

The RUB/Nedd8 conjugation pathway is required for early development in *Arabidopsis*

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The related-to-ubiquitin (RUB) protein is post-translationally conjugated to the cullin subunit of the SCF (SKP1, Cullin, F-box) class of ubiquitin protein ligases. Although the precise biochemical function of RUB modification is unclear, studies indicate that the modification is important for SCF function. In *Arabidopsis*, RUB modification of CUL1 is required for normal function of SCF^{TIR1}, an E3 required for response to the plant hormone auxin. In this report we show that an *Arabidopsis* protein called RCE1 functions as a RUB-conjugating enzyme *in vivo*. A mutation in the *RCE1* gene results in a phenotype like that of the *axr1* mutant. Most strikingly, plants deficient in both *RCE1* and *AXR1* have an embryonic phenotype similar to *mp* and *bdl* mutants, previously shown to be deficient in auxin signaling. Based on these results, we suggest that the RUB-conjugation pathway is required for auxin-dependent pattern formation in the developing embryo. In addition, we show that RCE1 interacts directly with the RING protein RBX1 and is present in a stable complex with SCF. We propose that RBX1 functions as an E3 for RUB modification of CUL1.

Keywords: auxin/CUL1/RCE1/RUB conjugation/RUB modification

Introduction

The ubiquitin–protein conjugation pathway regulates diverse cellular functions by promoting the ubiquitylation of protein substrates (Pickart, 2001). The pathway consists of three proteins, or protein complexes, called ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3). The SCFs make up one important class of E3 ligases (Deshaies, 1999). SCFs consists of four subunits: an F-box protein responsible for substrate binding, SKP1 (ASK in *Arabidopsis*), the RING protein RBX1/ROC1/HRT1 and CUL1 (Gray *et al.*, 1999). Biochemical and structural studies show that the CUL1 subunit functions as a scaffold for the complex (Zheng *et al.*, 2002). SKP1 and the F-box protein form a subcomplex that binds near the N-terminus of CUL1, while the RBX1 subunit binds sequences near the C-terminus (Furukawa *et al.*, 2000; Zheng *et al.*, 2002).

RBX1 also binds E2, thus bringing E2 in close proximity to the substrate (Deshaies, 1999; Zheng *et al.*, 2002).

The *Arabidopsis* genome encodes ~700 F-box proteins, suggesting a very broad role for SCFs in plants (Gagne *et al.*, 2002). So far, SCF complexes have been directly implicated in response to the plant hormones auxin (SCF^{TIR1}) (Gray *et al.*, 1999) and jasmonate (SCF^{COI1}) (Xu *et al.*, 2002). In addition, genetic studies have implicated F-box proteins in circadian rhythm (Nelson *et al.*, 2000; Somers *et al.*, 2000), senescence (Woo *et al.*, 2001), apical dominance (Stirnberg *et al.*, 2002), flower and meristem development (Ingram *et al.*, 1997; Samach *et al.*, 1999) and phytochrome signaling (Dieterle *et al.*, 2001).

The related-to-ubiquitin protein (RUB), called Nedd8 in some species, is conjugated to the SCF subunit CUL1 through a series of steps similar to ubiquitin conjugation (Yeh *et al.*, 2000; Hellmann and Estelle, 2002). A single RUB molecule is linked to a specific lysine residue near the C-terminus of the protein (Yeh *et al.*, 2000). Genetic studies in diverse species indicate that cullin modification is required for SCF function. In fission yeast, the modification is essential for viability while in mouse and *Caenorhabditis elegans*, a defect in the Nedd8 conjugation pathway results in embryonic lethality (Osaka *et al.*, 2000; Tateishi *et al.*, 2001; Kurz *et al.*, 2002). So far, the precise role of RUB modification is unclear. *In vitro*, the modification increases SCF activity (Morimoto *et al.*, 2000; Podust *et al.*, 2000; Read *et al.*, 2000; Wu *et al.*, 2000). However, genetic studies in *Arabidopsis* and fungi show that both decreased and increased levels of RUB–CUL1 have a negative effect on SCF function (Lyapina *et al.*, 2001; Schwechheimer *et al.*, 2001; Gray *et al.*, 2002). These results suggest that a cycle of RUB conjugation and removal is required for SCF activity *in vivo*.

In *Arabidopsis*, RUB conjugation is accomplished by a heterodimeric RUB-activating enzyme composed of the AXR1 and ECR1 proteins and a RUB-conjugating enzyme called RCE1 (del Pozo and Estelle, 1999; del Pozo *et al.*, 2002; Dharmasiri and Estelle, 2002). Whether or not there is a RUB-specific E3 activity is uncertain. Recent results suggest that the RBX1 protein may provide this function. Overexpression of RBX1 in budding yeast and in *Arabidopsis* results in an increase in RUB–CUL1 formation (Kamura *et al.*, 1999; Gray *et al.*, 2002).

In this paper, we describe a genetic and biochemical analysis of the RCE1 protein. Our results show that severe disruption of the RUB conjugation pathway in *Arabidopsis* causes a seedling lethal phenotype characteristic of a defect in auxin signaling (Hardtke and Berleth, 1998; Hamann *et al.*, 2002). In addition, we show that RCE1 interacts directly with RBX1 and is in a stable complex

with the SCF. Based on these results, we propose that RBX1 functions as an E3 for RUB modification of CUL1.

Results

Expression of the *RCE1* gene

In *Arabidopsis*, the heterodimeric RUB-activating enzyme is composed of the AXR1 and ECR1 proteins (del Pozo *et al.*, 1998, 2002; del Pozo and Estelle, 1999). Previous studies have shown that *AXR1* and *ECR1* are expressed throughout the life cycle of the plant with particularly high expression levels in dividing and elongating cells (del Pozo *et al.*, 2002). To investigate the pattern of *RCE1* expression, we first examined expression in various plant tissues by RNA blotting. As indicated in Figure 1A, *RCE1* RNA accumulates to substantial levels in all plant organs examined. To further characterize *RCE1* expression, we constructed an in-frame fusion between *RCE1* and the β -glucuronidase (*GUS*) gene. The construct, including a 1.5 kb DNA fragment from the promoter region of *RCE1*, was introduced into *Arabidopsis* plants, and 16 lines were examined for expression of GUS. All of the lines had a similar pattern of GUS staining. In young seedlings, GUS staining was strongest in the elongation zone of the root and at the shoot–hypocotyl junction (Figure 1B). Significant staining was also observed in root hairs. In older seedlings, intense staining was observed at the shoot apex, the root tip and the site of lateral root initiation (Figure 1C–E). In leaves, particularly strong staining was observed in the veins and trichomes (Figure 1F and G). The results of *in situ* hybridization studies also indicated strong expression in the inflorescence and floral meristems (data not shown). These results indicate that *RCE1* is broadly expressed in a manner similar to that of *AXR1* and *ECR1*.

Loss of *RCE1* results in altered morphology

Mutations in the AXR1 subunit of the RUB-activating enzyme result in diverse defects in morphology (del Pozo *et al.*, 1998). Similar defects are observed in plants expressing a dominant-negative form of the ECR1 subunit (del Pozo *et al.*, 2002). To investigate the role of *RCE1* in plant growth and development, we searched the available collections of insertion lines for mutations in *RCE1*. One line from the IMA collection contains a *Ds* insert 371 nucleotides upstream of the ATG for *RCE1*. This mutation, named *rce1-1*, is recessive and confers a characteristic phenotype (described below). RNA blot analysis indicates that *RCE1* RNA levels are significantly reduced in plants homozygous for the *rce1-1* allele (Figure 2A). However, some transcript is still present, indicating that *rce1-1* is not a null mutation. To confirm that the *rce1-1* mutation is responsible for the phenotype described below, we constructed a *35S::Myc-RCE1* fusion gene and introduced it into *rce1-1* plants. The transgene restored normal morphology to mutant plants indicating that the observed defects are caused by a reduction in *RCE1* levels (data not shown).

Based on genetic segregation data, the *rce1* mutation does not significantly disrupt gametophyte or embryo development. However, *rce1* plants exhibit a variety of growth defects throughout development. When grown on the surface of vertically orientated agar medium, the roots of mutant seedlings appeared to wander over the surface of the agar, suggesting a defect in gravitropism. To test this possibility, wild-type and mutant roots were grown in a vertical orientation for 6 days and turned 90° to a horizontal orientation. After 8 h, the angle from the vertical axis was measured. The results in Figure 2B show that *rce1-1* seedlings have a reduced response to the change in the gravity vector. The *axr1-12* mutant has a similar defect.

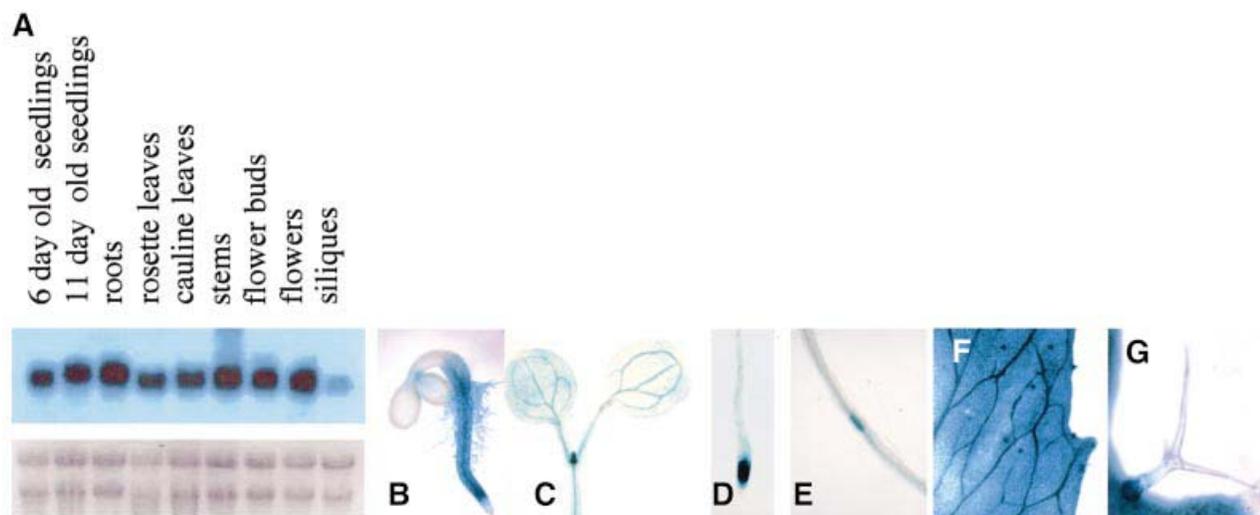


Fig. 1. Pattern of *RCE1* gene expression analyzed by RNA blot and GUS staining. (A) RNA blot showing expression of *RCE1* in all the tissues examined. The lower panel shows an ethidium bromide stained gel to demonstrate equal loading. (B–G) GUS staining in *RCE1::GUS* plants. These plants carry a translational fusion between *RCE1* and GUS under control of the *RCE1* promoter. (B) GUS staining in 4-day-old light grown seedling. (C) Shoot apical region of a 7-day-old light grown seedling. (D) Root apex of a 10-day-old seedling. (E) Root segment from a 10-day-old seedling showing GUS staining in lateral root primordium. (F) Primary leaf from 10-day-old seedling. (G) A magnified view of a trichome from (F) showing intense staining at the basal region.

Rosette and inflorescence morphology are also altered in the *rce1* mutant (Table I; Figure 2C and D). In general, organ length is reduced throughout development. The rosette leaves of *rce1* plants are smaller than those of *Ler* leaves, with shorter petioles and rounder, crinkled leaf blades. The inflorescence is shorter and more highly branched than *Ler*, a phenotype that is quite similar to *axr1* plants. Finally, the length of the floral organs is reduced (data not shown), resulting in the production of a shorter silique (Table I).

RCE1 is required for RUB modification of CUL1

In a previous study, we showed that RCE1 promotes RUB modification of CUL1 *in vitro* (del Pozo and Estelle, 1999). To show that this is also the case *in vivo*, we examined the status of CUL1 in wild-type and *rce1-1* plants by immunoblotting. Figure 3A shows that the level of RUB-CUL1 in the mutant is significantly reduced compared with *Ler* plants. To further investigate the effects of loss of RCE1 on the pathway, we crossed *rce1-1* into a line that carries the *35S-RBX1* transgene. Overexpression of RBX1 results in a dramatic increase in RUB-CUL1 levels (Gray *et al.*, 2002). The results in Figure 3B show that the loss of RCE1 suppresses the effect of increased RBX1 levels on RUB1-CUL1, confirming

that RCE1 acts upstream of RBX1 in the RUB conjugation pathway.

The *rce1* mutants are deficient in auxin and jasmonate response

A number of studies have shown that SCF function is sensitive to the level of RUB-CUL1 modification (Liakopoulos *et al.*, 1999; Morimoto *et al.*, 2000; Osaka *et al.*, 2000; Podust *et al.*, 2000; Read *et al.*, 2000; Gray *et al.*, 2001, 2002; Lyapina *et al.*, 2001; Schwechheimer *et al.*, 2001). In *Arabidopsis*, the best-characterized SCF complex is SCF^{TIR1}, which is required for auxin response (Gray *et al.*, 1999). To determine whether the *rce1-1* plants are deficient in auxin response, we first examined the effects of auxin on root growth. In our standard root growth assay, we found that *rce1-1* is significantly resistant to the synthetic auxin 2,4-D compared with the parental *Ler* line (Figure 4A). The *axr1-12* mutant was included in this experiment for comparison. To confirm that the auxin response defect is related to a reduction in RCE1 levels, we also tested *rce1-1*, *35S::Myc-RCE1* plants for auxin response. Figure 4B shows that the transgene restores normal auxin response to *rce1-1* plants. Another well-characterized effect of auxin is the induction of lateral root formation (Himanen *et al.*, 2002). To determine the effect of *rce1-1* on this process,

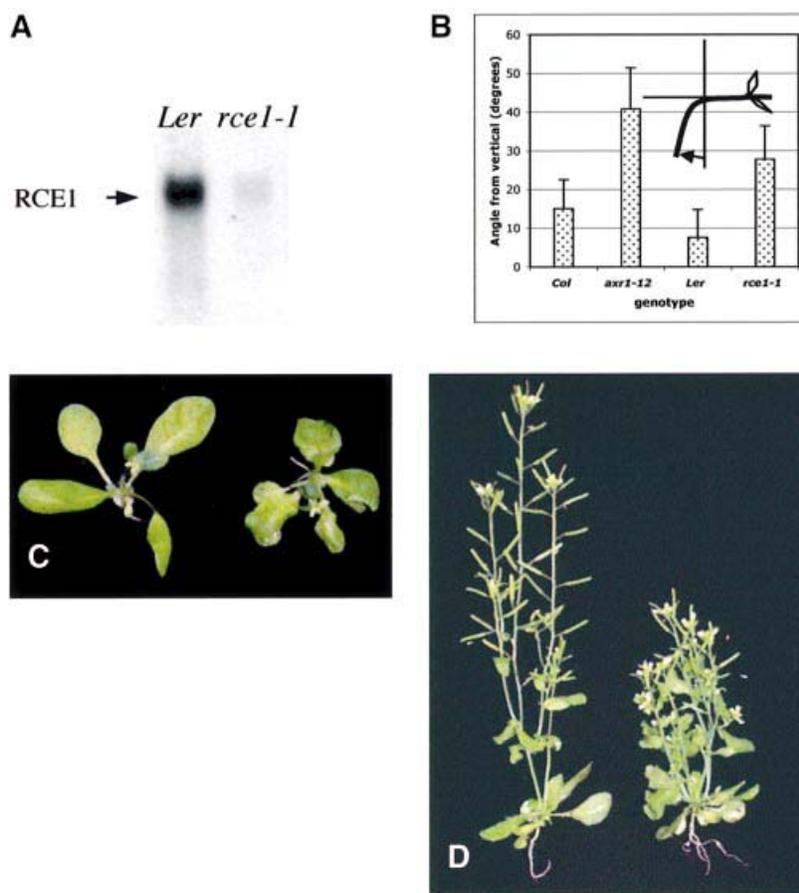


Fig. 2. A mutation in *RCE1* confers morphological defects similar to auxin-resistant mutants. (A) RNA blot showing expression of *RCE1* in 6-day-old wild-type and *rce1-1* seedlings. (B) Response of 6-day-old seedling roots to gravity. (C) Twenty-one-day-old rosettes. (D) Thirty-six-day-old plants. For both (C) and (D), *Ler* is on the left and *rce1-1* is on the right.

Table I. Morphological analysis of *rce1-1*

	<i>Ler</i>	<i>rce1-1</i>
Mature plant height (cm)	22.7 ± 1.8	8.3 ± 1.3
Number of primary inflorescences per plant	2.4 ± 0.9	6.1 ± 1.0
Length of leaf blade (cm)	1.3 ± 0.2	0.7 ± 0.1
Length of petiole (cm)	0.7 ± 0.1	0.4 ± 0.07
Length of silique (cm)	1.3 ± 0.1	0.6 ± 0.09

we transferred 6-day-old seedlings to medium with 85 nM 2,4-D and counted lateral roots after 3 days. As shown in Figure 4C, *rce1-1* seedlings produced fewer lateral roots in response to auxin than the wild type.

To determine whether these growth defects were accompanied by changes in auxin-regulated gene expression, we examined expression of the auxin-response gene *IAA2* in wild-type and *rce1-1* seedlings. Figure 4D shows that induction of this gene is reduced in *rce1-1* plants. Similarly, expression of the auxin-responsive GUS reporter *BA3-GUS* is nearly absent in the *rce1-1* background (Figure 4E). All of these results clearly demonstrate that *RCE1* is required for auxin response.

So far, the only known SCF substrates in *Arabidopsis* are the Aux/IAA proteins (Gray *et al.*, 2001). To determine whether the *rce1-1* mutation affects degradation of these proteins, we measured the half-life of the AXR2/IAA7 protein in a pulse–chase experiment. The results of a representative experiment are shown in Figure 4E. Based on three experiments, the half-life of AXR2/IAA7 is 7.99 ± 1.96 min and 12.26 ± 1.97 min for *Col* and *rce1-1*, respectively (Figure 4F). These results indicate that degradation of AXR2/IAA7 is impaired in the *rce1-1* mutant.

In *Arabidopsis*, SCF^{COI1} is required for response to the plant hormone jasmonic acid (JA) (Xie *et al.*, 1998; Xu *et al.*, 2002). Several recent studies have shown that the *axr1* mutants are moderately resistant to JA, suggesting that RUB modification of CUL1 is also required for optimal SCF^{COI1} function (Gray *et al.*, 2002; Schwechheimer *et al.*, 2002; Tiryaki and Staswick, 2002; Xu *et al.*, 2002). We examined the response of the *rce1-1* mutant and find that it also displays reduced JA response, confirming that RUB modification of CUL1 is required for normal SCF^{COI1} function (Figure 4G).

The RUB conjugation pathway is required during embryogenesis

The phenotypes of the *axr1* and *rce1* mutants indicate that the RUB conjugation pathway has an important role in plant growth and development. However, *rce1* is not a null mutation, and a related protein called RCE2 may also provide some RUB E2 activity (S.Dharmasiri and M.Estelle, unpublished data). Similarly, a gene closely related to *AXR1* is present in the *Arabidopsis* genome, suggesting that the *axr1-12* mutant may retain some RUB E1 activity (N.Dharmasiri and M.Estelle, unpublished data). Indeed the *axr1-12* mutation reduces but does not eliminate RUB modification of CUL1 (Figure 5A). To determine the effects of a more severely impaired RUB conjugation pathway, we crossed the *axr1-12* mutant to

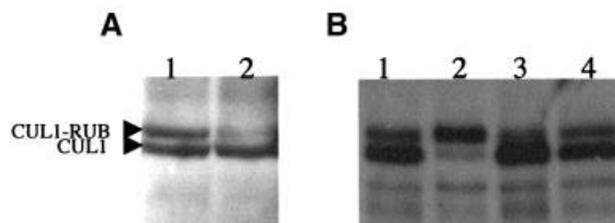


Fig. 3. RCE1 is required for RUB modification of CUL1. Ten micrograms of total proteins were loaded in each lane. Proteins were blotted and treated with α -CUL1 antiserum. (A) Protein blot of *Ler* (lane 1) and *rce1-1* (lane 2). (B) Protein blot of *Col-0* (lane 1), *35S::RBX1-2B RCE1* (lane 2), *rce1-1* (lane 3) and *35S::RBX1-2B rce1-1* (lane 4).

rce1-1 plants and examined the phenotype of the F₂ generation. The results are shown in Figure 5A. Strikingly, the *RCE1* gene is dosage sensitive in an *axr1* background. Homozygous *axr1-12* seedlings with a single *RCE1* gene had a seedling lethal phenotype. After germination, these seedlings did not produce any leaves and soon died. Homozygous *axr1-12 rce1-1* seedlings had an even more severe phenotype. Double mutant seedlings typically had two cotyledons but lacked all basal structures including the hypocotyl and root. This phenotype is very similar to that of the *monopteros* (*mp*), *bodenlos* (*bdl*) and *auxin resistant 6* (*axr6*) seedlings (Berleth and Jurgens, 1993; Hamann *et al.*, 1999b; Hobbie *et al.*, 2000). Recent studies implicate MP, BDL and AXR6 in auxin regulation of pattern formation during embryogenesis (Hardtke and Berleth, 1998; Hamann *et al.*, 2002).

The effects of each genotype on CUL1 modification are shown in Figure 5B. Surprisingly, significant amounts of modified CUL1 are present in each of the lines, including the double mutant. However, the level of unmodified CUL1 increases in the mutant backgrounds so that the relative level of RUB–CUL1 is decreased in each case (Figure 5C). Thus, a defect in RUB conjugation results in an increase in stability of the CUL1 protein.

Auxin is known to play an important role in vascular development (Berleth *et al.*, 2000). To determine whether the *axr1-12 rce1-1* double mutants exhibit defects in vascular structure, we examined the cotyledons of these seedlings. Both *axr1-12* and *rce1-1* cotyledons showed only slightly reduced vascular development (Figure 5D). In contrast, vascular development was severely deficient in *axr1-12 rce1-1* plants. Typically, one short vascular strand was visible in each cotyledon of these seedlings. Again, this phenotype is similar to that observed in *mp*, *bdl* and *axr6* seedlings (Berleth and Jurgens, 1993; Hamann *et al.*, 1999a; Hobbie *et al.*, 2000).

RCE1 interacts directly with RBX1 and is part of a stable complex with the SCF

Recent studies have shown that overexpression of RBX1 dramatically increases the level of RUB–CUL1 (Kamura *et al.*, 1999; Gray *et al.*, 2002). Based on these results, we have suggested that RBX1 functions as an E3 for RUB modification of CUL1 (Gray *et al.*, 2002). If this is the case, RBX1 should directly bind RCE1. To test this possibility, we added ³²P-labeled RBX to glutathione beads carrying GST–RCE1. The results in Figure 6A show that labeled RBX1 is retained on the beads, indicating a

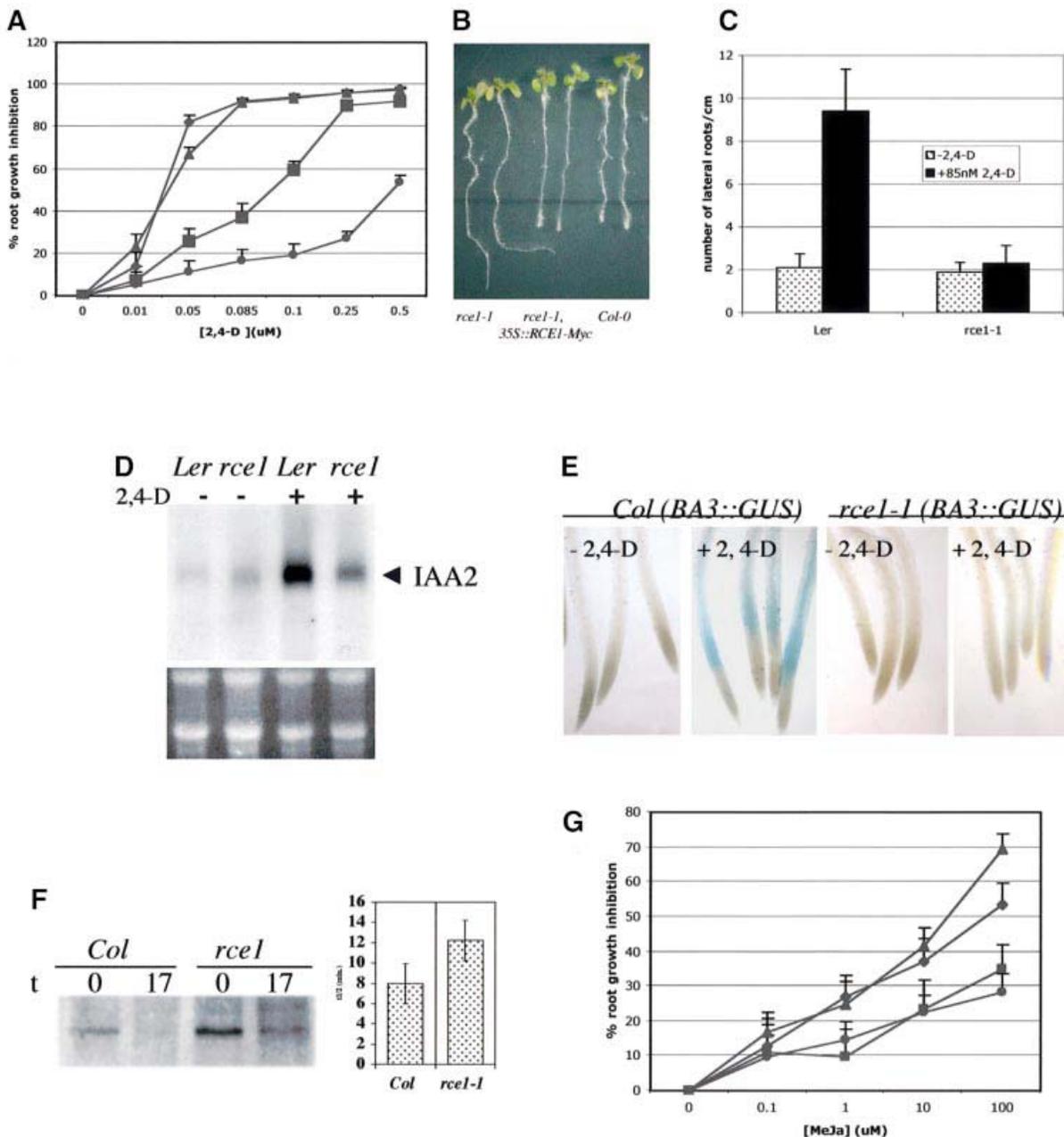


Fig. 4. *RCE1* is required for auxin and jasmonate response. (A) Inhibition of seedling roots on media containing 2,4-D. *Col* (diamonds), *Ler* (triangles), *rce1-1* (squares) and *axr1-12* (circles). (B) Growth of *rce1-1*, *rce1-1*, 35S::Myc-*RCE1* and *Col-0* roots on medium containing 85 nM 2,4-D. Black line represents the position of the root tip at time of transfer to auxin medium. (C) Lateral root formation in seedling roots in response to 85 nM 2,4-D. (D) RNA blot showing the induction of *IAA2* in 6-day-old seedlings treated with or without 20 μ M 2,4-D for 60 min. The ethidium bromide stained gel is shown in the bottom panel. (E) GUS staining of 7-day-old seedlings. The seedlings were treated with or without 20 μ M 2,4-D for 2 h. (F) Pulse-chase analysis of *IAA7/AXR2* in *Col-0* and *rce1-1* seedlings. The half-lives represent the mean of three separate experiments \pm SD. (G) Inhibition of seedling roots by methyl jasmonate. *Col-0* (diamonds), *Ler* (triangles), *rce1-1* (squares) and *axr1-12* (circles).

specific interaction between RBX1 and RCE1. To determine whether RCE1 interacts with SCF complexes, we used the 35S::Myc-*RCE1* line. As described above, this transgene restored a wild-type phenotype to *rce1-1* plants, indicating that the Myc-*RCE1* protein is functional. Initially, we used 35S::Myc-*RCE1* plants to determine whether GST-RBX1 interacts with Myc-*RCE1*. The results in Figure 6B show that GST-RBX1 interacts with Myc-*RCE1* in plant extracts, consistent with our results using purified proteins. To show that CUL is also present

in a complex with RCE1, we immunoprecipitated CUL1 from extracts prepared from 35S::Myc-*RCE1* seedlings. Immunoblotting with anti-Myc antibody revealed the presence of Myc-*RCE1* in the immunoprecipitate (Figure 6B). Taken together these results indicate that RCE1 interacts with a complex that contains at least the CUL1 and RBX1 proteins.

To confirm and extend these results, we performed GST pull-down experiments with GST-*RCE1* to demonstrate an interaction with a complex containing an F-box protein.

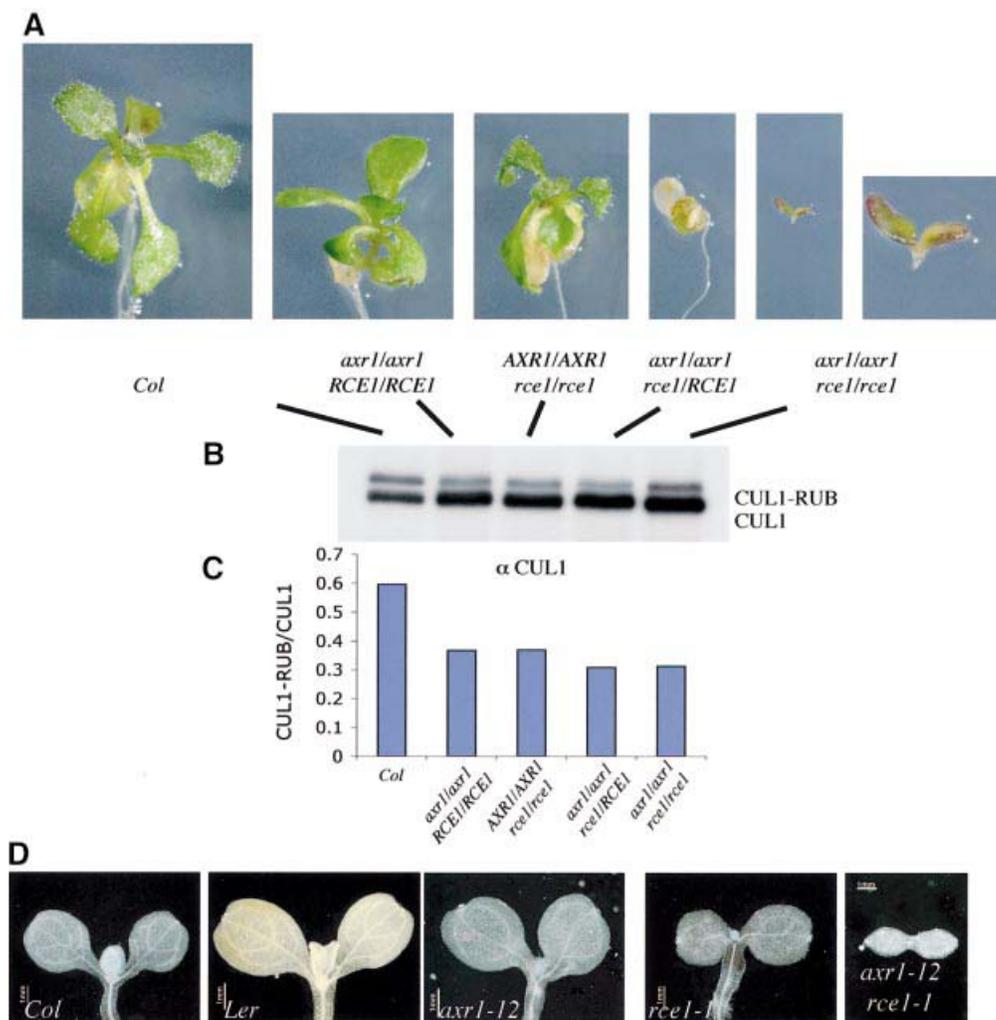


Fig. 5. The RUB conjugation pathway is required during embryogenesis. **(A)** Phenotypes of wild-type and mutant seedlings. The *rce1-1 axr1-12* double mutant seedlings were 6 days old when photographed. These seedlings died within 10–12 days of germination. All other seedlings were 12–14 days old when photographed. **(B)** Protein blot showing the levels of unmodified and modified CUL1 for each genotype. Total proteins were extracted from 6-day-old seedlings and immunoblotted with α -CUL1 antiserum. Ten micrograms of protein was loaded in each lane. **(C)** Ratio of RUB-CUL1 to CUL1 from **(B)** determined using NIH image. **(D)** Vascular patterning in cotyledons of 6-day-old seedlings.

Extracts were prepared from *Arabidopsis* lines expressing the *TIR1-Myc* transgene. Figure 6C shows that GST-RCE1 pulled down the TIR1-Myc protein from plant extracts. This experiment was also performed with another F-box protein that is closely related to TIR1 called leucine rich repeat F-box 1 (LRF1). As shown in Figure 6C, GST-RCE1 also pulled down LRF1-Myc from plant extracts. These results indicate that RCE1 interacts with complete SCF complexes.

Since RCE1 is associated with the SCF, we wondered whether AXR1 might also be in this complex. First we asked whether AXR1 interacts with Myc-RCE1 by immunoprecipitating AXR1 from *35S::Myc-RCE1* seedlings. Figure 6D shows that Myc-RCE1 co-immunoprecipitates with AXR1, indicating that RCE1 forms a stable complex with AXR1. We next attempted to demonstrate an interaction between AXR1-ECR1 and the SCF by reciprocal co-immunoprecipitation of AXR1 and CUL1, and GST pull-down experiments. However, we were unable to detect an interaction using either of these approaches.

Discussion

The conjugation of RUB/Nedd8 to cullin proteins is a highly conserved process that occurs in all eukaryotes (Yeh *et al.*, 2000). As in the ubiquitin pathway, RUB conjugation requires a RUB-activating enzyme and a RUB-conjugating enzyme. In *Arabidopsis*, the activating enzyme is a heterodimer consisting of the AXR1 and ECR1 proteins (del Pozo *et al.*, 1998, 2002). Loss of the AXR1 subunit results in a reduction in RUB-modified CUL1 and a variety of growth defects, many of which appear to be caused by stabilization of SCF substrates. In this study, we show that the RCE1 protein is also required for RUB modification of CUL1, consistent with its proposed role as RUB-E2 (del Pozo and Estelle, 1999). Insertion of a *Ds* element adjacent to *RCE1* results in a phenotype that is very similar to that of the *axr1* mutant, including stabilization of Aux/IAA proteins and a reduction in auxin response.

Among animals, RUB is known to be essential for viability in *C.elegans*, *Drosophila melanogaster* and mice

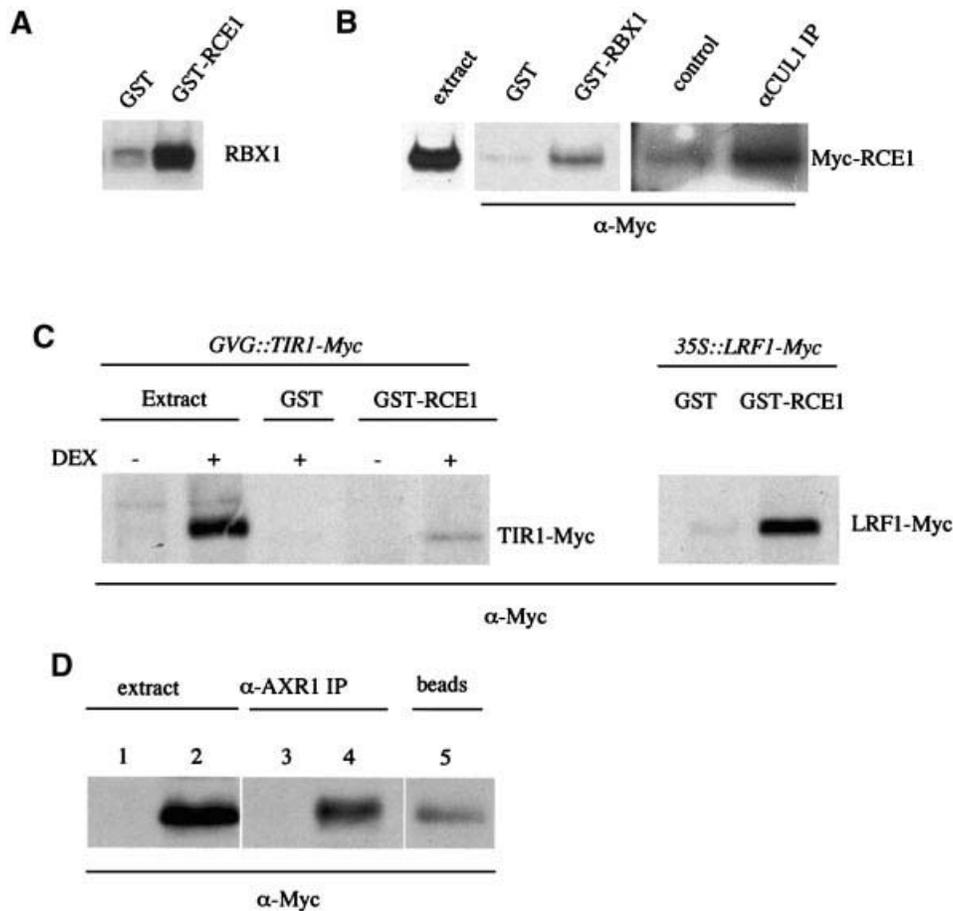


Fig. 6. RCE1 interacts with the SCF and the RUB activating enzyme. (A) [³²P]RBX1 (20 μ l) was added to glutathione beads loaded with 3 μ g of either GST or GST-RCE1. After washing, proteins retained on the beads were analyzed by SDS-PAGE. (B) Protein extracts were prepared from *35S::Myc-RCE1* seedlings. Proteins were recovered after pull-down with GST-RBX1 or immunoprecipitation with α -CUL1 antiserum and analyzed by immunoblot using α -Myc antiserum. (C) GST-RCE1 pull-downs were performed using protein extracts prepared from *GVG::TIR1-Myc* or *35S::LRF1-Myc* seedlings. Recovered proteins were analyzed by immunoblot using α -Myc antiserum. (D) Protein extracts from *35S::Myc-RCE1* seedlings were immunoprecipitated with α -AXR1 antiserum and immunoblotted with α -Myc antibody. Lanes 1 and 3, Columbia extract; lanes 2, 4 and 5, *35S::Myc-RCE1* extract.

(Tateishi *et al.*, 2001; Kurz *et al.*, 2002; Ou *et al.*, 2002). Nedd8-deficient *Drosophila* mutants arrest as first-instar larvae, while in *C.elegans* and mice, mutations that affect Nedd8 conjugation result in embryo lethality. In *Arabidopsis*, an assessment of the biological importance of RUB conjugation has been hindered by genetic redundancy. Strong *axr1* alleles lack the AXR1 protein, but a closely related gene called *AXL1* provides partial RUB-E1 activity (N.Dharmasiri and M.Estelle, unpublished data). Here we show that the combination of *axr1-12* and *rce1-1* results in an embryonic phenotype that is strikingly similar to that of the *mp*, *bdl* and *axr6* mutants (Berleth and Jurgens, 1993; Hamann *et al.*, 1999a; Hobbie *et al.*, 2000). In the developing embryo, MP/ARF5 has an essential role in the establishment of the apical-basal axis and in vascular differentiation. Recent studies suggest that MP function depends on degradation of the Aux/IAA protein BDL/IAA12 (Hamann *et al.*, 2002). Thus, either the loss of MP/ARF5 (as in *mp*) or the stabilization of BDL/IAA12 (as in the gain-of-function *bdl* alleles) results in similar defects in embryogenesis. Recently, we have shown that the *AXR6* gene encodes CUL1 (H.Hellmann, L.Hobbie and M.Estelle, unpublished data), suggesting

that the *axr6* phenotype is caused by stabilization of BDL/IAA12 and perhaps other Aux/IAA proteins. The fact that *axr1-12 rce1-1* seedlings have a similar phenotype is consistent with the proposal that RUB modification of CUL1 is required for degradation of Aux/IAA proteins during embryogenesis.

So far the precise function of RUB modification has remained elusive. *In vitro* experiments indicate that cullin modification increases activity of the SCF and related cullin-based E3 complexes (Morimoto *et al.*, 2000; Podust *et al.*, 2000; Read *et al.*, 2000; Wu *et al.*, 2000). Other evidence suggests that the modification may be important for recruitment of the E2 enzyme to the complex (Wu *et al.*, 2002). Recent studies have also shown that removal of RUB from CUL1 is required for normal SCF function (Lyapina *et al.*, 2001; Schwechheimer and Deng, 2001; Schwechheimer *et al.*, 2001; Gray *et al.*, 2002). RUB deconjugation is accomplished by the COP9 signalosome (CSN), a multi-subunit complex related to the lid subcomplex of the proteasome (Schwechheimer and Deng, 2001). A reduction in CSN levels causes accumulation of RUB-CUL1 and a phenotype that is similar to the *axr1* mutants (Schwechheimer *et al.*, 2001). These results

indicate that *in vivo*, SCF function requires a cycle of RUB conjugation and removal. Similar results have been obtained in fungal species (Lyapina *et al.*, 2001). In our experiments, we find that the combination of *axr1* and *rce1* does not dramatically alter the steady-state level of RUB–CUL1. However, because the total amount of CUL protein increases, the relative amount of RUB–CUL1 is decreased. These results suggest that a reduction in activity of the RUB conjugation pathway results in stabilization of CUL1. It is possible that the modification is required for SCF disassembly and CUL1 degradation. Alternatively, a fraction of CUL1 may be degraded during normal SCF function. If the SCF is not functioning properly due to a defect in the RUB pathway, less CUL1 will be consumed. It is also important to note that unknown substrates of RUB conjugation may exist. A reduction in RUB modification of these proteins may account for aspects of the phenotype.

Although the effects of mutations in components of the CSN and the RUB conjugation pathway are similar, they are not identical. Mutants that completely lack the CSN do not have an embryonic defect whereas the *axr1 rce1* double mutant has a severe embryonic defect. Apparently RUB conjugation is important very early in the life cycle of the plant whereas RUB de-conjugation and other potential functions of the CSN are not required until after germination.

The basic characteristics of an E3 are the ability to bind the E2 enzyme and the substrate, thus promoting transfer of ubiquitin from one to the other. In the case of RUB modification of CUL1, RBX1 appears to have these characteristics. It binds both RCE1 and CUL1, and when overexpressed, promotes RUB modification of CUL1. However, additional biochemical experiments are required to confirm that RBX1 does function as the RUB E3. Nevertheless, the possibility that RBX1 might be the RUB E3 raises some interesting questions. In the context of SCF E3 function, RBX1 is known to recruit the ubiquitin E2 to the complex. Since RCE1 is closely related to ubiquitin E2s, it seems likely that both proteins bind the same site on RBX1. If this is correct, competition between RCE1 and the ubiquitin E2 may have a role in regulation of the SCF. Our results also indicate that RCE1 is present in a stable complex with the SCF. In the future, it will be interesting to determine whether the ubiquitin E2 and the CSN are in this complex as well, and if they are, how their various activities are coordinated.

Materials and methods

Plant material and growth conditions

The *rce1-1* seeds were obtained from the Nottingham *Arabidopsis* Stock Center (NASC). All other mutants and transgenic lines were in *Colombia* ecotype. Seeds were surface sterilized and grown on *Arabidopsis thaliana* medium + 1% sucrose (ATS) plates under 16 h light/8 h dark conditions at 22°C. For root growth assays, 5- to 6-day-old seedlings were transferred onto ATS plates with or without 2,4-D or methyl jasmonate (Bedoukian Research, CT). Root lengths and number of lateral roots were measured after 3–5 days depending on the experiment. All protein extracts were generated using 6- to 8-day-old seedlings grown in liquid ATS medium in flasks under constant shaking.

RCE1 constructs and plant transformation

To prepare the *RCE1-GUS* reporter gene construct, a 1.5 kb segment from the promoter region immediately upstream of the translation initiation site

of the *RCE1* gene was ligated to the 0.55 kb *RCE1* cDNA carrying the entire open reading frame. This construct was inserted directly into the pBI101.1 plant transformation vector (Jefferson *et al.*, 1987), in frame with the GUS protein at the C-terminus of the fusion. The expression pattern was studied in 16 independent transgenic lines. To construct Myc-tagged RCE1, a *Myc* cassette was removed from the *pGEM* vector and ligated to the 5' end of the *RCE1* cDNA lacking the first ATG. This construct was inserted into *pROKII* binary vector carrying the 35S promoter. Both *RCE1-GUS* reporter and *Myc-RCE1* constructs were transformed into *Agrobacterium tumefaciens* line GV3101. Plant transformations, and GUS assays were performed as described previously (del Pozo *et al.*, 2002). To confirm function of the Myc-RCE1 fusion protein, the 35S::Myc-RCE1 transgene was introduced into *RCE1 (Col-0)* by transformation and crossed into *rce1-1 (Ler)* plants.

The *GST-RCE1* construct was prepared by inserting the 0.55 kb *RCE1* cDNA into *pGEX4T-3* vector. GST–RCE1 was purified using glutathione beads according to standard protocols. Other proteins used in this study were GST–RBX1 (Gray *et al.*, 2002), GST–IAA7 (Gray *et al.*, 2001) and TIR1-myc (Gray *et al.*, 1999).

Pull-down assays, immunoprecipitations and pulse-chase analysis

For pull-down and immunoprecipitation assays, proteins were extracted from 6-day-old seedlings into buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.25% Tween-20, 1 mM PMSF and 5 mg/ml protease inhibitor cocktail (Roche). All pull-downs and immunoprecipitations were performed using 1 mg total proteins, according to standard protocols. Monoclonal α -Myc was from BabCo (Richmond, CA). The AXR1 and CUL1 antibodies have been described previously (del Pozo *et al.*, 1998; Gray *et al.*, 1999). Protein blot analyses were performed according to standard protocols and detected with ECL (Amersham). Pulse-chase analysis was performed using 6-day-old seedlings as described previously (Gray *et al.*, 2002).

In vitro interaction between RBX1 and RCE1

GST–RBX was labeled with 32 P using protein kinase A and treated with thrombin to release labeled RBX. Supernatant containing labeled RBX was incubated with benzamide beads (to remove thrombin) and glutathione beads (to remove any remaining GST–RBX), then 20 μ l of labeled RBX was incubated with 3 μ g of GST–RCE1 for 12 h in 200 μ l of binding buffer [50 mM Tris–Cl pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.1 mM dithiothreitol (DTT) and protease inhibitor cocktail]. Beads were washed three times with 1 ml of the binding buffer before analysis by SDS–PAGE.

RNA gel blot analysis

To study the expression of *RCE1*, total RNA was extracted from seedlings growing in liquid culture, or from adult tissues. For the expression of *IAA2* gene, 6-day-old *rce1-1* and wild-type (Landsberg) seedlings were treated with or without 20 μ M 2,4-D for 60 min. Total RNA was extracted using Tri-reagent (Sigma), and 10 μ g RNA was loaded on each lane. The entire coding region of *RCE1* cDNA or *IAA2* cDNA was used as the probe.

Seedling vascular patterns

Seedlings grown on ATS medium for 7 days were fixed in ethanol:acetic acid:water (6:3:1), and cleared in Hoyers solution (Liu and Meinke, 1998). The vascular patterns were photographed using dark field optics.

Acknowledgements

The authors would like to thank Dr Seth Davis for drawing our attention to the *rce1-1* line and the NASC for providing this line. This work was supported by National Institutes of Health Grant RO1-GM43411 and National Science Foundation Grant 0115870 to M.E.

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Received November 22, 2002; revised February 19, 2003;
accepted February 27, 2003