Mutation of \textit{E1-CONJUGATING ENZYME-RELATED1} Decreases RELATED TO UBIQUITIN Conjugation and Alters Auxin Response and Development$^{[C][W][OA]}$

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The ubiquitin-like protein RELATED TO UBIQUITIN (RUB) is conjugated to CULLIN (CUL) proteins to modulate the activity of Skp1-Cullin-F-box (SCF) ubiquitylation complexes. RUB conjugation to specific target proteins is necessary for the development of many organisms, including Arabidopsis \textit{(Arabidopsis thaliana)}. Here, we report the isolation and characterization of \textit{e1-conjugating enzyme-related1-1} (\textit{ecr1-1}), an Arabidopsis mutant compromised in RUB conjugation. The \textit{ecr1-1} mutation causes a missense change located two amino acid residues from the catalytic site cysteine, which normally functions to form a thioester bond with activated RUB. A higher ratio of unmodified CUL1 relative to CUL1-RUB is present in \textit{ecr1-1} compared to wild type, suggesting that the mutation reduces ECR1 function. The \textit{ecr1-1} mutant is resistant to the auxin-like compound indole-3-propionic acid, produces fewer lateral roots than wild type, displays reduced adult height, and stabilizes a reporter fusion protein suggesting that the mutation reduces \textit{Ecr1} function. The \textit{ecr1-1} mutant is resistant to the auxin-like compound indole-3-propionic acid, produces fewer lateral roots than wild type, displays reduced adult height, and stabilizes a reporter fusion protein that is degraded in response to auxin, suggesting reduced auxin signaling in the mutant. In addition, \textit{ecr1-1} hypocotyls fail to elongate normally when seedlings are grown in darkness, a phenotype shared with certain other RUB conjugation mutants that is not general to auxin-response mutants. The suite of \textit{ecr1-1} molecular and morphological phenotypes reflects roles for RUB conjugation in many aspects of plant growth and development. Certain \textit{ecr1-1} elongation defects are restored by treatment with the ethylene-response inhibitor silver nitrate, suggesting that the short \textit{ecr1-1} root and hypocotyl result from aberrant ethylene accumulation. Further, silver nitrate supplementation in combination with various auxins and auxin-like compounds reveals that members of this growth regulator family may differentially rely on ethylene signaling to inhibit root growth.

Ubiquitin and members of the ubiquitin-like protein family modify the stability, localization, activity, or other characteristics of target proteins (for review, see Schwartz and Hochstrasser, 2003). One such ubiquitin-like protein, RELATED TO UBIQUITIN (RUB/NEDD8), is conjugated to CULLIN (CUL) subunits of ubiquitin E3 ligase complexes (Liakopoulos et al., 1998) to regulate E3 activity (for review, see Schwechheimer and Calderón Villalobos, 2004). The RUB/NEDD8 conjugation pathway is essential for viability in mice (Tateishi et al., 2001), \textit{Caenorhabditis elegans} (Jones and Candido, 2000), and Arabidopsis \textit{(Arabidopsis thaliana)}; Bostick et al., 2004).

In plants, RUB is activated by an E1-like heterodimer of \textit{E1-CONJUGATING ENZYME-RELATED1} (\textit{ECR1}; orthologous to human UBA3) and AUXIN RESISTANT1 (\textit{AXR1}; orthologous to human APPBP1; del Pozo et al., 1998), then transferred to the E2-like RUB1-CONJUGATING ENZYME1 (\textit{RCE1}; orthologous to human UBC12; del Pozo and Estelle, 1999; Gray et al., 2002; Huang et al., 2004). RUB transfer to the CUL1 target protein is facilitated by the RING-H2 protein RBX1/ROC (Gray et al., 2002). RUB modification of Skp1-Cullin-F-box (SCF) complexes is necessary for many plant developmental processes, including response to auxin (del Pozo et al., 2002), an essential phytohormone.

Auxin influences embryonic development, promotes apical dominance, inhibits root elongation, promotes lateral root proliferation, and affects many other aspects of plant development (for review, see Woodward and Bartel, 2005). The gross morphological effects are achieved through regulation of cell elongation and division (Campanoni and Nick, 2005). A plethora of endogenous and synthetic molecules elicit auxin-like effects in plant bioassays (for review, see Woodward and Bartel, 2005). Auxins and auxin-like compounds are utilized commercially as herbicides and to achieve adventitious rooting of cuttings for clonal plant propagation, with various compounds showing different efficacies depending on the application and species (Hartmann et al., 1990).
Auxin is perceived by the Leu-rich repeat-containing F-box protein TRANSPORT INHIBITOR RESPONSE1 (TIR1) and the closely related AUXIN SIGNALING F-BOX (AFB) proteins (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005). Auxin binding promotes TIR1/AFB interaction with Aux/IAA repressor proteins (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005), bringing the Aux/IAA repressors to the SCFTIR1/AFB complex for ubiquitylation and subsequent proteasomal degradation (Gray et al., 2001).

Auxin resistance is conferred by disruption of any of several SCFTIR1/AFB subunits, including TIR1 (Ruegger et al., 1998), AFB2 (Dharmasiri et al., 2005b), AFB3 (Dharmasiri et al., 2005b), CUL1/AXR6 (Hellmann et al., 2003; Quint et al., 2005), and ASK1 (Gray et al., 1999), as well as the SCF-regulatory protein SGT1b (Gray et al., 2003). Likewise, reduction of RUB gene expression (Bostick et al., 2004) or mutation of the RUB activation and conjugation protein-encoding gene AXR1 (Lincoln et al., 1990), RCE1 (Dharmasiri et al., 2003b), or RBX1 (Gray et al., 2002; Schwechheimer et al., 2002) causes auxin resistance. Overexpression of a catalytically inactive version of ECR1 confers dwarfism, floral abnormalities, and reduced expression of auxin-inducible genes, though roots of these plants have not been shown to be auxin resistant (del Pozo et al., 2002).

RUB conjugation is thought to modulate SCF activity by releasing CUL proteins from inhibition by the CULLIN-ASSOCIATED AND NEDDYLATION-DISSOCIATED (CAND1/ETA2) protein; mutation of the Arabidopsis CAND1 gene confers auxin resistance (Cheng et al., 2004; Chuang et al., 2004; Feng et al., 2004). RUB is removed from CUL by the COP9 signalsome complex, and mutation of COP9 signalsome components also confers auxin resistance (Schwechheimer et al., 2001; Dohmann et al., 2005). The related phenotypes of mutants defective in either RUB conjugation or removal suggest that a dynamic cycle of RUB conjugation and cleavage is necessary for proper SCF function.

Here, we report the characterization of a mutant deficient in the RUB conjugation pathway that we isolated from an Arabidopsis mutant screen for reduced auxin responsiveness. This mutant, ecr1-1, has overlapping and distinct phenotypes compared to mutants defective in other components needed for RUB modification. The ecr1-1 phenotypes confirm the keen sensitivity of auxin responsiveness to SCF misregulation.

RESULTS

Indole-3-Propionic Acid Is Active in Auxin Bioassays

One widely employed auxin bioassay is inhibition of root elongation by exogenous auxin. In Arabidopsis, both indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are effective inhibitors of primary root elongation; however, indole-3-carboxylic acid (ICA) is not (Woodward, 2005; Woodward and Bartel, 2005). To compare the efficacy of the structurally related compound indole-3-propionic acid (IPrA) to other indolic carboxylic acids, we examined Arabidopsis seedling root elongation on various concentrations of these compounds. We found that IPrA effectively inhibited root elongation (Fig. 1A) at similar dosages to IBA (Zolman et al., 2000); micromolar concentrations of these auxins were necessary to elicit the root elongation inhibition

Figure 1. Auxin activity of IPrA. A, Root length of wild-type Arabidopsis seedlings and indicated mutants grown for 8 d at 22°C on unsupplemented medium or with the indicated concentrations of IAA, IPrA, IBA, or 2,4-D. Bars represent means ± se; n ≥ 8. B, Root lengths of Blue Lake bush bean seedlings grown for 8 d at 22°C on various concentrations of ICA, IAA, IPrA, or IBA. Points represent means ± se; n ≥ 3. C, Lateral root number per centimeter of primary root length from seedlings grown for 4 d on unsupplemented medium, then transferred to the indicated conditions and grown for an additional 4 d. Bars represent means ± se; n ≥ 10.
achieved by approximately 100-fold less IAA. The auxin-resistant mutant \textit{axr1} (Estelle and Somerville, 1987; Lincoln et al., 1990) was resistant to root elongation inhibition by IPrA (Fig. 1A), further suggesting that IPrA is an effective auxin. Additionally, IPrA inhibited root elongation in \textit{Phaseolus vulgaris} similarly to IAA and IBA (Fig. 1B), revealing that IPrA responsiveness is not unique to Arabidopsis.

In addition to inhibiting root elongation, auxins induce lateral root proliferation (Woodward and Bartel, 2005); IBA is particularly effective in this bioassay (Zolman et al., 2000). We found that wild-type Arabidopsis seedlings were similarly responsive to IPrA and IBA in the production of lateral roots following transfer to auxin-containing medium (Fig. 1C).

Auxin-response mutants are not equally impaired in responses to different auxins, and we noted distinctions among previously characterized auxin-response mutants treated with IPrA. For example, the auxin influx carrier mutant \textit{aux1} (Marchant et al., 1999) is quite resistant to the root elongation inhibition caused by IAA and the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D; Fig. 1; Lincoln et al., 1990), but only minimally impaired in IPrA- and IBA-responsive root elongation inhibition (Fig. 1A) and lateral root promotion (Fig. 1C). Moreover, \textit{axr1-3} (Lincoln et al., 1990), which was resistant to all tested auxins in root elongation inhibition (Fig. 1A), responded to IBA, but not IPrA, in the lateral root promotion assay (Fig. 1C).

\section*{Isolation and Auxin Response Characterization of an IPrA-Resistant Mutant}

To identify additional components important for IPrA response, we conducted a mutant screen and isolated Arabidopsis seedlings that displayed IPrA-resistant root elongation. We screened 43,750 progeny of 29 pools of ethyl methanesulfonate (EMS)-mutagenized seeds (Lehle Seeds), 18,000 progeny of six pools of fast neutron-bombarded seeds, and 48,000 progeny of 16 pools of \(\gamma\)-irradiated seeds. We isolated 59 IPrA-resistant putative mutants; 23 were fertile and produced auxin-resistant progeny.

We chose one mutant, \textit{ecr1-1}, from these IPrA-response screens for further study because it was differentially defective in responses to various auxins in a pattern distinct from previously isolated auxin-response mutants. Although this mutant had a short root on unsupplemented medium, \textit{ecr1-1} roots were longer than wild type when grown on certain concentrations of IPrA (Figs. 1A and 2). In contrast, there were no tested concentrations of IAA, IBA, or 2,4-D on which \textit{ecr1-1} roots were significantly longer than wild type (Fig. 1A; data not shown). Like \textit{axr1-3}, \textit{ecr1-1} roots were largely unresponsive to IPrA induction of lateral roots. However, the mutant was not resistant to all auxins in the lateral root assay; for example, \textit{ecr1-1} lateral roots were induced by IBA (Fig. 1C).

\section*{An IPrA-Resistant Mutant Is Defective in \textit{ECR1}}

To determine the molecular basis for the mutant phenotypes, we employed recombination mapping to localize the IPrA resistance mutation to the top arm of chromosome 5 (Fig. 3A). When we sequenced candidate genes within the mapping interval, we found a mutation consistent with the EMS mutagen in the fourth exon of \textit{ECR1} (Fig. 3, A and B), which encodes a protein biochemically implicated in auxin response (del Pozo et al., 1998; del Pozo and Estelle, 1999; del Pozo et al., 2002). The missense mutation causes a Leu to Phe change two amino acids away from the catalytic Cys (Fig. 3, A and B) to which activated RUB is linked (del Pozo et al., 1998). We expressed a wild-type \textit{ECR1} cDNA behind the cauliflower mosaic virus 35S promoter in the \textit{ecr1-1} mutant. This construct restored normal root elongation and IPrA responsiveness to the mutant (Fig. 2) and rescued the lateral root formation defect (Fig. 1C), indicating that the mutation we identified in \textit{ECR1} was responsible for the mutant phenotypes we observed.

As illustrated in Figure 3C, \textit{ECR1} functions as a heterodimer with \textit{AXR1} to activate the two RUB proteins in Arabidopsis (del Pozo et al., 1998; Bostick...
Figure 3. Mapping, cloning, and expression of ECR1. A, Recombination mapping using the IPrA resistance phenotype was used to localize the mutation to a region on Arabidopsis chromosome 5. Fractions represent the number of recombination events over the total chromosomes examined at each molecular marker. A candidate gene within the region, ECR1, was sequenced and found to harbor a C to T mutation causing a Leu to Phe missense two amino acids away from the catalytic Cys for RUB conjugation (indicated with an asterisk). B, Active site regions of various putative and characterized E1-like enzymes were aligned using MegAlign (DNA Star) with the ClustalW method. Black shading indicates amino acids conserved in a majority of sequences; gray indicates conservation of functionally similar amino acids in a majority of sequences. The arrow indicates the missense mutation in ecr1-1, and an asterisk marks the catalytic Cys, as in A. Sequences are from Arabidopsis (At), Oryza sativa (Os), Homo sapiens (Hs), Mus musculus (Mm), Danio rerio (Dr), Pichia pastoris (Pp), Drosophila melanogaster (Dm), Caenorhabditis elegans (Ce), Neurospora crassa (Nc), and Saccharomyces cerevisiae (Sc). C, Schematic showing the activation of two characterized Arabidopsis RUB proteins (Bostick et al., 2004) by the ECR1-AXR1 heterodimer and subsequent transfer of RUB to RCE1 and ultimately to the target protein CUL1, where RUB modification modulates SCF activity. D, Expression of components of the RUB conjugation pathway in various tissues. Genevestigator (Zimmermann et al., 2004) data suggest that expression of all components is fairly even across the examined tissues. Bars represent mean level ± se; n = 320 for whole seedlings (S), 139 for inflorescence (I), 577 for rosettes (L), 187 for roots (R), and 42 for cell suspension (C); data from November 2005 Genevestigator update.
et al., 2004). Compiled microarray data from Genevestigator (Zimmermann et al., 2004) show that expression of the components of the RUB activation machinery, as well as a RUB target protein-encoding gene, CUL1, is fairly ubiquitous throughout Arabidopsis tissues (Fig. 3D). Both RUB genes, as well as ECR1 and AXR1, are most highly expressed in suspension cells (Fig. 3D).

Because ECR1 and AXR1 form a heterodimer (del Pozo et al., 1998), we sought to explore the genetic interaction between AXR1 and ECR by isolating an ecr1 axr1 double mutant. We crossed ecr1-1 to the partial loss-of-function axr1-3 mutant and used PCR to determine the genotypes of the F2 progeny. We did not recover any homozygous ecr1-1/ecr1-1 axr1-3/axr1-3 mutants from 45 F2 plants. We also examined progeny of an ecr1-1/ECR1 axr1-3/axr1-3 plant and recovered 17 ecr1-1/ECR1 axr1-3/axr1-3 and 11 ECR1/ECR1 axr1-3/axr1-3 seedlings but again failed to recover any homozygous double mutants. We conclude that the ecr1-1 axr1-3 double mutant confers embryonic or gametophytic lethality, consistent with the necessity of RUB modification for early embryo development previously demonstrated by the early lethality of the rub1 rub2 double mutant (Bostick et al., 2004).

Cullin Modification Is Defective in ecr1-1

A crystal structure of the human UBA3 and APPBP1 dimer (orthologous to Arabidopsis ECR1 and AXR1) has been characterized (Walden et al., 2003), allowing us to examine the potential effects of the ecr1-1 missense mutation on protein structure. In silico superimposition of a Phe at the position occupied by Ile in wild-type UBA3 (analogous to the Leu present in Arabidopsis ECR1; Fig. 3B) suggests that a Phe would cause a steric clash with a neighboring α-helix (residues 225–239), likely causing displacement of the active site Cys adjacent to the missense change (Fig. 4A). Because the ecr1-1 phenotype is recessive (Supplemental Fig. S1), we infer that this mutation results in reduced ECR1 function.

To characterize the molecular consequences of the ecr1-1 mutation, we examined CUL1 protein modification by RUB using immunoblotting with an α-CUL1 antibody (Gray et al., 1999). We included cop9 and axr1 as controls. The cop9-1 mutant is defective in removal of RUB from CUL1 and therefore preferentially accumulates CUL1-RUB (Schwechheimer et al., 2001; Fig. 4B). Conversely, less CUL1-RUB is observed in axr1 mutants (del Pozo et al., 2002). Like in axr1, we found hyperaccumulation of unmodified versus RUB-modified CUL1 in ecr1-1 mutant seedlings (Fig. 4B). CUL1 accumulation and modification were restored to wild-type patterns by introduction of wild-type ECR1 in ecr1-1 (35S-ECR1; Fig. 4B).

A Negative Regulator of Auxin Response Is Stabilized in ecr1-1

Defects in RUB activation or conjugation can lead to auxin resistance by compromising function of the SCF^{TIR1} complex necessary for the degradation of auxin-signaling repressor Aux/IAA proteins (for review, see Woodward and Bartel, 2005). Therefore, we examined in vivo degradation of a heat-inducible Aux/IAA-β-glucuronidase fusion protein, AXR3NT-GUS (Gray et al., 2001) in ecr1-1. This fusion protein is rapidly degraded in wild type and partially stabilized in axr1 (Gray et al., 2001).

Immediately after a 2-h heat shock, AXR3NT-GUS levels were higher in ecr1-1 and axr1-3 roots than in wild type (Fig. 5). After a 20-min recovery, higher levels persisted in both mutants (Fig. 5). These results indicate that stabilization of AXR3NT-GUS in ecr1-1 is similar to the stabilization observed in axr1-3 and...
again suggest that ecr1-1 confers a reduction in ECR1 function. After 200 min of recovery, GUS staining was minimally detectable in all genotypes (Fig. 5), indicating that AXR3NT-GUS degradation was slowed, but not blocked, in the mutants. Although AXR3NT-GUS was stabilized in both ecr1-1 and axr1-3, the pattern of GUS accumulation in the root was slightly different, with prominent root tip staining in axr1-3 and more distal staining in ecr1-1 (Fig. 5). The subtle differences in spatial localization of AXR3NT-GUS accumulation may reflect slight distinctions in the location of greatest auxin-response perturbation in the mutants.

Pleiotropic ecr1-1 Phenotypes

Like axr1-3, ecr1-1 hypocotyls grown in the light were shorter than wild type both at 22°C and at 28°C (Fig. 6A), a temperature at which auxin accumulates (Gray et al., 1998). These defects in ecr1 hypocotyl elongation in the light were rescued by wild-type ECR1 expression (Fig. 6A).

Several mutants compromised in auxin signaling, including tir1 and axr1, produce fewer lateral roots than wild type (Hobbie and Estelle, 1995; Timpte et al., 1995; Ruegger et al., 1998). Because auxin promotes lateral root proliferation, this phenotype is often considered to be a result of decreased auxin response. ecr1-1, like axr1-3, produced fewer lateral roots than wild type both at 22°C and at 28°C, and this phenotype was restored by wild-type ECR1 expression (Fig. 6B).

After seedlings were transferred to soil, ecr1-1 plants resembled wild type in morphology, time to flowering,
and fertility (data not shown). However, mature ecrl-1 shoots, like axr1-3 plants, were shorter than wild type at maturity (Fig. 6C).

**Certain ecrl-1 Phenotypes Are Altered by Blocking Ethylene Perception**

SCF complexes influence many aspects of plant signaling and development; therefore, we examined additional aspects of development and hormone responses in ecrl-1. Because ECR1 dimerizes with AXR1 (del Pozo et al., 1998), we compared ecrl-1 to the partial loss-of-function mutant axr1-3 (Leyser et al., 1993). We also included the auxin-response mutants tir1 (Ruegger et al., 1998) and aux1 (Marchant et al., 1999). Of these mutants, only ecrl-1 had a shorter root than wild type when grown on unsupplemented medium (Fig. 1A). In contrast to axr1-3, ecrl-1 hypocotyls were notably short when grown in darkness (Fig. 7A). Moreover, ecrl-1 hypocotyls and roots retained sensitivity to the elongation-inhibitory effects of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), whereas axr1-3 and aux1 roots are partially ACC resistant (Fig. 7A). ecrl-1 seedlings grown in the dark on unsupplemented medium displayed a characteristic triple response normally associated with ethylene treatment: an exaggerated apical hook accompanied by a short hypocotyl and root (Fig. 6A). These defects are reminiscent of ethylene-overproducing mutants, such as eto1 (Guzman and Ecker, 1990).

Ethylene perception can be blocked by silver ions, which may bind the ethylene receptor in place of the normal copper cofactor and thereby prevent signaling (Rodriguez et al., 1999; Zhao et al., 2002). We found that the short hypocotyl of ecrl-1 could be rescued by supplementing the growth medium with AgNO₃ (Fig. 7B). Similarly, we could rescue the root elongation defect of light-grown ecrl-1 seedlings by AgNO₃ supplementation (Fig. 8A). These results are consistent with the possibility that ecrl-1 defects in root elongation in the light and hypocotyl elongation in the dark result from ethylene overproduction. Interestingly, rub1 rub2 RNAi lines and rec1 mutant seedlings overproduce ethylene, whereas axr1 seedlings have slightly reduced ethylene levels (Bostick et al., 2004; Larsen and Cancel, 2004); our data suggest that ecrl-1 more closely resembles rub and rec1 than axr1 in ethylene physiology.

The observation that ecrl-1 elongation defects could be restored by silver ion supplementation allowed us to revisit the apparent auxin specificity of ecrl-1 that we had originally noted; that is, ecrl-1 appears resistant to root elongation inhibition by IPrA but not by the more commonly studied auxins IAA, IBA, and 2,4-D (Fig. 1A). We therefore repeated auxin-responsive root elongation assays with and without AgNO₃ supplementation to allow assessment of auxin responses without complications from possible ethylene overproduction, which could synergistically inhibit root elongation and thereby mask auxin resistance. In the presence of 10 μM AgNO₃, ecrl-1 was markedly resistant
to root elongation inhibition not only by IPrA (Fig. 8B), but also by 2,4-D (Fig. 8C) and IAA (Fig. 8E). Similarly, IAA resistance of the tir1-1 mutant, which is not dramatic in the absence of silver ions (Fig. 8D), is obvious in the presence of 10 μM AgNO3 (Fig. 8E).

Auxin induces ethylene biosynthesis in many plants (Yang and Hoffman, 1984); indeed, most of the Arabidopsis ACS (ACC synthase) genes are induced following auxin treatment (Yamagami et al., 2003; Tsuchisaka and Theologis, 2004). Although the auxin-induced inhibition of pea root elongation appears not be mediated by ethylene (Eliasson et al., 1989), we observed that silver ion supplementation dramatically muted the inhibition of wild-type Arabidopsis root elongation by IAA. In fact, 60 nM IAA had no measurable effect on wild-type root elongation the presence of 10 μM AgNO3 (data not shown), and even 150 nM IAA was not sufficiently inhibitory to wild type to gauge auxin resistance of the various mutants (Fig. 8D). To inhibit wild-type root elongation by 80% in the presence of 10 μM AgNO3 required 1.2 μM IAA (Fig. 8E), nearly 10-fold more than the level (150 nM) needed to inhibit wild-type elongation by 80% in the absence of AgNO3 (Fig. 8D). Interestingly, the inhibition of wild-type root elongation by IPrA and 2,4-D was less sensitive than IAA to amelioration by AgNO3 (Fig. 8, B–E).

DISCUSSION

In a mutant screen for reduced response to the auxin-like compound IPrA, we identified ecrl-1, a mutant carrying a single amino acid change in a RUB activation enzyme. IPrA is structurally similar to the endogenous auxins IAA and IBA. IPrA has not been described in modern analyses of endogenous plant compounds but has been isolated from Arabidopsis root exudates (Walker et al., 2003). We found that exogenous IPrA exerts auxin-like effects on seedling development, both inhibiting primary root elongation and promoting lateral root proliferation (Fig. 1). The concentration of IPrA required to achieve similar effects was higher than for IAA but slightly lower than IBA (Fig. 1).

We initially considered that IPrA might function as an IAA precursor and that a screen for mutants specifically deficient in IPrA responses might uncover enzymes necessary for IPrA conversion to IAA, as with other IAA precursors, such as IBA (Adham et al., 2005; Zolman et al., 2007), indole-3-acetonitrile (Normanly et al., 1997), and several auxin conjugates (Bartel and Fink, 1995; Davies et al., 1999). However, we did not recover such mutants, consistent with the possibility that IPrA acts as an auxin without modification. Indeed, in vitro studies have revealed that IPrA promotes

Figure 8. Inhibiting ethylene responses uncovers ecrl-1 resistance to commonly used auxins. A, Root lengths of wild-type Arabidopsis seedlings and indicated mutants grown for 8 d at 22°C on unsupplemented medium or medium supplemented with 10 μM AgNO3. Bars represent mean root lengths ± se; n ≥ 10. B to D, Relative root lengths versus no auxin. Bars represent mean percent elongation (compared to no auxin controls) ± se; n ≥ 10.
the interaction between AXR2/IAA7 and the TIR1 auxin receptor in a concentration-dependent manner, with an activity level severalfold lower than that of IAA (N. Dharmasiri and M. Estelle, personal communication). Of course, it remains possible that IPRa is converted to IAA in plants but that the genes required for this conversion are essential, unusually small, or redundantly encoded, and thus inaccessible to our forward genetic approach.

To identify components necessary for IPRa response, we isolated Arabidopsis mutants deficient in root elongation inhibition by exogenous IPRa. One mutant, *ecr1-1*, causes a missense mutation in the gene encoding ECR1, a RUB-activating enzyme (Fig. 3). Based on the crystal structure of the human ECR1 ortholog UBA3 (Walden et al., 2003), the bulky Phe replacing a Leu near the catalytic Cys in *ecr1-1* is likely to contact an adjacent a-helix, disrupting the active site structure (Fig. 4A). Indeed, the reduced fraction of RUB-modified wild-type the ability to rescue phenotypes by reintroduction of notypes (Supplemental Fig. S1; data not shown), and an adjacent coding ECR1, a RUB-activating enzyme (Fig. 3). Based with a loss-of-function mutation in other components of the RUB modification system, (Figs. 1A and 2), loss-of-function mutants defective in catalytically-inactive version, ECR1C215A (del Pozo et al., 2002), which could be caused by decreased RUB modification or stabilization of unmodified CUL1. Also like *ecr1-1* (Fig. 6C), adult ECR1C215A are shorter than wild type (del Pozo et al., 2002). Unlike *ecr1-1*, roots of ECR1C215A plants respond normally to auxin (del Pozo et al., 2002); however, IPRa assays have not been reported.

Interestingly, the molecular specificity of auxin resistance differs in various RUB pathway mutants. The initial report of *axr1* noted that this mutant is much more resistant to 2,4-D than to IAA (Estelle and Somerville, 1987). We initially found that *ecr1-1* displayed apparent resistance only to IPRa (Fig. 1), whereas *axr1*, *rec1*, and *rub1 rub2* RNAi lines are clearly resistant to 2,4-D (Estelle and Somerville, 1987; Leyser et al., 1993; Dharmasiri et al., 2003b; Bostick et al., 2004; Larsen and Cancel, 2004). The short root of *ecr1-1* on unsupplemented medium (Figs. 1A and 2) distinguishes this mutant from other auxin-response mutants and may mask resistance to some auxins. Indeed, when we restored normal root elongation to *ecr1-1* by treatment with silver ions, which inhibit ethylene responses, we were able to detect substantial *ecr1-1* resistance not only to IPRa, but also to IAA and 2,4-D (Fig. 8).

Differential response to different auxin-like compounds is not unique to RUB pathway mutants. For example, the auxin receptor mutant *tir1-1* (Ruegger et al., 1998) also displays molecule specificity, with greatest apparent resistance to the synthetic auxin 2,4-D in root elongation assays (Fig. 8). In vitro, however, TIR1 displays a marked preference for the natural auxin IAA over 2,4-D (Dharmasiri et al., 2003a; Kepinski and Leyser, 2005), suggesting that discriminatory mutant phenotypes do not necessarily reflect receptor affinity differences. In addition, the *antiauxin resistant1* mutant displays resistance to 2,4-D and p-chlorophenoxyisobutyric acid, but not IAA or 1-naphthaleneacetic acid (Rahman et al., 2006). Moreover, a gain-of-function mutation in PDR9, which encodes a pleiotropic drug resistance transporter, reduces responsiveness to 2,4-D, but not IAA, IBA, or p-chlorophenoxyisobutyric acid (Ito and Gray, 2006). At a molecular level, different auxins induce partially distinct changes in transcript abundance (Pufky et al., 2003). It will be interesting to learn whether any of these physiological and molecular distinctions can be traced to auxin-ethylene interactions. For example, even though *tir1* does not display a short root in the absence of supplementation, we found that the apparent degree of *tir1-1* resistance to IAA was markedly enhanced in the presence of silver ions (Fig. 8E). Our results with silver ion supplementation suggest that at least some of the auxin specificity displayed by different auxin-response mutants may reflect different efficiencies with which various auxins induce ethylene biosynthesis; this possibility will likely be an interesting area for future investigation. Notably, an uncharacterized *ecr1* mutant allele recently was reported in a screen for resistance to the auxin-like compound sirtinol (Dai et al., 2005), again supporting the idea that different auxin-like molecules may allow isolation of novel alleles of auxin-response genes. Together, these mutants provide tools for understanding differences in binding, transport, metabolism, and other characteristics that may contribute to bioactivity differences among various auxin-like compounds.

The *ecr1-1* mutant developmental phenotypes demonstrate a critical role for RUB conjugation in many aspects of plant development. Other mutations impacting the RUB conjugation pathway produce morphological effects similar to *ecr1-1*. Like *ecr1-1* (Fig. 7, A and B), *rub1 rub2* RNAi lines and *rec1* mutant seedlings have shorter hypocotyls than wild type when grown in darkness, and this elongation defect is restored by treatment with silver ions (Bostick et al., 2004; Larsen and Cancel, 2004). Direct measurement of ethylene in *rub1 rub2* RNAi lines and *rec1* mutant seedlings confirms that these mutants overproduce ethylene (Bostick et al., 2004; Larsen and Cancel, 2004). This overproduction
might result from stabilization of particular ACS mutants, which carry out the rate-limiting step in ethylene biosynthesis. Intriguingly, CUL3 can be RUB modified (Figueroa et al., 2005) and is a component of a ubiquitin-protein ligase that can include ETO1, which targets ACS5 for ubiquitin-dependent degradation (Wang et al., 2004). ACS enzyme activity (Woeste et al., 1999) and ACS5 protein levels (Wang et al., 2004) are enhanced in eto1 mutants, and it will be interesting to learn whether ACS5 is similarly stabilized in ccr1, ccr1, or rub mutants. It remains mysterious why axr1 displays opposite ethylene phenotypes, ethylene resistance (Timpire et al., 1995) and less ethylene production than wild type (Bostick et al., 2004), compared to other RUB pathway mutants. The abundance of F-box proteins in Arabidopsis (Gagne et al., 2002) suggests that SCF regulation will impact a multitude of plant processes, and auxin-response mutant screens continue to provide a key entree into understanding SCF and regulatory processes affecting CUL-containing E3 complexes, including the RUB activation and conjugation pathway.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (Arabidopsis thaliana seeds were surface sterilized by incubation in 30% (v/v) household bleach, 0.01% (v/v) Triton X-100 for 12 min, followed by two washes in sterile water and suspension in sterile 0.1% (w/v) agar (Last and Fink, 1988). Seedlings were grown on plant nutrient medium (PN; Haughton and Somerville, 1986) solidified with 0.6% agar and supplemented with 0.5% (w/v) Suc (PNS) unless indicated. Plates were sealed with Leukopor surgical tape (LekTek) to permit gas exchange. Seedlings were grown in Percival incubators (model CU-36 L) under continuous illumination (25–45 μE m⁻² s⁻¹) at 22°C under yellow light filters for 8 d unless indicated. All ccr1-1 assays were conducted with mutant lines that had been backcrossed to the parental Col-0 line twice. Lateral root induction by auxin was tested after stratifying seeds for 1 d at 4°C, growing seedlings on un-supplemented growth medium for 4 d, then transferring seedlings to the indicated conditions for four additional days of growth. Each root was measured, and then the number of lateral roots was counted using a dissecting microscope.

To test dark development and ethylene responses, seeds were plated on ACC- or control plates, grown under yellow light for 1 d, then covered in foil and grown in darkness at 22°C for four additional days. Seedlings were photographed and hypocotyls were measured using a Leica MZFLIII dissecting microscope equipped with a digital camera.

Mutant Isolation and Recombination Mapping

A screen for resistance to IPrA was performed on progeny of mutagenized pools of the Columbia-0 (Col-0) accession from EMS, γ-irradiation (60 krad), and fast neutron-bombardment (60 Gy). Seeds were grown on PNS supplemented with 5 μM IPrA. After approximately 8 d, seedlings with aberrantly long roots were removed and aseptically transferred to PNS for recovery for several days, then grown to maturity in soil. Progeny of fertile M2 putative mutants were rescreened on IPrA and IAA.

Mutant Isolation and Recombination Mapping

Phytohormone Response Characterization

PNS plates were supplemented with hormones dissolved in 50% (v/v) or 100% ethanol as follows: 1 or 5 mM ACC, 1 mM 2,4-D, 100 mM ICA, 1 mM IAA, 100 mM IBA, and 10 or 100 mM IPrA. Seeds were surface sterilized and stratified at 4°C overnight before plating. Plates were incubated at 22°C under yellow light filters for 8 d unless indicated. All ccr1-1 assays were conducted with mutant lines that had been backcrossed to the parental Col-0 line twice. Lateral root induction by auxin was tested after stratifying seeds for 1 d at 4°C, growing seedlings on un-supplemented growth medium for 4 d, then transferring seedlings to the indicated conditions for four additional days of growth. Each root was measured, and then the number of lateral roots was counted using a dissecting microscope.

To test dark development and ethylene responses, seeds were plated on ACC-containing or control plates, grown under yellow light for 1 d, then covered in foil and grown in darkness at 22°C for four additional days. Seedlings were photographed and hypocotyls were measured using a Leica MZFLIII dissecting microscope equipped with a digital camera.

Mutant Rescue

E3R1 was PCR amplified from the U13340 cDNA clone (Yamada et al., 2003) with the primers ECR1xhol (5′-CTCGAGACACAGAAGAGAACACATACGACACACG-3′) and pUNI51Nol (5′-CTCAATTGAGATGATTACGACACGAA-3′) using 0.75 units Accuprime Pfu Taq polymerase (Invitrogen) in 50-μL reactions. The amplification product was cloned into pCR4-TOPO (Invitrogen); derived cleaved amplified polymorphic sequences (dCAPS; Michaels and Amasino, 1998; Neff et al., 1998) primers ECR1-1 (5′-TTCGGTTGTGATTCCTTCTTCACCG-3′) and ECR1-2 (5′-GACATACAGAAAGAATCCATACATAC-3′), and the resultant product was digested with Hinfl, which cut the wild-type, but not the mutant, product. The axr1-3 mutation was tracked using PCR amplification with primers AXR1-Acc1 (5′-AACATTACGGTGAATGGCAAAAGAAC-3′) and AXR1-1 (5′-TCTCTATGTACCTTCTTCAGC-3′). The resultant product was digested with AccI, which cuts the wild-type, but not the mutant, product.
Antibody (Santa Cruz Biotechnology). Secondary antibodies were detected

Immunoblotting

Seeds (25) of the indicated genotypes, including only dark-colored (presumed homozygous) copy-1 seeds from a heterozygous parent, were surface sterilized and germinated in 25 μL of sterile water for 3 d in white light at 22°C. One volume of extraction buffer (0.1 x Tris-HCL, pH 6.8, 20% glycerol, 4% SDS) was added, and seedlings were homogenized with a mechanical pestle and then incubated on ice. Homogenates were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was recovered, and 1.8 μL of diithiothreitol was added to a 16-μL sample. Samples were heated to 80°C for 8 min and returned immediately to ice.

Samples were loaded on a 10% Bis-Tris NuPage gel (Invitrogen) and electrophoresed in MOPS buffer containing antioxidant, as suggested by the manufacturer. Amperage was increased gradually to 110 mA over the first 30 min; total run time was 2 h. Proteins were transferred to Hybond nitrocellulose membrane (Amersham Biosciences) at 24 V for 1 h. The membrane was blocked in 5% (w/v) powdered milk in TTBS (0.1% [v/v] Tween 20, 100 mM Tris-Cl, pH 7.5, 150 mM NaCl; Ausubel et al., 1999) for 2 h at room temperature with rocking. Rabbit α-CUL1 antibody (Gray et al., 1999), kindly provided by Mark Estelle, was diluted 1:1,000 in 5% powdered milk TTBS. The membrane was incubated overnight at 4°C with rocking, then washed three times in 5% powdered milk TTBS for at least 5 min at room temperature. Horseradish peroxidase-linked goat anti-rabbit antibody (1:500; Santa Cruz Biotechnology) in 5% powdered milk TTBS was added and rocked at room temperature for 1 h. The membrane was washed again as above. The membrane was visualized using LumiGLO reagent (Cell Signaling). The procedure was repeated twice with similar results. To monitor sample loading, the membrane was reprobed with a 1:1,000 dilution of a mouse monoclonal antibody raised against spinach Hsc70 (Stressgen Bioreagents SPA-817) followed by a 1:1,000 dilution of horseradish peroxidase-linked goat α-mouse secondary antibody (Santa Cruz Biotechnology). Secondary antibodies were detected using LumiGLO reagent (Cell Signaling Technology).

Supplemental Data

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

Supplemental Figure S1. ccr1-1 is a recessive mutation. The histogram shows root lengths of 6-d-old progeny of wild-type, heterozygous ccr1-1, and homozygous ccr1-1 plants grown on 3 μM iPRA under yellow light at 22°C.

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An Arabidopsis el-conjugating enzyme-related1 Mutant


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