

# AXL and AXR1 have redundant functions in RUB conjugation and growth and development in Arabidopsis

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## Summary

Cullin-RING ubiquitin-protein ligases such as the Skp1, cullin, F-box protein (SCF) have been implicated in many growth and developmental processes in plants. Normal SCF function requires that the CUL1 subunit be post-translationally modified by related to ubiquitin (RUB), a protein related to ubiquitin. This process is mediated by two enzymes: the RUB-activating and RUB-conjugating enzymes. In Arabidopsis, the RUB-activating enzyme is a heterodimer consisting of AXR1 and ECR1. Mutations in the *AXR1* gene result in a pleiotropic phenotype that includes resistance to the plant hormone auxin. Here we report that the *AXL* (*AXR1*-like) gene also functions in the RUB conjugation pathway. Overexpression of *AXL* in the *axr1-3* background complements the *axr1-3* phenotype. Biochemical analysis indicates that *AXL* overexpression restores CUL1 modification to the wild-type level, indicating that AXR1 and AXL have the same biochemical activity. Although the *axl* mutant resembles wild-type plants, the majority of *axr1 axl-1* double mutants are embryo or seedling lethal. Furthermore, the *axl-1* mutation reveals novel RUB-dependent processes in embryo development. We conclude that AXR1 and AXL function redundantly in the RUB conjugating pathway.

**Keywords:** auxin, embryogenesis, related to ubiquitin, Skp1, cullin, F-box protein, ubiquitin.

## Introduction

The phytohormone auxin (indole-3-acetic acid or IAA) is an essential regulator of plant growth and development from embryogenesis to senescence. To understand how plants respond to auxin at the cellular and molecular level, several mutant screens have been conducted in the model plant Arabidopsis, and a number of auxin-resistant mutants have been identified. One of these mutants, *auxin resistant1* (*axr1*) (Lincoln *et al.*, 1990), has a pleiotropic phenotype that includes defects in root gravitropism, lateral root formation, root hair growth, apical dominance, plant height and fertility. Molecular and biochemical studies indicate that *AXR1* encodes a subunit of a heterodimeric RUB (related to ubiquitin, Nedd8 in animals)-activating enzyme (Leyser *et al.*, 1993; del Pozo *et al.*, 1998). The other subunit in this enzyme is called ECR1 (del Pozo *et al.*, 1998).

The ubiquitin–proteasome pathway consists of three proteins or protein complexes called the ubiquitin-activating

enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin protein ligase (E3). The E3s are responsible for the specificity of the pathway, and constitute very large families in plants and other eukaryotes (Pickart, 2001). One superclass of E3s are the multisubunit cullin-RING ligases (also called CRLs), each of which contains a cullin protein, a RING protein and a substrate receptor protein (Petroski and Deshaies, 2005). The best-known CRLs are the SCF (Skp1, cullin, F-box protein) E3s, in which the F-box protein functions as the substrate receptor. SCFs are found in all eukaryotes and are responsible for the degradation of a wide variety of proteins involved in many different cellular processes (Petroski and Deshaies, 2005). In Arabidopsis, SCFs have been implicated in senescence (Woo *et al.*, 2001), apical dominance (Stirnberg *et al.*, 2002), circadian rhythm (Nelson *et al.*, 2000; Somers *et al.*, 2000), flower and meristem development (Samach *et al.*, 1999; Zhao *et al.*, 1999)

(Ingram *et al.*, 1997), phytochrome A signaling (Dieterle *et al.*, 2001), self-incompatibility (McClure, 2004) and phytohormone signaling (Gray *et al.*, 1999; McGinnis *et al.*, 2003; Ruegger *et al.*, 1998; Sasaki *et al.*, 2003; Xie *et al.*, 1998). The best-characterized SCF in plants is SCF<sup>TIR1</sup>, which is implicated in auxin signaling. SCF<sup>TIR1</sup> and the related E3s SCF<sup>AFB1</sup>–SCF<sup>AFB3</sup> promote the degradation of the Aux/IAA transcriptional repressors in an auxin-dependent manner (Dharmasiri *et al.*, 2003a; Gray *et al.*, 1999; Gray *et al.*, 2001). Recent studies have shown that auxin binds directly to TIR1 to promote interaction between the SCF and the Aux/IAA substrate (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005).

Like ubiquitin, RUB is conjugated to other proteins by E1 and E2 enzymes. In Arabidopsis, these proteins are the AXR1-ECR1 heterodimer and RCE1, respectively (del Pozo *et al.*, 1998, 2002). Studies in Arabidopsis as well as many other species indicate that the function of the RUB conjugation pathway is to attach RUB to members of the cullin family of proteins, including the CUL1 subunit of the SCF. In the case of the SCF, RUB modification of CUL1 is required for normal E3 function (Petroski and Deshaies, 2005). Thus, mutations in components of the RUB conjugation pathway, such as *axr1* and *rce1*, result in stabilization of the Aux/IAA proteins and decreased auxin response (Dharmasiri *et al.*, 2003b; del Pozo *et al.*, 1998). Furthermore, the double mutant *axr1 rce1* is severely affected in RUB modification of CUL1, and displays an early seedling lethal phenotype (Dharmasiri *et al.*, 2003b). This phenotype is similar to that of quadruple mutants lacking the TIR1/AFB auxin receptors, as well as *bdl/iaa12* and *mp/arf5* mutants, suggesting that RUB modification of CUL1 is essential for the degradation of SCF<sup>TIR1</sup> targets and normal embryogenesis (Dharmasiri *et al.*, 2003b; Weijers and Jurgens, 2005). Plants that are deficient in the related RUB1 and RUB2 proteins are arrested early in embryogenesis, confirming the importance of RUB conjugation to plant growth and development (Bostick *et al.*, 2004). The *axr1*, *rce1* and *rub* mutants are also affected in other hormone processes, including the jasmonic acid (JA) response and the regulation of ethylene biosynthesis, underscoring the general role of RUB conjugation in SCF function (Dharmasiri *et al.*, 2003b; Larsen and Cancel, 2004).

Although the RUB conjugation pathway is clearly important for plant growth and development, null *axr1* alleles are viable (del Pozo *et al.*, 1998). A search of the Arabidopsis genome identified *AXL* (*AXR1-like*), which encodes a protein with a high level of similarity to AXR1. Here we report on the characterization of *AXL*. Our studies show that *AXL* has a similar function to AXR1 in the RUB conjugation of CUL1. Genetic studies indicate that *AXL* and *AXR1* are functionally redundant. Thus, the loss of both genes results in severe effects on plant growth and development, confirming that the RUB conjugation pathway is essential for viability.

## Results

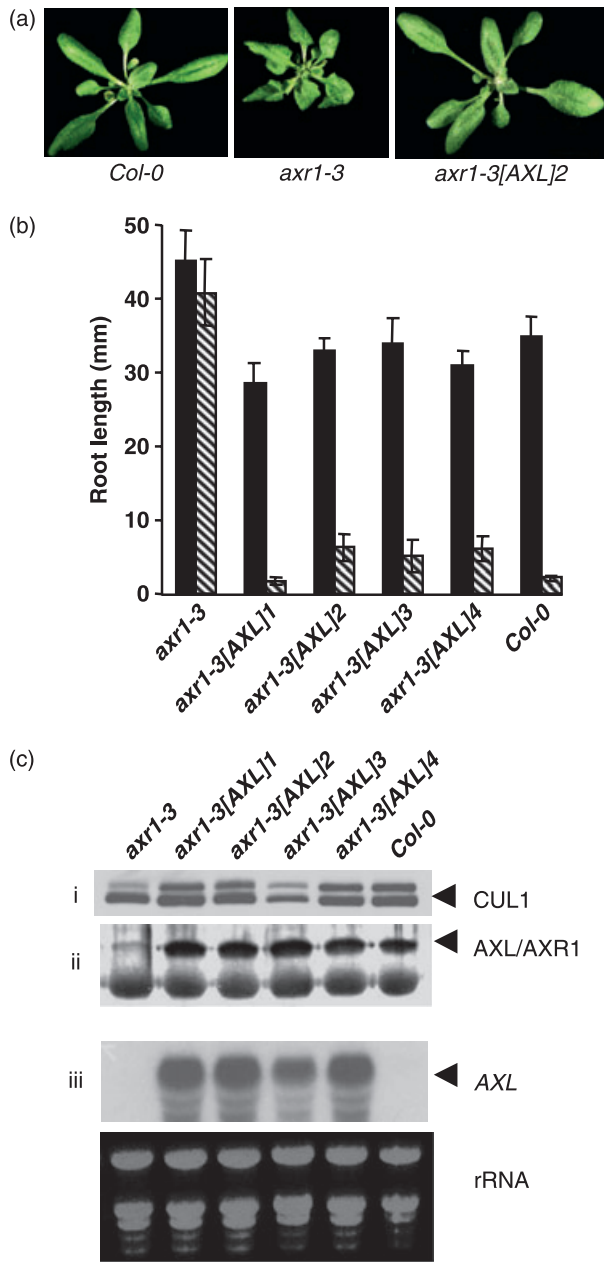
### *The AXL gene can replace AXR1*

The relatively weak phenotype of the *axr1* mutants, compared with the embryo lethal phenotype of mutants that are deficient in the *RUB1* and *RUB2* genes, prompted us to search for a second gene that can provide RUB-activating activity (Bostick *et al.*, 2004). The Arabidopsis genome contains a single gene (*At2 g32410*) with strong similarity to *AXR1*, which we named *AXL* (*AXR1-LIKE*). The protein encoded by this gene is 80% identical to AXR1. Based on this similarity, we hypothesized that *AXL* may also be part of a RUB E1 enzyme. To investigate this, we generated a construct in which the *AXL* cDNA was placed adjacent to the *CaMV35S* promoter, and introduced this construct into *axr1-3* plants. Four transgenic lines were selected for further characterization. In all four lines, the wrinkled rosette leaf phenotype characteristic of *axr1-3* plants was restored to a wild-type appearance (Figure 1a). In addition, the *35S:AXL* transgene rescued other aspects of the *axr1-3* phenotype, including inflorescence height and fertility (data not shown). Moreover, the transgene restored normal auxin sensitivity to the roots of *axr1-3* seedlings (Figure 1b). Consistent with these results, each line had a high level of *AXL* RNA and *AXL* protein (Figure 1c).

Previous studies have shown that the level of RUB-CUL1 is reduced in the *axr1* mutants (del Pozo *et al.*, 1998). Thus, we investigated whether overexpression of *AXL* leads to increased levels of modified CUL1. As expected, RUB modification of CUL1 is decreased in *axr1-3* plants as a result of reduced RUB E1 activity (Figure 1c). However, the level of RUB-CUL1 was restored in each transgenic line relative to wild-type levels, indicating that *AXL* can replace AXR1 in the RUB conjugation pathway.

### *AXL gene expression*

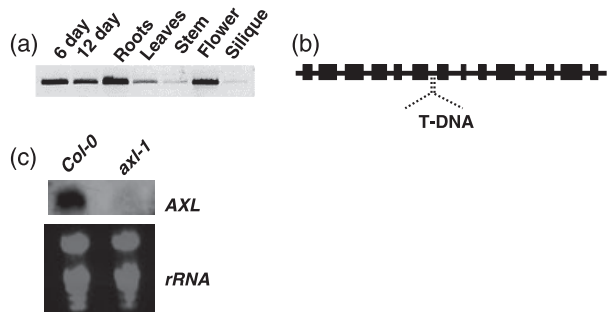
To investigate the pattern of *AXL* expression we performed a reverse transcription polymerase chain reaction (RT-PCR) experiment using RNA isolated from various tissues (Figure 2a). The abundance of *AXL* mRNA was extremely low compared with *AXR1* mRNA (data not shown). Although *AXL* RNA was detected in all the tissue types examined, root tissues showed the highest RNA abundance. However, at least 50 PCR cycles were required to obtain a band detectable by standard agarose gel electrophoresis. The relatively low level of expression of *AXL* in all tissues is also confirmed by microarray analysis (Schmid *et al.*, 2005) (<http://www.weigelworld.org/resources/microarray/AtGenExpress>). Notably, in these studies, the expression patterns of *AXR1* and *AXL* are very similar, but *AXR1* is consistently expressed at a level that is higher than *AXL* expression, by about one order of magnitude. Furthermore, a query of available microarray data



**Figure 1.** Overexpression of the *AXL* gene rescues the defects exhibited by *axr1-3* mutant plants.

(a) Rosettes of *Col-0*, *axr1-3* and *axr1-3 [35S::AXL]* seedlings. (b) Root growth of *Col-0*, *axr1-3* and four transgenic lines of *axr1-3 [35S::AXL]* seedlings on medium containing 85 nM 2,4-D. Seedlings (5 days old) growing on vertical agar plates were transferred on to 2,4-D containing medium, and root elongation was measured after 3 days. Error bars represent SD. (c) *AXL* overexpression increases RUB-CUL1 levels in *axr1-3* plants. (i) Immunoblot of CUL1 in *Col-0*, *axr1-3* and *axr1-3 [35S::AXL]* lines. (ii) Immunoblot of AXR1/AXL. (iii) RNA blot of *AXL* mRNA. An ethidium bromide stained RNA gel is shown in the bottom panel.

using GeneInvestigator shows that *AXL* is expressed at a low level under all conditions examined (Zimmermann *et al.*, 2005).



**Figure 2.** Expression of *AXL* in *Col-0* and *axl-1* mutant plants.

(a) RT-PCR of *AXL* mRNA in *Col-0* plants in 6- and 12-day-old seedlings, roots from 12-day-old seedlings, and rosette leaves, inflorescence stems, flowers, and siliques from 27-day-old plants. (b) Structure of the *AXL* gene. Boxes represent exons. The position of the T-DNA insertion in the *axl-1* mutant is indicated. (c) RNA blot showing reduced *AXL* mRNA levels in 6-day-old *axl-1* plants compared with *Col-0* seedlings. An ethidium bromide stained RNA gel is shown in the bottom panel.

#### Isolation of a T-DNA insertion line in the *AXL* gene

To investigate the function of *AXL*, we screened a T-DNA insertion mutant population in the *Wassilewskija* (*Ws*) ecotype, and identified a line carrying a single T-DNA insertion in *AXL*, designated *axl-1*. Like *AXR1*, the *AXL* gene consists of 14 exons and 13 introns. The exon/intron boundaries in the two genes are conserved, indicating that the two genes were produced by a relatively recent duplication event. The T-DNA insertion is located in the 6th intron (Figure 2a). RNA blot analysis indicated that *axl-1* is lacking full-length *AXL* mRNA (Figure 2c).

Despite the fact that *axl-1* appears to be a null allele, homozygous *axl-1* plants do not exhibit an obvious mutant phenotype. The rosettes are normal, and mutant plants bolt at the same time as the wild-type plants, producing a normal and fully fertile inflorescence. In contrast, even the moderately auxin-resistant *axr1-3* mutant displays abnormal floral features such as short stamens, reduced fertility and short siliques (Lincoln *et al.*, 1990). However it is important to note that *axl-1* plants may exhibit a phenotype under conditions that we have not examined, possibly reflecting a novel function of the *AXL* protein.

#### The *axl-1* and *axr1-12* mutations have synergistic effects on plant growth and development

To determine if *AXL* might function redundantly with *AXR1*, we generated a double mutant line between *axl-1* and *axr1-12*, a strong *AXR1* allele. The resulting F<sub>1</sub> plants were similar to wild type in appearance (data not shown). The segregating F<sub>2</sub> population was studied in detail to investigate the effect of *AXR1* and *AXL* gene dose on plant morphology. The *AXR1/axr1-12*, *axl-1/axl-1* plants were similar to wild type

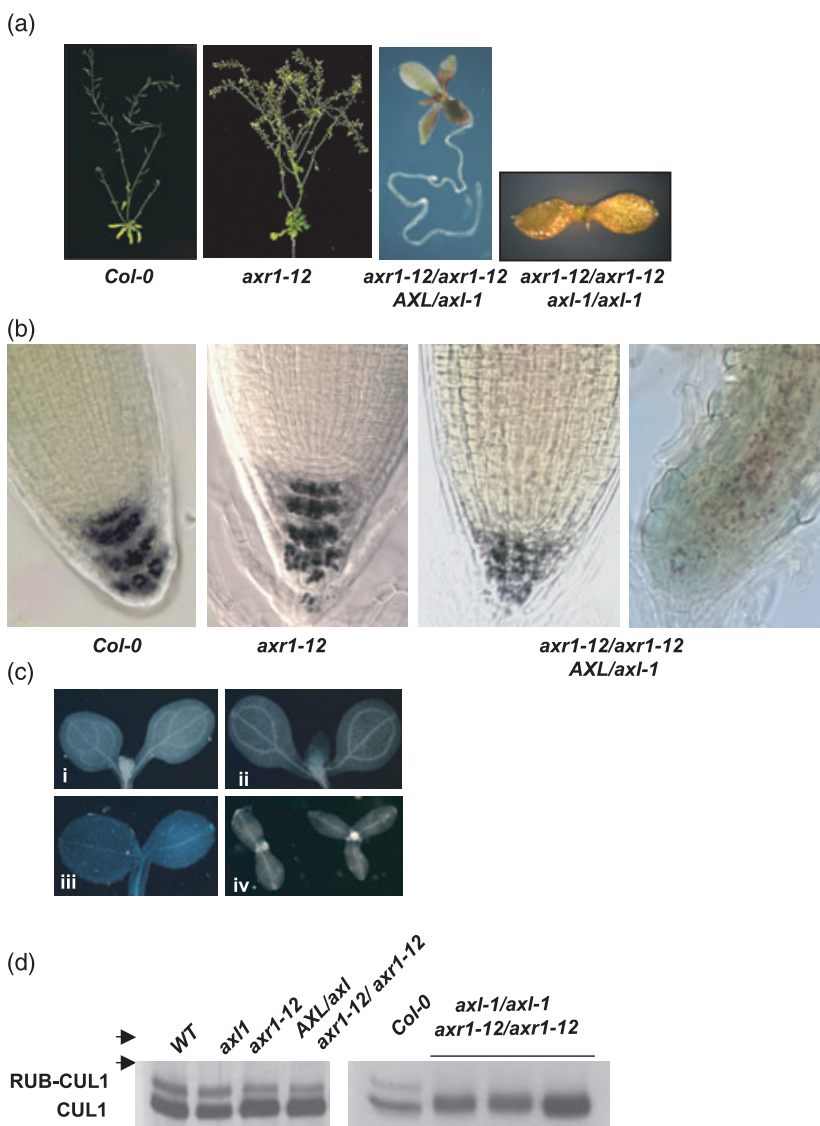
with respect to overall morphology and fertility (data not shown). However, as shown in Figure 3a, homozygous *axr1-12* plants that are heterozygous for *axl-1* display severe growth defects. These seedlings developed short, slow growing roots compared with wild-type roots, and were arrested at the late seedling stage.

In addition, plants that were homozygous for both mutations displayed severe growth and developmental defects. The phenotype of these plants resembled the *bdl/iaa12* and *mp/arf5* mutants (Berleth and Jurgens, 1993; Hamann *et al.*, 1999) (Figure 3a). These seedlings did not segregate at the expected 1/16 ratio. Instead, we identified three double mutant seedlings in 176 F<sub>2</sub> plants, suggesting the homozygous double mutants are arrested during embryogenesis. A further characterization of embryo defects in the *axr1 axl-1* double mutant is described below.

Auxin has been implicated in the patterning of the root meristem, and previous studies have shown that the *axr1* mutants display a reduction in the number of columella cells (Sabatini *et al.*, 1999). Similarly, the loss of the auxin receptors TIR1 and AFB1, AFB2 and AFB3, has a pronounced effect on the development and maintenance of the root meristem (Dharmasiri *et al.*, 2005b). To determine the contribution of AXL to the root meristem, we stained wild-type, *axl-1*, *axr1-12* and *AXL/axl-1 axr1-12/axr1-12* seedling roots with Lugol solution and examined root meristem organization. The *axl-1* single mutant did not display a defect in meristem organization (data not shown). As described previously, we frequently observed a slight decrease in the number of columella columns in *axr1-12* seedlings compared with wild type (Figure 3b) (Sabatini *et al.*, 1999). In contrast, the removal of a single AXL copy resulted in

**Figure 3.** AXR1 and AXL have redundant functions in plant development.

(a) The two left-hand panels show 27-day-old *Col-0* and *axr1-12* plants. The other panels show 12-day-old *axr1-12 axr1-12*, *AXL/axl-1* and *axr1-12 axr1-12, axl-1 axl-1* seedlings. These seedlings did not progress further.  
 (b) Lugol stained meristems from *Col-0*, *axr1-12* and *axr1-12 axr1-12, AXL/axl-1* seedlings. All seedlings are 6 days old, except for the seedling on the far right, which is 12 days old.  
 (c) Cotyledon vascular patterns in 6-day-old seedlings: (i) *Col-0*, (ii) *axr1-12*, (iii) *axr1-12 axr1-12, AXL axl-1* and (iv) *axr1-12 axr1-12, axl-1 axl-1*.  
 (d) Immunoblot of CUL1 in 6-day-old *Col-0*, *axl-1*, *axr1-12* and double mutant seedlings.



dramatic changes in the columella. The columella of *AXL/axl-1 axr1-12/axr1-12* root tips is highly disorganized during early seedling root growth, and is completely disrupted at the time when root growth ceases (Figure 3b).

Auxin is also known to play a role in vascular patterning in plants (Berleth *et al.*, 2000), and loss of AXR1 and RCE1 activities results in defects in vascular development (Dharmasiri *et al.*, 2003b). To investigate the impact of loss of AXL on vascular patterning, we examined the cotyledons of *axr1-12 axl-1* double mutants. As predicted, *axr1-12 axl-1* double mutants showed greatly reduced vascular development similar to that in *mp*, *bdl*, *axr6* and the *axr1-12 rce1* double mutant (Dharmasiri *et al.*, 2003a; Hamann *et al.*, 1999; Hellmann *et al.*, 2003; Przemeck *et al.*, 1996) (Figure 3c).

In summary, although not apparent in the *axl-1* single mutant, the *axr1 axl-1* double mutant reveals an important function for AXL throughout plant development that is redundant with the function of AXR1.

#### *RUB modification of CUL1 is affected in axr1 axl-1 double mutants*

To investigate the relationship between seedling lethality and RUB modification of CUL1, the level of RUB-CUL1 was examined in wild type and in various mutant genotypes. As shown in previous studies, the *axr1-12* mutant exhibits reduced RUB modification of CUL1 (Figure 3d) (Dharmasiri *et al.*, 2003a). However, the *axl-1* mutant has a normal level of RUB-CUL1 that is in keeping with the lack of any growth defect in these plants. Plants with the genotype *AXL/axl-1 axr1-12/axr1-12* had a slight but consistent reduction in RUB-CUL1 levels (Figure 3d), which is consistent with the severe phenotype displayed by these seedlings. However, the relatively rare homozygous double mutant seedlings are completely deficient in RUB-modified CUL1 (Figure 3d).

#### *Auxin response is reduced in axr1-12 axl-1 double mutants*

In order to study the role of AXL on auxin response, we determined the effects of auxin on root growth in single and double mutants. As expected, the *axr1-12* mutant was clearly resistant to auxin (Figure 4a). In contrast, the *axl-1* mutant was very similar to the *Ws* parental line. However, the *axr1-12/axr1-12 AXL/axl-1* line was nearly completely resistant to auxin, a much more severe phenotype than for *axr1-12* alone. This result demonstrates that AXL functions in auxin response.

Previous studies have shown that AXR1 is also required for the JA response (Tiryaki and Staswick, 2002). This requirement is probably related to SCF<sup>CO11</sup>, an E3 known to be required for JA signaling (Xie *et al.*, 1998). To determine if AXL is also required for the JA response, we performed root growth assays with our mutant lines (Figure 4b). As for

auxin, we found one *axl-1* gene dramatically reduces the JA response in *axr1-12* plants.

To obtain additional evidence for a role for AXL in auxin signaling, we also examined expression of the auxin-regulated genes *IAA2*, *IAA5* and *IAA7*. The induction of *IAA2* and *IAA5* was dramatically reduced in *axr1-12/axr1-12 AXL/axl-1* plants compared with the wild type (Figure 4c), and was also reduced compared with *axr1-12*. Induction of *IAA7* was also reduced, although to lesser extent.

Previous studies show that defects in SCF<sup>TIR1</sup> and/or SCF<sup>AFB</sup>s stabilize the Aux/IAA proteins in Arabidopsis (Gray *et al.*, 2001; Dharmasiri *et al.*, 2005b). To determine whether Aux/IAA degradation in response to auxin treatment is altered in the *axl-1* mutants we crossed the *HS::AXR3NT-GUS* transgene into *axl-1* and *AXL/axl-1 AXR1/axr1-12* lines and screened for homozygous *GUS axl-1 axr1-12* plants. The resulting seedlings were heat shocked for 2 h at 37°C to induce GUS expression, and AXR3-GUS stability in the presence of auxin was tested using GUS histochemical assays. As shown in Figure 4d AXR3-GUS is degraded in wild type, but is relatively stable in *axr1-12* plants. In *axl-1 axr1-12* double mutants GUS staining is even stronger than for *axr1-12*, suggesting a strong correlation between Aux/IAA stability and the severe growth and developmental defects of double mutant plants.

#### *The RUB conjugation pathway is required during early embryogenesis*

The AXR1 protein accumulates throughout plant development, starting very early during embryogenesis in the zygote (del Pozo *et al.*, 2002). However, it is not known whether its function is required at this early stage. Because AXR1 appears to be the major RUB E1 (see above), we first investigated embryo development in the null allele *axr1-12*, and found no deviation from the wild type (data not shown). As AXR1 shares a number of redundant functions with AXL, we next examined embryo development in the *axl-1* single and *axr1-12 axl-1* double mutants. No embryo defects were found in *axl-1* ( $n = 92$ ). In contrast, very strong embryo defects were observed in homozygous *axl-1* plants segregating the *axr1-12* mutation. These phenotypes fell into two classes based on the severity of their defects. Embryos in one class (7% of total embryos;  $n = 116$ ) were very severely affected and displayed developmental arrest after a few rounds of, often abnormal, cell divisions. Examples of such embryos are shown in Figure 5, and show aberrant planes of cell division (Figure 5e), bloating of cells (Figure 5f) and occasionally failure to complete cell walls (Figure 5g).

A larger fraction of double mutant embryos (19% of total embryos;  $n = 116$ ) were less severely affected, with patterning defects reminiscent of *bdl*, *mp* and *tir1 afb2 afb3* mutants (Dharmasiri *et al.*, 2005b) (Figure 5b,d). As the percentage of abnormal embryos is close to 25%, the double

**Figure 4.** The *axl-1* mutation enhances auxin response defects in *axr1-12* mutant plants.

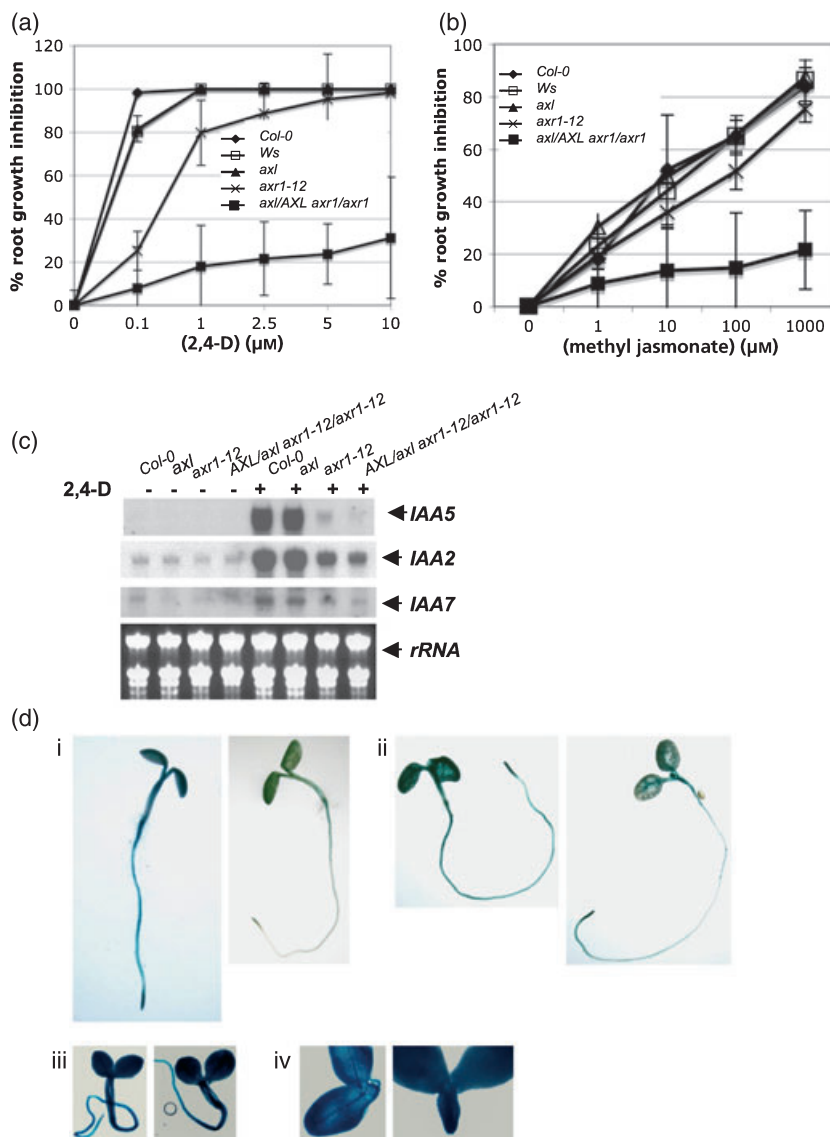
(a) Percentage of root growth inhibition in response to increasing levels of 2,4-D.

(b) Percentage of root growth inhibition in response to increasing levels of methyl jasmonate. In both (a) and (b) ♦, *Col-0*; ▲, *Ws*; □, *axl1*; ×, *axr1-12*; ■, *axr1-12 axr1-12*, *AXL/axl-1*. Error bars represent SE.

(c) *Aux/IAA* transcript levels in response to auxin. Seedlings (6 days old) were incubated in liquid growth medium with or without 20 μM 2,4-D for 60 min prior to RNA extraction. Lanes 1 and 5 (*Col-0*), 2 and 6 (*axl-1*), 3 and 7 (*axr1-12*), and 4 and 8 (*AXL axl-1, axr1-12 axr1-12*). The blot was probed with radiolabelled *IAA5*, *IAA2* and *IAA7* cDNA probes. An ethidium bromide stained RNA gel is shown at the bottom.

(d) Stability of AXR3NT-GUS fusion protein in *Col-0*, *axr1-12* and *axr1-12 axl-1* double mutant seedlings. The seedlings were heat treated at 37°C for 2 h and histochemically stained for GUS activity at 0 min (left) and 60 min (right) after heat treatment:

(i) *Col-0 HS::AXR3NT-GUS*; (ii) *axr1-12 HS::AXR3NT-GUS*; (iii) *axr1-12 axr1-12, AXL axl-1 HS::AXR3NT-GUS*; (iv) *axr1-12 axr1-12, axl axl-1 HS::AXR3NT-GUS*.



mutants appear to have a fully penetrant phenotype, without clear evidence for gametophytic defects. It is unclear what causes the variability in phenotype. As the rootless double mutant seedlings described above are found at a rate of 1–2%, it is likely that most of the double mutant embryos in the weaker class are either arrested late in embryo development or do not germinate. Nonetheless, AXR1 and AXL share redundant functions in early embryogenesis, and their activities are required for proper cell division and embryo patterning.

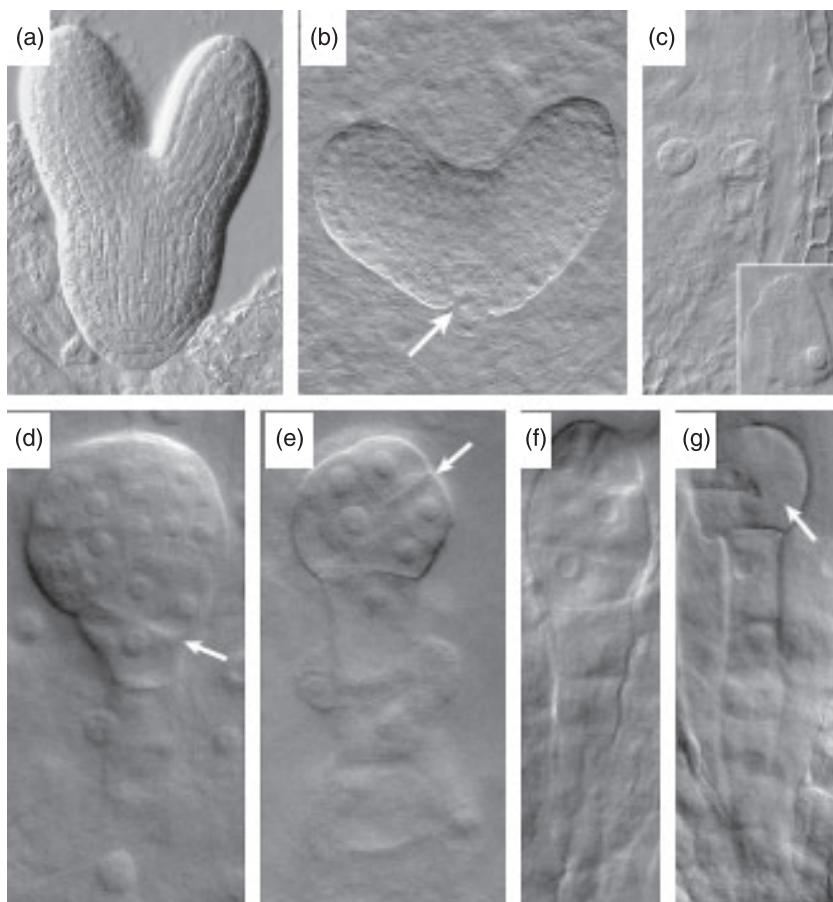
**Discussion**

In addition to ubiquitin, eukaryotic organisms contain several ubiquitin-like proteins (Ubls) that are post-translationally conjugated to a variety of other proteins (Kerscher *et al.*, 2006). Ubls have been implicated in diverse cellular pro-

cesses including transcription, cell cycle control and autophagy (Kerscher *et al.*, 2006). In the case of RUB/Nedd8, attachment of the Ubl to the cullin subunit of CRLs is required for CRL activation. The best-characterized CRL, the SCF complex, is involved in a wide variety of hormonal and developmental pathways in Arabidopsis (Moon *et al.*, 2004). As in other organisms, modification of the CUL1 subunit of the SCF by RUB is necessary for normal function of the E3 complex. In this report we show that the AXL protein functions in RUB activation, and that the RUB conjugation pathway is essential for viability in Arabidopsis.

*The AXL protein is part of a RUB E1*

RUB is covalently attached to the cullin through the action of a heterodimeric activating enzyme and a RUB-specific E2 enzyme. In Arabidopsis, these enzymes are called AXR1-



**Figure 5.** Phenotypes of *axr1 axl-1* double mutant embryos.

(a) Wild-type embryo at early torpedo stage.

(b) *axr1-12 axl-1* embryo from the same silique as the wild-type embryo in (a), showing the absence of the root primordium (arrow).

(c) Arrested *axr1-12 axl-1* mutant embryo from the same silique as the embryos in (a) and (b). The inset shows a different region of the same seed with an endosperm nodule.

(d–g) Examples of *axr1 axl-1* double mutant embryos.

(d) Globular-stage embryo with aberrant hypophysis division (arrow indicates plane of cell division).

(e) Dermatogen-stage embryo with irregular cell division planes (arrow).

(f) Octant-stage embryo with erroneous division of the uppermost suspensor cell.

(g) Arrested embryo with incomplete cell division (arrow).

Embryos in (a)–(c) and (d)–(g) were recorded with the same magnification, respectively.

ECR1 and RCE1 respectively. Both the *axr1* and *rce1* mutants display severe growth defects as well as reduced auxin sensitivity (Dharmasiri *et al.*, 2003b; del Pozo *et al.*, 2002). However, both mutants produce some RUB-CUL1, suggesting that they are not completely deficient in RUB-activating and -conjugating activity, respectively. Consistent with this, the *axr1-12 rce1* double mutant is more severe than either single mutant, with respect to both RUB-CUL1 levels and development. The *AXL* gene produces a protein that is 80% identical to *AXR1*, suggesting that it may also function in a RUB E1 enzyme. Indeed, overexpression of *AXL* in the *axr1-3* mutant increases the level of RUB-CUL1 to wild-type levels, and restores the wild-type phenotype to mutant plants, strongly suggesting that *AXL* also functions in a heterodimeric RUB-activating enzyme. As there is only a single *ECR1* gene in *Arabidopsis*, it is likely that *AXL* interacts with *ECR1* to form this enzyme. Interestingly, the *Arabidopsis* genome also has two closely related genes that encode RUB-conjugating enzymes. One of these, called *RCE1*, is expressed at a relatively high level, and has an important role in RUB conjugation (Dharmasiri *et al.*, 2003b). The second, *RCE2*, has not been characterized, but like *AXL* is expressed at a low level throughout development (Schmid *et al.*, 2005).

#### *The RUB-activating enzyme is essential for viability in Arabidopsis*

Genetic studies in a wide variety of eukaryotes indicate that the RUB/Nedd8 pathway is essential for viability (Kerscher *et al.*, 2006). The one exception is the yeast *Saccharomyces cerevisiae*, in which defects in the pathway have little or no effect on cell growth. In *Arabidopsis*, single mutants in *AXR1* and *RCE1* have severe defects, but are nonetheless viable (Dharmasiri *et al.*, 2003b). However, plants that are homozygous for mutations in both genes die as young seedlings. Similarly, plants with mutations in two of the three *RUB* genes (*RUB1* and *RUB2*) are embryo lethal (Bostick *et al.*, 2004). Our results with the *axl-1* mutant confirm that RUB conjugation is required for normal embryogenesis. The *axr1-12 axl-1* mutant also exhibits an embryo or early seedling lethal phenotype, and appears to completely lack RUB-CUL1.

#### *The axl-1 mutation reveals novel RUB-dependent processes in embryo development*

Interestingly, the early embryo arrest of the *axr1-12 axl-1* double mutant allows the definition of additional develop-

mental events during which RUB-dependent CRL activity is required. So far, a series of mutant genotypes has been reported that progressively affect CUL1 levels or the RUB modification of CUL1. Intriguingly, all mutants analyzed so far are arrested either at or directly after the first cell division, or progress normally to the globular stage and fail to initiate a root. The *cul1* knock-out mutant is zygote lethal (Shen *et al.*, 2002), and weaker alleles either cause rootless seedlings or pleiotropic post-embryonic defects (Hellmann *et al.*, 2003; Moon *et al.*, 2007; Quint *et al.*, 2005). Likewise, *rub1 rub2* mutants are arrested at the two-cell stage of embryogenesis, and RNA knock-down causes pleiotropic post-embryonic defects (Bostick *et al.*, 2004). In the case of the RUB conjugation pathway, the earliest defects reported so far are in *axr1rce1* mutants (Dharmasiri *et al.*, 2003b). These double mutant seedlings lack a root and, when examined microscopically, the earliest defect observed was the stage when the root is initiated (22% of 51 embryos in homozygous *axr1-12* plants segregating *rce1-1*) (data not shown). Hence, it is unclear if RUB-dependent CRL activity is required after the first cell division and before root initiation. In addition, *AXR1*, *RCE1* and *CUL1* are all expressed broadly in early embryos at stages where no mutant phenotypes have been reported (Dharmasiri *et al.*, 2003b; del Pozo *et al.*, 1998, 2002; Shen *et al.*, 2002). These results suggest that there are two checkpoints in early embryogenesis where CRL activity is critical for progression. Alternatively, it is possible that the full spectrum of RUB-dependent CRL activity has not yet been revealed, perhaps because of functional redundancy. Indeed, the severe *axr1 axl-1* double mutant phenotype displays defects in stages ranging from the zygote to the dermatogen stage. These defects include aberrant cell division planes, abnormal swelling of cells and incomplete cell divisions. Although some effects may be indirect consequences of defects at earlier stages, these results show that many processes during early development require RUB modification of CULLINs, and that RUB modification is required throughout plant embryogenesis.

## Experimental procedures

### Plant material, growth conditions and treatments

The *axl* mutant is in the *Ws* ecotype, whereas *axr1-3* and *axr1-12* mutants are in the *Columbia* (*Col-0*) ecotype. The *HS::AXR3NT-GUS* has been described previously by Gray *et al.* (2001). Surface-sterilized *Arabidopsis* seeds were germinated on *Arabidopsis thaliana* salts (ATS) medium [1% sucrose, 5 mM KNO<sub>3</sub>, 2.5 mM KH<sub>2</sub>PO<sub>4</sub> (pH 5.6), 2 mM MgSO<sub>4</sub>, 2 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 50 μM CuSO<sub>4</sub>, 1 μM ZnSO<sub>4</sub>, 0.2 mM NaMoO<sub>4</sub>, 10 μM NaCl, and 0.01 μM CoCl<sub>2</sub>] with 0.8% agar. Plates were placed vertically in a growth chamber at 22°C under continuous light. Where necessary, 8–10-day-old seedlings were transferred to soil and grown at 22°C under continuous light.

For root growth assays, 5-day-old seedlings were transferred to ATS medium with or without hormones, and were grown vertically in a growth chamber at 22°C under continuous light for the

designated times. To study auxin-induced gene expression, seedlings were grown on ATS for 6 days, transferred to liquid ATS medium and grown for indicated times with or without auxin. Seedlings were harvested at the end of treatments and used for RNA extraction.

### Generation of transgenic lines

The *AXL* cDNA was cloned into pCR 2.1 vector and the correct sequence was confirmed by DNA sequencing. To construct the *AXL* overexpression lines, *AXL* cDNA was placed behind the *CaMV 35S* promoter in the binary vector *pROKII* and the plasmid construct was introduced into *A. tumefaciens* strain *GV3101*. *Arabidopsis* mutant line *axr1-3* was transformed by using the floral-dip method to generate the *axr1-3(AXL)* lines.

### Isolation of T-DNA insertion line and generation of double mutants

The T-DNA insertion mutant line for the *AXL* gene (*axl-1*) was isolated by screening the University of Wisconsin mutant lines (*Ws*) using the gene-specific primer *AXL1F* (5'-CCACAACTTGCA-ACCAGTTGAA-3') together with the T-DNA left border primer *JL202* (5'-CATTITATAATAACGCTGCGGACATCTAC-3'). After selecting the mutant line, the T-DNA insertion site was confirmed by sequencing the PCR product using the *JL202* primer.

To generate *axl-1 axr1-12* double mutants, homozygous *axl-1* plants were crossed with *axr1-12* lines. As the double mutant is seedling lethal, resulting F<sub>2</sub> progeny were genotyped by PCR to isolate lines that are homozygous for the *axl-1* mutation and heterozygous for the *axr1-12* mutation. Progeny from these plants were used for the experiments to characterize the phenotype of various genotypes. The *HS::AXR3NT-GUS* line was introduced to the parent heterozygous mutant line by crossing.

### Northern, RT-PCR and protein blot analysis

To determine the expression of the *AXL* gene, total RNA was isolated from 100 mg of tissue collected from 10–12-day-old *Arabidopsis* seedlings using Tri-Reagent (Sigma, <http://www.sigmaaldrich.com>). Total RNA (10 μg) was separated on formaldehyde-agarose gel and transferred to nylon membrane. The blot was hybridized to the <sup>32</sup>P-labeled *AXL* cDNA probe. Hybridization and washing of the blot was performed according to standard conditions and exposed to X-ray film. To analyze the expression of *AXL* in different tissues or developmental stages, total RNA was isolated from tissues using the Tri-Reagent. cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, <http://www.invitrogen.com>) and oligo dT primer. The reverse transcription product (1 μl) was used as the template DNA to amplify the *AXL* sequence using specific primers running 50 cycles of PCR.

The expression of *Aux/IAA* genes in mutant plants was determined using 6-day-old seedlings treated with or without 20 μM 2,4-D for 1 h. Total RNA was extracted, separated and blotted as describe above. Blots were hybridized to probes prepared from cDNAs of *IAA2*, *IAA5* or *IAA7*.

To analyze *AXL* protein abundance and the status of the CUL1 protein in various genetic backgrounds, total protein was isolated from 10–12-day-old seedlings using a denaturing buffer containing 125 mM Tris (pH 8.8), 1% sodium dodecyl sulphate (SDS), 10% glycerol and 50 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. Total protein was estimated by the Bradford method. Total protein (20 μg) was separated by SDS-

polyacrylamide-gel electrophoresis and transferred onto polyvinylidene difluoride membrane and incubated with affinity-purified either anti-AXR1 or anti-CUL1 antibody. Protein bands were visualized by horseradish peroxidase conjugated rabbit IgG using enhanced chemiluminescence (ECL; Pierce, <http://www.piercenet.com>).

#### GUS staining and analysis

HS::AXR3NT-GUS seedlings (6 days old) were heat shocked at 37°C for 2 h in liquid ATS medium. The seedlings were collected by filtration and transferred into new medium. Samples were collected immediately (0 min) and after 60 min to stain for GUS activity (Jefferson *et al.*, 1987).

#### Analysis of root tip morphology and cotyledon vascular patterns

Wild-type and mutant seedlings grown on vertical ATS media were stained with Lugol solution (Sigma) for 5 min for root morphology analysis. The seedlings were mounted on Hoyer's medium and observed under the microscope (Meinke *et al.*, 1994). To examine cotyledon vascular patterns, seedlings were first fixed in ethanol:acetic acid:water (6:3:1) by vacuum infiltration and mounted on Hoyer's solution for visualization under dark field optics.

#### Microscopy

For embryo phenotypic analysis, *axr1-12*, *axl-1* and plants segregating *axr1-12* and *axl-1* or *axr1-12* and *rce-1*, were grown, and (in the case of *axr1-12*) pre-selected for the homozygous *axr1-12* phenotype. Developing seeds were collected from several siliques of 5–10 independent plants, and directly mounted in a 8:3:1 (w:v:v) mixture of chloral hydrate, water and glycerol. Embryos were observed with a Nikon microscope (Nikon, <http://www.nikon.com>) equipped with Differential Interference Contrast optics and a Zeiss Axiocam camera (Zeiss, <http://www.zeiss.com>). Digital photos were recorded using Zeiss AXIOVISION software and processed using Adobe PHOTOSHOP CS2 (Adobe, <http://www.adobe.com>).

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