Identification of *Arabidopsis* SUMO-interacting proteins that regulate chromatin activity and developmental transitions

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Posttranslational modification of proteins by small ubiquitin-like modifier (SUMO) plays essential roles in eukaryotic growth and development. Many covalently modified SUMO targets have been identified; however, the extent and significance of noncovalent interactions of SUMO with cellular proteins is poorly understood. Here, large-scale yeast two-hybrid screens repeatedly identified a surprisingly small number of proteins that interacted with three *Arabidopsis* SUMO isoforms. These SUMO-interacting proteins are nuclear and fall into two main categories: six histone or DNA methyltransferses or demethylases and six proteins that we show to be the evolutionary and functional homologs of SUMO-targeted ubiquitin ligases (STUbLs). The selectivity of the screen for several methylases and demethylases suggests that SUMO interaction with these proteins has a significant impact on chromatin methylation. Furthermore, the *Arabidopsis* STUbLs (AT-STUbLs) complemented to varying degrees the growth defects of the *Schizosaccharomyces pombe* STUbL mutant *rfp1/rfp2*, and three of them also complemented the genome integrity defects of this mutant, demonstrating that these proteins show STUbL activity. We show that one of the AT-STUbLs least related to the *S. pombe* protein, AT-STUbL4, has acquired a plant-specific function in the floral transition. It reduces protein levels of CYCLING DOF FACTOR 2, hence increasing transcript levels of CONSTANS and promoting flowering through the photoperiodic pathway.

SIM | Slx5/Slx8 | Nucleolus | Flowering time | CDF

In addition to genetic, transcriptional, and posttranscriptional mechanisms that regulate gene function, posttranslational modification of proteins plays crucial roles in shaping protein structure and function, and hence in cellular and organismal development. Substantial evidence suggests that modification by small protein modifiers such as ubiquitin (Ub) and small Ub-like modifier (SUMO) is essential for eukaryotic growth and development (1). In *Arabidopsis*, inactivation of genes encoding SUMO1 and SUMO2, the SUMO activating enzyme (SAE, or E1), or SUMO conjugating enzyme (SCE, or E2) is embryolethal (2). Although SUMO ligases (E3) and proteases are encoded by multigene families, inactivation of some of these genes causes gross developmental and physiological defects (3–7), highlighting the crucial need for the proper regulation of protein SUMOylation. To elucidate how SUMO mediates these essential functions, attempts were made to characterize the nature of interactions of SUMO and cellular proteins. This led to the identification of a large number of covalently modified targets (8–12). However, SUMO also interacts with proteins noncovalently (13). Because only a few SUMO-interacting proteins (SIPs) have been identified in yeast and metazoans (14–18) and none has been identified in plants so far, the extent and significance of noncovalent SUMO interactions is still poorly understood. Here, we describe the systematic identification and characterization of SIPs in *Arabidopsis*.

Covalent attachment of SUMO to target proteins requires the SUMO E1, E2, and E3 and results in the formation of an isopeptide bond between a lysine residue in the substrate and the C-terminal glycine of mature SUMO (19). A canonical SUMO attachment lysine (ΨKXE/D) is preceded by a hydrophobic amino acid (Ψ) and followed by acidic residues (20). SUMO, attached to cellular proteins, could also recruit other proteins through noncovalent interactions (13, 14). These SIPs recognize SUMO by virtue of a short sequence motif (SUMO-interaction motif, or SIM) that is rich in hydrophobic amino acids (21, 22). Variations of the SIM include "type a" SIM containing a stretch of three to four hydrophobic residues and three to four acidic residues, and "type b" SIM containing the sequence I/VDL/T (14). Additionally, analysis of the SIM (IIVLSDSD) of the transcriptional coregulator Daxx suggests that phosphorylation of its second serine regulates SUMO isoform selective binding (23). The functional consequences of noncovalent interactions of SUMO and the importance of the SIM in this process are still being explored. In all cases studied, regulated recruitment of SIPs controls important biological outputs. For example, the base-excision repair enzyme THYMINE DNA GLYCOSYLASE (TDG) is covalently modified by SUMO-1 at K-330, whereas noncovalent interactions between its C-terminal β-sheet (β_6) and SUMO's α-helix (α_1) and β-strand (β_2) are essential for its release from DNA (24). SUMO also recruits the Srs2 helicase to SUMOylated proliferating cell nuclear antigen, which consequently inhibits recombination repair during DNA replication (15, 25). Noncovalent SUMO interactions may also regulate covalent SUMO conjugation and target discrimination. For example, autoSUMOylation of human SCE (Ubc9) at K-14 causes increased activity toward the transcription factor Sp100 and reduced activity toward RanGAP1, whereas its activity toward HDAC4, E2-25K, PML, or TDG is not altered (26). A SIM in Sp100 is required for its

Significance

Protein activity is often regulated by posttranslational modifications. In eukaryotes, proteins covalently modified by small ubiquitin-like modifier (SUMO) interact with other proteins that bind SUMO noncovalently. Here we describe the systematic identification and characterization of *Arabidopsis thaliana* proteins that noncovalently bind SUMO. The identity of these proteins reveals the cellular and developmental processes likely to be regulated through SUMO modification. Some of these processes are widely conserved among eukaryotes, whereas others are involved in specific aspects of plant development such as the initiation of flowering. These data will facilitate detailed studies of the many roles of SUMO in the regulation of plant growth and development.

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enhanced SUMOylation, likely through specific recruitment of Ubc9~SUMO.

Perhaps the best-studied SIPs are a group of RING-type Ub E3 ligases that target polySUMOylated proteins for degradation by the proteasome [SUMO-targeted ubiquitin E3 ligases (STUbLs) or Ub ligases for SUMO conjugates (Uls)] (14, 16–18). STUbLs have been suggested as an alternative route to reverse SUMO modification of proteins, the primary route being deconjugation by SUMO proteases (14, 16, 18). They also highlight important cross-talk between the SUMO and Ub systems because they mediate a novel form of regulated protein turnover stimulated by polySUMO chains (18). STUbLs in the fission yeast (Rfp1 and Rfp2), budding yeast (Hex3/Slx5-Slx8), and mammals (RNF4) are implicated in the maintenance of genome integrity (17, 27–30). RNF4 is also required for global DNA demethylation and found to interact with TDG and to enhance its repair activity (31). Consistent with an essential role during development, RNF4 knockout mice are embryo-lethal, and mutations of the *Drosophila* STUbL (Degringolade) cause early embryonic arrests (31, 32). These and other reports suggest major and probably conserved roles for SIPs during eukaryotic development. Nonetheless, a systematic screen to identify SIPs in a higher multicellular eukaryote is still lacking.

Here, we used three SUMO isoforms as separate baits in large-scale yeast two-hybrid (Y2H) screens to identify *Arabidopsis* SIPs. A surprisingly small number of proteins repeatedly interacted with at least one SUMO. These include six RING-type Ub E3 ligases that we show to be the evolutionary and functional homologs of STUbLs and six proteins involved in the regulation of chromatin state by the reversible methylation and demethylation of histones and DNA. The identification of six proteins implicated in the control of chromatin methylation suggests that this process is heavily regulated by SUMO. Five *Arabidopsis* STUbLs (AT-STUbLs) can to varying degrees complement the growth defect phenotype of the *Schizosaccharomyces pombe rfp1/rfp2* STUbL mutant, and three of them can also complement the genome integrity phenotype of this mutant, demonstrating that they retain STUbL activity. AT-STUbLs have likely evolved plant-specific functions. Mutations in AT-STUbL4, for example, delay flowering consistent with a role for this gene in promoting floral transition. We identified CYCLING DOF FACTOR 2 (CDF2) as a likely target of AT-STUbL4, and we discuss how the degradation of this repressor of CONSTANS (CO) expression may explain the regulation of floral transition by AT-STUbL4.

Results and Discussion

Identification of *Arabidopsis* SUMO-Interacting Proteins. Using three SUMO isoforms as baits in large-scale Y2H screens, we identified an unexpectedly small number of SIPs (Fig. 1 and Table S1). In addition to the SUMO E2 (AT-SCE1) and E3 (AT-SIZ1), 12 proteins were isolated repeatedly (7–21 times) and interacted with SUMO1, 2, or 3 only through regions that span a putative SIM (Fig. 1 *A* and *B* and Table S1). We identified six histone or DNA methyltransferases (MT) or demethylases (DMT) and six Ub E3 ligases. The first group of proteins includes the SET DOMAIN GROUP 8 (SDG8) histone MT, a Jumonji-type histone DMT (JMJ-type), a protein required for transcriptional gene silencing and maintenance of DNA methylation (MOM1), a DNA MT (CMT2), and two proteins that possess nuclease activity (DEMETER "DME" and Like-DME) and act by removing 5′methylcytosine (Fig. 1 *A* and *B* and Table S1). The second group of proteins comprises six RING-type Ub E3 ligases that we show here to be plant homologs of STUbLs. All Y2H clones identified for each protein contained at least one putative SIM, suggesting that a SIM is required for interaction with SUMO (Fig. 1 *A* and *B*). However, we also confirmed the importance of the putative SIM in full-length AT-STUbL1 for the interaction with SUMO1 (Fig. 1*C*). The functional importance of the SIM was also assessed both in yeast and in plants (discussed below), and together with functional complementation of the *S. pombe* STUbL mutant *rfp1/rfp2* (17) (Fig. 2, discussed below) these experiments support that we identified bona fide *Arabidopsis* SIPs.

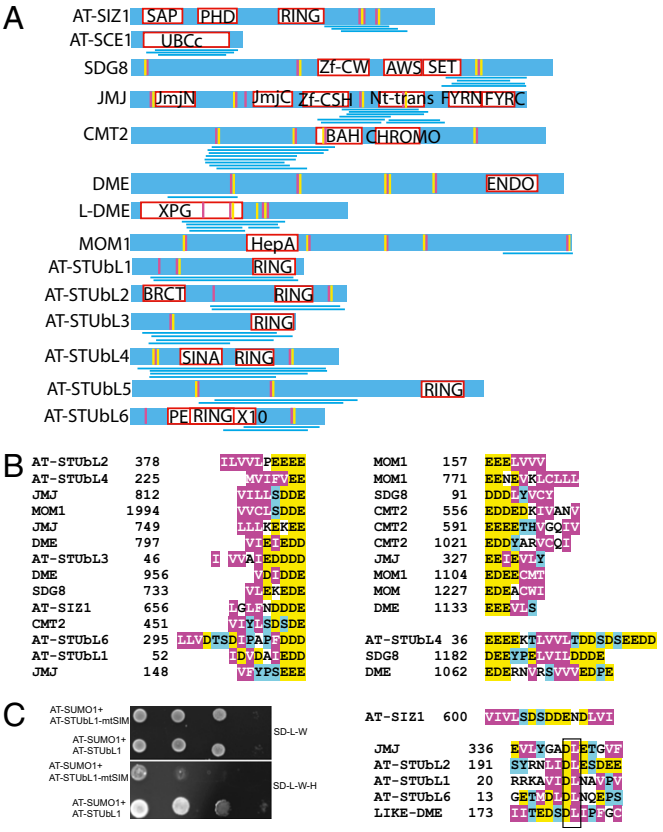

Fig. 1. Identification of *Arabidopsis* SIPs by Y2H screening. (*A*) Depiction of full-length SIPs showing domains in squares (domain definition is available at www.ncbi.nlm.nih.gov/cdd/), SIMs as vertical stripes (one purple stripe represents type-b SIM and purple-yellow stripes represent type-a SIM). Clones that interacted with the SUMO bait are represented as lines. Clones that span exactly the same region are represented once. (*B*) Sequence alignments of the *Arabidopsis* SIM. Highly hydrophobic residues are in purple, less hydrophobic residues in blue, and acidic residues in yellow. We identified type-a SIM (*Left*), type-a SIM but in the inverted orientation (*Upper Right*), a SIM that contains a hydrophobic core flanked by acidic residues or an acidic core flanked by hydrophobic residues (*Middle Right*), and a type-b SIM (*Bottom Right*). (*C*) Interaction of SIPs and SUMO requires the SIM. Both SUMO1 and full-length AT-STUbL1 or AT-STUbL1 in which the SIM was mutated were cotransformed into yeast cells, spotted on dropout media lacking tryptophane (W) and leucine (L) (*Upper*) or W, L, and histidine (H) (*Lower*).

Six chromatin MTs and DMTs were identified, suggesting that SUMO interaction plays a general and conserved role in this process. A SUMOylation "wave" causing simultaneous multisite modification of a group of proteins stabilizes physical interactions between them and is required for DNA repair (33). A similar effect of SUMOylation acting through a protein group might occur in chromatin methylation. For example, SUMOylation of transcription factors may recruit and/or regulate interactions of these chromatin MTs and DMTs (34). Notably, both DNA and histone MTs interact with SUMO. CHG methylation by CMT3 (closest homolog of CMT2) depends on histone H3K9 dimethylation, and CMT3 interacts with H3K9me2-containing nucleosomes, suggesting that recruitment of CMT3 to chromatin may require prior function of histone MTs (35). Whether this interaction is coordinated by SUMO remains to be explored. Also, these SIPs mediate both attachment (MTs) and removal (DMTs) of methyl groups and play essential roles in plant

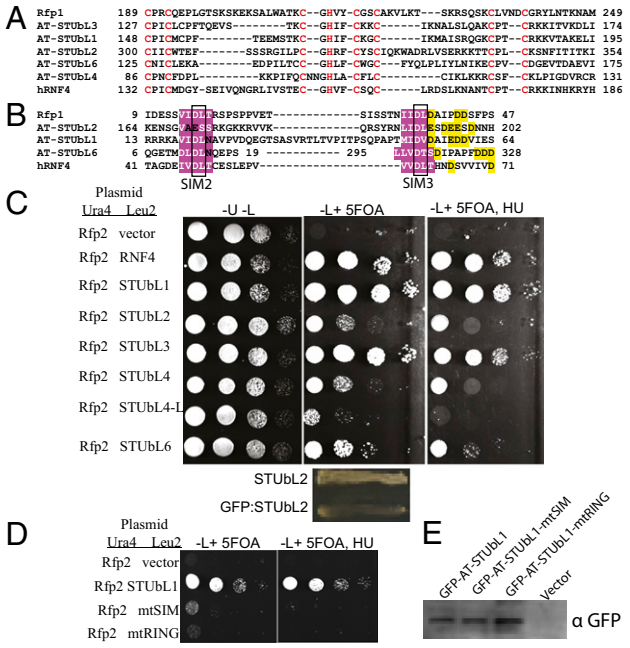

Fig. 2. *Arabidopsis*, yeast, and mammalian STUbLs are functionally and evolutionarily conserved. (*A*) Sequence alignment of the RING domain of the *Arabidopsis* (AT-STUbLs), human (hRNF4), and fission yeast (Rfp1) homologs. The eight metal-binding residues of RING domain are in red. (*B*) Sequence alignment of regions of Rfp1, hRNF4, and three AT-STUbLs elucidating the conservation of the SIMs of these proteins. The SIM (type b) is in purple, and the central "DL" residues are boxed. Acidic residues present close to the SIM to help orient it with SUMO β_2-strand are highlighted in yellow. SIM2 and SIM3 refer to the middle two SIMs of RNF4. (*C*) Functional complementation of the growth and genome integrity phenotypes of *S. pombe rfp1/rfp2* mutant. *rfp1/rfp2*, kept alive by a URA4 plasmid encoding Rfp2, was transformed with a LEU2 plasmid encoding a putative AT-STUbL and selected on media lacking Uracil (U) and Leucine (L) (*Left*). Complementation was tested on media lacking L but containing 5′FOA to eliminate cells containing the URA4 plasmid and select for cells containing the LEU2 plasmid (*Center*). Growth of AT-STUbL2 is enhanced when the N-terminal GFP tag was removed (*Lower Center*). The compromised genome integrity phenotype of *rfp1/rfp2* cells caused by 10 mM HU is also reverted in cells expressing AT-STUbL1, 3, and 6 (*Right*). (*D*) Functional complementation of *rfp1/rfp2* by AT-STUbLs requires both the SIM and the RING. The SIM or RING motifs of AT-STUbL1 were mutated, and growth was tested as before. (*E*) Immunoblot analysis of protein extracts from the strains shown in *D* and probed with anti-GFP antibody. All strains express AT-STUbL1 or mutant AT-STUbL1 at similar levels.

development (36–39). They may regulate the expression of common target genes in a dynamic and antagonistic manner, as is the case for *FLOWERING LOCUS C* (*FLC*), which is regulated by both SGDG8 (MT) and the Jmj-type protein REF6 (DMT) (37–39). SUMO might contribute to this dynamic regulation. DME is a DNA glycosylase that functions in a manner similar to TDG (40). Covalent and noncovalent SUMO interactions play a central role in TDG function by reducing its DNA binding activity and increasing its enzymatic turnover (24, 41). The identification of DME in this study suggests that this role may also be conserved for *Arabidopsis* TDG (e.g., DME). Additionally, in mammals RNF4 interacts with TDG to regulate global DNA methylation levels (31). We identified both STUbLs and chromatin modifiers (including DME) in one screen by their interaction with SUMO, corroborating prior evidence for interaction of TDG and RNF4 and implying that this role in global DNA methylation may be conserved throughout eukaryotes.

Evolutionary and Functional Conservation of STUbLs. STUbLs in yeast and mammals localize to the nucleus, to foci formed at sites of DNA breaks, and are required for genome integrity (18, 29–32). We assessed whether the RING proteins identified here are evolutionary and functional homologs of yeast and mammalian STUbLs. The putative *Arabidopsis* STUbLs share a conserved RING domain with *S. pombe* Rfp1/Rfp2 and human RNF4 (Fig. 2*A*), and three of them contain "type-b" SIMs located within the N-terminal 40 aa of Rfp1/Rfp2 (Fig. 2*B*) (the remaining AT-STUbLs contain "type-a" SIM; Fig. 1*B*) (14, 17). Previous phylogenetic analyses of *Arabidopsis* RING domains suggest that all of the AT-STUbLs identified here contain RING-HCa type domains (42) and cluster within only one of 54 RING domain clusters (43). A simpler phylogenetic analysis of the *Arabidopsis*, yeast, and mammalian STUbLs illustrated the relatedness of these proteins (Fig. S1). This analysis suggested that AT-STUbL1 and 3 are most related, an observation that was previously suggested by the phylogenetic analysis of all *Arabidopsis* RING proteins (42) and that is consistent with their similar activities in a yeast complementation assay described below.

We then tested whether the *Arabidopsis* proteins could complement the growth defect and compromised genome integrity phenotypes of *S. pombe rfp1/rfp2* (17). These cells survive owing to the expression of Rfp2 from a URA4+ plasmid (17). AT-STUbL1, 3, and, to a lesser extent, 6 were able to rescue the growth defect of *rfp1/rfp2* cells on media containing 5′ fluoroorotic acid (5′FOA), which kills cells expressing URA4. These proteins also complemented the genome integrity phenotype of *rfp1/rfp2* cells when grown on media containing 5′FOA and the replicative stress agent hydroxyurea (HU), although again AT-STUbL6 was less efficient in doing so (Fig. 2*C*). AT-STUbL2 shows homology to a BRCT domain at its N terminus (Fig. 1*A*), and although N-terminal GFP fusions complemented the growth defect of *rfp1/rfp2* cells only partially (Fig. 2*C*, *Center*), efficient growth was observed when a nontagged form of AT-STUbL2 was used (Fig. 2*C*, *Lower*). Neither form of AT-STUbL2 could restore the genome integrity phenotype efficiently. AT-STUbL4 could only support weak growth on 5′FOA, suggesting that it partially complements the *rfp1/rfp2* mutation but neither it nor a highly related protein (AT-STUbL4-Like) was able to support growth on HU. We designated AT-STUbL4 as a putative STUbL because, although it only weakly rescued *rfp1/rfp2*, this protein interacted with SUMO in the Y2H, contained both SIM and RING domains, and is phylogenetically related to other STUbLs. Stable cDNA clones could not be isolated for AT-STUbL5 and hence it was not characterized in this study. These results suggest that the activities of AT-STUbL1, 3, and 6 are most similar to *S. pombe Rfp1/Rfp2*, whereas AT-STUbL2 and 4 are less similar in activity. To assess the functional relevance of the SIM and RING, each of these domains in AT-STUbL1 was mutagenized and the ability of the mutant forms to support growth of the *rfp1/rfp2* cells on 5′FOA and HU was assessed. The mutant forms could rescue neither the growth nor the genome integrity defect of *rfp1/rfp2* cells (Fig. 2 *D* and *E*), indicating that both the SIM and the RING are required for these functions.

***Arabidopsis* SIPs Are Nuclear Proteins.** The histone and DNA MTs and DMTs identified here function in the nucleus and all SIPs studied so far are nuclear proteins (34). We thus assessed the subcellular localization of AT-STUbLs by expressing them in tobacco epidermal pavement cells as N-terminal fusions with YFP. These proteins exclusively localize to the nucleus, but AT-STUbL3 also to the cytoplasm (Fig. 3 and Fig. S2). Interestingly, we observed different localization patterns within the nucleus. All proteins localize to the nucleoplasm; however, whereas AT-STUbL1, 3, and 4 had intense fluorescence in the nucleolus, AT-STUbL2 and 6 were excluded from the nucleolus (Fig. 3 *A* and *B*). This suggests that all SIPs identified in this study are nuclear proteins and corroborates roles proposed for the nucleolus as a center for several SUMO-related activities (44). AT-STUbL4 showed a unique pattern where in addition to the nucleoplasm and the nucleolus it localizes also to discrete speckles (three to nine per nucleus, but occasionally we found a nucleus with no

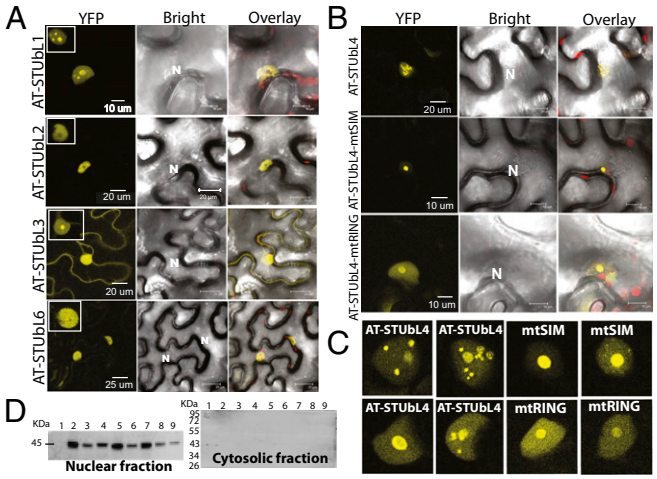

Fig. 3. Subcellular localization of AT-STUbLs in plant cells. AT-STUbLs were expressed in tobacco cells as YFP fusions, and fluorescence was visualized using confocal microscopy. (*A*) Two nuclei are shown for AT-STUbL1, 2, 3, and 6. (*B* and *C*) AT-STUbL4 and its SIM and RING mutant forms. Five nuclei of AT-STUbL4 and three nuclei of either mutant form are shown. (*D*) AT-STUbL4 or its mutant forms were stably expressed in *Arabidopsis* as fusions with 3xHA tag, and immunoblot analysis of nuclear and cytosolic extracts was performed using anti-HA antibody. Lanes: 1, *at-stubl4*; 2–4, three different lines expressing AT-STUbL4 with a mutated SIM; 5–7, three different lines expressing AT-STUbL4 with a mutated RING; 8 and 9, two lines expressing wild-type AT-STUbL4.

speckles) (Fig. 3 *B* and *C*). We tested the effect of mutagenizing the SIM and the RING of AT-STUbL4 on its subcellular localization. Mutations of the RING completely abolished AT-STUbL4 localization to the speckles, and mutations of the SIM also strongly reduced speckle localization (Fig. 3 *B* and *C*). The nature of these speckles is unclear, but we conclude that the RING and the SIM are required either for their formation or for the proper localization of AT-STUbL4 to them. Thus, interaction of AT-STUbL4 with SUMOylated proteins (through the SIM) and other proteins (through the RING) is likely required for these processes.

We tested whether proteins with nuclear functions are overrepresented among those identified because they contained a putative SIM. Use of a consensus type-a SIM as a query identified 656 proteins (2% of the proteome). Although sequence and structural context is important for SUMO interaction (45), the proteins identified computationally included several members of each functional group of proteins identified by Y2H, supporting the idea that they are enriched for proteins that interact with SUMO. No specific biological processes were differentially represented among the 656 proteins, but they were highly and significantly enriched in nucleic-acid-binding proteins, transcription factors, and proteins with transferase activities (e.g., MTs) (Fig. S3 and Dataset S1), suggesting that the great majority of these proteins are nuclear and likely involved in the regulation of chromatin state and transcriptional control.

AT-STUbL4 Is Involved in Floral Transition. The MTs and DMTs described above regulate a variety of biological processes and are important for plant growth and development (36, 37, 39). We assessed the functions of AT-STUbLs by investigating transfer DNA (T-DNA) insertion mutants and generating lines overexpressing these genes. Consistent with their proposed redundant functions, insertion mutants and overexpressors of AT-STUbL1, 2, 3, and 6 did not exhibit visible phenotypes under normal growth conditions. By contrast, AT-STUbL4 was found to be involved in the floral transition. The *at-stubl4* mutant flowered later than wild-type plants, as determined by the number of rosette leaves at flowering, with approximately six or approximately four more leaves under long-day (LD) or short-day (SD) conditions (Fig. 4*A*). The larger difference in leaf number for plants grown in LD is consistent with a possible role of AT-STUbL4 in photoperiodic flowering. The AT-STUbL4 overexpressor lines, however, flowered earlier than wild-type plants (approximately two or approximately five fewer leaves in LD or SD) (Fig. 4*B*). Furthermore, overexpression of a 3xHA-tagged version of AT-STUbL4 was found to revert the late-flowering phenotype of *at-stubl4* mutants to that of wildtype (Fig. 4*C*). These results suggest that AT-STUbL4 contributes to the promotion of flowering in *Arabidopsis*. We then tested whether SIM or RING mutations of AT-STUbL4 also complement the late-flowering phenotype of *at-stubl4*. Consistent with a functional role for these domains in floral transition, these mutant forms were unable to complement the late-flowering phenotype of *at-stubl4* efficiently (Fig. 4*D*). Because these mutations also abolish the localization of AT-STUbL4 to subnuclear speckles (Fig. 3), they establish a correlation between AT-STUbL4 activity in flowering and its capacity to be recruited to or to form speckles.

CYCLING DOF FACTORs Are Likely Targets of AT-STUbL4. Two approaches to identify AT-STUbL4 targets that may regulate floral transition were used. First, the expression profiles of four genes that integrate information from different flowering-time pathways were compared in wild-type and *at-stubl4* mutants (46). Whereas expression of *FLC* and *GI* was unchanged across a diurnal time course, the levels of *CO* and *FT* mRNAs were generally lower in *at-stubl4* (Fig. 5). This is particularly the case for *CO*, where *at-stubl4* seedlings produce about half the amount of *CO* mRNA of wild-type plants 12 and 20 h after dawn (ZT12 and ZT20). Similarly, the characteristic "peak" of *CO* expression at ZT12 that marks the significant rise in *CO* mRNA evening expression specifically in LD (46) is abolished in *at-stubl4* (Fig. 5*C*). This twofold reduction in *CO* evening expression is reflected in the levels of expression of its downstream target, *FT*, so that an approximately threefold reduction of *FT* mRNA is observed in *at-stubl4* at ZT20 (Fig. 5*D*). This pattern of expression may

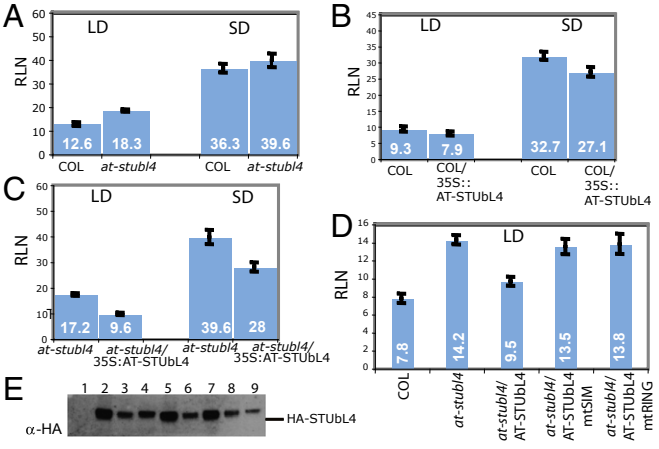

Fig. 4. AT-STUbL4 is involved in floral transition. (*A–D*) Flowering time estimates determined in rosette leaf number (RLN). (*A*) The T-DNA insertion mutant (*at-stubl4*) (*Materials and Methods*) of AT-STUbL4 is late-flowering compared with wildtype (COL) in LD and SD conditions. (*B*) Transgenic plants overexpressing AT-STUbL4 flower earlier than wild-type plants. (*C*) The late-flowering phenotype of the *at-stubl4* mutant is rescued by a transgene expressing AT-STUbL4. (*D*) SIM or RING mutant forms of AT-STUbL4 do not rescue the late-flowering phenotype of the *at-stubl4* mutant. (*E*) SIM or RING mutant forms of AT-STUbL4 are expressed efficiently in these transgenic plants as suggested by immunoblot analysis of nuclear extracts made from these plants and probed with the anti-HA antibody. Lane 1, *at-stubl4*; lanes 2–4, three different lines expressing AT-STUbL4 with a mutated SIM; lanes 5–7, three different lines expressing AT-STUbL4 with a mutated RING; lanes 8 and 9, two lines expressing wild-type AT-STUbL4.

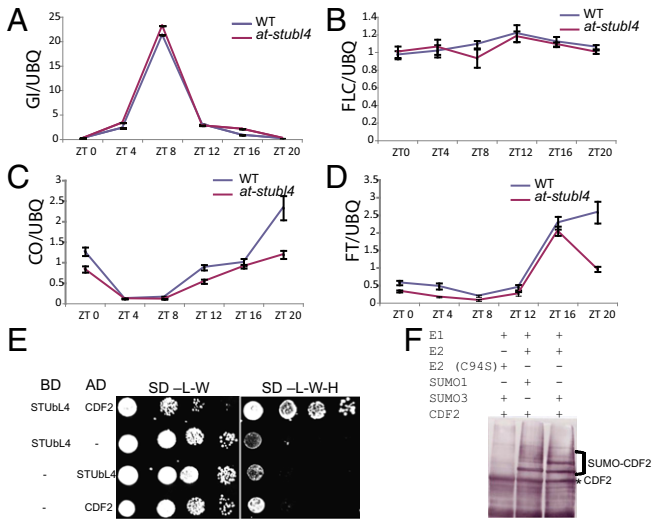

Fig. 5. Identification of AT-STUbL4 targets. (*A–D*) Expression of four flowering-time genes (*GI, FLC, CO*, and *FT*) determined by real-time PCR showing that expression of *CO* and *FT* but not *GI* or *FLC* is dampened during the end of the day in *at-stubl4*. (*E*) Y2H assay suggesting that CDF2 physically interacts with AT-STUbL4. AD, the GAL4 activation domain; BD, the GAL4 binding domain. (*F*) SUMOylation assay suggesting that CDF2 is indeed SUMOylated. *E. coli* cells expressing the SAE (E1), SCE (E2), or a catalytically inactive version of the E2 (C94S) and either SUMO1 or SUMO3 were transformed with an expression plasmid encoding a thioredoxin-6xHis–tagged CDF2 and SUMOylation was performed as before. Immunoblot analysis using anti-thioredoxin antibodies detect unmodified CDF2 (asterisk) and SUMO-modified CDF2 (bracket).

explain the late-flowering phenotype of *at-stubl4* and suggests that a protein acting upstream of *CO* to repress *CO* expression may be stabilized by the *at-stubl4* mutation.

Second, AT-STUbL4 was used as bait in the Y2H assay and identified CDF2 as an interacting partner (Fig. 5E). CDF2 acts redundantly with other CDFs to repress *CO* transcription (47). Degradation of these proteins toward the end of the day is essential to release the transcriptional repression they exert on *CO* and hence to promote flowering (47, 48). The effects detected on the pattern of *CO* expression in the *at-stubl4* mutant are consistent with our finding that AT-STUbL4 and CDF2 physically interact in the Y2H system and, together, these results suggest CDF2 as a likely target of AT-STUbL4.

As described above, STUbLs recognize SUMOylated proteins, leading to their ubiquitination and degradation. CDF2 was identified previously in a screen for *Arabidopsis* SUMOylated proteins and shown to be SUMOylated in vivo (10). We therefore tested SUMOylation of CDF2 in *Escherichia coli* strains in which the SUMO conjugation pathway was engineered (9) and found that it is SUMOylated efficiently by SUMO3 (Fig. 5*F*). Taken together, these results are consistent with a model whereby SUMOylated forms of CDF2 are targeted for ubiquitination by AT-STUbL4.

Overexpression of AT-STUbL4 in SUC2::HA-CDF2 Plants Suppresses Their Late-Flowering Phenotype and Reduces CDF2 Protein Levels. To establish genetic and molecular evidence for the role of AT-STUbL4 in regulating CDF2 protein levels, a homozygous line expressing a 3xHA-tagged version of CDF2 from the *SUCROSE TRANSPORTER 2* (*SUC2*) promoter (9, 47) was used. These plants flower considerably later than wild-type plants (~45 compared with ~15 rosette leaves at flowering in LD) owing to increased levels of CDF2 protein and decreased levels of *CO* mRNA (47). A construct expressing AT-STUbL4 under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter was introduced into *SUC2:HA-CDF2* plants by transformation and the flowering time of the resulting plants scored at the T1 stage and in homozygous T2 plants. Suppression of the late-flowering phenotype of *SUC2::HA-CDF2* plants was readily observed in T1 plants (Fig. S4), and some lines even flowered at times similar to when wild-type plants flowered. The levels of HA-CDF2 largely correlated with flowering time estimates so that *SUC2::HA-CDF2* lines that flowered early contained dramatically reduced levels of the HA-CDF2 protein (Fig. S4). T2 lines homozygous for both transgenes were selected after self-fertilization of the T1 plants (Fig. 6). Many of these lines flowered much earlier than the parental *SUC2::HA-CDF2* line and contained much-reduced levels of HA-CDF2 that broadly correlated with the flowering time of individual lines (Fig. 6 *A* and *B*). The *CaMV35S::AT-STUbL4* transgene expressed much higher levels of *AT-STUbL4* mRNA than wild-type plants in all lines tested, consistent with the early flowering time and the reduced levels of HA-CDF2 observed (Fig. S5). Importantly, when the *CaMV35S::AT-STUbL4* transgene was segregated away from *SUC2:HA-CDF2* in a T1 line (line 16, homozygous for *SUC2:HA-CDF2* but heterozygous for *CaMV35S::AT-STUbL4*) both the flowering time and HA-CDF2 levels reverted back to those of the *SUC2::HA-CDF2* parent (Fig. 6). Together with the altered expression pattern of *CO* expression in *at-stubl4* and the evidence for physical interaction between AT-STUbL4 and CDF2, these results suggest that CDF2 is a likely target of AT-STUbL4.

CDFs are proposed to form a repressor complex on the *CO* promoter to dampen *CO* expression until they are degraded toward the end of the day, which is essential for *CO* evening expression and for photoperiodic flowering (47, 48). This is at least in part mediated by the F-box protein FKF1 as part of a complex whose formation is stimulated by blue light (49).

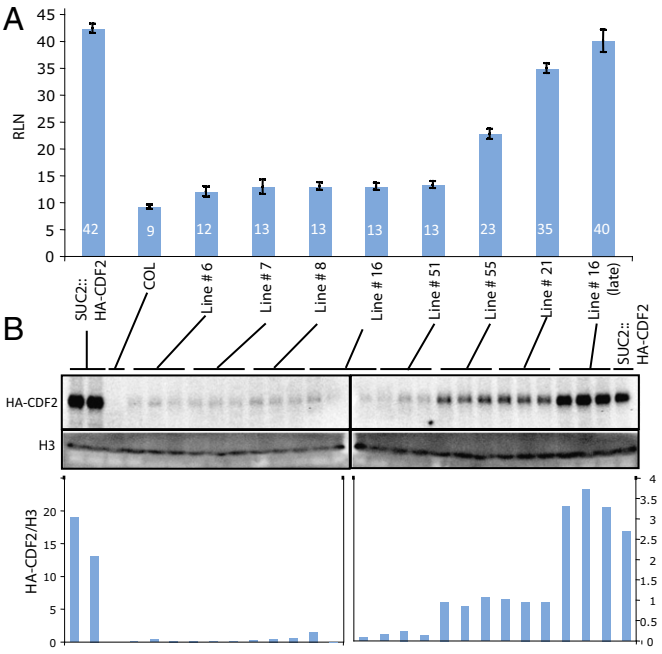

Fig. 6. AT-STUbL4 regulates floral transition by reducing the levels of the floral repressor CDF2. AT-STUbL4 was transformed into an *Arabidopsis* homozygous line expressing 3xHA-tagged CDF2 from the SUC2 promoter. (*A*) Flowering time scored as total rosette leaf number (RLN) at flowering of the parental SUC2::HA-CDF2 line, and of double transformants (SUC2::HA-CDF2/35S::AT-STUbL4; lines 6, 7, 8, 16, 51, 55, and 21). 35S::AT-STUbL4 suppresses the late-flowering phenotype of the parental SUC2::HA-CDF2 line. Also, late-flowering segregants of line 16 that lost the 35S::AT-STUbL4 transgene flower at levels similar to their parental line. (*B*) HA-CDF2 protein levels are much reduced in double-homozygous lines compared with the parental SUC2::HA-CDF2 line or double-homozygous lines that lost their 35S::AT-STUbL4 transgene, as suggested by immunoblot analysis of nuclear extracts probed with anti-HA antibody.

Mutations of FKF1 and related proteins delay CDF degradation (47, 48). It will be interesting to determine whether AT-STUbL4 is involved in CDF evening turnover, and how its activity is related to that of FKF1.

Conclusions

In this report we assessed the extent of noncovalent interactions of SUMO and cellular proteins of a higher multicellular eukaryote. *Arabidopsis* SIPs are nuclear proteins that regulate chromatin state by reversible methylation and demethylation of histones and DNA, and STUbLs that target SUMOylated proteins for degradation. Some AT-STUbLs functionally complement the growth and genome integrity phenotypes of the corresponding yeast mutant, suggesting a close functional relatedness. Other AT-STUbLs have evolved plant-specific functions and are involved in the control of transcription of genes involved in the floral transition. Our study provides a description of AT-STUbLs and necessary resources to study the roles of noncovalent interactions of SUMO in plant growth and development.

Materials and Methods

An extended discussion of materials and methods is given in *SI Materials and Methods*. All primers used in this study are described in Table S2. Y2H screens were performed using SUMO1, 2, and 3 as baits against whole-plant cDNA library. Complementation of the *S. pombe rfp1/rfp2* mutant was assessed on media supplemented with 5′FOA or 5′FOA and 10 mM HU. To assess subcellular localization, YFP fusions of AT-STUbLs were expressed transiently in tobacco leaves and visualized using confocal microscopy. pAlligator-2 was used to assess the function of AT-STUbLs in *Arabidopsis* using Agrobacterium-mediated transformation.

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