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Arabidopsis AXR6 encodes CUL1 implicating SCF E3 ligases in auxin regulation of embryogenesis

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The *AXR6* gene is required for auxin signaling in the *Arabidopsis* embryo and during postembryonic development. One of the effects of auxin is to stimulate degradation of the Aux/IAA auxin response proteins through the action of the ubiquitin protein ligase SCF^{TIR1}. Here we show that *AXR6* encodes the SCF subunit CUL1. The *axr6* mutations affect the ability of mutant CUL1 to assemble into stable SCF complexes resulting in reduced degradation of the SCF^{TIR1} substrate AXR2/IAA7. In addition, we show that CUL1 is required for lateral organ initiation in the shoot apical meristem and the inflorescence meristem. These results indicate that the embryonic *axr6* phenotype is related to a defect in SCF function and accumulation of Aux/IAA proteins such as BDL/IAA12. In addition, we show that CUL1 has a role in auxin response throughout the life cycle of the plant.
Keywords: *Arabidopsis*/auxin/cullin/IAA/SCF

Introduction

The plant hormone auxin regulates diverse aspects of development throughout the plant life cycle (Davies, 1995). In *Arabidopsis* seedlings, auxin response depends on the ubiquitin protein ligase SCF^{TIR1} (Ruegger *et al*., 1998; Gray *et al*., 1999, 2001). SCF E3s consist of four subunits: an F-box protein (FBP) responsible for substrate binding, SKP1 (ASK in *Arabidopsis*), the RING protein RBX1/ROC1/HRT1 and CUL1. Biochemical and structural studies show that the CUL1 subunit functions as a scaffold, binding RBX1 and a SKP1–FBP dimer (Zheng *et al*., 2002). RBX1 also binds the ubiquitin-conjugating enzyme (E2) thus bringing E2 in close proximity to the substrate. There are over 700 FBPs encoded by the *Arabidopsis* genome suggesting that SCFs have a prominent role in cellular regulation in plants (Gagne *et al*., 2002; Kuroda *et al*., 2002).

SCF function requires modification of CUL1 by the ubiquitin-related protein RUB (Nedd8 in some species) (Yeh *et al*., 2000; Hellmann and Estelle, 2002). In *Arabidopsis*, RUB modification requires a heterodimeric E1 enzyme composed of AXR1 and ECR1, and a RUB1-conjugating enzyme called RCE1 (del Pozo *et al*., 1998, 2002). RUB is conjugated to a conserved lysine residue near the C-terminal region of CUL1 (del Pozo *et al*., 1998, 2002). Although the function of the modification is unknown, recent genetic results suggest that dynamic cycling of CUL1 between the unmodified and modified forms is required for normal SCF activity (Lyapina *et al*., 2001; Schwechheimer *et al*., 2001; Gray *et al*., 2002).

The only known substrates of SCF^{TIR1} are the Aux/IAA proteins (Gray *et al*., 2001). These short lived, nuclear proteins consist of four conserved domains. Domains III and IV are required for dimerization between Aux/IAA proteins and with members of a second family of transcriptional activators called the auxin response factors (ARFs) (Reed, 2001). The Aux/IAA proteins repress ARF function through this interaction (Reed, 2001). In *Arabidopsis* seedlings, auxin relieves this repression by promoting binding of the Aux/IAA proteins by SCF^{TIR1}, resulting in their ubiquitination and degradation (Gray *et al*., 2001; Tiwari *et al*., 2001; Zenser *et al*., 2001). Domain II of the Aux/IAA protein is necessary and sufficient for interaction with the SCF (Ramos *et al*., 2001). Dominant mutations within domain II prevent the interaction and stabilize the affected protein resulting in a reduction in auxin response (Hellmann and Estelle, 2002).

Studies of the *bodenlos* (*bdl*), *monopteros* (*mp*) and *hobbit* (*hbt*) mutants suggest that auxin response in the embryo requires regulated protein degradation (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998; Hamann *et al*., 1999, 2002). The *mp* and *bdl* mutants have a similar phenotype characterized by cell division defects as early as the two-cell stage of embryogenesis. Mutant embryos complete embryogenesis but lack basal embryonic structures including the hypocotyl and root meristem. The *hbt* mutants display early cell division defects that are similar to those of *mp* and *bdl* (Willemsen *et al*., 1998). However, *hbt* seedlings are relatively normal except for the absence of the root meristem (Willemsen *et al*., 1998). The *MP* gene encodes ARF5 while *BDL* encodes IAA12 (Hamann *et al*., 2002). The *mp* phenotype is caused by loss of ARF5 while *bdl* is a gain-of-function mutation in domain II similar to other mutations known to prevent interaction with SCF^{TIR1}. Based on these results Hamann *et al*. (2002) have proposed that BDL/IAA12 represses MP/ARF5 during embryogenesis. The *HBT* gene encodes a subunit of the anaphase promoting complex (APC), another class of ubiquitin protein ligase (Blilou *et al*., 2002). The

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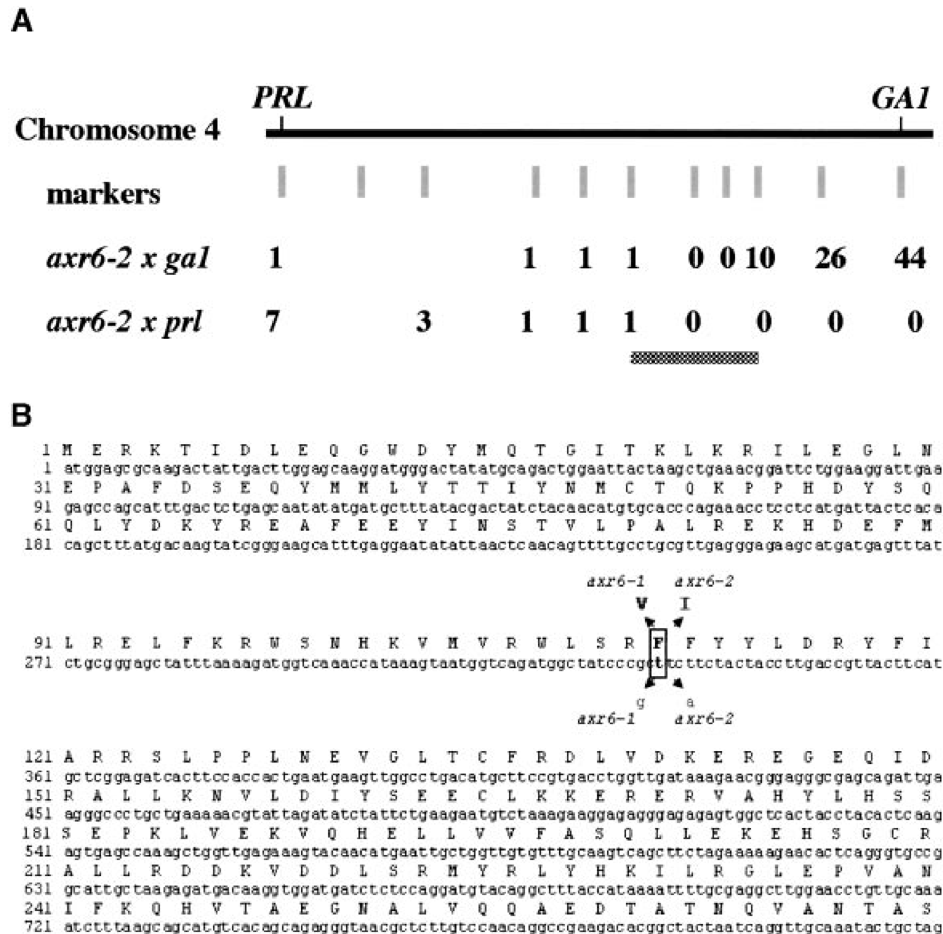

Fig. 1. Mapping of the *AXR6* gene. (**A**) *AXR6* was mapped using crosses between *axr6-2* (*Col-0* background) and two mutants in the *Ler* background, *ga1* and *prl*. The number of recombinants at each position for each cross are indicated. The mutation was localized to a region of ~80 kb (indicated by the gray horizontal bar) on the short arm of chromosome 4 containing *CUL1*. (**B**) Sequencing of CUL1 in two independent alleles, *axr6-1* and *axr6-2*, showed mutations in the same base at position 336. The mutations result in substitution of phenylalanine with valine and isoleucine in *axr6-1* and *axr6-2*, respectively.

Aux/IAA protein IAA17 is stabilized in *hbt* mutants suggesting that Aux/IAA proteins are substrates of APC in the embryo. Thus it is possible that APC or SCF (or both) are responsible for degradation of BDL/IAA12 and other Aux/IAA proteins during embryogenesis.

The phenotype of the auxin-resistant mutant *axr6* closely resembles that of *mp* and *bdl* (Hobbie *et al*., 2000). Here we show that *AXR6* encodes the SCF subunit CUL1. The mutations result in stabilization of the AXR2/IAA7 protein suggesting that the *axr6* embryonic phenotype is caused by accumulation of Aux/IAA proteins, probably including BDL/IAA12. Furthermore, a reduction in CUL1 levels in transgenic lines leads to defects in organ initiation similar to those observed in mutants deficient in auxin transport (Okada *et al*., 1991). These results suggest that SCF-mediated degradation of the Aux/IAA proteins is important for early events in embryogenesis and diverse growth process throughout plant development.

Results

AXR6 gene encodes SCF subunit CUL1

In a previous report, we showed that the *AXR6* gene is required for auxin response throughout plant growth and development (Hobbie *et al*., 2000). To further explore the function of *AXR6* we isolated the gene using a map-based approach. Earlier mapping studies had shown that *AXR6* is located on chromosome 4 near *PRL*. Additional recombinants were generated by crossing *axr6-2* (*Col-0*) to *ga1* and *prl* (*Ler*) (Figure 1A). The *AXR6* gene was localized to an ~80 kilobase (kb) interval on chromosome 4. Among the genes in this region is *CUL1*. Since SCF^{TIR1} is required for auxin response, we sequenced *CUL1* from *axr6-1* and *axr6-2* mutants. Both alleles carry a base substitution at the same position in the coding region of *CUL1*. The mutations result in replacement of phenylalanine 111 with valine (*axr6-1*) or isoleucine (*axr6-2*) (Figure 1B). Based on the crystal structure of human SCF^{SKP2}, Phe111 is within a region of CUL1 that interacts with the SKP1–FBP module (Zheng *et al*., 2002).

Shen *et al*. (2002) used two *CUL1* T-DNA insertion mutants (*cul1-1* and *cul1-2*) to show that loss of CUL1 results in lethality at the zygote stage of embryogenesis. We isolated two new T-DNA insertion lines from the Wisconsin collection. The new alleles, called *cul1-3* and *cul1-4*, each have a T-DNA insert in exon 15 of the *CUL1* gene (Figure 2A). Like *cul1-1* and *cul1-2*, the new alleles are recessive embryo lethal mutations (data not shown).

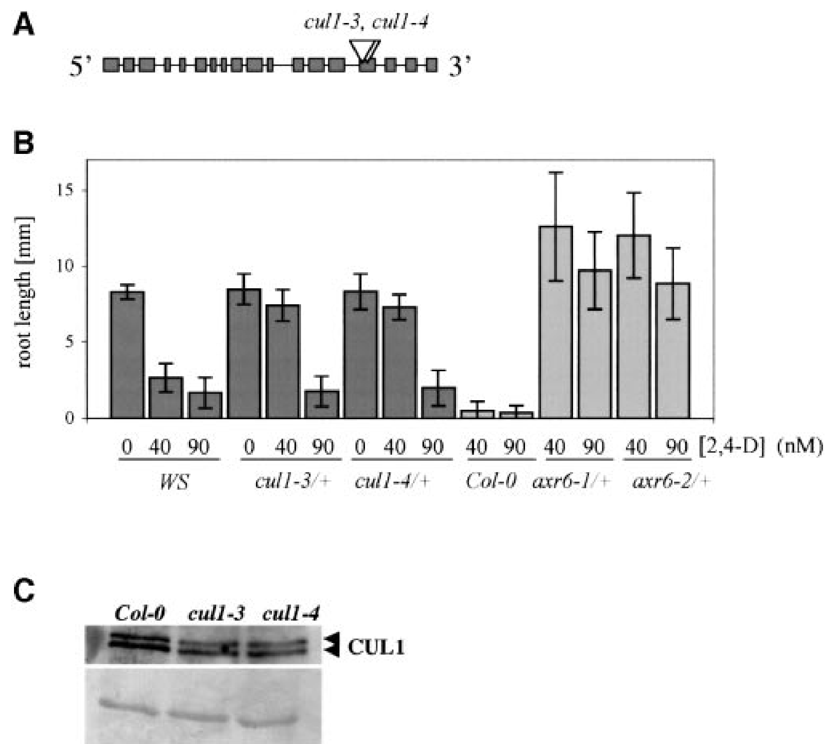

Fig. 2. *CUL1* T-DNA insertion mutants are auxin resistant. (**A**) Two independent T-DNA insertion lines, *cul1-3* and *cul1-4* [*Wasselewskja* (*WS*) background], were identified carrying insertions in exon 15 of the gene. (**B**) Response of mutant and wild-type seedlings to auxin. At least 15 seedlings were tested for each genotype. Error bars represent standard deviation. (**C**) Heterozygous *cul1-3/+* and *cul1-4/+* plants have reduced CUL1 levels. Ten-day-old seedlings were used. The loading control (lower panel) shows an unidentified protein visualized by Poinceau staining.

Table I. Analysis of progeny from cross of *axr6* × *cul1-4*

	axr6-1/+	*axr6-2/+*	*cul1-4/+*	Col-0	*axr6-1/+* × *cul1-4/+*	*axr6-2* × *cul1-4/+*
Viable seed	100	100	228	100	95	221
Inviable seed	0	0	72	0	31	80
Total	100	100	300	100	126	301

Heterozygous plants are normal in appearance but display a mild auxin-resistant phenotype (Figure 2B). This defect is associated with reduced levels of CUL1 (Figure 2C). Additional cross-reacting proteins were not observed in *cul1-3* or *cul1-4* plants indicating that auxin resistance was not related to accumulation of a truncated version of CUL1 (data not shown). This result indicates that auxin response is unusually sensitive to CUL1 levels. However, it is also important to note that *cul1-3*/+ and *cul1-4*/+ are much less auxin resistant than *axr6*/+ plants (Figure 2B).

To confirm that the *axr6* mutations are in the *CUL1* gene, heterozygous *axr6-1* and *axr6-2* plants were crossed to heterozygous *cul1-4* plants. In each case ~25% of the progeny seed were not viable, showing that *axr6* does not complement the embryo-lethal phenotype of *cul1-4* (Table I). Of the viable seedlings, the expected 33% displayed the *axr6*/+ auxin resistant phenotype (data not shown).

Degradation of the AXR2/IAA7 protein is impaired in axr6 mutants

Heterozygous *axr6-1*/+ plants are deficient in auxin induction of the *BA3–GUS* reporter gene indicating that AXR6/CUL1 is required for this auxin response (Hobbie *et al*., 2000). We also examined transcript levels of the *IAA5* and *IAA7* primary auxin response genes. We found that accumulation of both in response to auxin is reduced in the mutants compared with wild type (Figure 3A). In the case of *IAA7*, RNA levels were also reduced in the absence of exogenous auxin. These results confirm that AXR6/CUL1 is required for auxin regulation of gene expression. We also show that the *CUL1* RNA level does not change in response to auxin and is not affected in the *axr6* mutants (Figure 3A).

Since SCF-dependent degradation of Aux/IAA proteins is required for auxin response in seedlings, we speculated that this process might be impaired in the *axr6* mutants (Gray *et al*., 2001). To test this possibility, the half-life of IAA7 was determined in wild-type and heterozygous *axr6* seedlings. The results show that IAA7 is more stable in seedlings heterozygous for either *axr6-1*or *axr6-2* compared with wild-type seedlings (Figure 3B and C). This difference is statistically significant (P <0.05 for each mutant compared with wild type; Student's t-test).

CUL1 levels are increased in axr6 plants

To explore the effects of the *axr6* mutations on CUL1 function, we examined CUL1 in mutant plants by

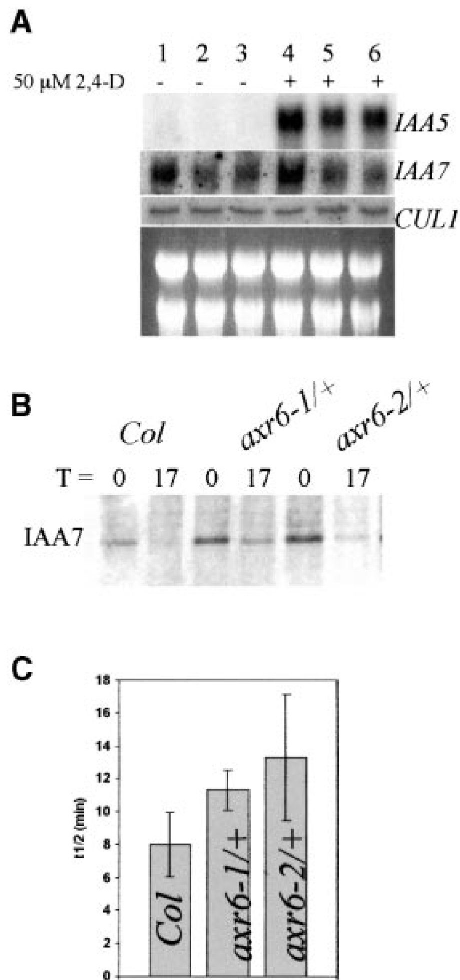

Fig. 3. The *axr6* mutants are deficient in auxin-induced gene expression and degradation of AXR2/IAA7. (**A**) RNA blot analysis shows that auxin-regulated expression of *IAA5* and *IAA7* is reduced in *axr6* mutants. Seven-day-old seedlings were treated with buffer or 50 μM 2,4-D for 1 h before RNA extraction. *CUL1* expression is not regulated by auxin and is not altered in *axr6-1* or *axr6-2* plants. *Col-0* (lanes 1 and 4), *axr6-1*/+ (lanes 2 and 4), *axr6-2*/+ (lanes 3 and 6). (**B**) Pulse–chase analysis in 7-day-old seedlings showed that stability of the IAA7 protein is increased in *axr6-1* and *axr6-2*. (**C**) The half-life of IAA7 is higher in the *axr6* mutants. Values presented are the averages of three independent experiments ± SD. (P <0.05 for each mutant compared with wild type; Student's *t*-test.)

immunoblotting. Each mutation causes an increase in CUL1 protein levels (Figure 4A). Since *CUL1* RNA level is not affected by the mutations (Figure 3A), this difference must reflect a post-transcriptional change. A fraction of the CUL1 protein is modified by the ubiquitin-related protein RUB1 (del Pozo *et al*., 2002). Despite the increase in total CUL1 levels in the *axr6* mutants, the amount of RUB-modified CUL1 is similar to wild type. The relative level of RUB-CUL1 may be decreased because the mutant proteins are poor substrates for RUB modification. To test this possibility a rabbit reticulocyte lysate was used to perform *in vitro* RUB modification of mutant and wild-type CUL1. The results in Figure 4B show that the mutant proteins are effective substrates for RUB modification in this system. However, it is still possible that in the context of the plant cell, RUB modification of CUL1 is impaired. Alternatively, the mutant proteins may be subject to increased RUB deconjugation, perhaps catalyzed by the COP9 signalosome (CSN) (Schwechheimer *et al*., 2001).

Genetic studies indicate that decreased RUB-CUL1 levels result in reduced SCF function (del Pozo *et al*., 2002). Thus the *axr6* phenotype may be caused by a decrease in the relative levels of RUB-CUL1. To test this model, we used a line that carries a *35S::RBX1* transgene. Over-expression of *RBX1* results in increased levels of RUB-CUL1 (Gray *et al*., 2002). Surprisingly, the effects of this change are similar to those of decreased RUB-CUL1, including stabilization of Aux/IAA proteins. To examine the effects of increased RBX1 levels in an *axr6* background, we crossed *axr6-2* to the *35S:RBX1* line (Gray *et al*., 2002). F_2 plants that were homozygous for the *axr6-2* mutation and carrying the *35S:RBX1* transgene were recovered and analyzed by western blot (Figure 4C). These plants had increased levels of RUB-modified CUL1. However, no effect on the morphology of homozygous *axr6-2* seedlings was observed, suggesting that decreased RUB-CUL1 levels do not cause this phenotype (data not shown).

axr6 mutations affect the interaction between CUL1 and ASK1

Based on the structure of mammalian SCF^{SKP2}, phenylalanine 111 of CUL1 lies adjacent to residues that contribute directly to SKP1-FBP binding (Zheng *et al*., 2002). To determine if the mutations affect the interaction between CUL1 and ASK1, we tested for co-immunoprecipitation of the two proteins (Figure 4D). As expected, more CUL1 was recovered from mutant extracts than wild-type extracts. However, we observed less ASK1 in the CUL1 immunoprecipitate compared with wild type despite the presence of similar levels of ASK1 in the extract (Figure 4D and E). We conclude that the mutations affect the ability of CUL1 to form a stable complex with ASK1.

RBX1 binds cullin near its C-terminus (Furukawa *et al*., 2000; Zheng *et al*., 2002). We used an RBX1 antibody to examine RBX1 levels in plant extracts and CUL1 immunoprecipitates. Surprisingly, RBX1 levels are higher in *axr6* seedlings than in wild type (Figure 4F). Consistent with this difference, and the higher level of CUL1 in these extracts, we found increased levels of RBX1 in the CUL1 immunoprecipitate from mutant plants. Thus the mutations do not affect the interaction between CUL1 and RBX1. However, RBX1 appears to be more stable in mutant plants.

The IAA7 protein is a substrate for SCF^{TIR1} and interacts with the complex in plant extracts prepared from seedlings. As a further test of the effects of the *axr6* mutations on CUL1 function, we performed a GST–IAA7 pulldown experiment from wild-type and *axr6* extracts. Figure 4G shows that less CUL1 is pulled down from *axr6* extracts than from wild-type extracts, despite an increase in CUL1 levels. Since IAA7 interacts with CUL1 indirectly through the ASK-TIR1 module, these results indicate that less CUL1 is assembled into SCF^{TIR1} complexes in *axr6* plants.

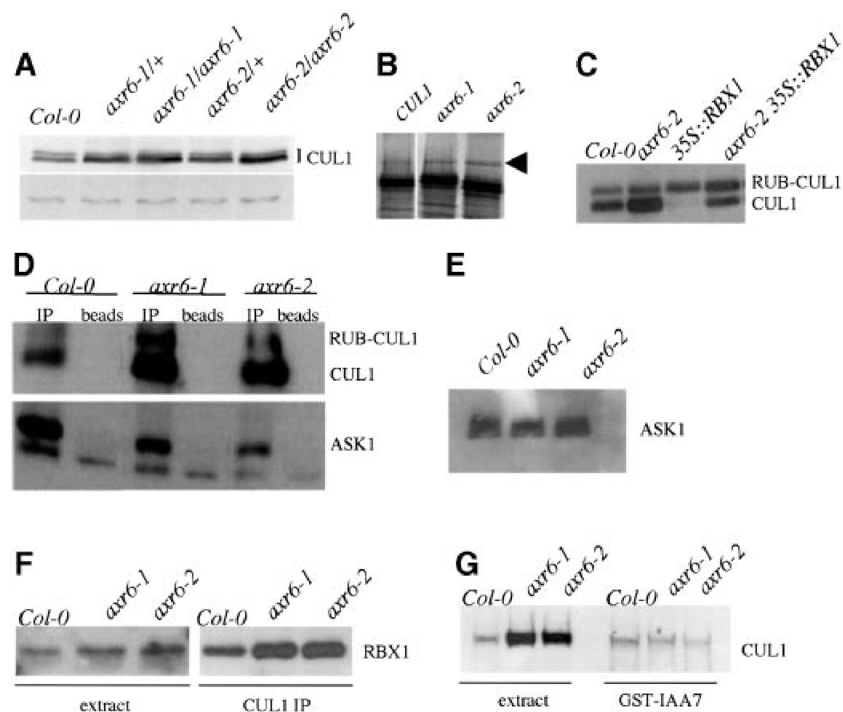

Fig. 4. The *axr6-1* and *axr6-2* mutations alter CUL1 levels and formation of stable SCF complexes. (**A**) CUL1 levels in wild-type and mutant seedlings determined by western blot with antibody against CUL1. The lower panel shows an unidentified cross-reacting protein used as a loading control. (**B**) *In vitro* translation and GST–RUB1 modification of CUL1, AXR6-1 and AXR6-2 proteins in rabbit reticulocyte lysates. Arrow indicates GST–RUB1 modified cullin. (**C**) Effect of *RBX1* over-expression on CUL1 levels in *axr6* plants determined by western blot. (**D**) Co-immunoprecipitation of CUL1 (upper panel) and ASK1 (lower panel) from *Col-0* and homozygous *axr6-1* or *axr6-2* seedlings. Proteins were immunoprecipitated with CUL1 antibody and analyzed by western blot. The ASK1 antiserum detected an unknown protein that was recovered after incubation with beads alone. (**E**) ASK1 levels in *Col-0* and homozygous *axr6-1* or *axr6-2* extracts determined by western blot using α-ASK1 antibody. (**F**) Co-immunoprecipitation of CUL1 and RBX1 in *Col-0* and mutant seedlings. (**G**) CUL1 levels in GST-IAA7 pulldowns from *Col-0* and homozygous *axr6* seedlings determined by western blot.

Table II. Recovery of pin phenotype after transformation of *Col-0* and *tir1-1* plants with *CUL1* and CUL^{K682M} constructs

Construct/genotype	No. of transformants	No. of co-suppressor [*n* (%)]	Plants with pin inflorescence [*n* (%)]
CUL1/Col-0	105	26 (24.8)	2 (1.9)
$CUL1^{K682M}$/*Col-0*	117	39 (33)	2 (1.7)
CUL1/tir1-1	129	30 (23)	11 (8.5)
$CUL1^{K682M}$/*tir1-1*	135	61 (45)	15 (11.1)

Reduction in CUL1 levels results in severe shoot meristem defects

To learn more about the function of CUL1 throughout plant development, we attempted to overexpress the protein in *Col-0* plants. Two *35S::CUL1* constructs were used containing either the wild-type cDNA or a mutant form in which lysine 682, the site of RUB attachment, is changed to methionine. Over 100 transformants were generated with each construct. No lines with increased expression of either gene were recovered suggesting that CUL1 overexpression is severely detrimental to the plant. However, 25–30% of the lines exhibited defects in development (Table II). Figure 5A illustrates the range of phenotypes observed in T_1 plants. Most seedlings exhibited a severe phenotype similar to seedlings 3 through 5 in Figure 5A. The severity of the phenotype correlated with the level of CUL1 protein, suggesting that the phenotype is caused by co-suppression of *CUL1* expression (Figure 5B). A variety of defects were observed. The hypocotyls of affected T_1 seedlings were two to three times longer than *Col-0* seedlings (Figure 5C), but had the same number of cells (data not shown), indicating an increase in cell size. Root growth was reduced and no lateral roots were observed in these seedlings (Figure 5C). Subsequent growth was slow and most seedlings died before they developed leaves. The occasional survivor produced a compact inflorescence (Figure 5D and E). These plants produced seed but only a small fraction of the T_2 population (typically <2%) displayed the severe co-suppressor phenotype. These lines were not analyzed further.

In addition to the severely affected T_1 plants, ~2% of the transformed lines displayed a less severe but completely heritable phenotype (Table II). Each of the lines had a similar phenotype regardless of the transgene. Leaf initiation was slower and irregular compared with *Col-0*

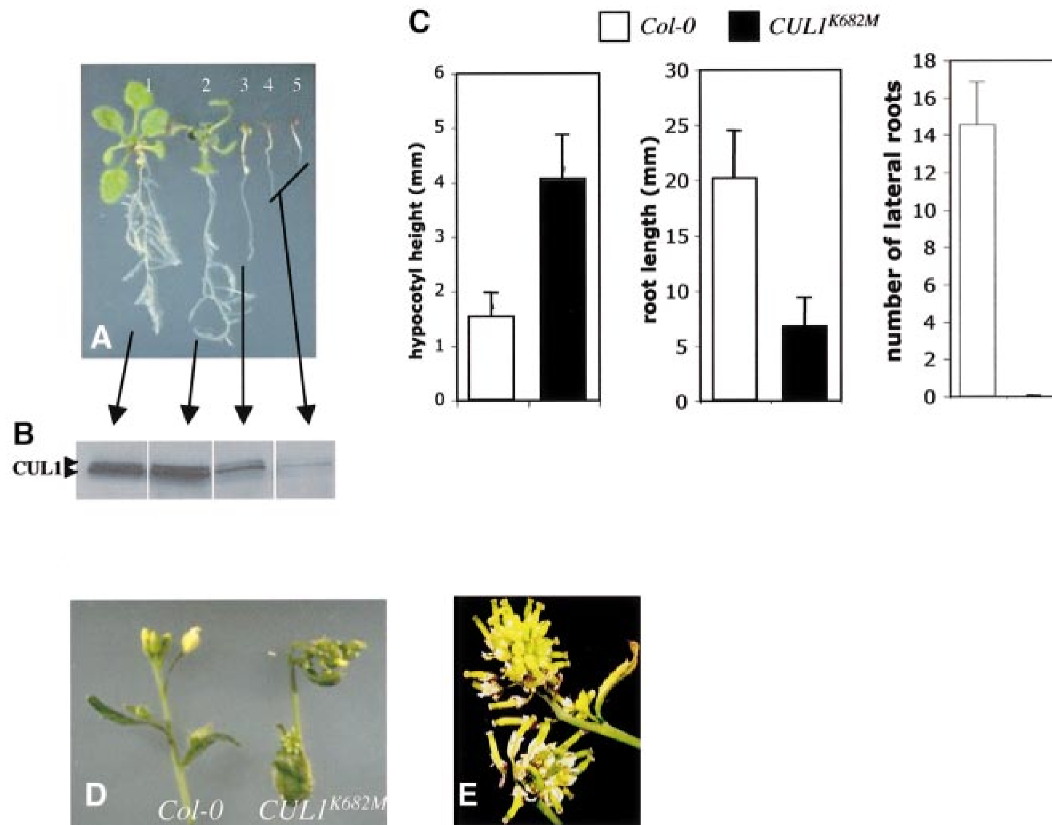

Fig. 5. Reduced CUL1 levels lead to severe developmental defects. *Arabidopsis Col-0* plants were transformed with a *CUL1* or *CUL1*^{K682M} cDNA under the control of the *35S* promoter from cauliflower mosaic virus. (**A**) Approximately 25% of the transgenic lines displayed growth defects of varying severity. The plant labeled '1' is untransformed *Col-0*. The others are transgenic for *35S::CUL1*^{K682M}. (**B**) CUL1 levels in transgenic lines from (A) determined by western blot; 30 µg total protein were loaded in each lane. (**C**) Three-day-old kanamycin-resistant *35S::CUL1*^{K682M} seedlings with elongated hypocotyls were transferred to minimal medium lacking antibiotics and compared with *Col-0* seedlings 12 days after transfer. Transgenic seedlings also displayed reduced root elongation and almost no lateral roots. (**D**) Surviving plants developed strongly distorted and compact inflorescences. (**E**) Compact inflorescence from a mature *35S::CUL1*^{K682M} plant. The frequency and severity of defects observed with the *35S::CUL1* construct were similar to those shown here.

plants (Figure 6A). After producing a few leaves, the shoot apical meristem (SAM) ceased organogenesis and formed a short pin-shaped structure similar to the pin meristem observed in the *mp*, *pinformed1* (*pin1*) and *pinoid* (*pid*) mutants (Figure 6B) (Okada *et al*., 1991; Bennett *et al*., 1995; Przemeck *et al*., 1996). In most seedlings, the SAM did not develop further. To determine if these defects were associated with changes in meristem structure we prepared paraffin sections from 9-day-old *Col-0* and *35S:CUL1*^{K682M} seedlings and immunolocalized CUL1 (Figure 6C). The SAM of *35S:CUL1*^{K682M} seedlings was enlarged compared with *Col-0*. As expected, the levels of CUL1 were reduced in the transgenic lines compared with *Col-0* (Figure 6C). This was confirmed by western blotting (Figure 6D). After SAM arrest, the axillary buds developed, resulting in the formation of a 'normal looking' rosette. When these plants flowered, many of the inflorescences also formed a pin-like structure (Figure 6E). These results show that CUL1 is required for normal organ initiation from the SAM and inflorescence meristem

Pin formation is enhanced by mutations in AXR1 or TIR1

Modification of CUL1 by the ubiquitin-related protein RUB is important for auxin response (Hellmann and Estelle, 2002). To determine if this is the case during organ initiation, we crossed the *35S:CUL1*^{K682M} line to the *axr1-13* mutant, known to be deficient in RUB-CUL1 conjugation (del Pozo *et al*., 2002). All of the F_1 plants displayed the pin phenotype. Strikingly, F_2 seedlings that were homozygous for *axr1-13* and either hetero- or homozygous for *35S:CUL1* developed a pin-shaped meristem immediately after germination and did not develop further (Figure 6F). Direct transformation of *axr1-3* with *35S:CUL1* also produced dramatically affected seedlings. In this case, ~10% of the transformants resemble homozygous *axr6* or *mp* plants with severe defects in basal structures (Figure 6G). These results indicate that RUB modification of CUL1 is essential for organ initiation.

A reduction in auxin response is a striking feature of the *axr1* phenotype. However, the RUB conjugation pathway is probably required for normal function of many different SCFs. To determine if the defects in organ initiation observed in the *35S:CUL1* lines are related to auxin response and SCF^{TIR1} in particular, we introduced the *35S:CUL1* transgenes into *tir1* plants. The frequency of transformants with a pin inflorescence in the *tir1* background was ~5-fold higher than in wild type (Table II). Since TIR1 is required for the auxin-dependent degradation of the Aux/IAA proteins, the defect in organ

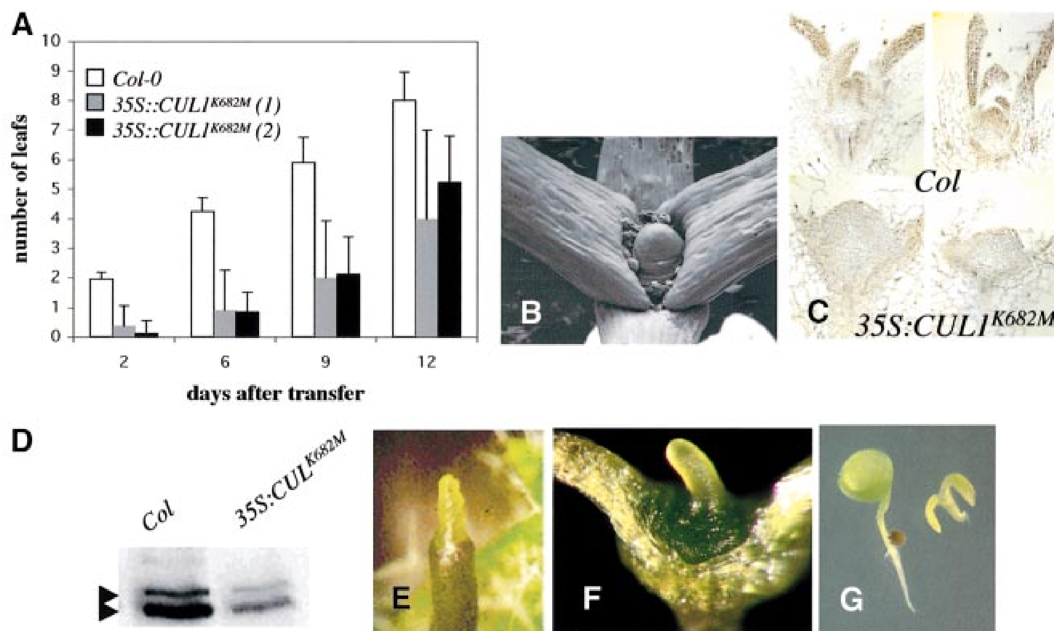

Fig. 6. CUL1-deficient plants display defects in meristem growth and lateral organ initiation. (**A**) Stable CUL1-deficient plants exhibit slow and irregular leaf development. Leaf number was counted at intervals in *Col-0* and two independent *35S::CUL1*^{K682M} lines. (**B**) Scanning electron micrograph of the SAM from a *35S::CUL1* seedling showing the pin phenotype. (**C**) Immunolocalization of CUL1 in *Col-0* and *35S::CUL1*^{K682M} 9-day-old seedlings. Images from two different seedlings are shown for each genotype. The brown staining represents CUL1 protein. (**D**) Protein blot showing reduced CUL1 levels at the apex of 21-day-old plants. Thirty micrograms of total protein extract were loaded in each lane. (**E**) Pin-like meristem from inflorescences of *35S::CUL1*^{K682} plants. (**F**) Homozygous *axr1-13* carrying the *35S::CUL1*^{K682} transgene generated by crossing a stable CUL1-deficient line(*35S::CUL1*^{K682}) to *axr1-13*. All homozygous *axr1-13* seedlings with at least one transgene had this phenotype. (**G**) Example of a T_1 *axr1-3* plant transformed with the *35S:CUL1* construct. Approximately 10% of the T_1 seedlings had this phenotype.

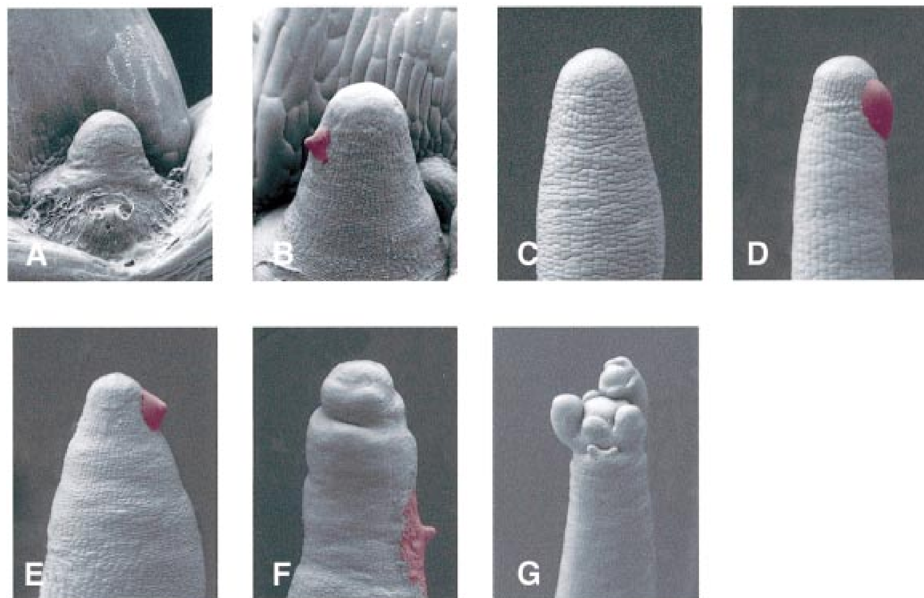

Fig. 7. Response of CUL1-deficient pins to exogenous auxin. (**A**) Two-week-old *35S::CUL1*^{K682} seedling with two cotyledons and two true leaves (one removed) and a central pin. (**B**) Arrested SAM 3 days after application of 1 mM IAA in lanolin paste (red). (**C**) Inflorescence pin on CUL1-deficient plant. (**D**) Inflorescence pin 3 days after local treatment with 1 mM IAA (red). (**E**) Inflorescences pin 3 days after local treatment with 1 mM IAA (red). (**F**) Floral pin 7 days after local treatment with 1 mM IAA (red). (**G**) Spontaneous flower formation on an inflorescence pin after prolonged growth.

initiation is probably related to stabilization of these proteins.

35S::CUL1 pin meristems are auxin insensitive

The failure to initiate lateral organs in *pin1* is related to a defect in auxin transport. Auxin application to the flank of the *pin1* meristem results in normal organ initiation suggesting that localized auxin accumulation is required for organ initiation (Reinhardt *et al*., 2000). To see if CUL1 deficient plants respond to auxin similarly, we applied IAA to the meristems of *35S::CUL1* plants. A total of 59 SAMs were examined. Of 32 pin SAMS that were

not treated, nine recovered and formed new leaves. The other 23 SAMs terminated without further organogenesis. IAA was applied to the flanks of 27 defective SAMs. Twenty terminated without formation of additional leaves (Figure 7A and B). Seven SAMs recovered, but of these only two initiated an organ at the site of IAA application. We conclude from these results that pin SAMs on CUL1-deficient plants are insensitive to auxin. Similarly, application of IAA to the flanks of pin inflorescence meristems on *35S::CUL1* plants did not induce organ formation (n = 25) (Figure 7C–F). Occasionally a meristem resumed flower formation after prolonged growth (Figure 7G). These results indicate that the pin phenotype is related to a defect in auxin response rather than transport. This is consistent with a model in which accumulation of Aux/IAA proteins prevents organ formation in *35S::CUL1* lines.

Discussion

Recent advances in our understanding of auxin action have demonstrated that auxin regulation of transcription involves two families of proteins (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). The ARF transcription factors bind AuxRE promoter elements and either activate or repress transcription depending on the ARF. The short-lived Aux/IAA proteins negatively regulate ARFs, probably through a direct interaction (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). In *Arabidopsis* seedlings, auxin promotes degradation of Aux/IAA proteins by stimulating an interaction between the Aux/IAA proteins and the ubiquitin protein ligase SCF^{TIR1} (Gray *et al*., 2001; Ramos *et al*., 2001; Tiwari *et al*., 2001). In this study we show that decreased AXR6/CUL1 function results in auxin-related defects during embryogenesis and throughout plant development. These results indicate that auxin and SCF-dependent degradation of Aux/IAA proteins is a general aspect of auxin response.

CUL1 is required throughout the plant life cycle

Animal cullins function as subunits in several classes of ubiquitin protein ligases (Deshaies, 1999; Winston *et al*., 1999; Pickart, 2001). There are six known cullin proteins in humans (Winston *et al*., 1999). Cul1 interacts with SKP1–FBP to form SCFs while Cul2 and Cul5 assemble into a second large group of E3s all of which also contain the Elongin BC complex. Cul3 interacts with RBX1 (also called Roc1) to form an E3 that functions to regulate the level of cyclin E and possibly other proteins (Singer *et al*., 1999; Winston *et al*., 1999). In *Arabidopsis* there are at least five genes encoding canonical cullins called CUL1, CUL2, CUL3A, CUL3B and CUL4 (Shen *et al*., 2002). Because of sequence divergence, orthologous relationships between the plant and human cullins are difficult to discern. *CUL1* is expressed throughout the life cycle of the plant from the zygote to the pollen (Farras *et al*., 2001; del Pozo *et al*., 2002; Shen *et al*., 2002). However, because null mutations in *CUL1* result in embryonic arrest at the zygote stage, the function of CUL1 later in development has been impossible to evaluate (Shen *et al*., 2002). The phenotype of the *axr6* mutants, and the CUL1-deficient lines described here, indicate that CUL1 is required for auxin-dependent patterning in the embryo and auxin response throughout the life of the plant. It is also important to note that CUL1 is very likely to be part of many different SCF complexes with diverse physiological roles (Xu *et al*., 2002).

axr6 mutations affect SCF complex formation

The *axr6* mutations affect the same residue near the N-terminus of CUL1, a region known to interact with the SKP1–FBP module (Zheng *et al*., 2002). Co-immunoprecipitation experiments indicate that the mutations alter the interaction between the *Arabidoipsis* SKP1 ortholog, ASK1, and CUL1, but not between RBX1 and CUL1. Consistent with these findings, the results of GST–IAA7 pulldown experiments indicate that less SCF^{TIR1} is present in *axr6* extracts.

The mutations also stabilize CUL1 and decrease the relative levels of RUB-modified CUL1. When the level of RUB-CUL1 is increased by introducing the *35S::RBX1* transgene, CUL1 continues to accumulate to higher levels than the wild-type control suggesting that increased stability of cullin is not directly related to decreased RUB modification. Rather, we suspect that increased CUL1 levels are related to reduced incorporation of CUL1 into active SCF complexes. Some cullin degradation may be a normal consequence of SCF activity. If this is the case, CUL1 that is not assembled into an active SCF may be more stable. This hypothesis may also explain why RBX1 is more abundant in *axr6* plants. A similar effect on CUL1 levels is observed in plants that are severely deficient in the RUB/Nedd8 conjugation pathway (Dharmasiri *et al*., 2003). Seedlings that are deficient for the RUB-activating enzyme AXR1-ECR1, and the RUB-conjugating enzyme RCE1, accumulate high levels of CUL1 (Dharmasiri *et al*., 2003). In this case, the defect is related to SCF function rather than assembly. It is not clear why *axr6* mutants also have decreased relative RUB-CUL1 levels. One possibility is that CUL1 modification requires ASK1–FBP binding. This would also explain why increased levels of RBX1 in the *axr6* mutants are not associated with increased RUB-CUL1 formation as in the wild type (Figure 4C and F). Alternatively, the mutations cause increased removal of RUB, perhaps by the CSN (Schwechheimer *et al*., 2001).

The *axr6* mutations are homozygous seedling lethals and result in auxin resistance in heterozygous plants. Because triploid plants with the genotype *axr6-2/AXR6/AXR6* are auxin resistant, Hobbie *et al*. (2000) proposed that the *axr6-2* allele has a gain-of-function quality. Our results confirm this hypothesis. Heterozygous *cul1-3* and *cul1-4* plants are slightly auxin resistant, but display no other defects, *axr6-1*/+ and *axr6-2*/+ have a higher level of auxin resistance and a pronounced post-embryonic phenotype (Figure 2) (Hobbie *et al*., 2000). It seems likely that this genetic behavior is related to accumulation of defective CUL1. Mutant CUL1 interacts normally with RBX1. Because RBX1 binds the ubiquitin E2, it is possible that both RBX1 and E2 become limiting in *axr6*/+ plants. In addition, CUL1 has been shown to interact with the CSN (Schwechheimer *et al*., 2001). The accumulation of defective CUL1 may disrupt the function of this complex.

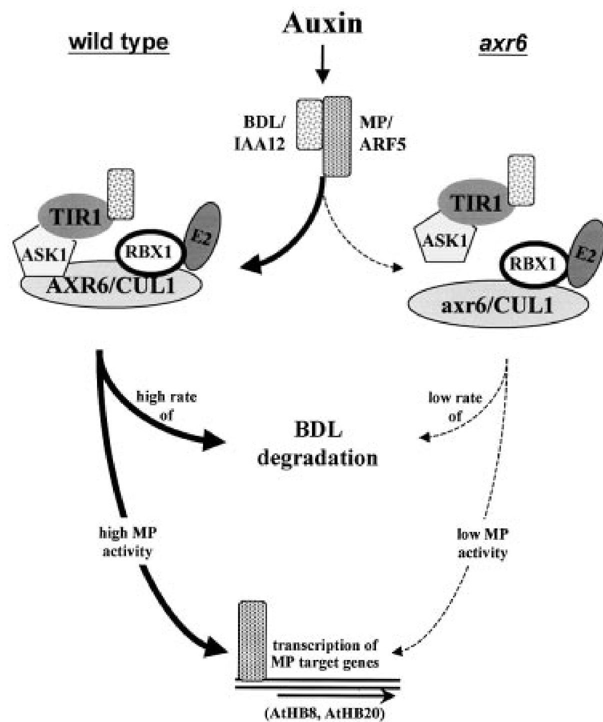

Fig. 8. Role of *AXR6/CUL1* during embryonic development. In wild-type plants (left side) embryonic development requires auxin-dependent degradation of BDL/IAA12 leading to transcription of MP target genes (Mattsson *et al*., 2003). MP transcriptional activators can act either as monomers or dimers (Tiwari *et al*., 2003). Possible target genes include *AtHB8* and *AtHB20* (Mattsson *et al*., 2003). In contrast, the *axr6* mutations affect the interaction between ASK1 and CUL1 and reduce the number of functional SCF complexes (right side). As a result BDL–MP heterodimers are stabilized. Low levels of MP activity lead to abnormal embryonic development.

CUL1 is required for auxin signaling in the embryo

A striking aspect of the *axr6* phenotype is its similarity to the *mp* and *bdl* phenotypes. All three mutations affect the orientation of cell division as early as the two-cell stage of the embryo and they each lack the SAM, hypocotyl and root meristem and display vascular defects (Berleth and Jurgens, 1993; Hamann *et al*., 1999; Hobbie *et al*., 2000). These similarities suggest that *AXR6*, *MP* and *BDL* function in the same pathway during embryogenesis. The molecular characterization of the gene products provides strong support for this view. *MP* encodes ARF5 while *BDL* encodes IAA12 (Hardtke and Berleth, 1998; Hamann *et al*., 2002). These two proteins interact in a yeast two-hybrid test and are co-expressed during early embryogenesis, consistent with a model in which BDL negatively regulates MP function (Hamann *et al*., 2002). Our demonstration that *AXR6* encodes CUL1 strongly suggests that degradation of BDL/IAA12 requires the action of an SCF. A model describing the role of these proteins is presented in Figure 8. We propose that the *axr6* mutations affect the levels of functional SCF resulting in abnormal accumulation of BDL and subsequent repression of MP function. It is also possible that an APC containing the HBT protein contributes to BDL/IAA12 degradation (Blilou *et al*., 2002). However, the *axr6* phenotype suggests that one or more SCF complexes are primarily responsible for degradation of these repressors during embryogenesis. This view is also consistent with the phenotype of the recently described *axr1 rce1* double mutant (Dharmasiri *et al*., 2003). These plants also have a seedling phenotype that is similar to *axr6*, *bdl* and *mp*. The *axr1* and *rce1* mutations affect SCF function indirectly by reducing the levels of RUB-modified CUL1. The APC also has a cullin-related subunit, but this protein is not modified by RUB (Yeh *et al*., 2000).

The identity of MP-regulated genes is unclear. In a recent study, Mattsson *et al*. (2003) showed that expression of the HD-ZIP genes *AtHB8* and *AtHB20* is increased in plants overexpressing MP (Mattsson *et al*., 2003). *AtHB8* has been implicated in vascular development and may be an MP target in provascular cells (Baima *et al*., 2001; Mattsson *et al*., 2003). Similarly, the identity of the FPB involved in BDL destruction is unknown. The *TIR1* gene is expressed during embryogenesis but the *tir1* mutants do not have an embryonic phenotype (Gray *et al*., 1999). However, genetic studies indicate that TIR1 and several closely related FBPs have overlapping functions leaving open the possibility that SCF^{TIR1} and/or close relatives are involved in Aux/IAA degradation during embryogenesis (N.Dharmasiri, S.Dharmasiri and M.Estelle, unpublished data).

CUL1 is required for auxin signaling during postembryonic development

The postembryonic phenotype of *axr6* plants indicates that CUL1 and SCF complexes are required for normal auxin response throughout development (Hobbie *et al*., 2000). Heterozygous *axr6* plants have fewer lateral roots, decreased root gravitropism and shorter but more numerous inflorescences. Since these plants retain one wild-type *CUL1* gene they presumably retain significant CUL1 function. A further reduction in CUL1 activity may result in additional defects. Indeed transgenic lines with reduced CUL1 levels have novel phenotypes not observed in the *axr6* or *cul1* knockout lines. For example, light-grown T_1 seedlings with reduced CUL1 levels had much longer hypocotyls suggesting that a negative regulator of light signaling was accumulating in these seedlings. Defects in root growth and inflorescence elongation were also observed.

The most dramatic defects are in meristem growth and organ initiation. In young CUL1-deficient seedlings, the SAM was enlarged compared with the control. This may be related to the much slower rate of leaf initiation in these plants resulting in accumulation of cells in the meristem. After producing several leaves the SAM arrested in a pin-like structure. At this point axillary meristems produced additional rosette leaves and eventually an inflorescence. However, the inflorescence meristem also failed to initiate lateral organs, and terminated in a pin-like structure similar to that observed in the *pin* and *pid* mutants. Unlike these mutants, the pin meristems on CUL1-deficient plants were insensitive to applied auxin indicating a defect in auxin response (Reinhardt *et al*., 1998) (D.Reinhardt and C.Kuhlemeier, unpublished data).

Based on studies of organ initiation in *Arabidopsis* and tomato, Reinhardt and co-workers proposed that organ initiation requires the local accumulation of auxin (Reinhardt *et al*., 2000; Kuhlemeier and Reinhardt, 2001; Reinhardt and Kuhlemeier, 2001). Since auxin

induces degradation of Aux/IAA proteins and the frequency of plants with a pin inflorescence increases ~5-fold in a *tir1* background, the failure to initiate lateral organs is probably related to accumulation of Aux/IAA proteins. Hence, our results are consistent with the hypothesis of Rheinhardt and co-workers and further suggest that auxin promotes organ initiation by stimulating degradation of Aux/IAA proteins at the sites of initiation.

RUB conjugation pathway is required for meristem function

The *axr1* mutants are deficient in the RUB activating enzyme AXR1-RCE1 and have reduced RUB-CUL1 levels (del Pozo *et al*., 2002). The consequences of this change are decreased auxin and jasmonate response and diverse defects in growth and development (Lincoln *et al*., 1990; del Pozo *et al*., 2002; Tiryaki and Staswick, 2002; Xu *et al*., 2002). However, gross defects in meristem structure and function have not been observed, leaving open the possibility that the RUB pathway is not required in this tissue. We find that the effects of reduced CUL1 levels were dramatically enhanced in an *axr1* background indicating that the pathway is required in the meristem. The lack of meristem defects in the *axr1* mutant is probably due to genetic redundancy. The *Arabidopsis* genome contains a gene that is closely related to *AXR1*, that we have called *AXR1 RELATED 1* (*AXL1*). Plants that carry mutations in both genes have an embryo lethal phenotype (N.Dharmasiri and M.Estelle, unpublished data).

Genetic screens have led to the recovery of many genes that function in auxin response. So far, these genes fall into three groups: members of the *ARF* gene family (*MP/ARF5*, *NPH4/ARF7*, *ETT/ARF3*), members of the *Aux/IAA* gene family [*BDL/IAA12*, *AXR2/IAA7* and many others, see Reed (2001) for a review], and genes that function in SCF-dependent protein degradation (*AXR1*, *AXR6*, *TIR1*). Each of these genes is likely to have specific functions in auxin response except for *AXR1* and *AXR6*, both of which have a general role in SCF function. The fact that mutations in *CUL1* result in striking auxin-related phenotypes is indicative of the importance of the SCF for auxin response and the central role of auxin in plant growth and development.

Materials and methods

Plant material

Arabidopsis thaliana plants were grown in soil and in sterile culture at 20–23°C with continuous light. For culture experiments, seeds were surface sterilized for 10 min in 30% (v/v) bleach, 0.01% Triton X-100, washed three times with sterile water and spread on plates using 0.1% agar. Auxin resistance was scored on day 5 of growth on minimal medium containing different concentrations of 2,4-dichlorophenoxyacetic acid. Analyses of segregating populations and identification of auxin resistant plants were done as described in Hobbie *et al*. (2000).

Generation of 35S::CUL1 Arabidopsis plants

CUL1 overexpression constructs were generated by cloning CUL1 cDNA or mutated CUL1 cDNA carrying a point mutation in the RUB modification site (del Pozo *et al*., 1999) behind a *35S CaMV* promoter into the binary vector pROKII using *Sac*I/*Kpn*I sites. Transgenic *Arabidopsis* plants were generated using a floral dip protocol according to Clough and Bent (1998).

Mapping

Heterozygous *axr6-2* (*Columbia*) plants were crossed into *Landsberg erecta* mutants, *ga1* and *prl*. Selection, analysis and DNA isolation from F_3 families was performed as described in Hobbie *et al*. (2000).

RNA expression analyses

Col-0, *axr6-1* and *axr6-2* seedlings were pre-selected for 5 days on minimal medium containing 40 nM 2,4-D. Auxin-resistant seedlings were then transferred to medium without 2,4-D and grown for 2 additional days. For 2,4-D induction, plants were transferred to liquid minimal medium containing 50 µM 2.4-D for 1 h. RNA extraction, gel electrophoresis and blotting were done using standard techniques.

In vitro translation and RUB modification

CUL1 cDNA was cloned into the *Sma*I site of pBluescript (±) (Stratagene) and used for introduction of *axr6-1* and *axr6-2* point mutations by a Stratagene mutagenesis kit with complementary primer pairs (*axr6-1mut*, 5′- GGTCAGATGGCTATCCCGCGTCTTCTACTACCTTGACCG; *axr6-1complement*, 5′-CGGTCAAGGTAGTAGAAGACGCGGGATAGCCATCTGACC; *axr6-2mut*, 5′-GGTCAGATGGCTATCCCGCATCTTCTACTACCTTGACCG; *axr6-2complement*, 5′-CGGTCAAGGTAGTAGAAGATGCGGGATAGCCATCTGACC). Coupled transcription/translation reactions of *CUL1* and mutant cDNAs (1 mg each) were performed in the TNT-T7 coupled system (Promega, Madison, WI) using [^{35}S]trans-labeled methionine (ICN). After a 90 min reaction time, GST–RUB1, 3 mM ATP, 0.1 mM DTT, 5 mM $MgCl_2$ and 10 U/ml inorganic pyrophosphatase were added to the reaction and the mixtures were incubated at 25°C for 15 min. Reactions were stopped by adding 4× SDS/DTT loading buffer and boiling for 10 min. Proteins were resolved on an SDS–PAGE/10% acrylamide gel. Products were detected by autoradiography.

Protein extraction, immunoprecipitations and GST pulldowns

Proteins were extracted from plant tissue using a standard extraction buffer [100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% (v/v) Igepal]. Samples were left 10 min on ice before centrifugation for 15 min in a microcentrifuge. Supernatant containing 30 µg protein was used for western blot analysis while up to 3 mg protein was used for immunoprecipitation (IP) or pulldown experiments. For IPs and pulldowns, extracts were incubated at 4°C with primary antibody or GST fusion proteins. Proteins were recovered from the extract by adding protein agarose beads (Sigma, St Louis, MO) or glutathionine beads (Sigma) and incubating for 1 h. Beads were washed four times with extraction buffer before loading on a SDS–PAGE/10% acrylamide. Detection of proteins on nitrocellulose membrane was done using an ECL kit (Amersham, Arlington Heights, IL).

Pulse–chase experiments

axr6/+ seedlings were identified by growth on medium containing 2,4-D. Wild-type and mutant seedlings were incubated for 3.5 h in liquid sugar-free minimal medium supplemented with 200 µCi of trans-labeled [^{35}S]methionine. After washing with water, seedlings were either directly frozen in liquid nitrogen or incubated for 17 min with 1 mM cold methionine/cysteine (Sigma) and cycloheximide (125 µg/ml) before freezing in liquid nitrogen. Protein extraction, immunoprecipitation and detection of AXR2/IAA7 were done as described previously (Gray *et al*., 2002).

Immunolocalization of CUL1

Paraffin embedded 9-day-old seedlings grown in sterile culture were sectioned (10 µm) and transferred to Superfrost Plus Micro Slides (VWR). Before incubation with the primary antibody, sections were deparaffinized in xylene, rehydrated in an decreasing ethanol series, treated for 20 min with 0.2 M HCl and protease K (1 µg/ml) and were re-fixed in a freshly prepared 4% paraformaldehyde solution. Sections were incubated overnight at 4°C with an affinity purified αCUL1 as primary antibody. Detection of the primary antibody was done using a secondary biotin-conjugated antibody and biotin–avidin detection kit with peroxidase activity (Vector Laboratories, Burkingame, CA).

Exogenous application of auxin and scanning electron microscopy

For local treatments of apices, IAA (Fluka, Buchs, Switzerland) 100 mM stock solutions in DMSO were dissolved in a pre-warmed (50°C) paste consisting of lanolin with 2.5% paraffin (Merck) according to Reinhardt *et al*. (2000) to give a final concentration of 1 mM IAA. The paste was

manually applied directly to *Arabidopsis* inflorescence apices on the intact plant. For microscopic analysis, apices were viewed with an S-3500N variable pressure scanning electron microscope from Hitachi (Tokyo, Japan), equipped with a cool stage. Lanolin paste in digital images was pseudocolored for clarity.

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