain of SRA-RING and SRA-SET proteins has been shown to be a methylated DNA binding domain (Johnson *et al.*, 2007; Unoki *et al.*, 2004; Woo *et al.*, 2007). The presence of this domain in a proteome correlates with the presence of methylated DNA in a genome. For example, SRA domain-containing proteins are not predicted from the annotated genomes of *Drosophila melanogaster*, *Caenorhabditis elegans*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* by BLAST and SMART searches, and CpG methylation has not been reliably detected in their DNA. The SET domain associated with the SRA domain is recognized to be required for protein methyltransferase activity, most notably, but not exclusively, onto histone lysyl ϵ-amino groups (Marmorstein, 2003; Ng *et al.*, 2007; Trievel *et al.*, 2002). Those organisms lacking SRA domains contain SET domain proteins, as well as methylated histones. *C. elegans* protein Maternal-effect sterile protein 4 (MES-4) and *D. melanogaster* protein Mes4 have either/both a PHD and/or RING domain(s), and the N-terminal MES-4 PHD is required for chromosome association (Bender *et al.*, 2006). A SET domain in association with a RING and/or PHD without an SRA domain is also observed in human and mouse proteins, but not in plants. Thus, it appears that the SRA domain is specific to organisms with cytosine methylation, whereas SET domains are more widespread.

The ORTH PHD fits the PHD consensus (SMART, $e = 1.77e^{-05}$), including the spacing limitations, nature of the metal ligands and the characteristic tryptophan residue one residue N-terminal to the seventh metal ligand. Here, we present *in vitro* data to support the hypothesis that the PHD does not function as an E3 ligase. However, it remains to be determined if the PHD can function as an E3 *in vivo*, or possesses E3 ligase activity with an untested E2. Recently, Liu *et al.* (2007) reported that ORTH2/VIM1 PHD was required and sufficient for interaction with NtSET in yeast two-hybrid assays. Thus, the ORTH/VIM PHD may be a protein interaction domain.

It is interesting that both loss of *ORTH2/VIM1* (Woo *et al.*, 2007) and expression of *35S::ORTH1* or *35S::GFP-ORTH2* lead to a loss of DNA methylation. A similar phenomenon was seen with *GL2* (*GLABRA2*) expression, where *35S::GL2* had the same phenotype as a loss-of-function line (Ohashi *et al.*, 2002). The mechanism responsible is not understood. One possibility is that there are decreased rather than increased *ORTH* mRNA levels, caused by co-suppression in the 35S expression lines. However, endogenous *ORTH1* mRNA levels were not substantially changed in the GFP-ORTH1 lines tested, thereby eliminating this as a mechanism. Another possibility is that overexpression leads to a dominant-negative effect, for example, by sequestering other proteins. The large GFP tag is not responsible for the dominant-negative effect, because expression of untagged ORTH1 also results in a late-flowering phenotype, but overexpression of untagged and tagged ORTH proteins could reduce the free levels of other proteins. Finally, expression of hUHRF1 is regulated through the cell cycle, with the highest expression just before the S phase (Miura *et al.*, 2001). It is possible that the phenotype is derived from *35S*-driven expression throughout the cell cycle.

In this study, overexpression of either *ORTH1* or *ORTH2* phenocopy the *vim1* mutant at the *Cen180* repeats, implicating both proteins in the same pathway. However, the *35S::ORTH* lines have a more severe phenotype than *vim1*, as these lines lose CpG methylation additionally at *FWA*, as well as at the heterochromatic *Cen180* repeats. This result implicates the ORTH family members in activities outside of centromeric DNA methylation maintenance. In addition to affecting flowering time, overexpression of ORTH2/VIM1 affects root growth (Liu *et al.*, 2007).

Although the target for ubiquitylation by the ORTH/VIM family of proteins remains uncertain, it is clear that this family has an important role in DNA methylation and subsequent gene regulation. Whereas single insertion lines have no obvious macroscopic phenotype (data not shown and Liu *et al.*, 2007; Woo *et al.*, 2007), T-DNA insertions in *ORTH2/VIM1* have decreased methylation at centromeric repeats, and the *vim1-2* allele exhibited increased centromeric decondensation by fluorescence *in situ* hybridization (FISH) (Woo *et al.*, 2007). The generation of multiple knockout lines in ORTH family members will be required to further elucidate ORTH function *in vivo*. Additional work to understand the intricacies of how ORTHs regulate DNA modifications will not only prove valuable in plants, but will also aid in the understanding of the orthologous chordate proteins.

Experimental procedures

Identification of Arabidopsis ORTH proteins

The RING domain of Arabidopsis CIP8 was used in BLAST searches against the complete non-redundant Arabidopsis genome (TAIR, http://www.arabidopsis.org; also see Stone *et al.*, 2005). BLAST searches identified five proteins each with two RING domains, a PHD, and an SRA domain, as well as one protein with only a single RING and SRA domain. The SMART database was used to analyze retrieved sequences (Simple Modular Architecture Research Tool, version 4.0, http://smart.embl-heidelberg.de) followed by manual inspection to confirm the presence of the complete PHD/RING/SRA domains.

Phylogenetic analysis

The ClustalX program was used to generate an alignment of the ORTH protein sequence. The alignment was generated using a PAM350 protein matrix, with gap opening and gap extension penalty parameters of 35.0 and 0.75, respectively, in pairwise alignment, and 15.0 and 0.3, respectively, in the multiple alignments (Thompson *et al.*, 1997). The MacClade sequence editor (Sinauer Associates Inc., http://www.sinauer.com) was used to manually edit the alignment. The phylogenetic trees were created by PAUP* 4.0 (Sinauer Associates, Inc.) using the neighbor-joining method with 1000 bootstrap replicates.

Cloning and mutagenesis

Arabidopsis ORTH cDNAs were cloned by reverse transcription (RT) reactions, followed by PCR to amplify the predicted open reading frame (ORF) and recombined into Gateway pDONR vector (Invitrogen, Carlsbad, CA, USA). RNA was isolated from either 10-day-old *Arabidopsis thaliana* ecotype Col-0 seedlings or floral tissue from 6- to 7-week-old plants using Qiagen RNeasy plant RNA extraction kit (Qiagen, http://www.qiagen.com) following manufacturer's instructions. The DNA sequence was identical to the predicted ORF (http://www.arabidopsis.org). ORTHs were expressed in bacteria as GST fusions from pDEST15-based plasmids (Invitrogen, http://www.invitrogen.com). Quik-Change site-directed mutagenesis (Stratagene, http://www.stratagene.com) was used to make a series of point mutations in RING-domain metal–ligand codons. Truncations were made with gene-specific primers with in-frame stop codons using cDNA clones as templates, and the sequences were verified. The positions of truncations (nucleotide relative to ATG) are as follows: PHD only, nt 198; PHD and N-terminal RING, nt 675; no C-terminal RING, nt 1476. The protein with the SRA and C-terminal RING domains contains nucleotides from 676 to stop. *ORTH1* and *ORTH2* coding regions were recombined into the pGWB6 binary transformation vector for localization studies (Nakagawa *et al.*, 2007). Overexpression of tagless ORTH1 was achieved by recombination into pYL TAP C-t, but with a stop codon present (Rubio *et al.*, 2005).

Protein expression and purification

GST-ORTH fusions were expressed in *E. coli* strain BL21 AI or BL21-pLysS. Transformed cells were grown at 37°C for 2–3 h, or to an OD_{600} of 0.4–0.6 before induction with 0.2% arabinose or 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), respectively, for 2–3 h at 25°C. Cells were harvested by centrifugation and were then lysed in a buffer containing 25 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100. For purification, glutathione agarose (Sigma-Aldrich, http://www.sigmaaldrich.com) was added to cleared lysates and incubated for 1 h at 4°C. Beads were then washed four times with wash buffer containing 25 mM Tris–HCl, pH 7.5, 300 mM NaCl, 0.1% Triton X-100. GST fusion proteins were eluted with elution buffer containing 25 mM Tris–HCl, pH 8.1, 50 mM glutathione and 0.1% Triton X-100. Glycerol was added to the eluted protein

to a final concentration of 40%. Proteins were stored at −80°C until needed.

In vitro *ubiquitylation assay*

Ubiquitylation reactions (30 μl) containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.05 mM $ZnCl_2$, 1 mM ATP, 0.2 mM dithiothreitol, 10 mM phosphocreatine, 0.1 unit creatine kinase (Sigma-Aldrich), 50 ng yeast E1 (Boston Biochem, http://www.bostonbiochem.com), 250 ng of recombinant Arabidopsis E2 (Kraft *et al.*, 2005), 500 ng of GST-ORTH and 2 μg ubiquitin (Sigma-Aldrich) were incubated at 30°C for 2 h. For histone ubiquitylation assays, 2 μg of crude bovine nucleosomes (Sigma-Aldrich) were added to the ubiquitylation reaction. Reactions were stopped by adding 6 μl of 5× SDS-PAGE sample buffer [125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerin, 4% (w/v) SDS and 10% (v/v) β-mercaptoethanol], and were then analyzed by SDS-PAGE followed by western blotting using ubiquitin and/or GST antibodies, as described previously (Stone *et al.*, 2005).

Plant growth conditions and GFP visualization

All transgenes were expressed in ecotype Arabidopsis (*A. thaliana*) ecotype Columbia (Col). Seed was surface-sterilized with 30% (v/v) bleach and 0.1% (v/v) Triton X-100, and were grown on GM containing 1% (w/v) agar with 1× MS salts (Sigma-Aldrich) under continuous light. Seedling roots were examined for GFP expression using a Nikon Optiphot 2 fluorescent microscope with a Nikon Super High Pressure Mercury Lamp power supply, model HB-10101AF (Nikon, Melville, NY, USA). Images were captured by a QImaging Retiga 1300 camera and QCapture 2.68.2 software (Quantitative Imaging Corporation, http://www.qimaging.com).

FWA expression

Six micrograms of RNA extracted from adult leaf tissue (Trizol; Invitrogen) was converted to cDNA (First Strand; Invitrogen). *FWA* transcript levels were analyzed by semiquantitative real-time PCR, using primers JP3130 (CCCACCAAGATCTGAAGTCC) and JP3133 (CAGGTGCAATGGTGGTGTAT), and internal primer M12 (CCTTCG GGATTTTCGATAGTGCCA). *ACTIN* was used to standardize cDNA levels, and was quantified with primers JP2452 (TCGTGGTGGTGA GTTTGTTAC) and JP2453 (CAGCATCATCACAAGCATCC), and visualized with Brillant SYBR Green QPCR Master Mix (Stratagene).

Southern blot analysis

DNA was extracted from leaves or flowers of recently bolted plants using a slightly modified cetyl trimethylammonium bromide (CTAB)/sarkosyl method (Bernatzky and Tanksley, 1986). For *Cen180* Southern blots, 0.5–1 μg of DNA was digested with *Hpa*II overnight and run for 4 h on a 1% agarose gel, and was then transferred to membrane. The membrane was incubated overnight with radiolabeled probe for *Cen180* bp repeats (Vongs *et al.*, 1993). After washing, the membrane was exposed to film for 4 h. *FWA* Southern blots were performed as previously described (Chan *et al.*, 2004).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of ORTH/UHRF proteins.

Figure S2. Overexpression of GFP-OLK does not cause a late-flowering phenotype.

Figure S3. Overexpression of ORTH1 causes a late-flowering phenotype.

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