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Characterization of *Arabidopsis* and Rice DWD Proteins and Their Roles as Substrate Receptors for CUL4-RING E3 Ubiquitin Ligases [W]

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A subset of WD40 proteins that contain a DWD motif (for DDB1 binding WD40) is reported to act as substrate receptors for DDB1-CUL4-ROC1 (for Damaged DNA Binding 1–Cullin 4–Regulator of Cullins 1) based E3 ubiquitin ligases in humans. Here, we report 85 *Arabidopsis thaliana* and 78 rice (*Oryza sativa*) proteins containing the conserved 16–amino acid DWD motif. We show by yeast two-hybrid and in vivo coimmunoprecipitation that 11 *Arabidopsis* DWD proteins directly interact with DDB1 and thus may serve as substrate receptors for the DDB1–CUL4 machinery. We further examine whether the DWD protein PRL1 (for Pleiotropic Regulatory Locus 1) may act as part of a CUL4-based E3 ligase. PRL1 directly interacts with DDB1, and *prl1* and *cul4cs* mutants exhibited similar phenotypes, including altered responses to a variety of stimuli. Moreover, AKIN10 (for *Arabidopsis* SNF1 Kinase Homolog 10) was degraded more slowly in cell extracts of *prl1* and *cul4cs* than in cell extracts of the wild type. Thus, both genetic and biochemical analyses support the conclusion that PRL1 is the substrate receptor of a CUL4-ROC1-DDB1-PRL1 E3 ligase involved in the degradation of AKIN10. This work adds a large new family to the current portfolio of plant E3 ubiquitin ligases.

INTRODUCTION

Eukaryotes use the ubiquitin–proteasome system to control selective protein degradation. Since this regulates processes ranging from cell division to cell death, it is essential to understand how the specificity of protein degradation is determined (Hershko and Ciechanover, 1998; Smalle and Vierstra, 2004).

Proteins are targeted for proteasomal degradation by ubiquitination via the successive activities of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3), which binds the substrate and thus determines the specificity of the entire pathway (Hochstrasser, 1996; King et al., 1996). Accordingly, understanding selective protein degradation entails identifying and characterizing the substrate receptors.

Eukaryotic cells contain many different E3 ubiquitin ligases. The most abundant families are based on cullin scaffolding proteins that provide a framework for assembling the selective ubiquitination machinery (Schwechheimer and Calderon, 2004; Petroski and Deshaies, 2005; Thomann et al., 2005). Members of the cullin family have two modules assembled on the cullin protein. One is a RING finger domain protein, ROC1 (also known as RBX1 and Hrt1), which binds a C-terminal domain in cullins and recruits the E2 enzyme; the other is a substrate-recognition complex. A remarkable aspect of cullin-RING E3 ligases is that each cullin can assemble into many distinct cullin RING–dependent ligases by interacting with various proteins (Petroski and Deshaies, 2005). CUL1 uses an N-terminal domain to bind a linker protein, SKP1 (for S-phase Kinase-Associated Protein 1), which binds various F-box proteins that recruit specific substrates (Feldman et al., 1997; Skowyra et al., 1997; Zheng et al., 2002). CUL2 and CUL5 use a heterodimeric linker complex containing elongins B and C to bind VHL box or SOCS box proteins that target various substrates differentially to the CUL2-ROC1 or CUL5-ROC2 catalytic cores using additional protein–protein interaction modules (Kamura et al., 1998, 2001, 2004; Stebbins et al., 1999; Zhang et al., 1999). Without a linker, CUL3 uses its N-terminal domain to bind proteins with a conserved 100-residue BTB domain, which then target various substrates to the CUL3-ROC1 catalytic core via additional protein–protein interaction domains (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003). Cullins form the largest family of E3 ligase complexes in plants and control the ubiquitination of a wide variety of substrates; there are 694 potential F-box and 80 BTB genes in *Arabidopsis thaliana*, while the number of BTB genes has expanded to 149 in rice (*Oryza sativa*) (Gagne et al., 2002; Gingerich et al., 2005, 2007).

Identifying the substrate receptors of CUL4-based E3 ligases has been more challenging. *CUL4*, originally identified in

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Caenorhabditis elegans (Kipreos et al., 1996), is present as a single gene in *C. elegans*, *Arabidopsis*, *Drosophila melanogaster*, and *Schizosaccharomyces pombe* and has two closely related paralogs in mammals, *CUL4A* and *CUL4B* (Kipreos et al., 1996). Deletion of *CUL4* caused decondensation of chromosomes in fission yeast (Osaka et al., 2000) and massive DNA replication in *C. elegans* embryos (Zhong et al., 2003), while targeted disruption of the mouse *CUL4A* gene resulted in embryonic lethality (Li et al., 2002). Similarly, two recent studies showed that lowering CUL4 expression in *Arabidopsis* resulted in many defects, including constitutive photomorphogenesis, altered light-regulated gene expression, reduced lateral root formation, and aberrant stomatal and vascular tissue development (Bernhardt et al., 2006; Chen et al., 2006).

CUL4, like other cullin-based E3 ligases, binds ROC1/RBX1 to recruit the E2 ubiquitin-conjugating enzyme. It has also been shown to bind adaptor proteins that link CUL4 to substrate receptors. DDB1 has been identified as one such adaptor, which can associate other proteins and substrate receptors. For example, DDB1 was shown to be an essential component for targeted ubiquitination of CDT1 (for Cdc10-dependent transcript 1), which is a licensing factor for DNA replication, by CUL4-ROC1 (Hu et al., 2004). In plants, coimmunoprecipitation assays indicate it associates with CONSTITUTIVELY PHOTOMORPHOGENIC10 (COP10) and DE-ETIOLATED1 (DET1) to form the COP10-DET1-DDB1 (CDD) complex in *Arabidopsis* (Yanagawa et al., 2004). In turn, genetic studies and in vitro reconstitution assays showed that the CDD complex binds CUL4-ROC1/RBX1 to form a complex that associates with COP1 to help repress photomorphogenesis (Chen et al., 2006).

Recent studies in mammalian cells have identified many proteins containing WD40 repeats that associate with CUL4-DDB1 complexes (Angers et al., 2006; He et al., 2006; Higa et al., 2006a; Jin et al., 2006). Moreover, it has been shown for several of these proteins that a functional E3 ligase is formed when they bind the CUL4-ROC1/RBX1-DDB1 complex. For example, the proteolysis of CDT1 after DNA replication and in response to DNA damage is contingent on CDT2 binding to the CUL4-DDB1 complexes (Banks et al., 2006; Higa et al., 2006b; Jin et al., 2006; Ralph et al., 2006; Kim and Kipreos, 2007). Similarly, DDB2 must bind CUL4-DDB1 complexes for the ubiqutination of histone H2A at UV-damaged DNA sites, and mutations that prevent this binding cause a form of *Xerodema pigmentosum* (Kapetanaki et al., 2006). These WD40 proteins are thus good candidates for the substrate receptors of CUL4-based E3 ligases.

Most WD40 proteins associated with CUL4-DDB1 in animals contain a conserved motif within the WD40 repeats required for this interaction. This motif, named the DWD box (also known as WDXR and DxR) consists of 16 amino acids. There are four highly conserved residues: Asp-7 (or Glu), Trp-13 (or Tyr), Asp-14 (or Glu), and Arg-16 (or Lys). Several other positions are also consistently occupied by residues with similar properties, including hydrophobic residues at positions 1, 2, 10, 12, and 15 and small residues at positions 3, 4, and 5 (He et al., 2006). Arg-16 is essential, since several different proteins containing the DWD motif no longer bind DDB1 when Arg-16 is mutated to another residue. For example, DDB2 cannot bind DDB1 in a *X. pigmentosum* group E patient whose Arg-16 of DDB2 was mutated to His (Shiyanov et al., 1999). Similarly, mutation studies of WDR (WD40-repeat protein) 23, which is also known as DCAF11 (DDB1- and CUL4-associated factor 11), and H326 (DCAF8) have confirmed that Arg-16 in the DWD motif is necessary for binding DDB1 (Angers et al., 2006). Models of the structures of two DWD proteins, Hs CDT2 and CSA (for Cockayne syndrome protein A), showed that Arg-16 of the DWD motif is exposed on the surface of the bottom face of the β propeller and is thus available for interacting with DDB1 (Jin et al., 2006).

Proteins containing the conserved 16-residue DWD motif have been detected in many species, including human, mouse, *Drosophila*, *C. elegans*, *S. pombe*, *S. cerevisiae*, and *Arabidopsis*, suggesting that their role is highly conserved in eukaryotes (He et al., 2006). Their potential importance in plants is shown by the fact that several *Arabidopsis* proteins known to regulate various stages of development contain DWD motifs, including COP1, TGF-β RECEPTOR INTERACTING PROTEIN (TRIP-1), VERNALIZATION INDEPENDENCE3 (VIP3), FY (the homolog of yeast polyadenylation factor, Pfs2p), and PLEIOTROPIC REGULATORY LOCUS1 (PRL1) (Nemeth et al., 1998; Jiang and Clouse, 2001; Simpson et al., 2003; Zhang et al., 2003; Chen et al., 2006).

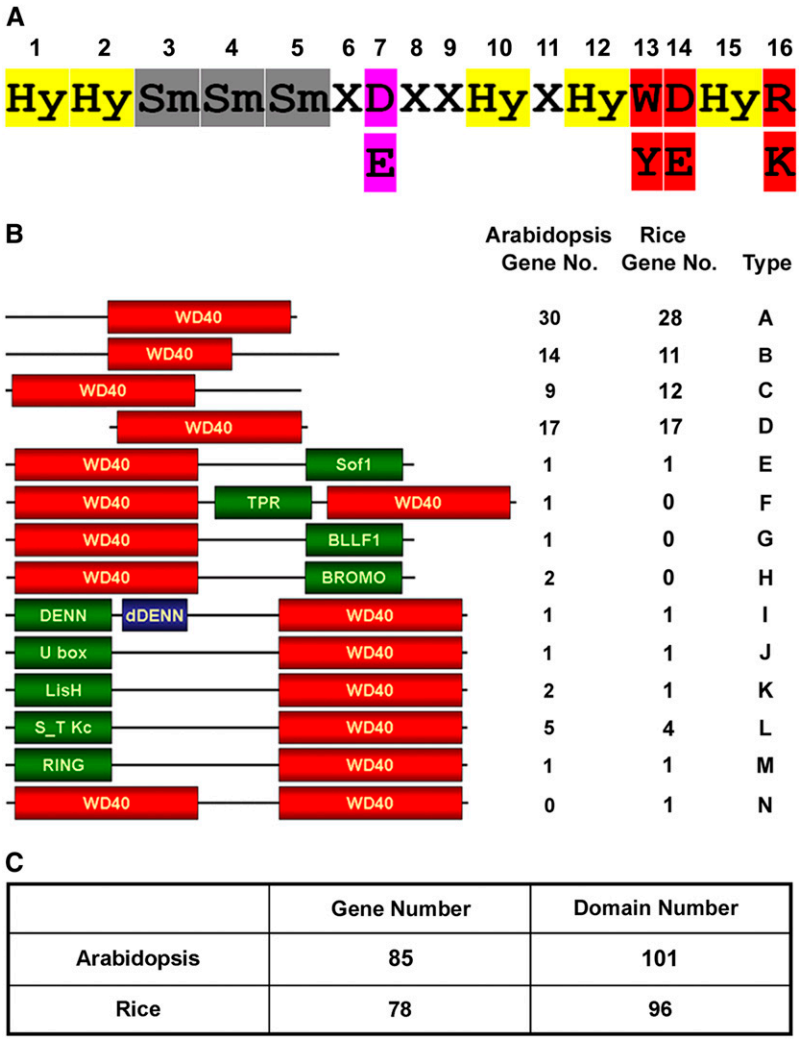
Here, we report the characterization of DWD proteins and their possible roles in CUL4-RING E3 ligases in *Arabidopsis* and rice. We also present evidence that the representative DWD protein PRL1 functions as the substrate receptor for the degradation of AKIN10 by a CUL4-DDB1–based E3 ligase in *Arabidopsis*.

RESULTS

Comparative Analysis of *Arabidopsis* and Rice DWD Proteins

To elucidate the possible roles of DWD proteins in plants, we searched the *Arabidopsis* and rice genomes for proteins containing this motif (Figure 1A). Since all DWD motifs are found within WD40 repeats, we first retrieved all annotated *Arabidopsis* WD40 proteins (Figure 1B) and then searched the WD40 repeats for the DWD motif manually and using the DWD sequence as the pattern input for the MyHits program (http://myhits.isb-sib.ch/cgi-bin/pattern_search; Pagni et al., 2004). A detailed description of our search is provided in Supplemental Methods online. In total, 85 putative DWD proteins with 101 DWD domains were found in *Arabidopsis* (Figure 1C; see Supplemental Figure 1 online). Sixty-nine had one DWD domain and 16 had two, consistent with the report that DWD proteins usually possess one and sometimes two but rarely three DWD motifs (He et al., 2006). In addition, 32 of these DWD proteins also had WD40 repeats that showed high similarity to the first 14 amino acids of the DWD motif but lacked the Arg/Lys at position 16 needed to bind DDB1 (see Supplemental Figure 1 online). We designated this additional sequence the DWD△R(16) domain. Next, we generated a phylogenetic tree for the *Arabidopsis* DWD motifs using ClustalW in MEGA3.1 (Kumar et al., 2004). To select representative proteins for further studies, the DWD phylogenetic tree was divided into subgroups A1, A2, B, C, and D (Figure 2A).

We also identified 78 putative DWD proteins and 96 DWD motifs in the *O. sativa* genome (see Supplemental Methods online for search details). Fourteen rice DWD proteins had two



	Gene Number	Domain Number
Arabidopsis	85	101
Rice	78	96

Figure 1. Core Sequence of the DWD Motif and the Structure of *Arabidopsis* DWDs.

(A) Conserved amino acids of DWD motifs in various DWD proteins. Hy, hydrophobic amino acids; Sm, small amino acids.
(B) Structure of DWD proteins in *Arabidopsis* and rice. Each type is labeled A to N corresponding to groups in Supplemental Figures 1 and 2 online. S_T Kc, catalytic domain of Ser/Thr protein kinase.
(C) Numbers of DWD proteins and DWD motifs in *Arabidopsis* and rice.

highly conserved DWD motifs, and two rice proteins had three DWD motifs. In addition, 41 rice DWD proteins also contained DWD△R(16) motifs adjacent to the DWD motifs (see Supplemental Figure 2 online). A phylogenetic tree of putative DWD motifs in rice was generated as in *Arabidopsis* and divided into subgroups E1, E2, E3, E4, F, and G (Figure 2B). We also searched for *Arabidopsis* homologs of each rice DWD protein using BLAST and found that for 58 rice DWD proteins, the *Arabidopsis* protein with the highest similarity (all had >33% identical and 45% similar amino acids) was also a DWD protein, showing that the DWD motif is highly conserved in plants (see Supplemental Table 1 online).

Fifteen *Arabidopsis* and nine rice DWD proteins contained additional conserved domains that might be involved in interacting with substrate proteins (Figure 1B). *Arabidopsis* At5g67320 and At1g73720 and rice Os07g22220 contain a LisH motif that aids microtubule dimerization and thus helps regulate microtubule dynamics (Emes and Ponting, 2001). *Arabidopsis* At4g28450 and rice Os01g13730 have Sof1 domains that are a component of the nucleolar rRNA processing machinery and are therefore essential for cell growth (Jansen et al., 1993). The TPR1 domain, which mediates protein–protein interaction, was detected in *Arabidopsis* At5g10940. Five *Arabidopsis* (At4g29380, At2g46340, At3g15354, At1g53090, and At4g11110) and four rice DWD proteins (Os01g52640, Os02g55340, Os05g49590, and Os11g10640) possess the catalytic domain of Ser/Thr protein kinases. Proteins containing two types of E2-dependent ubiquitination-related domains were detected: *Arabidopsis* At2g33340 and rice Os10g32880 contain the U-box domain, while *Arabidopsis* At2g32950 and rice

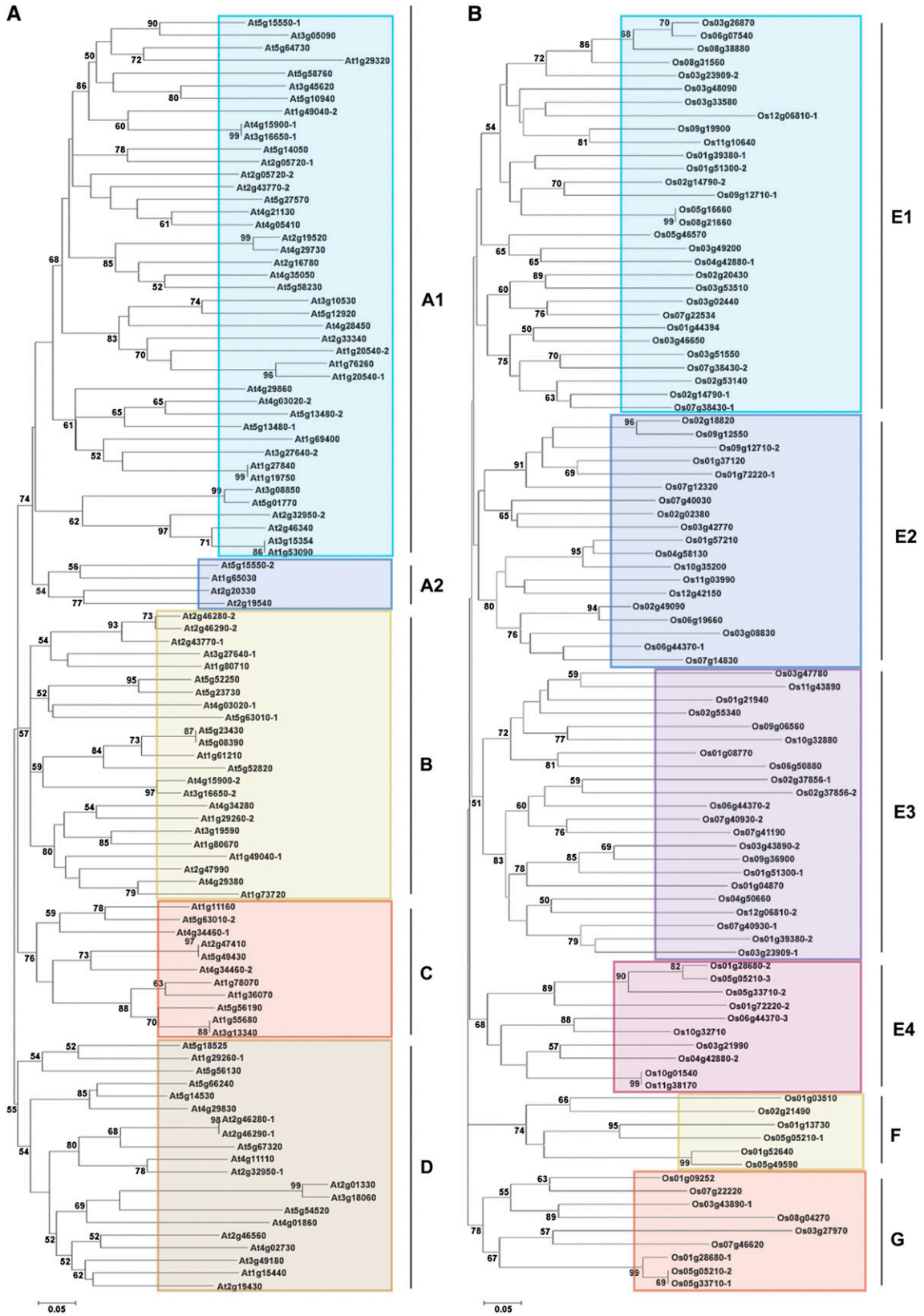


Figure 2. Phylogenetic Trees Based on DWD Motifs in *Arabidopsis* and Rice.

Os02g53140 contain the RING domain. *Arabidopsis* At1g49040 and rice Os01g39380 contain the DENN domain implicated in the regulation of mitogen-activated protein kinase signaling pathways (Levivier et al., 2001). *Arabidopsis* At2g47410 and At5g49430 have a BROMO domain that binds acetylated histones (Owen et al., 2000), and *Arabidopsis* At1g61210 contains the BLLF1 domain, which shows homology with the Epstein-Barr virus major glycoprotein, BLLF1 (Janz et al., 2000). Unlike human DWD proteins, no proteins containing F-box or SOCS domains were detected in either plant (He et al., 2006). The finding of these additional modules is consistent with the hypothesis that the DWD motif binds DDB1 while other portions of the protein may bind substrates. It is therefore surprising that more additional modules were not detected. This may indicate that many DWD proteins bind their substrates with motifs that have not yet been identified.

Selected Putative Plant DWD Proteins Can Interact with the DDB1 Protein

To investigate whether plant DWDs are the substrate receptors for the CUL4-DDB1 E3 ligase machinery, we first tested whether they can bind DDB1. Representative *Arabidopsis* proteins from each subgroup of the DWD phylogenetic tree were tested for their ability to bind DDB1a by yeast two-hybrid assay (Figure 3). Although there was some β-galactosidase activity with the empty vector, activity increased at least twofold when DWD proteins were used as prey. Furthermore, β-galactosidase activity was almost abolished when another negative control, green fluorescent protein (GFP), was used as prey. These interactions were confirmed by growth on Leu-deficient media (Figure 3). To test whether the DWD motif was needed to bind DDB1a, we mutated the Arg/Lys-16 of the two DWD motifs of At4g15900 (R290 and R332) to His. β-galactosidase activity using this mutant form as prey was threefold lower than the wild type and similar to that of the empty vector (Figure 3). These data indicate that the selected DWD proteins can bind DDB1 and that this binding requires the DWD motif.

To test whether DWD proteins bind DDB1 in planta, transgenic plants overexpressing FLAG-DDB1b in the background of *ddb1a* (FLAG-DDB1b/*ddb1a*) were prepared, and interactions between selected DWD proteins and DDB1b were assayed by coimmunoprecipitation with anti-FLAG antibodies (Figure 4A). DWD proteins PRL1 (At4g15900), TRIP-1 (At2g46280), and FY (At5g13480) were efficiently retrieved from the DDB1b immunocomplex together with CUL4, a positive control for DDB1 binding, whereas the negative control RPN6, 26S proteasome regulatory subunit 6 (At1g29150, subunit 9 of the 19S proteasome) was not. Moreover, PRL1 was efficiently retrieved from the CUL4 immunocomplex in FLAG-CUL4 transgenic lines, whereas RPN6 was not, showing that PRL1 associates with CUL4 in vivo (Figure 4B).

Since antibodies were not available for the other DWD proteins tested in Figure 3, we generated MYC-tagged constructs for the DWD proteins At3g45620, At4g35050, At1g65030, At2g19430, and At4g29830 and transformed them into FLAG-DDB1b/*ddb1a* lines. As shown in Figure 5, all of these proteins were coimmunoprecipitated with FLAG-DDB1b, with the exception of At1g04140, which has a motif similar to DWD except that it has Asn rather than Asp at position 14 (WNAK *cf* WDXR/K). These results confirm that DWD proteins identified by database analysis bind DDB1 in vivo and thus may help mediate interactions between the CUL4–DDB1 complex and its substrates. They also show that interactions between DWD proteins and DDB1 are dependent on the DWD motif.

prl1 and *cul4cs* Have a Stunted Phenotype as Adults and Small Cotyledons with Elevated Anthocyanin Levels as Seedlings

PRL1 is a WD40 protein that has been previously shown to control responses to glucose and several hormones (Nemeth et al., 1998; Smeekens, 2000; Rolland et al., 2006). Since PRL1 (At4g15900) binds DDB1 (Figures 3 and 4A) and CUL4 (Figure 4B), it seems possible that it may serve as a substrate receptor for the DDB1–CUL4 E3 ubiquitin ligase machinery. This hypothesis predicts that the absence of either PRL1 or CUL4 should have similar effects on PRL1-mediated signaling; therefore, we compared the development of *prl1* (Nemeth et al., 1998) and *cul4cs* (CUL4 cosuppression line), which has reduced levels of CUL4 (Chen et al., 2006). Both 3-d-old *cul4cs* and *prl1* seedlings had smaller cotyledons and more anthocyanin in the aerial parts than the wild type; however, the roots of *prl1* were shorter, whereas those of *cul4cs* were about the same length as the wild type (Figure 6A). Adult 5-week-old plants of *prl1* and *cul4cs* showed similar stunted phenotypes, including smaller leaves and siliques than the wild type (Figures 6B to 6D).

prl1 and *cul4cs* Are Hypersensitive to Sugar and Several Hormones and Show Transcriptional Derepression of Several Sugar-, Abscisic Acid-, and Cytokinin-Responsive Genes

To further test the hypothesis that PRL1 functions as a substrate receptor for a CUL4-based E3 ligase, we compared the effects of sucrose, glucose, abscisic acid (ABA), cytokinin, and ethylene on the growth of *cul4cs* and *prl1* seedlings. We confirmed that *prl1* is hypersensitive to these regulators, and *cul4cs* also showed hypersensitivity to many of these signaling molecules. For example, cotyledon growth of both mutants was more inhibited than that of the wild type by the addition of 6% sucrose, 4% glucose, or 0.1 μM ABA (Figures 7A, 7B, and 7D). Similarly, root growth of both mutants was also more strongly inhibited than

Figure 2. (continued).

(A) A phylogenetic tree constructed from the 101 *Arabidopsis* DWD motifs and divided into subgroups A1, A2, B, C, and D. Numbers indicate percentage values after 1000 replications; values below 50% are not shown. The bar represents the amino acid substitutions per site for a unit branch length.
(B) Phylogenetic tree generated with the 96 rice DWD motifs divided into subgroups E1, E2, E3, E4, F, and G. Gene names are from The Institute for Genomic Research (TIGR) Rice Genome Annotation.

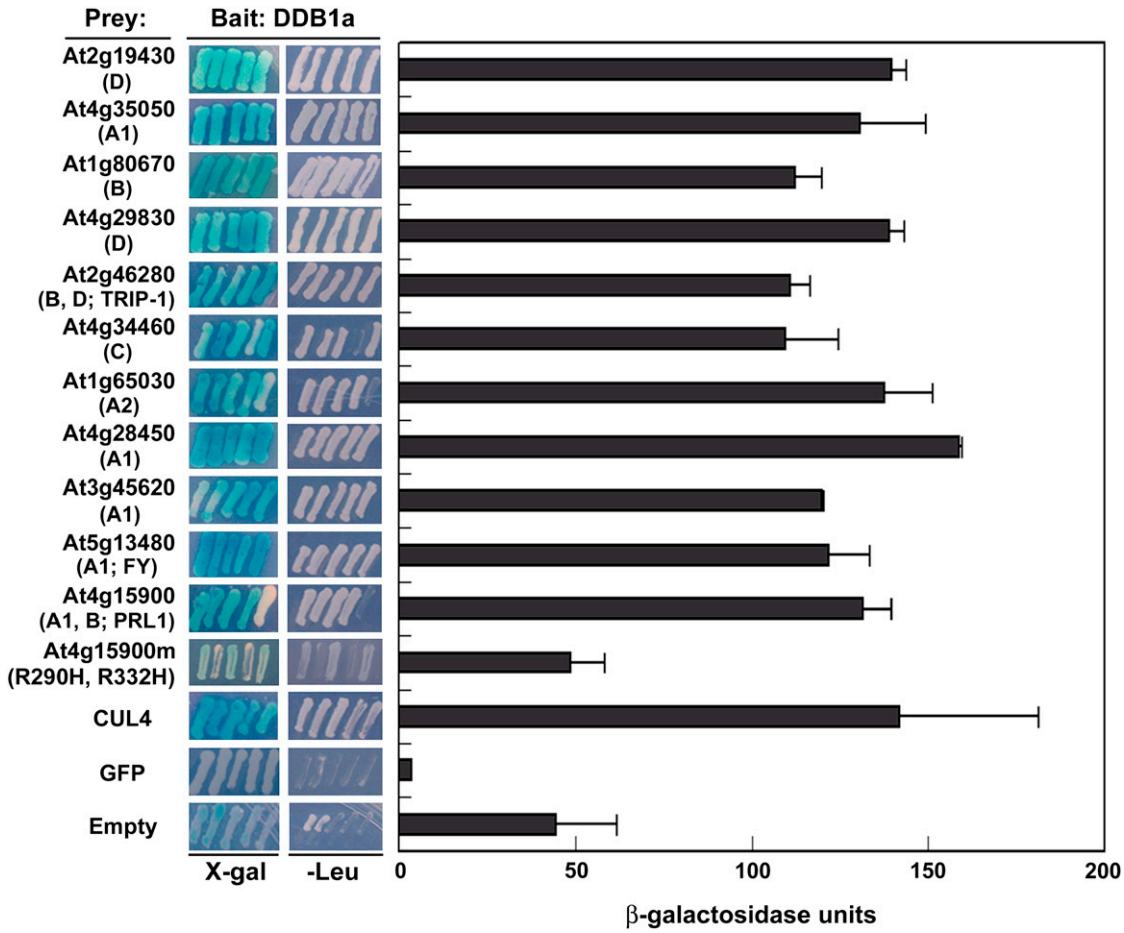


Figure 3. Interactions between DDB1a and *Arabidopsis* DWD Proteins Detected by Yeast Two-Hybrid Assays.

Growth of yeast strains harboring the indicated DWD protein as prey and DDB1a as bait on X-gal and plates lacking leucine is shown, together with their β-galactosidase activity. CUL4 was used as a positive control since it is known to bind DDB1. Empty vector and GFP proteins were used as negative controls. Values are means ± SD ($n = 3$). At4g15900m is an At4g15900 mutant whose Arg residues at positions 290 and 332 were changed into His residues.

that of the wild type by these three treatments and by 0.5 μM kinetin (Figure 7F). However, unlike *prl1*, we did not detect any differences between wild-type and *cul4cs* seedlings treated with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Figures 7E and 7G). For all treatments, the response of *cul4cs* was less pronounced than that of *prl1*, so this lack of response may indicate that the threshold for an effect had not been attained due to the weak nature of the *cul4cs* line. Alternatively, it may indicate that PRL1 is involved in ethylene signaling via an alternative mechanism that does not involve CUL4.

Since it was previously reported that genes regulated by sucrose, ABA, and cytokinin are transcriptionally derepressed in *prl1* mutants (Nemeth et al., 1998), we next compared the expression of several sugar-, ABA-, and cytokinin-responsive genes in 3-d-old *cul4cs*, *prl1*, and wild-type seedlings under normal growth conditions (Figure 8). We detected more signal for chalcone synthase (*CHS*), a glucose-inducible gene, in *prl1* and *cul4cs* compared with the wild type, consistent with the marked accumulation of anthocyanins in these mutants. Similarly, we detected more signal for sucrose synthase (*ASUS1*), alcohol dehydrogenase (*ADH*), and ribulose-1,5-bis-phosphate carboxylase/oxygenase small subunit (*RBCS*) in these mutants, in keeping with their hypersensitivity to sugar, ABA, and cytokinin. By contrast, more signal for ACC oxidase 2 (*ACO2*) was only detected in *prl1* seedlings, consistent with the lack of any visible differences between wild-type and *cul4cs* seedlings treated with ACC.

PRL1 Protein Levels Are Lower in *cul4cs* Than in the Wild Type

Next, we checked the amounts of PRL1 and CUL4 proteins in 3-d-old *prl1* and *cul4cs* seedlings and confirmed that *prl1* mutants had less PRL1 and *cul4cs* had less CUL4 than the wild type (Figure 9A). Unexpectedly, there was less PRL1 in *cul4cs* than in the wild type, whereas the level of CUL4 in *prl1* was normal. We therefore checked *PRL1* gene expression in *cul4cs* and did not detect any differences in amounts of *PRL1* mRNA in *cul4cs* and the wild type (Figure 9B). This result shows that the reduced level of PRL1

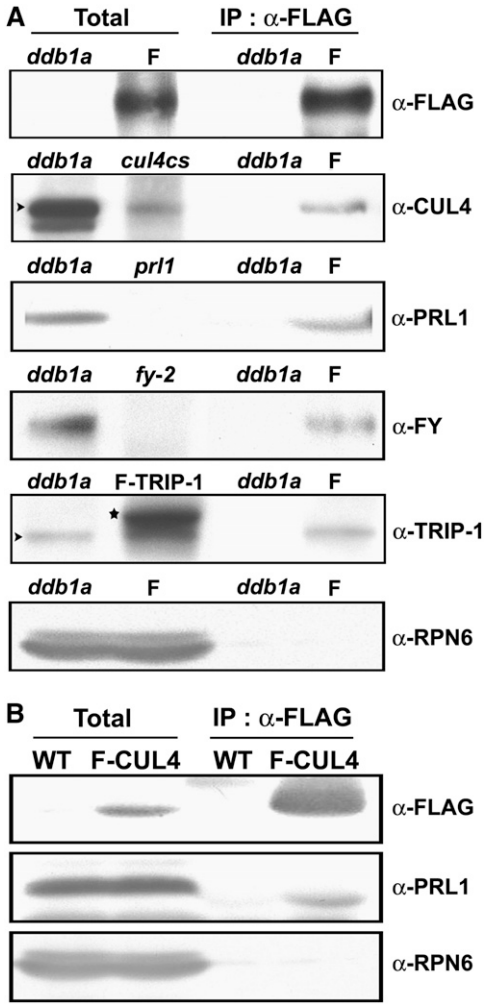


Figure 4. In Vivo Interaction between DDB1b and DWD Proteins and between CUL4 and PRL1.

(A) In vivo interaction of DDB1b with DWD proteins. Total protein (left two lanes) or extracts immunoprecipitated for FLAG (right two lanes) from transgenic plants overexpressing FLAG-DDB1b detected with antibodies to the indicated DWD proteins (right labels). Total, 5% of the crude extract used for coimmunoprecipitation assays; F, FLAG-DDB1b/*ddb1a* transgenic plants; F-TRIP-1, FLAG-TRIP-1 trangenic plants. The arrowhead on the α-CUL4 panel represents the position of the CUL4 protein. The asterisk and arrowhead on the anti-TRIP-1 panel indicate the positions of FLAG-TRIP-1 and endogenous TRIP-1 protein, respectively. The immunoblot using anti-RPN6 was used as a negative control.

(B) In vivo interaction of CUL4 with PRL1. As above, total protein (left two lanes) or extracts immunoprecipitated for FLAG (right two lanes) from transgenic plants overexpressing FLAG-CUL4 detected with antibodies to the indicated DWD proteins (right labels). Total, 5% of the crude extract used for coimmunoprecipitation assays; F-CUL4, FLAG-CUL4 trangenic plants. The immunoblot using anti-RPN6 was used as a negative control.

protein in *cul4cs* occurs posttranscriptionally and suggests that the maintenance of PRL1 proteins may depend on CUL4. As shown in Figures 7A and 7B, the phenotypic difference between the wild type and *cul4cs* was alleviated in 7-d-old seedlings compared with 3-d-old seedlings (Figure 6A), although cotyledon growth was still reduced in *cul4cs*. To check if this alleviation was associated with accumulation of PRL1, we monitored the level of PRL1 in 7-d-old *cul4cs* seedlings and found that PRL1 expression is comparable in 3- and 7-d-old *cul4cs* seedlings (Figure 9C). Therefore, the phenotypic differences between wild-type and *cul4cs* 3-d-old seedlings might reflect effects of reduced CUL4 expression unrelated to the decreased level of PRL1 protein. They might indicate that the action of the CUL4-DDB1-PRL1 complex is more effective in early stages of seedling development.

PRL1 and CUL4 Are Necessary for Degradation of AKIN10 (SnRK1)

Previous work using yeast two-hybrid assays has shown that PRL1 binds *Arabidopsis* AKIN10 and AKIN11, two members of the SnRK1 (for Snf1-related protein kinase 1) family of protein kinases involved in metabolic signaling and that PRL1 inhibits their protein kinase activity in vitro (Bhalerao et al., 1999). Moreover, it was subsequently shown that AKIN10 and AKIN11 interact with SKP1/ASK1 (for S-phase Kinase-associated Protein 1/*Arabidopsis* SKP1-like 1), a component of the SCF E3 ligase complex and that this interaction was inhibited by PRL1 (Farras et al., 2001). PRL1 was therefore proposed to compete with SKP1 for binding to the C-terminal regulatory domain of AKIN10 and AKIN11. However, since PRL1 is a DWD protein shown to bind both DDB1 (Figure 4) and AKIN10 and AKIN11 (Bhalerao et al.,

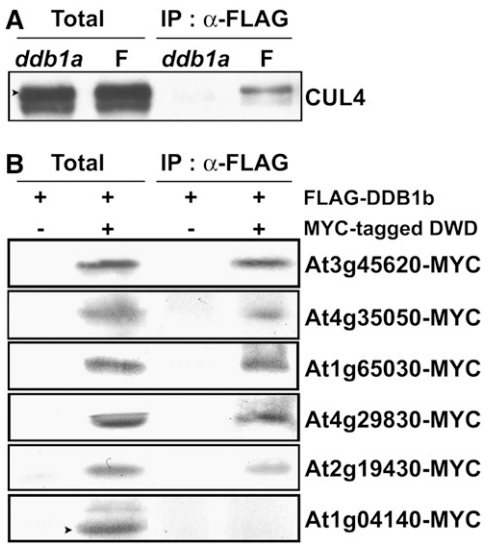


Figure 5. Coimmunoprecipitation of DDB1b and *Arabidopsis* DWDs in FLAG-DDB1b/*ddb1a*/MYC-DWD Transgenic Plants.

(A) Coimmunoprecipitation of DDB1b and CUL4 in FLAG-DDB1b/*ddb1a* transgenic plants. Arrowhead indicates CUL4 protein. Total, 5% of the crude extract used for coimmunoprecipitation assays; F, FLAG-DDB1b/*ddb1a* transgenic plants.

(B) Coimmunoprecipitation of DDB1b and *Arabidopsis* DWDs in FLAG-DDB1b/*ddb1a*/MYC-DWD transgenic plants. Arrowhead on At1g04140-MYC panel indicates the position of At1g04140-MYC. Total, 5% of the crude extract used for coimmunoprecipitation assays.

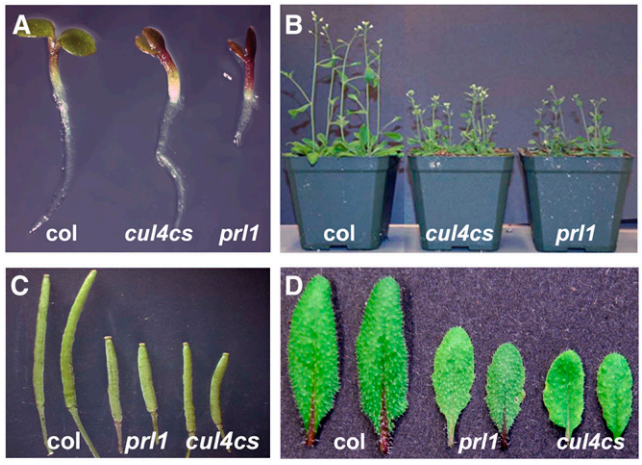


Figure 6. Phenotypic Comparison of *cul4cs* and *prl1* at the Seedling and Adult Stages.

(A) Three-day-old light-grown wild-type (Columbia [col]), *cul4cs*, and *prl1* seedlings.
(B) Five-week-old wild-type, *cul4cs*, and *prl1*.
(C) Wild-type, *prl1*, and *cul4cs* siliques.
(D) Rosette leaves from wild-type, *prl1*, and *cul4cs* plants.

1999), an alternative hypothesis is that PRL1 might regulate the amount of AKIN10 and AKIN11 by tagging them for destruction via the DDB1–CUL4 ubiquitin ligase machinery.

Therefore, a cell-free protein degradation assay was performed to examine whether AKIN10 stability was affected by CUL4 and PRL1 levels (Figures 10A and 10B). His_6-AKIN10 was gradually degraded when incubated with total protein extracts of wild-type plants. However, this degradation was blocked by MG132, a proteasome-specific inhibitor, indicating that AKIN10 was destroyed via the 26S proteasome pathway (Figure 10A). By contrast, His_6-AKIN10 was degraded more slowly in extracts from *prl1* and *cul4cs* plants (Figure 10B). To confirm that AKIN10 was more stable in *prl1* and *cul4cs* mutants, the endogenous levels of AKIN10 protein were checked in extracts of 3-d-old wild-type, *cul4cs*, and *prl1* using AKIN10-specific antibody (Baena-Gonzalez et al., 2007). As shown in Figure 10C, AKIN10 levels in the wild type were lower than those in *cul4cs* and *prl1* seedlings. Taken together, these data support a conclusion that AKIN10 degradation depends on PRL1 and CUL4 and that AKIN10 degradation is mediated by a CUL4-based E3 ligase that uses PRL1 as a substrate receptor.

DISCUSSION

Plants Have Many Proteins That Contain a Highly Conserved 16–Amino Acid DWD Motif That Binds DDB1

Ubiquitination of specific proteins is essential for selective protein degradation, which enables cells to adjust appropriately to a variety of signals and eliminate proteins that are no longer needed or deleterious (Pintard et al., 2004). Specific ubiquitination in plants is primarily regulated by E3 ubiquitin ligases based on Cullins 1, 2, 3, or 4, and all four have been linked with many cellular processes. Unlike the other three cullins, the substrate-recruiting mechanism for Cullin 4 is poorly understood. Several recent papers indicate that in mammals, proteins containing a conserved 16–amino acid motif variously known as DWD, WDXR, DCAF, or CDW may be the substrate receptors for CUL4-based E3 ligases (Angers et al., 2006; Higa et al., 2006a; He et al., 2006; Jin et al., 2006). However, the substrate receptors for CUL4-based E3 ligases in plants remained unknown. In this study, we provide evidence that proteins containing DWD motifs function as substrate receptors for CUL4-based E3 ligases in plants.

We identified 85 *Arabidopsis* and 78 rice DWD proteins by searching each database for proteins containing the conserved 16–amino acid DWD motif that we initially defined based on bioinformatics analysis and computer modeling (He et al., 2006). Although several variants of this motif have been described, we chose this sequence because it contains the highly conserved WDXR submotif necessary for binding to CUL4-DDB1 in mammals (Angers et al., 2006; He et al., 2006; Higa et al., 2006a; Jin et al., 2006). All 11 proteins that we tested that contained this conserved motif bound DDB1 in yeast two hybrid or coimmunoprecipitation assays (Figures 3, 4A, and 5). By contrast, when the Arg residues at position 16 in the two DWD motifs in PRL1 (At4g15900) were mutated to His, it no longer bound DDB1, consistent with reports that mutating Arg-16 in mammalian DWD proteins abrogated binding to DDB1 (Angers et al., 2006; He et al., 2006; Jin et al., 2006). Moreover, At1g04140, a protein that contains a motif similar to DWD but lacks the Asp at position 14, also did not interact with DDB1 (Figure 5). We are therefore confident that the amino acid sequence we used defines the functional DDB1 binding motif and that all DWD proteins identified in this study are likely to bind the CUL4-DDB1 complex. However, additional proteins may also bind this complex, by analogy with studies in mammalian cells that identified a subset of DDB1 binding proteins lacking the canonical DWD motif (Angers et al., 2006; He et al., 2006; Higa et al., 2006a; Jin et al., 2006). Therefore, our inventory of DWD proteins probably underestimates the true number of CUL4-DDB1 binding proteins but illustrates that there are many proteins potentially capable of serving as the substrate receptors for CUL4-based ubiquitin ligases.

More than 75% of both the *Arabidopsis* and rice DWD proteins only contained a single DWD motif, suggesting that one copy is sufficient to bind DDB1 (Figure 1). However, some proteins had two copies, and two rice proteins had three. These additional DWD motifs may enhance DDB1 binding or may interact with other proteins. In addition, 32 of the 85 *Arabidopsis* DWD proteins and 41 of the 78 rice DWD proteins had a DWD△R(16) motif adjacent to the DWD motif (see Supplemental Figures 1 and 2 online). This motif is very similar to the DWD motif, but it lacks the Arg at position 16, and proteins containing only this motif cannot bind DDB1 (Figure 3). We were not able to define the role of this motif in this study; however, its prevalence suggests it serves an important function (see Supplemental Figures 1 and 2 online).

We divided the *Arabidopsis* and rice DWD proteins into several subfamilies based on variation in the DWD sequence (Figures 2A and 2B). All representatives of each *Arabidopsis* subfamily tested bound DDB1, showing that these differences in sequence did not

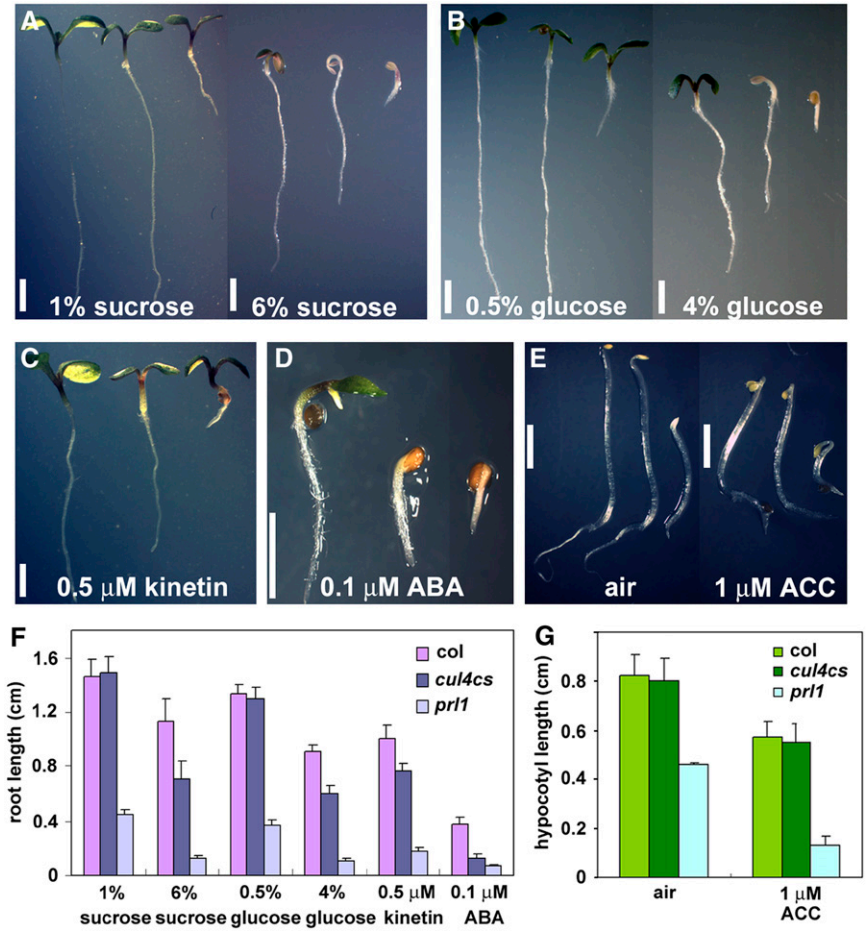


Figure 7. Phenotypes of Wild-Type, *cul4cs*, and *prl1* Seedlings Treated with Sucrose, Glucose, and Various Hormones.

(A) Effects of 6% sucrose on wild-type, *cul4cs*, and *prl1* light-grown seedlings.
(B) Effects of 4% glucose on light-grown seedlings.
(C) Effects of 0.5 μM kinetin on light-grown seedlings.
(D) Effects of 0.1 μM ABA on light-grown seedlings.
(E) Effects of 1 μM ACC on dark-grown seedlings. Bars = 2 mm in **(A)** to **(E)**.
(F) Root lengths of wild-type, *cul4cs*, and *prl1* seedlings after treatment with sucrose, glucose, cytokinin, or ABA. Values are means ± SD ($n = 20$).
(G) Hypocotyl lengths of wild-type, *cul4cs*, and *prl1* seedlings after treatment with ACC. Values are means ± SD ($n = 20$).

abrogate DDB1 binding (Figures 3, 4A, and 5). However, they might modulate the affinity of the DWD proteins for DDB1 or other proteins.

We identified *Arabidopsis* homologs of nearly all of the rice DWD proteins using BLAST, and for >74%, the most similar *Arabidopsis* counterpart was also a DWD protein (see Supplemental Table 1 online). Although we do not have functional studies showing that they are orthologous, their prevalence suggests that DWD proteins may use similar mechanisms (targeting specific substrates to DDB1-CUL-ROC1 E3 ligase) to perform their physiological functions in the two plants. In several cases, two rice proteins shared the same *Arabidopsis* relative, and the converse was also found, suggesting that the ancestral gene had been duplicated after their divergence. It is curious that there are fewer DWD proteins in rice than *Arabidopsis*, in contrast with BTB proteins where rice has nearly twice as many as *Arabidopsis* (Gingerich et al., 2007). This may indicate that after their divergence, rice expanded the BTB protein family to deal with new targets as the species evolved, whereas the DWD proteins and their targets have remained relatively static.

We found that four *Arabidopsis* DWD proteins that had been previously shown to regulate various developmental processes bound DDB1 in yeast two-hybrid and coimmunoprecipitation assays (Figures 3, 4A, and 5). All four had rice homologs that also contained DWD repeats, suggesting that they are conserved in the two species (see Supplemental Table 1 online). FY, the homolog of yeast polyadenylation factor, Pfs2p, has been proposed to cooperate with FCA (for Flowering time Control protein A) to repress FLC (for Flowering Locus C) by regulating the processing of the 3′ end of the *FLC* mRNA (Simpson et al., 2003;

Henderson et al., 2005). However, FY may also act independently of FCA since silencing the *FY* gene in *Nicotiana tabacum* caused abnormal growth, whereas silencing the *FCA* gene did not (Henderson et al., 2005). Thus, although FY has been proposed to mediate RNA 3′-end processing, based on its interactions with

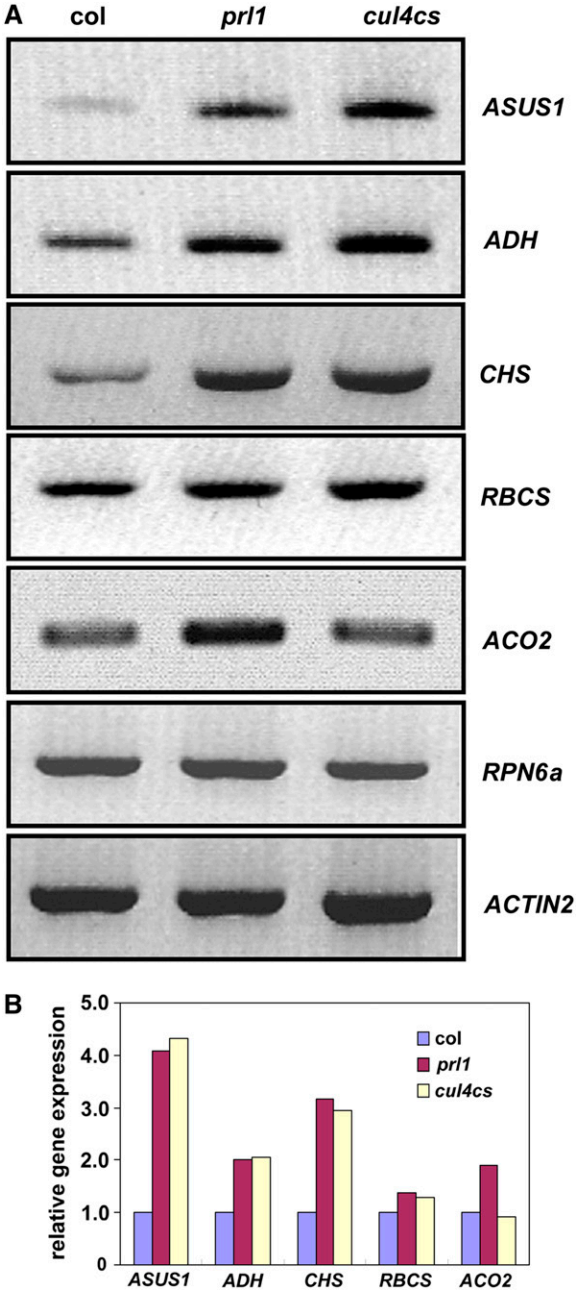


Figure 8. Transcriptional Derepression of Various Sugar-, Cytokinin-, and ABA-Responsive Genes in *prl1* and *cul4cs*.

(A) Expression of various sugar-, cytokinin-, and ABA-responsive genes in the wild type, *prl1*, and *cul4cs*.
(B) Relative expression of various sugar-, cytokinin-, and ABA-responsive genes in the wild type, *prl1*, and *cul4cs*.

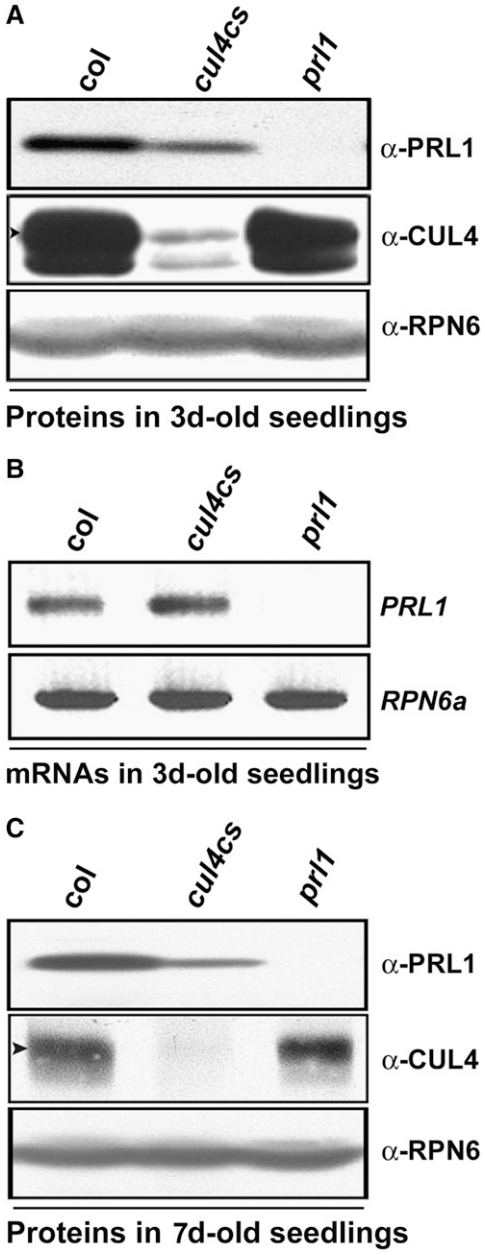


Figure 9. Comparison of PRL1 Protein and mRNA Levels in Wild-Type, *cul4cs*, and *prl1* Seedlings.

Total proteins (**[A]** and **[C]**) and RNA **(B)** were extracted from wild-type, *cul4cs*, and *prl1* seedlings. In **(B)**, total RNA was reverse-transcribed, and then PCR was performed with specific forward and reverse primers for *PRL1*. Anti-RPN6 and RT-PCR with *RPN6a*-specific primers were used as controls for equivalent loading for the protein and RT-PCR gels, respectively.
(A) PRL1 and CUL4 protein levels in 3-d-old wild-type, *cul4cs*, and *prl1* seedlings.
(B) *PRL1* mRNA levels in 3-d-old wild-type, *cul4cs*, and *prl1* seedlings.
(C) PRL1 and CUL4 protein levels in 7-d-old wild-type, *cul4cs*, and *prl1* seedlings.

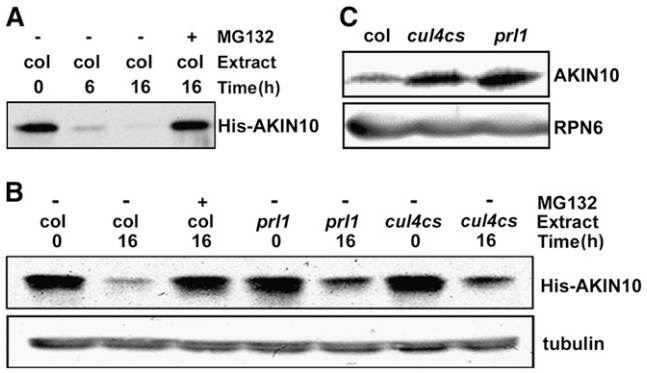


Figure 10. Degradation of His_6-Tagged AKIN10 in Cell Extracts of Wild-Type, *cul4cs*, and *prl1* Seedlings and the Expression Level of AKIN10 Protein in Wild-Type, *cul4cs*, and *prl1* Seedlings.

(A) Effect of MG132 on cell-free degradation of AKIN10. His_6-tagged AKIN10 protein was incubated in extracts (20 μg) of 3-d-old wild-type seedlings in the presence or absence of 10 μM MG132 for the indicated times at 30°C. His_6-tagged AKIN10 protein levels were determined by protein gel blotting with anti-His antibody.

(B) AKIN10 protein degradation in extracts of *prl1*, *cul4cs*, and wild-type seedlings. His_6-tagged AKIN10 proteins were incubated in extracts (20 μg) of 3-d-old *prl1*, *cul4cs*, and wild-type seedlings. His_6-tagged AKIN10 protein levels were determined by protein gel blotting with anti-His antibody. Equivalence of loaded extracts was determined by tubulin protein levels.

(C) The expression level of AKIN10 protein in 3-d-old wild-type, *cul4cs*, and *prl1* light-grown seedlings. Equivalence of loaded extracts was determined by RPN6 protein levels.

DDB1, it may also help target proteins for ubiquitination by the CUL4 complex (Figure 4A). VERNALIZATION INDEPENDENCE3 (VIP3) was previously known as a negative regulator of flowering (Zhang et al., 2003). However, *vip3* mutants had many developmental defects unrelated to flowering time, including smaller rosette leaves, stunted growth, and altered floral morphology (Zhang et al., 2003). Since VIP3 associates with DDB1 and *vip3* and *cul4cs* mutants have similar phenotypes, VIP3 may act in concert with the CUL4-DDB1 complex to regulate these processes (Zhang et al., 2003; Figure 5). TRIP-1 is proposed to regulate brassinosteroid-responsive genes *in Arabidopsis* by binding target proteins with its WD40 domain after phosphorylation by the BRASSINOSTEROID INSENSITIVE1 receptor Ser/Thr kinase (Jiang and Clouse, 2001; Ehsan et al., 2005). Thus, the interaction between DDB1 and TRIP-1 shown in our study (Figure 4A) suggests that the CUL4-based complex may help control brassinosteroid-responsive genes.

Many other plant proteins previously shown to affect various cellular processes also contain DWD motifs. For example, COP1 and the SPA (for Suppressor of *phytochrome A-105*) proteins have been implicated in the repression of photomorphogenesis. COP1 (At2g32950 and Os02g53140) acts as an E3 ubiquitin ligase to repress light signaling by targeting photomorphogenesis-promoting transcription factors for destruction. Recent studies indicate that CUL4 acts together with RBX1 and the CDD complex (for COP10, DDB1, DET1) to positively regulate COP1 activity (Yanagawa et al., 2004; Chen et al., 2006). Our data suggest that this interaction may be mediated by the DWD motif. In addition, all four *Arabidopsis* SPA proteins (At2g46340, At4g11110, At3g15354, and At1g53090) and rice SPA1 (Os05g49590) and SPA3 (Os01g52640) contain DWD motifs (see Supplemental Figures 1 and 2 and Supplemental Table 1 online). All four SPA proteins function in a COP1-containing complex to repress photomorphogenesis, and it has been reported that they can bind COP1 (Laubinger and Hoecker, 2003; Laubinger et al., 2004). Therefore, the presence of DWD motifs in SPA proteins suggests that they might bind the CUL4-based E3 complex to modulate COP1 activity.

Several other plant DWD proteins are homologous to mammalian proteins known to associate with DDB1-CUL4, including CDT2, WDR5, WDR12, WDR82, DDB2, and CSA. This suggests that these DWD proteins may perform similar functions in plant and animal cells. In addition, as described above, many plant DWD proteins contain additional motifs shown to mediate protein–protein interactions in other organisms and that may therefore help it bind its substrate (Figure 1B).

cul4cs and *prl1* Mutants Have Similar Phenotypes

To elucidate the roles of DWD proteins in plants, we chose PRL1 as a representative to study in greater detail. We found that PRL1 has two highly conserved DWD motifs and interacts with DDB1 in yeast two-hybrid and coimmunoprecipitation assays (see Supplemental Figure 1 online; Figures 3 and 4A). Moreover, adult *prl1* and *cul4cs* plants were much smaller than the wild type, and both seedlings showed similar sensitivity to various hormones and sugars, implying that PRL1 and CUL4 worked together in some signal transduction pathways (Figures 6 to 8).

However, there were also notable differences in the phenotypes of *cul4cs* and *prl1* mutants. For instance, although 3-d-old *cul4cs* and *prl1* seedlings had similar mutant shoot phenotypes, *prl1* roots were very short, whereas those of *cul4cs* were of normal length (Figure 6A). Similarly, *prl1* seedlings exhibited enhanced repression of hypocotyl elongation by ethylene, whereas *cul4cs* seedlings did not (Figures 7E and 7G). Moreover, more *ACO2* mRNA was detected in *prl1*, whereas it was comparable to the wild type in *cul4cs* (Figure 8).

This finding that reduction in PRL1 had a stronger effect than reduction in CUL4 is counterintuitive since CUL4 is a component of all CUL4-based E3 ligases, whereas PRL1 is presumably a component of only a fraction of those. One potential explanation for these differences between *cul4cs* and *prl1* is the weak nature of the *cul4cs* line. Under this hypothesis, the concentration of ACC used was not high enough to trigger an elevated response in *cul4cs*, by analogy with its response to sucrose and glucose where no major differences were observed between 7-d-old *cul4cs* and wild-type seedlings at low concentrations but *cul4cs* was significantly affected by 6% sucrose and 4% glucose (Figures 7A, 7B, and 7F). This may have occurred because enough CUL4 was present in *cul4cs* to cope with moderate but not with elevated levels of signaling molecules. An alternative is that PRL1 may be acting through some mechanism other than CUL4-DDB1 in ethylene signaling and perhaps other pathways. This is consistent with the findings that PRL1 interacted with many proteins in

yeast two-hybrid assays and inhibited the protein kinase activity of AKIN in vitro (Nemeth et al., 1998; Bhalerao et al., 1999).

CUL4 Influences the Level of PRL1

PRL1 levels were low in 3- and 7-d-old *cul4cs* seedlings (Figures 9A and 9C). Since PRL1 levels in *cul4cs* were regulated post-transcriptionally (Figure 9), the basal level of PRL1 in wild-type plants probably depends on the amount of CUL4, perhaps because PRL1 bound to CUL4 is protected from degradation. This suggests that an unidentified negative regulator targets excess PRL1 for destruction. The COP9 signalosome (CSN) is a candidate for this regulator since it has been reported to modulate the stability of various components of cullin complexes in mammals and in fission yeast (Wee et al., 2005; Cope and Deshaies, 2006; Denti et al., 2006). Moreover, CSN also helps modulate the stability of cullin proteins in plants (Wu et al., 2005; Chen et al., 2006; Gusmaroli et al., 2007). Accordingly, it will be of interest to determine whether CSN is also involved in regulating the stability of PRL1 in *Arabidopsis*.

PRL1 May Be the Substrate Receptor for a CUL4-Based E3 Ubiquitin Ligase

A potential reason for the pleiotropic effects of the *prl1* and *cul4cs* mutations is that PRL1 and CUL4 may modulate the activity of AKIN10 and AKIN11. PRL1 has previously been proposed to affect *Arabidopsis* development by inhibiting the protein kinase activity of AKIN10 and AKIN11 (Bhalerao et al., 1999; Farras et al., 2001). However, AKIN10 was degraded more slowly in extracts prepared from *prl1* or *cul4cs* cells, and AKIN10 levels in *cul4cs* and *prl1* were higher than those in the wild type (Figures 10B and 10C). This indicates that both PRL1 and CUL4 are needed for AKIN10 degradation, perhaps because PRL1 is delivering AKIN10 to the CUL4-DDB1 complex for degradation. Under this hypothesis, the growth reduction, accumulation of anthocyanin, and ABA, cytokinin, and sugar hypersensitivity exhibited by *cul4cs* and *prl1* mutants is largely due to increased AKIN10 and perhaps AKIN11 activity resulting from the absence of the PRL1-CUL4-DDB1 E3 ubiquitin ligase. However, we cannot exclude the possibility that other factors besides AKIN10 and AKIN11 are involved in phenotypes shared by *prl1* and *cul4cs* mutants, since PRL1 interacted with other proteins in addition to AKIN10 and AKIN11 in yeast two-hybrid assays (Nemeth et al., 1998).

The putative interaction between AKIN10 and the CUL4-based E3 ligase mediated by PRL1 is intriguing because it was previously reported that AKIN10 and AKIN11 could interact with SKP1/ASK1, a component of the SCF complex based on CUL1 (Farras et al., 2001). However, the AKINs were not ubiquitinated by the SCF E3 ligase but instead appeared to help SKP1/ASK1 associate other SCF ubiquitin ligase subunits into the 20S proteasomal complex (Farras et al., 2001). Nevertheless, since AKIN10 was degraded slowly in *prl1* and *cul4cs* extracts, we cannot exclude the possibility that AKIN10 levels are modulated by both the SCF complex and the CUL4-DDB1 machinery, by analogy with CDT1, whose degradation is mediated by either SCF^{SKP2} or CUL4-DDB1-CDT2 depending on the phase of the cell cycle (Kim and Kipreos, 2007).

DWD proteins play important roles in mammalian cells, including regulation of the cell cycle (Banks et al., 2006; Higa et al., 2006b; Jin et al., 2006; Ralph et al., 2006; Kim and Kipreos, 2007), chromatin remodeling (Higa and Zhang, 2007), and repair of UV-damaged DNA (Kapetanaki et al., 2006). By contrast, although several plant DWD proteins have been shown to play important roles, the functions of most are unknown. Characterization of the phenotypes of T-DNA insertion mutants in each *Arabidopsis* DWD gene will improve our understanding of the mode of action of CUL4-based ubiquitin ligases in selective protein degradation in plants.

METHODS

Plant Materials and Growth Conditions

The wild type, FLAG-CUL4, and FLAG-DDB1b/*ddb1a Arabidopsis thaliana* plants used in this study were of the Columbia-0 ecotype. FLAG-DDB1b transgenic lines were generated in the background of *ddb1a*. The *ddb1a* T-DNA mutant (SALK_041255) was obtained from the Arabidopsis Stock Center (http://www.arabidopsis.org/). Mutants and transgenic lines are as follows: *prl1* (Nemeth et al., 1998), *cul4cs* (Chen et al., 2006), *fy-2* (Henderson et al., 2005), and Flag-TRIP-1 (Ehsan et al., 2005). *Arabidopsis* seedlings were surface-sterilized with 75% ethanol and 0.02% Triton X-100 for 5 min, stored at 4°C for 3 to 5 d to break dormancy, and then plated on 1× Murashige and Skoog medium, including 1% sucrose and Gamborg's vitamins in continuous white light (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C. To obtain adult plants, 1-week-old seedlings were transferred to soil and grown under long-day conditions (16 h light/8 h dark) in a controlled-environment chamber at 22°C.

Phylogenetic Analysis

The 101 *Arabidopsis* and 96 rice (*Oryza sativa*) DWD motifs were aligned with ClustalW running in MEGA3.1 using the default settings (Gonnet matrix, gap opening penalty 10, gap extension penalty 0.1 for pairwise alignments and 10 and 0.2, respectively, for multiple alignments). A rooted phylogenetic tree based on the midpoint was generated using MEGA3.1 by the neighbor-joining method and the p-distance model (Kumar et al., 2004). Interior branch tests were conducted to assess statistical significance of the phylogenetic trees using 1000 replicates (Nei, 1996; Paoletti et al., 2007). Rice gene names are from the TIGR Rice Genome Annotation. For *Arabidopsis* genes with splice variants, only the representative gene model listed at The Arabidopsis Information Resource was used, and for rice genes with splice variants, only the variant listed as the ''Locus Name'' at TIGR was used.

RT-PCR

Total RNA was isolated from 3-d-old wild-type, *prl1*, and *cul4cs* seedlings and used for RT-PCR of each gene. Reverse transcription was performed on 5 μg of total RNA at 42°C with oligo(dT) primers using Superscript II reverse transcriptase (Invitrogen) in 20 μL. PCR was performed in a total volume of 25 μL containing 1 μL of the first-strand cDNA reaction products, 10 μM primers, 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxyribonucleotides, and 2.5 units of Taq polymerase (Qiagen) with specific forward and reverse primers for *ASUS1* (Martin et al., 1993), *ADH* (accession number M12196), *CHS* (Feinbaum and Ausubel, 1988), *RBCS* (accession number X13611), and *ACO2* (Raz and Ecker, 1999), respectively. Oligonucleotide sequences for the primer

pairs used are presented in Supplemental Table 2 online. Twenty-five thermal cycles were performed (30 s at 94°C, 30 s at 60°C, and 60 s at 72°C) in an automatic thermal cycler (Bio-Rad). PCR products were separated on 0.8% agarose gels, stained with ethidium bromide, and visualized under UV light. This assay was performed using RNA from at least two independent biological replicates. Extracts were normalized by the amounts of RT-PCR products with specific primers for *RPN6a* and *ACTIN2* genes. The values of amplicons obtained by RT-PCR analysis were determined with Image Gauge V3.12 software (Fuji). For normalization, the value for each amplicon was first divided by the value for the wild-type amplicon of that specific gene. This normalized value was then divided by the normalized value for the *ACTIN2* gene from that sample to correct for variations in amounts of cDNA.

Cell-Free Degradation Assay

Three-day-old light-grown seedlings were ground in liquid nitrogen and resuspended in a buffer (25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, and 10 mM NaCl) (Osterlund et al., 2000). Then, cell debris were removed by centrifugation. Purified recombinant His_6-AKIN10 (500 ng) and cell extracts (10 μg) were mixed in reaction buffer (25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 10 mM NaCl, and 10 mM ATP) and incubated at 30°C for the indicated times. The reactions were stopped by adding an equal volume of 2× protein gel-loading buffer. The samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with anti-His antibody (Santa Cruz Biotechnology). To confirm the equivalence of loaded extracts, tubulin levels in the cell extracts used for each reaction sample were detected with anti-tubulin antibody (Sigma-Aldrich).

Determination of Protein Concentrations

For immunoblot analysis, *Arabidopsis* tissues were homogenized in an extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 1× complete protease inhibitor [Roche]). The extracts were centrifuged twice at 13,000*g* for 10 min, and the protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad), and equal amounts of protein were loaded in each lane. All samples were resolved by SDS-PAGE and transferred to Immobilon membranes (Millipore). These were incubated in blocking buffer (1× PBS buffer including 0.1% Tween 20 and 5% dried nonfat milk) for 1 h at room temperature and then with the appropriate antibody overnight at 4°C and finally incubated another hour with secondary antibody with horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG. The proteins were visualized with film using the ECL Plus protein gel blotting detection system (Amersham) after extensive washing with washing solution, and their levels were estimated from the intensity of the bands.

Yeast Two-Hybrid Assay

The two-hybrid interaction assay in yeast was performed as described previously (Serino et al., 1999) except that the LexA fusion constructs and the activation domain fusion constructs were cotransformed into yeast strain EGY48 containing p80p-lacZ (Invitrogen). DDB1a was subcloned into pEG202 (Clontech) to make the bait construct, and fragments containing full-length cDNAs of various DWD genes and GFP were subcloned into pJG4-5 (Clontech). Transformants were confirmed by growth on SD/-His/-Trp/-Ura plates. Interactions between DDB1a and DWD proteins were monitored by measuring β-galactosidase enzyme activity after growing yeast strains in liquid culture using *o*-nitrophenyl-β-D-galactopyranoside as substrate according to the manufacturer's instructions (Clontech) and confirmed by growth on plates without leucine. Site-directed mutagenesis of At4g15900 (At4g15900m) was performed using the QuikChange Multi site-directed mutagenesis kit (Stratagene).

In Vivo Coimmunoprecipitation Assay

Arabidopsis tissues were ground in liquid nitrogen and then homogenized in protein extraction buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 0.05% Nonidet P-40, 2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1× complete cocktail of protease inhibitors [Roche]). The extracts were centrifuged twice at 13,000*g* for 10 min, and the protein concentration in the supernatant was determined by Bradford assay (Bio-Rad). For coimmunoprecipitation, 5 mg of total proteins were incubated for 4 h at 4°C with 30 μL of monoclonal anti-FLAG antibody immobilized onto agarose beads (Sigma-Aldrich). The precipitated samples were washed three times with the protein extraction buffer and then eluted twice with 0.1 M glycine, pH 2.8. Subsequent immunoblot assays were performed with anti-MYC or various anti-DWD antibodies.

Construction and Expression of His_6-AKIN10 Recombinant Protein

The full-length pAKIN10 cDNA clone was introduced into plasmid pET-28c (+) (Novagen) to generate His_6-AKIN10 fusion protein. The resulting plasmid was expressed in vitro using the EasyXpress protein synthesis kit (Qiagen). The fusion protein was purified by affinity chromatography using Ni^+-NTA resin according to the manufacturer's instructions (Invitrogen).

Generation of the *35S:DWD-MYC* Construct and Transformation into FLAG-DDB1b/*ddb1a* Transgenic Plants

Full-length cDNAs of various DWD genes were inserted into the corresponding sites of pMYC-fused pCR-BluntII vector. Then, *Bgl*II or *Xho*I fragments containing the inserted cDNA were subcloned into the binary vectors pJIM19 (gentamycin), pJIM19 (basta), or pCAMBIA 1390 (hygromycin), placing the DWD gene under the control of the cauliflower mosaic virus 35S promoter. The fusion gene constructs were transformed into *Agrobacterium tumefaciens* strain GB3101 by electroporation, and the resulting *Agrobacterium* strains were transformed into FLAG-DDB1b/*ddb1a* transgenic plants by the floral dip method (Clough and Bent, 1998). The seeds collected from the plants were selected on 1× Murashige and Skoog plates containing 200 mg L^{-1} gentamycin, 25 mg L^{-1} basta, or 50 mg L^{-1} hygromycin and 1% sucrose to obtain independent T1 transgenic lines. The presence of the transgene was confirmed by PCR with gene-specific primers.

Accession Numbers

The plant locus identifiers investigated in this article correspond to the Arabidopsis Genome Initiative codes for *Arabidopsis* and the Rice Annotation Project codes for rice: At5g46210 (*CUL4*), At1g29150 (*RPN6a*), At4g21100 (*DDB1b*), At4g05420 (*DDB1a*), At5g20830 (*ASUS1*), At5g13930 (*CHS*), At1g67090 (*RBCS*), At1g77120 (*ADH*), and At1g49240 (*ACTIN2*). Those without corresponding gene names are as follows: At3g27640 and Os03g49200 (CDT2 homologs), At4g02730 and Os07g38430 (WDR5 homologs), At5g15550 and Os07g40930 (WDR12 homologs), At5g66240 and Os07g40030 (WDR82 homologs), At5g58760 and Os01g04870 (DDB2 homologs), and At1g27840 and Os02g20430 (CSA homologs).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Alignment of DWD Motifs in *Arabidopsis*.

Supplemental Figure 2. Alignment of DWD Motifs in *O. sativa*.

Supplemental Figure 3. Phylogenetic Tree for All Rice and *Arabidopsis* DWD Proteins.

Supplemental Table 1. Rice DWD Proteins and Their Counterparts in *Arabidopsis*.

Supplemental Table 2. Oligonucleotide Sequences of the Primer Pairs Used in RT-PCR Experiments and to Confirm the Presence of the Transgene.

Supplemental Methods. Identifying *Arabidopsis* and Rice DWD Proteins.

Supplemental Data Set 1. Amino Acid Alignment Used to Construct the Phylogenetic Tree Presented in Figure 2.

Supplemental Data Set 2. Amino Acid Alignment Used to Construct the Phylogenetic Tree Presented in Supplemental Figure 3.

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