The *Arabidopsis* cullin AtCUL1 is modified by the ubiquitin-related protein RUB1

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The ubiquitin-like protein RUB1 is conjugated to target proteins by a mechanism similar to that of ubiquitin conjugation. Genetic studies in *Arabidopsis thaliana* have implicated the RUB-conjugation pathway in auxin response. The first step in the pathway is RUB activation by a bipartite enzyme composed of the AXR1 and ECR1 proteins. Ubiquitin activation is an ATP-dependent process that involves the formation of an AMP-ubiquitin intermediate. Here we show that RUB activation by AXR1-ECR1 also involves formation of an AMP-RUB intermediate and that this reaction is catalyzed by the ECR1 subunit alone. In addition, we identified an Arabidopsis protein called RCE1 that is a likely RUB-conjugating enzyme. RCE1 works together with AXR1-ECR1 to promote formation of a stable RUB conjugate with the Arabidopsis cullin AtCUL1 *in vitro*. Using a tagged version of RUB1, we show that this modification occurs *in vivo*. Because AtCUL1 is a component of the ubiquitin protein ligase SCF^{TIR1}, a complex that also functions in auxin response, we propose that RUB modification of AtCUL1 is important for auxin response.

The phytohormone auxin regulates a wide variety of cellular and physiological processes during plant development (1). Using a genetic approach in the model plant *Arabidopsis thaliana*, we have identified several genes that function in auxin action (2). Recessive mutations in one of these genes, called *AXR1,* result in a severe reduction in auxin response and diverse defects in growth and development (2). Recently, the molecular characterization of *AXR1* led to the proposal that auxin response involves the posttranslational modification of one or more proteins by an ubiquitin-related protein called RUB (3). The RUB family of proteins and their mammalian counterpart NEDD8 are ≈50–60% identical to ubiquitin. Studies in a number of species have shown that these proteins are conjugated to target proteins through the sequential action of RUB-activating and -conjugating enzymes in a manner that is similar to ubiquitin conjugation (4–9). The ubiquitin-activating enzyme (E1) is a single polypeptide of ≈110 kDa (10). In contrast, the RUB-activating enzyme consists of two smaller proteins corresponding to the N-terminal and C-terminal halves of E1. In Arabidopsis, these two subunits are AXR1 and ECR1 (9). ECR1 corresponds to the C-terminal half of the E1 enzyme and contains the cysteine that forms a thiolester bond with RUB1 whereas AXR1 is similar to the N-terminal half of E1 (9).

At present, the only known targets for RUB modification are members of the cullin protein family (5–7, 11). Cullins are subunits of E3 ubiquitin–protein ligase complexes called SCFs (for SKP1, CDC53 or cullin, F-box protein) (12). The function of the SCF is to facilitate transfer of ubiquitin from the ubiquitin-conjugating enzyme (E2) to the target. Unlike ubiquitin modification, conjugation of RUB to cullin does not appear to modify its metabolic stability. Rather, genetic experiments in *Saccharomyces cerevisiae* indicate that conjugation of Rub1p to the cullin Cdc53p affects the function of SCF^{CDC4}, an E3 that is required for degradation of the cell cycle regulator Sic1p (5).

Recent studies in our lab have demonstrated that an E3 complex called SCF^{TIR1} is required for auxin response in Arabidopsis. Mutations in *ASK1*, a *SKP1* ortholog, or *TIR1*, the F-box protein component of this SCF, result in reduced auxin response (13). These results, together with our studies of the *AXR1* gene, have led us to suggest that SCF^{TIR1} may be regulated by *AXR1*-dependent RUB modification. This hypothesis is supported by the finding that the *axr1* and *tir1* mutants display a synergistic interaction in the double mutant (14).

In this study, we continue our characterization of the RUB-conjugation pathway in Arabidopsis. We show that, like ubiquitin activation, RUB1 activation involves the formation of an adenylated intermediate. Surprisingly, this reaction is catalyzed *in vitro* by ECR1 in the absence of AXR1. We also report the identification of an Arabidopsis E2 protein, called RCE1 (RUB-conjugating enzyme) that forms a thiolester linkage with RUB1. Furthermore, we demonstrate that RUB1 is covalently attached to Arabidopsis AtCUL1 at a conserved lysine and that the AXR1-ECR1 and RCE1 enzymes can achieve this modification *in vitro*. Finally, we demonstrate that AtCUL1 is modified by RUB1 *in vivo*.

Experimental Procedures

Protein Expression and Purification. *AXR1* and *RCE1* cDNAs were cloned into the pQE expression plasmid by using the *Bam*HI-*Sac*I sites (Qiagen, Chatsworth, CA) and were transformed into the XL1-blue *Escherichia coli* strain. Both pQE-AXR1 and pQE-RCE1 cells were grown at 30°C to an OD at ≈1.2 and were induced with 1.5 mM IPTG for 4 hours at 30°C. These proteins were purified by using the His-6-tag kit from Qiagen, with neutralizing conditions as described in the manufacturer's manual. Proteins were liberated from the beads with 0.3 M imidazole in buffer T (50 mM Tris·HCl, pH 7.4/50 mM NaCl/0.1% Tween-20). The *ECR1* cDNA was cloned into the pQE vector, and the protein was expressed as described by del Pozo *et al.* (9). ECR1 was also expressed as a fusion protein with glutathione *S*-transferase (GST), using the plasmid pGEX2 (Amersham Pharmacia). The GST-ECR1 fusion protein was digested with thrombin to release ECR1. After purification, AXR1, ECR1, and RCE1 proteins were exchanged to buffer T by using Amicon-10 filters. The final concentration and purity of these proteins was determined by using the Micro BCA system (Pierce) and by Coomassie blue gel staining. Purified proteins were stored at −80°C in small aliquots.

The wheat UBA1 and Arabidopsis UBC1 proteins were expressed by using pET expression plasmids pET-UBA1 and pET-UBC1, respectively (15, 16) in XL1-Blue cells. Two hundred milliliters of culture cells were grown to OD at ≈0.8 and were induced with 1 mM IPTG for 4 hours. The cells were dissolved in 10 ml of buffer T plus 1 mM PMSF and were sonicated until lysis was complete.

Protein expression, purification, and labeling of RUB1 were

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Abbreviation: GST, glutathione *S*-transferase.

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performed as described in del Pozo *et al.* (9). Ubiquitin protein was also expressed as GST-UBQ and was purified and labeled as for RUB1. The concentrations of purified RUB1 and ubiquitin were determined by using the Micro BCA method.

Thiolester Reactions. Reactions were performed with total bacterial protein extracts containing recombinant proteins as described (9), except that ATP was removed from the sample by precipitation of proteins with (NH4)$_2$SO$_4$ at 100% of saturation. The pellet was dissolved in the same volume of buffer T. AXR1 (10 μl) and ECR1 (20 μl) extracts were used for 40-μl thiolester reactions. These reactions also contained 1.5 μM of ^{32}P-RUB1 and, unless otherwise specified, 5 mM ATP, 0.1 mM DTT, 10 mM MgCl$_2$, and 10 units/ml inorganic pyrophosphatase. When AMP-PNP was used, ATP was omitted from the reaction. The reactions were incubated for 20 min at room temperature and were analyzed as described (9).

AMP-RUB1 Formation. Two experiments were performed to establish the identity of the AMP-RUB1 intermediate. First, thiolester reactions were performed by using α-^{32}P-ATP or γ-^{32}P-ATP. These reactions contained 200 ng of purified AXR1 or ECR1, 2.5 μM cold RUB1, 1 mM ATP, 0.1 mM DTT, 10 mM MgCl$_2$, 10 units/ml inorganic pyrophosphatase, and 0.5 mM [α-^{32}P]ATP or [γ-^{32}P]ATP in 30 μl of buffer T. After 15 min of incubation at room temperature, the reaction was stopped with SDS/DTT loading buffer and was boiled for 15 min. The radioactive products were resolved on SDS/PAGE by using a 13% acrylamide gel.

In the second experiment, a reaction containing ECR1 (200 ng), 10 mM ATP, 0.1 mM DTT, 10 mM MgCl$_2$, 10 units/ml inorganic pyrophosphatase, and 2.5 μM ^{32}P-RUB1 in 80 μl of buffer T was incubated for 30 min at room temperature. To remove ATP from the reaction, proteins were precipitated with (NH4)$_2$SO$_4$ at 100% of saturation for 1 hour at 4°C. The sample was centrifuged at full speed in a microcentrifuge, and the pellet was dissolved in the same volume of buffer T. A 20-μl aliquot was incubated with or without 50 ng of H6-AXR1 protein, 0.1 mM DTT, 10 mM MgCl$_2$, and 5 units/ml inorganic pyrophosphatase. Half of the reaction was stopped with 4× SDS loading buffer and the other half with 4× SDS/DTT loading buffer and was boiled for 10 min. The radioactive samples were resolved on SDS/PAGE by using a 13% acrylamide gel.

Isolation of RCE1 and Formation of RUB1-RCE1 Thiolester. A search of the Arabidopsis database with the yeast Ubc12p sequence led to the identification of a genomic region (Z99708) and two expressed sequence tags with significant homology to Ubc12p. We used the expressed sequence tag cDNA (31B1T7) to screen a cDNA library prepared from etiolated seedlings (gift of J. Ecker, Univ. of Pennsylvania) as described by Ausubel *et al.* (17). Thiolester reactions containing purified AXR1 (50 ng), ECR1 (50 ng), RCE1 (30 ng), 5 mM ATP, 0.1 mM DTT, 10 mM MgCl$_2$, 10 units/ml inorganic pyrophosphatase, and 1.5 μM ^{32}P-RUB1 in 40 μl of buffer T were performed for 20 min at room temperature. Afterward, the reactions were stopped by adding 4× SDS loading buffer with or without DTT, and the products were resolved by SDS/PAGE using a 13% acrylamide gel.

Ubiquitin conjugation reactions contained 10 μl of *E. coli* extract prepared from UBA1-expressing cells and 10 ml of extract from UBC1-expressing cells or 30 ng of purified RCE1 in 40 μl of buffer T. These reactions also contained 3 mM ATP, 0.1 mM DTT, 10 mM MgCl$_2$, 10 units/ml inorganic pyrophosphatase, and 1.5 μM ^{32}P-ubiquitin. The reactions were performed at room temperature for 20 min and were stopped with 4× SDS loading buffer with or without DTT (400 mM). The products were separated by SDS/PAGE using a 13% acrylamide gel.

RUB1 Conjugation to AtCUL1. The AtCUL1 cDNA was cloned into pET30 vector (Novagen) in frame with the H6-tag sequence. The lysines at positions 692 and 722 were replaced with methionine (At-CUL1^{K692M} and At-CUL1^{K722M}) by using a mutagenesis kit from Stratagene. Coupled transcription/translation reactions of At-CUL1 and mutant cDNAs (1 μg each) were performed in the TNT-T7 coupled system (Promega) by using ^{35}S-methionine (ICN). After 120 min of translation at 30°C, the reactions were brought to 3 μM GST or GST-RUB1, 3 mM ATP, 0.1 mM DTT, 5 mM MgCl$_2$, and 10 units/ml of inorganic pyrophosphatase was added to the reaction. This mixture was incubated at 25°C for 30 additional min, and the reaction was stopped by addition of 4× SDS/DTT loading buffer and boiling for 10 min. Proteins were resolved on an SDS/PAGE/8.5% acrylamide gel, and the ^{35}S-products were detected by autoradiography.

To characterize RUB modification of AtCUL1 by Arabidopsis proteins, *in vitro* translated ^{35}S-H6-AtCUL1 was purified as described above for other H6-tagged proteins. Purified ^{35}S-H6-AtCUL1 was incubated with purified H6-AXR1 (50 ng), ECR1 (50 ng), H6-RCE1 (30 ng), 5 mM ATP, 0.1 mM DTT, 8 mM MgCl$_{2,}$ 3 μM GST-RUB1, and 10 units/ml of inorganic pyrophosphatase at room temperature for 30 min. The reactions were stopped with 4× SDS/DTT loading buffer and were boiled for 10 min. Proteins were resolved on an SDS/PAGE/8.5% acrylamide gel.

To determine whether RUB1 is attached to AtCUL1 *in vivo*, we cloned the RUB1 cDNA into the pET30a vector (Novagen) in frame with the 6× histidine and S-peptide. This construct (H6-S-RUB1) was cloned into the plant expression vector pROK2 (18). *Agrobacterium tumefaciens* harboring this construct were used to transform Arabidopsis ecotype Columbia with the vacuum infiltration method (19). Kanamycin-resistant T1 plants were selected by plating seeds on MS (Murashige and Skoos) medium supplemented with 1% sucrose and 50 μg/ml kanamycin. These plants were transferred to soil, and independent T2 homozygous lines were identified. Soluble protein from 5-day-old wild-type and H6-S-RUB1 transgenic seedlings (0.4 g of tissue) were extracted in 1 ml of buffer E [100 mM Tris·HCl, pH 7.5/400 mM (NH$_4$)$_2$SO$_4$/10 mM MgCl$_2$/1 mM EDTA/10% glycerol/1 mM PMSF/1× proteases inhibitor mixture (Boehringer Mannheim)] for 30 min at 4°C. The extracts were clarified by at centrifugation for 30 min at 4°C. The soluble fraction was precipitated with (NH$_4$)$_2$SO$_4$ at 50% of saturation. Proteins in the pellet were dissolved in 0.4 ml of binding buffer (50 mM NaPO$_4$, pH 8.0/400 mM NaCl/0.3% Triton X-100/1 mM PMFS/1× proteases inhibitor cocktail), and an aliquot (crude extract) was taken and mixed with SDS/loading buffer with 5% of β-mercaptoethanol. The extracts were incubated with prewashed nickel beads (Qiagen) in binding buffer for 3 hours at 4°C. Afterward, the beads were washed five times for 10 min each with 1.5 ml of washing buffer (50 mM NaPO$_4$, pH 6.0/400 mM NaCl/40 mM imidazole/0.3% Triton X-100/1 mM PMFS/1× proteases inhibitor cocktail). Proteins were liberated from the beads in binding buffer/1× SDS/loading buffer with β-mercaptoethanol and were boiled for 10 min.

Standard Molecular Biology Procedures. All standard molecular biology techniques were performed as described by Ausubel *et al.* (17). For Western blot analysis, proteins were resolved by SDS/PAGE using a 9% acrylamide gel and were transferred to nitrocellulose membrane (17). AtCUL1 antibody was affinity purified against bacterially expressed AtCUL1 bound to immobilon membrane (13) and used at 1:1,000 dilution. The S-peptide was detected by using the Novagen detection kit, following the manufacturer's instructions.

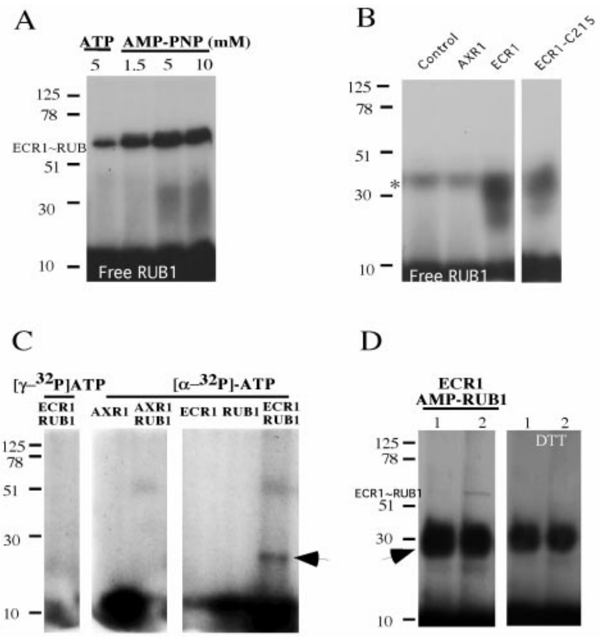


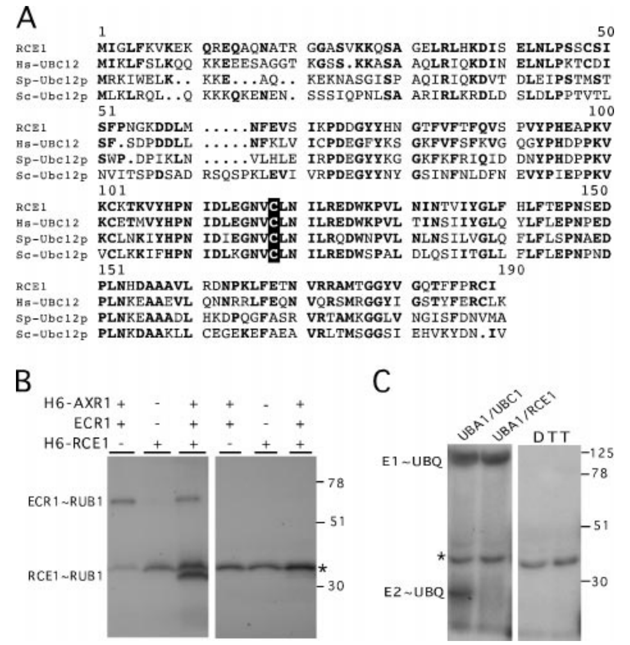
Fig. 1. RUB1-AMP is an intermediate product of RUB1 activation. (*A*) Thiolester reactions containing radiolabeled RUB1 and 5 mM ATP or different AMP-PNP concentrations were performed. These reactions contained protein extract prepared from bacteria expressing recombinant H6-AXR1 and H6-ECR1 (see *Experimental Procedures*). The reaction products were separated by SDS/PAGE in the absence of DTT. (*B*) Thiolester assay of ECR1 or AXR1 subunits. Radiolabeled RUB1 was used in thiolester reactions that contained 10 mM ATP and bacterial protein extract containing recombinant H6-AXR1, H6-ECR1, or H6-ECR1^{215A} proteins. The reactions were stopped with 4× SDS/DTT loading buffer. The DTT-resistant band at 25–35 kDa was formed when the reaction was performed with ECR1 or ECR1^{215A}. Asterisks indicate residual GST-^{32}P-RUB1 remaining after thrombin digestion. (*C*) Purified H6-AXR1 or ECR1 were incubated with [α-^{32}P]ATP or [γ-^{32}P]ATP and cold RUB1 protein. A DTT-resistant product at ≈25 kDa (arrow) was generated only when ECR1 was incubated with [α-^{32}P]ATP. The label at the bottom of the gel is unincorporated ATP. (*D*) AMP-^{32}P-RUB1 (arrow) was generated by incubation of ECR1 with ^{32}P-RUB1 and ATP. After removing the ATP by ammonium sulfate precipitation, either buffer (lane 1) or H6-AXR1 (lane 2) was added to the reactions.

Results

AMP-RUB1 Is an Intermediate Product of RUB1 Activation. Ubiquitin activation by the E1 enzyme is an ATP-dependent reaction that involves the formation of an AMP-ubiquitin intermediate noncovalently bound to the enzyme (10). Because E1 enzymes hydrolyze ATP between the α- and β-phosphoryl groups to form the AMP-ubiquitin intermediate, the ATP analogue AMP-PNP, which is β- and γ-nonhydrolysable, can substitute for ATP in the reaction (20). To investigate the possibility that AXR1-ECR1 activation of RUB1 has a similar mechanism, thiolester reactions containing AMP-PNP instead of ATP were performed (Fig. 1*A*). The formation of a DTT-sensitive ECR1-RUB1 conjugate under these conditions suggests that RUB1 activation proceeds through a RUB1-adenylate intermediate.

Previously, we reported that incubation of radiolabeled RUB1 with ATP, AXR1, and ECR1 resulted in the formation of a broad DTT-resistant band (≈25 kDa) migrating above free RUB1, in addition to the DTT-sensitive ECR1-RUB1 bond (9). The intensity of this 25-kDa broad band increased when higher concentrations of ATP were used in the reaction (data not shown). This ATP-dependence suggested to us that the broad band might be an adenylate-RUB1 intermediate generated during RUB1 activation. Because RUB activation is performed by a bipartite enzyme, we also wondered whether the ≈25-kDa species could be generated by AXR1 or ECR1 alone. To address these questions, radiolabeled RUB1 was incubated with AXR1 or ECR1 in thiolester reactions. Fig. 1*B* shows that formation of the RUB1-DTT-resistant species occurred with ECR1 alone but not AXR1, indicating that ECR1 is necessary and sufficient to bind RUB1 and ATP and generate this product. In addition, we tested the ability of the ECR1^{C215A} mutant to generate this species. In this mutant, the proposed active site cysteine is replaced by alanine, eliminating thiolester formation by the AXR1-ECR1 enzyme (9). In contrast, loss of this cysteine has no effect on formation of the 25-kDa product (Fig. 1*B*). To determine whether the ≈25-kDa species corresponded to AMP-RUB1, reactions with nonradiolabeled RUB1 and [α-^{32}P]ATP or [γ-^{32}P]ATP were performed. Purified AXR1 and ECR1 were used in these reactions to avoid background generated by bacterial proteins. Fig. 1*C* shows that ECR1 is able to form a DTT-resistant product at ≈25 kDa when the reaction is incubated with [α-^{32}P]ATP but not with [γ-^{32}P]ATP. AXR1 did not support the formation of this species. To address whether the generation of adenylate-α-^{32}P-RUB1 depended on ATP concentration, competition analysis with 15 mM nonradioactive ATP was performed. The addition of cold ATP dramatically reduced the formation of the 25-kDa DTT-resistant radiolabeled product (data not shown).

To further explore the role of ECR1 in activation, we set up



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A
             1                                                    50
RCE1         MIGLFKVKEK QREQAQNATR GGASVKKQSA GELRLHKDIS ELNLPSSCSI
Hs-UBC12     MIKLFSLKQQ KKEEESAGGT KGSS.KKASA AQLRIQKDIN ELNLPKTCDI
Sp-Ubc12p    MRKIWELK.. KKE...AQ.. KEKNASGISP AQIRIQKDVT DLEIPSTMST
Sc-Ubc12p    MLKLRQL..Q KKKQKENEN. SSSIQPNLSA ARIRLKRDLD SLDLPPTVTL
             51                                                  100
RCE1         SFPNGKDDLM .....NFEVS IKPDDGYYHN GTFVFTFQVS PVYPHEAPKV
Hs-UBC12     SF.SDPDDLL .....NFKLV ICPDEGFYKS GKFVFSFKVG QGYPHDPPKV
Sp-Ubc12p    SWP.DPIKLN .....VLHLE IRPDEGYYKG GKFKFRIQID DNYPHDPPKV
Sc-Ubc12p    NVITSPDSAD RSQSPKLEVI VRPDEGYYNY GSINFNLDFN EVYPIEPPKV
             101                                                 150
RCE1         KCKTKVYHPN IDLEGNVCLN ILREDWKPVL NINTVIYGLF HLFTEPNSED
Hs-UBC12     KCETMVYHPN IDLEGNVCLN ILREDWKPVL TINSIIYGLQ YLFLEPNPED
Sp-Ubc12p    KCLNKIYHPN IDIEGNVCLN ILRQDWNPVL NLNSILVGLQ FLFLSPNAED
Sc-Ubc12p    VCLKKIFHPN IDLKGNVCLN ILREDWSPAL DLQSIITGLL FLFLEPNPND
             151                                      190
RCE1         PLNHDAAAVL RDNPKLFETN VRRAMTGGYV GQTFFPRCI
Hs-UBC12     PLNKEAAEVL QNNRRLFEQN VQRSMRGGYI GSTYFERCLK
Sp-Ubc12p    PLNKEAAADL HKDPQGFASR VRTAMKGGLV NGISFDNVMA
Sc-Ubc12p    PLNKDAAKLL CEGEKEFAEA VRLTMSGGSI EHVKYDN.IV
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Fig. 2. Identification of an Arabidopsis RUB E2 enzyme. (*A*) Alignment of UBC12 proteins and RCE1. Amino acid sequences corresponding to the RCE1 protein in *Arabidopsis* (accession no. AF202771), human (Hs-UBC12), *S. pombe* (Sp-Ubc12p), and *S. cerevisiae* (Sc-Ubc12p) were aligned by using the PILEUP program of GCG. RCE1 contains the conserved cysteine (position 112) implicated in thiolester bond formation within the highly conserved UBC domain. The residues conserved between RCE1 and these proteins are labeled in bold. (*B*) Thiolester formation between RUB1 and the Arabidopsis E2, RCE1. Thiolester reactions containing purified H6-AXR1, ECR1, and radiolabeled RUB1 and with or without H6-RCE1 were performed. Half of the reactions were stopped with 4× SDS loading buffer for 10 min, and the other half were stopped with 4× SDS/DTT loading buffer and were boiled for 10 min. (*C*) RCE1 forms a thiolester with RUB1 but not ubiquitin. Thiolester reactions containing ^{32}P-ubiquitin, wheat E1 UBA1, and the Arabidopsis E2 UBC1 or H6-RCE1 were performed. Half of the reactions were stopped with 4× SDS loading buffer, and the other half were stopped with 4× SDS/DTT loading buffer and were boiled for 10 min. The asterisks indicate GST-^{32}P-RUB1 or GST-^{32}P-UBQ, which remained after thrombin digestion.

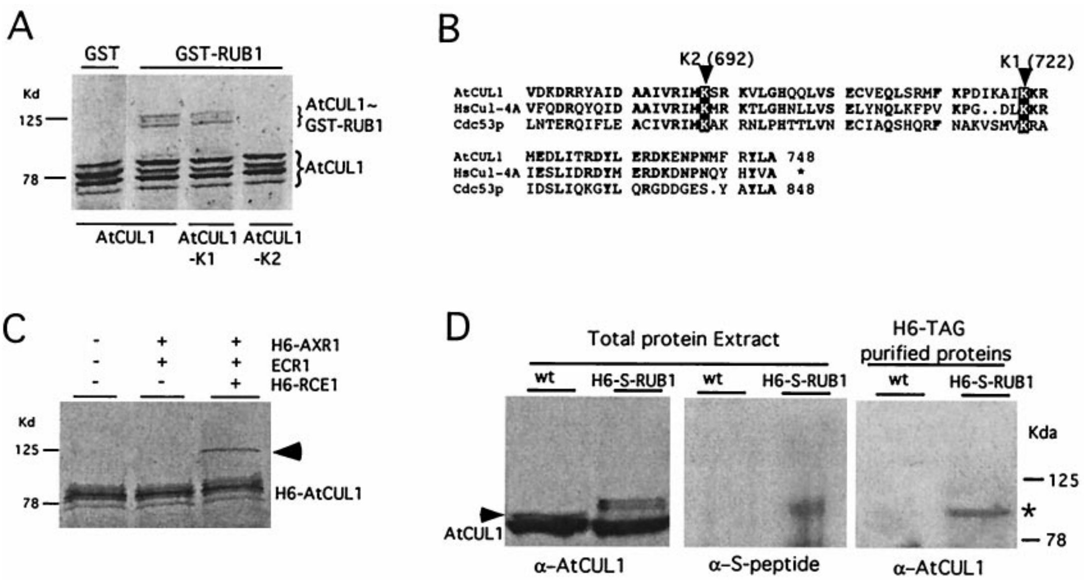

Fig. 3. Modification of AtCUL1 with RUB1. (*A*) AtCUL1 is modified with RUB1. *In vitro* translated ^{35}S-H6-AtCUL1 was incubated in the reticulocyte lysate with the GST or GST-RUB1. ^{35}S-H6-AtCUL1^{722M} or ^{35}S-H6-AtCUL1^{692M} were incubated with GST-RUB1 in the reticulocyte lysate. (*B*) Amino acids sequences corresponding to the C-terminal region of cullin proteins from Arabidopsis (AtCUL1), humans (HsCul 4A), and *S. cerevisiae* (Cdc53). K1(722) and K2(692) correspond to conserved lysines in this C-terminal region. The residues conserved between AtCUL1 and HsCul-4A or Cdc53p are labeled in bold. The asterisk indicates that HsCul-4A is a partial cDNA. (*C*) ^{35}S-H6- AtCUL1 was purified from the reticulocyte lyaste and was added to reactions that also contained H6-AXR1 and ECR1 or H6-AXR1, ECR1, and H6-RCE1. The arrow indicates the ^{35}S-H6-AtCUL1 protein modified with GST-RUB1. (*D*) Western blot analysis of protein extracts from wild-type and transgenic H6-S-RUB1 seedlings. Total protein extract was probed with the antibody against AtCUL1 or with the S-peptide detection kit (Novagen). Blot containing nickel-agarose purified proteins from wild-type and transgenic H6-S-RUB1 seedlings was probed with the antibody against AtCUL1. The arrow indicates the possible AtCUL1 modified with RUB1. The asterisk indicates the position of AtCUL1 modified with H6-S-RUB1.

a reaction with ECR1 identical to that analyzed in Fig. 1*B* and precipitated proteins with $(NH_4)_2SO_4$ to remove the ATP. When H6-AXR1 was added to the ATP-depleted reaction, the DTT-sensitive ECR1-RUB product was formed (Fig. 1*D*). This result indicates that the ATP-dependent part of RUB activation can be performed by ECR1, but AXR1 is required to form the thiolester bond between ECR1 and RUB. These results are consistent with formation of AMP-RUB by ECR1.

Identification of an Arabidopsis RUB E2 Enzyme. After the E1 enzyme activates ubiquitin, it is transferred to a ubiquitin-conjugating enzyme (E2) (10). For yeast Rub1p and human NEDD8, the E2s are Ubc12p and HsUbc12, respectively (6, 8). Based on sequence similarity to Ubc12p, we identified an Arabidopsis genomic sequence and two partial expressed sequence tags that might encode an Arabidopsis RUB-specific E2. Using one of the expressed sequence tags as a probe, we recovered several longer cDNA clones corresponding to this gene. The longest clone encodes a protein that is 61% identical to the human UBC12, 52% identical *to Schizosaccharomyces pombe* Ubc12p, and 42% identical to *S. cerevisiae* Ubc12p (Fig. 2*A*). Because another Arabidopsis E2 has already been named UBC12 (a homolog of yeast Ubc5p), we elected to call this new protein RCE1 (RUB-conjugating enzyme 1). Like all E2 enzymes, RCE1 has the highly conserved UBC domain with the active site cysteine residue located at position 112 (Fig. 2*A*) (21).

To test the possibility that RCE1 functions in the RUB1 conjugation pathway, purified H6-RCE1 was added to a RUB1 activation reaction containing purified H6-AXR1 and ECR1, ATP/DTT/$MgCl_2$, and ^{32}P-RUB1. A new DTT-sensitive RUB1-conjugate was detected when RCE1 was present in the reaction (Fig. 2*B*). This DTT-sensitive species has the molecular weight (≈34 kDa) expected for an RCE1-RUB1 conjugate. The DTT-sensitive nature of the product indicates that RUB1 is bound to RCE1 by a thiolester bond. Furthermore, appearance of the 34-kDa product depends on AXR1 and ECR1. To test the specificity of RCE1, thiolester reactions with radiolabeled ubiquitin containing the wheat E1 enzyme UBA1 plus the Arabidopsis E2 AtUBC1 or RCE1 were performed. In these reactions, we were able to detect the DTT-sensitive E1-ubiquitin and E2-ubiquitin products but not an RCE1-ubiquitin conjugate, suggesting that RCE1 is specific for RUB1 protein (Fig. 2*C*).

AtCUL1 Is a Target for RUB1 Conjugation. In yeast and humans, RUB1 is attached to members of the cullin family of proteins (5–7, 11). In a previous study, we identified the Arabidopsis cullin AtCUL1 and showed that it interacts with a SKP1 ortholog called ASK1 and the F-box protein TIR1 to form the SCF^{TIR1} complex (13). To explore the possibility that AtCUL1 might be modified by RUB1, we first used a rabbit reticulocyte lysate *in vitro* assay. Addition of GST-RUB1 to the lysate together with *AtCUL1* cDNA resulted in the generation of GST-RUB1-^{35}S-AtCUL1 conjugate that was DTT-resistant (Fig. 3*A*). In addition to full length AtCUL1, a series of slightly smaller proteins are synthesized in the lysate, presumably because of selection of alternative AUGs during translation initiation and/or premature termination of translation. In any case, all of these proteins are modified by GST-RUB.

Ubiquitin is covalently attached to target proteins through an isopeptide bond between the carboxyl terminal glycine of ubiquitin and a lysyl ε-amino groups on target proteins. A similar mechanism of conjugation has been proposed for the ubiquitin-like proteins (4). Osaka *et al.* (6) showed that a protein comprising the C-terminal 171 amino acids of HsCUL-4A was sufficient for the formation of a GST-NEDD8 conjugate, suggesting that the lysine involved in the linkage with NEDD8 is located in this region of HsCUL-4A. An alignment of the C-terminal regions of cullin proteins from Arabidopsis, humans, and yeast revealed two conserved lysines (K692 and K722 of AtCUL1) that might be sites of isopeptide bond formation (Fig.

3*B*). We replaced these two lysines with methionine by directed mutagenesis. Fig. 3*A* shows that AtCUL1^{K722M} is modified with GST-RUB1 but AtCUL1^{K692M} is not, indicating that lysine 692 is the site of RUB1 attachment. Recently, Wada *et al.* (11) showed that human Cul2 is also modified at this conserved lysine (K689 in Cul2), suggesting that the RUB/NEDD8 conjugation site is conserved among eukaryotic cullins.

In some cases, ubiquitin can be conjugated to acceptor proteins directly from the ubiquitin-conjugating enzyme E2 *in vitro* (10). However, this reaction normally requires the participation of a ubiquitin ligase (E3). To investigate whether AXR1-ECR1 and RCE1 are sufficient to conjugate RUB1 to AtCUL1, we performed thiolester reactions with these proteins. When ^{35}S-H6-tagged-AtCUL1 was synthesized in reticulocyte lysates, purified by using the H6 tag, and incubated with AXR1, ECR1, RCE1, and GST-RUB1, we detected a GST-RUB1-AtCUL1 conjugate (Fig. 3*C*). This result suggests that RUB1 modification of AtCUL1 does not require an E3, at least *in vitro*. However, we cannot exclude the possibility that an E3 activity copurifies with AtCUL1 from the reticulocyte lysate. More experiments are required to resolve this issue.

To determine whether RUB1 is conjugated to AtCUL1 *in vivo*, we generated transgenic plants that expressed a version of RUB1 containing the H6-S-peptide at its N terminus. Fig. 3*D* shows Western blots of either total protein extracts from wild-type and transgenic H6-S-RUB1 seedlings or proteins purified from these extracts by using nickel-Sepharose beads. As described previously, the AtCUL1 antibody detected two protein species migrating closely together in wild-type extracts (13). In extracts prepared from the transgenic line expressing tagged RUB, a new larger species is present. When this blot was stripped and reprobed with the S-peptide detection kit, the larger isoform was found in the transgenic lane only. To confirm that this protein is AtCUL1-modified by RUB1, we recovered H6-S-RUB1 by using the 6× histidine tag and performed a Western blot using anti-AtCUL1 (Fig. 3*D*). A single protein of the correct molecular weight was detected in the transgenic lane but not in the wild-type lane. This result indicates that RUB1 is covalently attached to AtCUL1 *in planta*.

Discussion

The RUB/NEDD8 proteins are conserved in plants, animals, and fungi, suggesting a fundamental role for these proteins in cellular metabolism (4). Despite this conservation, loss of Rub1p in *S. cerevisiae* has no detectable effect on cell appearance or growth. In contrast, genetic studies in Arabidopsis indicate that RUB conjugation is required for normal response to the plant hormone auxin (2). Loss of the AXR1 subunit of the RUB-activating enzyme results in dramatic and diverse effects on growth and development of the plant. To further characterize the RUB conjugation pathway in plants, we have studied the RUB-activating enzyme AXR1-ECR1, identified a candidate RUB-conjugating enzyme, and shown that the cullin AtCUL1 is a target of the pathway.

Activation of RUB/NEDD8 is accomplished by an enzyme composed of two subunits that resemble the two halves of a ubiquitin-activating enzyme (E1) (5–9). In Arabidopsis, this heterodimer consists of the AXR1 and ECR1 proteins (9). Activation of ubiquitin by the E1 enzyme is a well characterized reaction. In the presence of ubiquitin and ATP, E1 generates AMP-ubiquitin. Adenyl-ubiquitin is then used to form the ubiquitin thiolester while a second ubiquitin molecule is adenylated and remains associated with the E1 enzyme (10). In this report we show that RUB1 activation also involves generation of an AMP-RUB1 intermediate. Further, the ECR1 protein is able to generate the adenylate intermediate in the absence of AXR1 and without the participation of ECR1 cysteine 215. These results suggest that ECR1 can bind ATP and RUB1. Several groups have noted the presence of the putative nucleotide-binding site GXGXXG near the N terminus of Uba2p and Uba3p from yeast and human UBA3, as well as plant and animal E1 enzymes (6, 8, 22, 23) This sequence is also present in ECR1 and is the likely site of nucleotide binding. Once AMP-RUB is formed, AXR1 is presumably required for formation of the thiolester bond between RUB1 and ECR1. AXR1 may also be required to recognize and interact with RCE1.

It is noteworthy that, when the ubiquitin-activating enzyme is treated with iodoacetamide, the enzyme can generate the ubiquitin-AMP intermediate but is not able to form the thiolester bond (24). This result suggests that ubiquitin activation can be separated into two independent reactions: generation of ubiquitin-AMP, and formation of the thiolester bond. Because the C-terminal half of E1 has significant sequence similarity with ECR1, it is tempting to speculate that this region of E1 is responsible for formation of AMP-ubiquitin. Similarly, the transfer of ubiquitin to the active cysteine within E1 and thiolester bond formation and/or interaction with the E2 enzyme may require sequences in the N-terminal part of E1.

In fungi and animals, the RUB-conjugating enzyme is UBC12 (6, 8). *RCE1* encodes an Arabidopsis UBC12-like protein that forms a thiolester conjugate with RUB1 but not with ubiquitin, suggesting that RCE1 is an E2 for the RUB-conjugation pathway. Because there is at least one *RCE1*-related gene in the Arabidopsis genome, we cannot be sure that RCE1 functions downstream of AXR1-ECR1 *in vivo* (S. Dharmasiri and M.E., unpublished work). To address this question, we are currently screening for mutants in each of the *RCE* genes as well as generating transgenic lines with altered levels of wild-type and mutant RCE1.

To date, the only known targets for RUB/NEDD8 modification are members of the cullin family in yeast and humans. Our results show that the Arabidopsis cullin AtCUL1 is also modified by RUB. Further, we show that the modification occurs at lysine 692 near the C terminus of the protein. An examination of additional cullin sequences reveals a striking conservation of this lysine and adjacent residues (Fig. 4). In 15 cullins examined, the motif VRIMK is completely conserved, suggesting that this

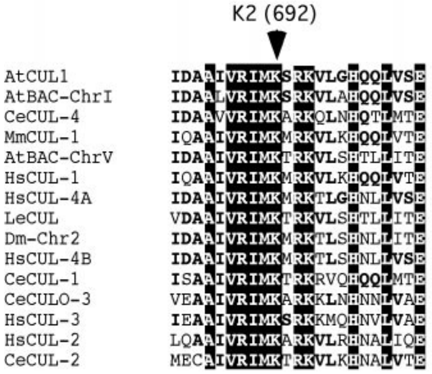

Fig. 4. The site of RUB-conjugation is highly conserved among cullins from diverse organisms. Residues conserved with AtCUL1 are in bold. Boxes indicate residues that are identical in all proteins shown. AtBAC-ChrI (*A. thaliana*, GenBank accession no. AC002330), CeCUL-4 (*Caenorhabditis elegans*, GenBank accession no. U58086), MmCUL-1 (*Mus musculus*, GenBank accession no. AF083216), At-BAC-ChrV (*A. thaliana*, GenBank accession no. AB025620), HsCUL-1 (*Homo sapiens*, GenBank accession no. AF062536), HsCUL-4A (*H. sapiens*, GenBank accession no. U58090), LeCUL (*Lycopersicon esculentum*, European Molecular Biology Laboratory accession no. Y16124), DmChr2 (*Drosophila melanogaster*, accession no. AC005473), HsCul-4B (*H. sapiens*, accession no. U58091), CeCUL-1 and -2 (*C. Elegans*, accession nos. U58083 and U58085), HsCUL3 and -2 (*H. Sapiens*, accession nos. U58089 and gbU83410), and CeCUL-2 (*C. Elegans*, accession no. U58084).

region is important for recognition by the RUB/NEDD8 E2 and/or E3 proteins. At this point, it is not clear whether all of these proteins are modified *in vivo*. However, the existence of the conserved VRIMK domain suggests this possibility. The involvement of an E3 enzyme in RUB conjugation is also uncertain. Lammer *et al.* showed that Rub1p modification of Cdc53p in yeast depends on Skp1, suggesting that an SCF complex may be required (5). In contrast, we find that AXR1-ECR1 and RCE1 are capable of modifying AtCUL1 *in vitro*, presumably without the presence of other factors. However, the yield of modified cullin in this reaction is very low, and it is possible that, in the presence of the appropriate E3, the reaction would proceed more efficiently.

AtCUL1 forms an SCF complex with the SKP1-related protein ASK1 and the F-box protein TIR1 (13). Because genetic studies show that $\mathrm{SCF}^{\mathrm{TIR1}}$ is also required for auxin response, we have proposed that RUB modification of Arabidopsis cullins might play a role in auxin regulation (3, 13). The results described in this report are consistent with this model. We show that AtCUL1 is a target for RUB modification both *in vitro* and *in vivo*. So far, the biochemical function of RUB modification has not been established. Among the possibilities are effects on assembly, localization, or activity of specific SCF complexes. Conjugation of another ubiquitin-related protein, called SUMO-1, has been shown to effect cellular localization of RanGAP1 and PML (25, 26). SUMO-1 is also conjugated to IκBα, but, in this case, the modification appears to preclude ubiquitin modification, thus preventing degradation of the protein (27). Further studies are required to explore these possibilities for RUB modification of cullin.

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