Related to Ubiquitin 1 and 2 Are Redundant and Essential and Regulate Vegetative Growth, Auxin Signaling, and Ethylene Production in Arabidopsis

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Related to Ubiquitin (RUB)/Nedd8 is a ubiquitin-like protein that covalently attaches to cullins, a subunit of the SCF (for Skp, Cdc53p/Cul1, and F-box protein) complex, an E3 ubiquitin ligase, and has been shown to be required for robust function of the complex. The effects of reducing protein levels for two Rub proteins, RUB1 and RUB2, were characterized in Arabidopsis thaliana. T-DNA insertional null lines homozygous at a single RUB-encoding locus were analyzed and found to have a wild-type phenotype. A double mutant was never recovered. More than one-quarter of the progeny from the self-fertilization of plants with a single functional RUB-encoding gene died as embryos at the two-cell stage. Outcrosses demonstrated reduced inheritance of the null allele from both the male and female parent. Hemiglutinin-tagged forms of RUB1 and RUB2 conjugate to the same cullin protein, CUL1, and produce the same conjugation pattern. To further understand the function of the RUB proteins, a construct designed to produce a double-stranded RUB1 mRNA was introduced into plants, and three lines with reduced levels of RUB1- and RUB2-encoding mRNA and RUB1/2 protein content were analyzed in detail. Mature plants were severely dwarfed, seedlings were insensitive to auxin in root assays, and dark-grown seedlings had a partial triple-response phenotype that was suppressed when seedlings were grown on ethylene perception or synthesis inhibitors. The dsrub lines produced threefold to fivefold more ethylene than the wild type. This study illustrates that RUB1 and RUB2 are genetically and biochemically redundant and demonstrates that RUB1/2 proteins are essential for early embryonic cell divisions and that they regulate diverse processes.

INTRODUCTION

Related to Ubiquitin (RUB), also known as Nedd8 (neuronal precursor cell expression developmentally downregulated in mammals and Schizosaccharomyces pombe), is a ubiquitin-like protein found in all eukaryotes. Rub/Nedd8 functions, like ubiquitin, through its covalent attachment to other proteins (Pan et al., 2004). Rub/Nedd8 from Homo sapiens, S. pombe, and Saccharomyces cerevisiae has been shown to be attached to members of a family of proteins first discovered in Caenorhabditis elegans called the cullins (Kipreos et al., 1996; Lammer et al., 1998; Hori et al., 1999; Osaka et al., 2000; Laplaza et al., 2004). Cullins are a small multigene family in fungi, mammals, and plants, and most have been identified as the scaffolding component of a ubiquitin E3 complex, either an SCF (named for the first three components identified: Skp, Cdc53p/Cul1, and F-box protein) (Zheng et al., 2002) or an SCF-like complex. These complexes serve as the substrate recognition component in the ubiquitin conjugation pathway, interacting with both the ubiquitin-charged E2 and the protein targeted for ubiquitylation. Rub1/Nedd8 and consequently its conjugation pathway are required, in addition to the ubiquitin pathway, for robust polyubiquitylation of substrates by SCF complexes in vitro (Read et al., 2000). Genetics studies in mice, nematodes, and fission yeast have suggested that this stimulation of SCF function is essential; cells with disruptions in either Rub/Nedd8 or components required for its activation have characteristic cell cycle abnormalities eventually leading to death (Osaka et al., 2000; Tateishi et al., 2001; Kurz et al., 2002). However, disruption of RUB1 and its conjugation pathway in S. cerevisiae has no effect on viability (Lammer et al., 1998).

In Arabidopsis thaliana, there are three Rub proteins. RUB1 and RUB2 differ only at amino acid 60, containing an Asn or Ala, respectively, whereas RUB3 has 16 amino acid substitutions throughout the protein. RUB1/2 expression can be readily detected with RT-PCR, and their mRNAs are present in all organs tested, whereas RUB3 mRNA level is lower and more restricted (Rao-Naik et al., 1998). The Rub conjugation pathway was discovered through the isolation of the auxin resistant mutant, axr1-12 (Estelle and Somerville, 1987); AXR1 is part of the AXR/ECR1 heterodimeric complex, which has been shown to activate RUB1 in vivo and in vitro and requires a Rub-specific conjugating enzyme, RCE1, to conjugate RUB1 to AtCUL1 (del Pozo and Estelle, 1999).

Recent data suggest that the Rub pathway is important in vegetative growth and embryo development. axr1-12 plants are dwarf and defective in auxin responses (Lincoln et al., 1990). An rce1 Dissociation insertional mutant and a plant expressing an
ECR1 dominant negative mutant protein are viable but exhibit multiple growth defects throughout the life cycle (del Pozo et al., 2002; Dharmasiri et al., 2003). axr1-12 rce1 mutants have a seedling lethal phenotype resembling monopteros and bodenlos, two previously described mutants altered in auxin signaling (Liscum and Reed, 2002; Dharmasiri et al., 2003). However, the full impact of the Rub pathway remains unclear because rce1 is not a null mutant and there are additional RCE1-like (Dharmasiri et al., 2003) and AXR1-like1 (named AXL1) (del Pozo et al., 2002) predicted proteins in the Arabidopsis genome that have not been characterized and whose roles are not known.

The gaseous hormone ethylene plays an important role in many processes in plants, such as fruit ripening, defense, response to wounding, and senescence (Schaller and Kieber, 2002), and its biosynthesis is highly regulated. The biosynthetic pathway unique to ethylene uses the methyl donor S-adenosyl Met and consists of only two enzymes: ACS (1-aminocyclopropane-1-carboxylic acid [ACC] synthase) and ACC (ACC oxidase). ACS genes produce 10 functional proteins, some have been demonstrated to be relatively short lived (Kim and Yang, 1992) and regulated at transcriptional (Yamagami et al., 2003) and posttranslational levels (Chae et al., 2003). ACC mRNA accumulates in response to ethylene, indicating that ethylene exerts positive feed-forward regulation (Petruzzelli et al., 2000).

To elucidate the role of Rub in Arabidopsis, the effect of eliminating or reducing Rub1 and Rub2 levels was analyzed. This study combined biochemical and genetic studies to demonstrate that Rub1 and Rub2 are redundant; both attached to CUL1 and only one functional allele expressing either Rub1 or Rub2 was required for viability. Reducing Rub expression using posttranscriptional gene silencing (PTGS) had severe effects on vegetative growth and conferred insensitivity to auxin in root assays. Strikingly, dark-grown seedlings with lower Rub1/2 levels had a partial constitutive triple response resulting from increased ethylene synthesis. Altogether these studies demonstrate that Rub1/2 are essential and regulate diverse processes throughout development.

RESULTS

rub1 and rub2 Plants Have a Wild-Type Phenotype

To understand the biological roles for Rub1 and Rub2 proteins in plants, the effect of eliminating their expression individually and collectively was analyzed through the isolation and characterization of insertional mutants in the loci encoding Rub1 and Rub2 proteins. Two independent T-DNA insertional mutagenesis lines were identified for the UBQ7 locus that encodes a ubiquitin-Rub1 dimer (Figure 1A, Rub1) (Rao-Naik et al., 1998). UBQ15 will be referred to here as the Rub1 locus for clarity, and the T-DNA alleles will be rub1-1 and rub1-2. Rub1-1 is in the Columbia (Col) ecotype, and the insert is in the first intron 447 bp downstream of the start codon. RT-PCR using Rub1-specific primers amplifies three low abundance cDNAs from mRNA isolated from rub1-1 homozygous plants, one the size of the endogenous band (Figure 1B, a in lanes 3 and 4, top panel), one smaller (Figure 1B, b in lane 3, top panel), and one larger (not visible in Figure 1, named band c). DNA sequences of the three bands were determined and all have alternative splicing in the Rub1 coding region, not recognizing the second intron acceptor site. Sequences from bands a and c are missing 27 nucleotides of exon 3, eliminating Rub1 amino acids 42 to 51. The sequence from band b is missing 287 nucleotides, including all of exon 3 and some of the 3’ untranslated region, and the predicted Rub1 protein lacks authentic sequence after amino acid 41. A second line containing a T-DNA in Rub1, rub1-2, is in the Wassilewskija (Ws) ecotype, and the insert is in the first intron, 128 nucleotides 3’ of the start codon. No DNA fragments were visible after PCR amplification of cDNA from mRNA from homozygous rub1-2 plants, confirming this as a null mutational allele for Rub1 mRNA (Figure 1B, lanes 9 to 12, top panel).

Two T-DNA insertional lines, rub2-1 and rub2-2, were identified in UBQ7, the locus encoding a ubiquitin-Rub2 dimer (Rao-Naik et al., 1998); both are in the Col ecotype. UBQ7 will be referred to as Rub2 for clarity. The T-DNA inserts are located 337 and 810 nucleotides, respectively, downstream of the ATG for Rub2 (Figure 1A, Rub2). Neither line produces a PCR product when cDNA was amplified with Rub2-specific primers (Figure 1B, lanes 1, 5, and 7, middle panel). Thus, both rub2-1 and rub2-2 are Rub2 null lines.

To determine if inactivation at one locus affected expression at the other nondisrupted locus, RT-PCR was used to detect mRNA for the intact Rub-encoding gene in each single insertion line (Figure 1B). There were no changes in the level of mRNA from Rub2 in rub1-1 and rub1-2 lines compared with a UBQ10 (polyubiquitin) control (Figure 1B, lanes 1, 3, 9, and 11, middle and bottom panels). Similarly, Rub1 mRNA is unchanged in rub2-1 and rub2-2 homozygous plants compared with the UBQ10 control (Figure 1B, lanes 1, 5, and 7, top and bottom panels). This indicates that loss of one Rub gene does not affect expression of the other. The growth of single homozygous lines was compared with wild-type siblings, and no phenotypic differences could be detected (data not shown).

RUB1 and RUB2 Are Genetically Redundant and a rub1 rub2 Plant Is Not Viable

To understand the functional relationship between these two highly similar proteins, rub2-1 homozygous plants were crossed to plants homozygous for one of the two insertional alleles for Rub1 (rub1-1 and rub1-2), and F2 plants were genotyped using PCR (data not shown). Because both rub2 insertion lines were null for Rub2 mRNA, the results from crosses with rub2-1 are described. A wild-type phenotype was observed for all plants with a single functional Rub1 or Rub2; Rub1/rub1 rub2 and Rub1 Rub2/rub2 plants for both rub1 alleles were indistinguishable from Rub1 Rub2 plants in vegetative growth (data not shown). This result supports the hypothesis that Rub1 and Rub2 proteins are redundant because one wild-type allele of Rub1 complements a homozygous rub2 plant and one wild-type allele of Rub2 complements a homozygous rub1 plant.

From 100 F2 individuals, no plant was found homozygous for insertions at both loci, although the expectation is only 1 in 16 plants would have this genotype (P < 0.01). To better test
whether double mutant plants were viable, the RUB1 and RUB2 genotypes of progeny from multiple F2 plants that should segregate double mutants at a higher frequency were determined. F2 plants with the genotypes RUB1/rub1 rub2 or rub1 RUB2/rub2 (for both rub1 alleles) were subsequently allowed to self-fertilize and their progeny genotyped. For this F3 population, the expected frequency of rub1 rub2 plants is higher at 25%. From 40 seedlings from a RUB1/rub1-2 rub2 parent, no rub1-2 rub2 plants were found (Table 1, line 1; P < 0.0005). From 71 seedlings from a RUB1/rub1-1 rub2 parent, no rub1-1 rub2 plants were found (data not shown; P < 0.0005). In 34 seedlings from a rub1-2 RUB2/rub2 parent, no rub1-2 rub2 plants were found (Table 1, line 2; P < 0.001).

Additional seedlings from the F3 generation were genotyped using the antibiotic resistance carried in the T-DNA. Of the antibiotic resistant seedlings, one-third should be homozygous for the T-DNA. From the self-fertilization of a RUB1/rub1-2 rub2 plant (kanamycin resistance segregates with the rub1-2 allele), 40 resistant seedlings were genotyped by PCR at the RUB1 locus and all were heterozygous for the wild-type and T-DNA alleles (Table 1, line 1; P < 0.0005). Similarly, 50 BASTA-resistant (segregating with the rub2 allele) seedlings from a rub1-2 RUB2/rub2 parent were genotyped by PCR at RUB2 and none were homozygous rub1 rub2 (Table 1, line 2; P < 0.0005). Altogether, from 100 F2 plants and more than 200 F3 seedlings genotyped by PCR, no rub1 rub2 plant was observed. These data strongly indicate that a rub1 rub2 plant is not viable. Because F2 and F3 seeds appeared to have 100% viability and no seedling lethality is seen after germination (data not shown), the loss of a double mutant plant must occur early in seed development.

There Is Reduced Inheritance of the T-DNA Alleles through Both Gametes That Results from Embryonic Death of a Proportion of Heterozygous Embryos

If only the rub1 rub2 developing seeds were not viable, then the progeny of a RUB1/rub1-2 rub2 or a rub1-2 RUB2/rub2 plant allowed to self-fertilize would segregate 2:1 for heterozygous and homozygous wild type at the heterozygous locus of the parent. However, the observation was that the percentage of heterozygous seedlings was much less than 67%, with only 15% heterozygous at the RUB1 locus and 44% heterozygous at the RUB2 locus. This suggests that the loss of a double mutant plant must occur early in seed development.

Figure 1. Authentic RUB1 and RUB2 mRNAs Are Eliminated in T-DNA Insertional Lines and Reduced in dsrub Lines.

(A) Genomic representations of RUB1 and RUB2 and of the dsrub construct introduced into plants. Introns are represented by lines and exons by boxes; shaded boxes encode RUB1 or RUB2 protein, black boxes encode ubiquitin, and open boxes represent T-DNA (not to scale). rub1-1 (Col) has a T-DNA insert at 447 bp in intron 1, relative to the ATG, and rub1-2 (Ws) has a T-DNA insert at 128 bp in intron 1. rub2-1 (Col) and rub2-2 (Col) have T-DNA inserts at 387 and 810 bp, respectively. dsrub lines were created with a construct containing the RUB1 open reading frame in opposite directions, separated by an intron, under transcriptional control of the 35S promoter of Cauliflower mosaic virus (CaMV35S).

(B) RT-PCR for RUB1, RUB2, and UBQ10 (polyubiquitin) with cDNA from Col (lanes 1 and 2), rub1-1 (lanes 3 and 4), rub2-1 (lanes 5 and 6), rub2-2 (lanes 7 and 8), Ws (lanes 9 and 10), and rub1-2 (lanes 11 and 12) seedlings. Asterisks indicate genomic PCR band for RUB2. The a and b are splice variants. Odd numbered lanes contain PCR reactions made from cDNA using reverse transcriptase, and even numbered lanes contain PCR reactions treated identically but with no reverse transcriptase added.

(C) RT-PCR with total RNA from Col (lane 1), dsrub-1 (lane 2), dsrub-2 (lane 3), dsrub-3 (lane 4), and the transgenic control line dsrub-con (lane 5) using primers for RUB1 (top panel), RUB2 (middle panel), and UBQ10 (bottom panel). Lane 6 is identical to lane 1, except reverse transcriptase was not included. PCR using the same primers on Col genomic DNA is shown in lane 7. The numbers indicate size markers in base pairs.
null alleles of RUB1/2 in Arabidopsis

Table 1. Segregation and inheritance of rub T-DNA alleles

<table>
<thead>
<tr>
<th>Female Parent</th>
<th>Male Parent</th>
<th>RUB1/rub1</th>
<th>rub1</th>
<th>rub2</th>
<th>RUB2/rub2</th>
<th>RUB1/rub1</th>
<th>RUB1</th>
<th>n</th>
<th>Het Freq. Exp. (%)</th>
<th>Het Freq. Obs. (%)</th>
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</thead>
<tbody>
<tr>
<td>RUB1/rub1</td>
<td>RUB1/rub1</td>
<td>188 (6, 142, 40)</td>
<td>1077 (9, 1009, 34)</td>
<td>1265</td>
<td>67</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rub1/rub2</td>
<td>RUB1/rub1</td>
<td>248 (6, 192, 50)</td>
<td>310 (28, 282)</td>
<td>558</td>
<td>67</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RUB1/rub1</td>
<td>Col</td>
<td>109 (109)</td>
<td>202 (202)</td>
<td>311</td>
<td>50</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Col</td>
<td>RUB1/rub1</td>
<td>26 (26)</td>
<td>421 (421)</td>
<td>447</td>
<td>50</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rub1/rub2</td>
<td>RUB2/rub2</td>
<td>31 (26, 6)</td>
<td>76 (57, 19)</td>
<td>107</td>
<td>50</td>
<td>29</td>
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<tr>
<td>Col</td>
<td>rub1 RUB2/rub2</td>
<td>110 (104, 6)</td>
<td>262 (246, 16)</td>
<td>372</td>
<td>50</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RUB1/rub1</td>
<td>rub1</td>
<td>63 (63)</td>
<td>110 (110)</td>
<td>173</td>
<td>50</td>
<td>36</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>RUB1/rub2</td>
<td>RUB1/rub1</td>
<td>5 (5)</td>
<td>257 (257)</td>
<td>262</td>
<td>50</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rub1/rub2</td>
<td>RUB2/rub2</td>
<td>46 (46)</td>
<td>120 (120)</td>
<td>166</td>
<td>50</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rub1/RUB2</td>
<td>rub1 RUB2/rub2</td>
<td>39 (39)</td>
<td>201 (201)</td>
<td>240</td>
<td>50</td>
<td>16</td>
<td></td>
<td></td>
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</tbody>
</table>

Bold numbers represent plants genotyped by PCR, italicized numbers were genotyped by antibiotic resistance, bold and italicized numbers were genotyped by T-DNA alleles. Complement.

Table 2. Seed Set for Siliques of RUB1 RUB2, rub1-1 RUB2/rub2, rub1-2 RUB2/rub2, RUB1/rub1-1 rub2, and RUB1/rub1-2 rub2 Plants

<table>
<thead>
<tr>
<th>Plant Genotype</th>
<th>Seed per Silique (mean ± SD)</th>
<th>n</th>
<th>P^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>rub1-1 RUB2/rub2</td>
<td>20.0 ± 5.8</td>
<td>80</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>rub1-2 RUB2/rub2</td>
<td>20.6 ± 4.4</td>
<td>60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RUB1/rub1-1 rub2</td>
<td>18.3 ± 5.2</td>
<td>102</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RUB1/rub1-2 rub2</td>
<td>23.4 ± 4.9</td>
<td>19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RUB1 RUB2</td>
<td>35.6 ± 7.5</td>
<td>87</td>
<td></td>
</tr>
</tbody>
</table>

^a Student’s t test, for same as RUB1 RUB2.

RUB2 locus (Table 1, lines 1 and 2). Neither percentage supports a 2:1 segregation (RUB2; P < 0.0005) or even a 1:1 ratio (RUB2; P < 0.005). This deviation from a standard inheritance pattern implies death of more than just the double null developing seed.

To confirm this hypothesis, the fertility of RUB1/rub1-1 rub2, RUB1/rub1-1 rub2, rub1-1 RUB2/rub2, and rub1-2 RUB2/rub2 plants after self-fertilization was determined and compared with the wild type. The number of seeds per silique for all four lines was significantly reduced compared with wild-type siblings and was greater than the 25% loss expected by loss of only the double mutant (Table 2). In addition, dissected siliques illustrated that there were no partially mature seeds and spaces were present (Figure 2A). This suggests that the loss of inheritance of the mutant allele occurs early, either as a result of female gametophyte or early embryo death.

To distinguish between these two possibilities, crosses were performed between RUB1/rub1-2 rub2 and Col plants and rub1-2 RUB2/rub2 and Col plants in both directions. The null alleles were successfully inherited from either gamete for both loci, although at a rate reduced from the expected 50% frequency (Table 1, lines 3 to 6). The crosses to Col illustrated that the frequency of inheritance of the rub1-2 allele was only 6% when inherited from the male, compared with 35% when inherited from the female (Table 1, lines 3 and 4). By contrast, the inheritance frequencies of the rub2 allele from either gamete and the rub1-2 allele from the female were all similar between 29 and 35% (Table 1, lines 3, 5, and 6). This dramatic decrease in inheritance of the rub1-2 allele from the male may explain the 15% frequency of heterozygous plants derived from the RUB1/ rub1-2 rub2 self-fertilized plant (Table 1, line 1).

Crossovers were also made between RUB1/rub1-2 rub2 and RUB1 rub2 plants and rub1-2 RUB2/rub2 and rub1-2 RUB2 plants to determine if the absence of one wild-type locus affects the transmission efficiency of the other. The genetic background did not have any effect on the inheritance of the T-DNA allele, when inherited from the female, from 35 to 36% for rub1-2 and from 29 to 28% for rub2-1 (Table 1, lines 3, 7, 5, and 9). A decrease in inheritance of inheritance was observed for both loci when inherited from the male plant, from 6 to 2% for rub1-2 and from 30 to 16% for rub2-1 (Table 1, lines 4, 8, 6, and 10). The data collected from outcrossing eliminated the possibility of a complete gametophytic lethal phenotype from either parent, although there was some implication of high male gametophytic lethality or low male competition competence from the RUB1/ rub1-2 rub2 plants.

To determine the terminal stage to which the female gametes or embryos develop, young siliques were dissected, cleared, and observed using differential interference contrast microscopy. In a silique dissected from either a RUB1/rub1-1 rub2 or a rub1-1 RUB2/rub2 plant, the female gametes were indistinguishable and were able to be fertilized (data not shown). Differences arose after fertilization. Within the same silique, approximately half of the ovules were small, and the embryo in these ovules never developed past the two-cell stage, whereas larger ovules had embryos beyond the eight cell stage (Figures 2B and 2C). Observations from older siliques showed that the smaller embryos were usually reenveloped by the ovule, which eventually collapsed (data not shown). Together with the genetic studies described above, this phenotype illustrated that the decrease in inheritance of the null alleles of RUB1 and RUB2 was from an arrest in embryonic development at the two-cell stage. Because there were more than just 25% of embryos with this phenotype, some of these embryos must have carried one wild-type allele of these two genes whose expression was not sufficient to complement.
Epitope-Tagged Versions of RUB1 and RUB2 Have Identical Conjugation Patterns and Conjugate to the Same Cullin

In addition to genetic evidence for RUB1 and RUB2 functioning redundantly in plants, this study looked at biochemical redundancy between these two proteins through their conjugation patterns. The Rub proteins function through their attachment to the cullin family of proteins. To visualize the two closely related proteins individually, hemagglutinin (HA)-tagged versions of RUB1 and RUB2 were expressed under a dexamethasone (dex)-inducible promoter. The conjugation of the epitope-tagged RUB1 and RUB2 was observed by SDS-PAGE and immunoblotting with anti-HA antibodies extracts from dex-induced plants. The conjugation patterns for 3HA-RUB1 and 3HA-RUB2 under the same induction condition were indistinguishable (Figure 3A, lanes 3 and 5), and the two strongest bands were in the area of the cullin proteins, around 100 kD, and the monomer, around 15 kD. Arabidopsis cullins are similar in size, between 75 and 92 kD, and therefore are difficult to distinguish on this gel. Some bands of lighter intensity were also observed, although their identity is still unknown. In addition, the conjugation pattern for 3HA-RUB1 and 3HA-RUB2 was unchanged by the addition of the plant hormone auxin (Figure 3A, lanes 2 and 4).

Previously, it was shown that RUB1 attaches to CUL1 in Arabidopsis plants (del Pozo and Estelle, 1999). To confirm that both 3HA-RUB1 and 3HA-RUB2 conjugate to the same cullin, the proteins were expressed for 17 h. This allowed for visualization of a triplet when extracts were reacted with anti–CUL1 antibodies, where the fastest migrating band was unconjugated CUL1 and the middle band was the endogenous RUBx–CUL1 (Figure 3B, lanes 1 and 2). The slowest migrating band was present after only extended dex treatment (cf. Figures 3B and 3C, lanes 1 and 2). Immunoprecipitation of the 3HA-RUBx–CUL1 complex were performed with antibodies against the epitope-tagged Rub proteins and were visualized with anti–CUL1 antibodies. This confirmed that the slowest migrating CUL1 immunoreactive band was indeed 3HA-RUBx–CUL1 (Figure 3B, lanes 3 and 4). This blot confirmed that both RUB1 and RUB2 conjugate to CUL1 when overexpressed and allowed for a marker to identify where this protein complex migrated in an SDS-PAGE.

Once the migration of the 3HA-RUBx–CUL1 complex was established, it was important to see if the conjugation of 3HA-RUB1 and 3HA-RUB2 occurred after short term induction. Immunoprecipitations were performed with extracts from seedlings only dex-treated for 2 h, as well as with extracts from seedlings that were mock treated. The slowest migrating CUL1 band is no longer visible from total seedling extracts (Figure 3C, lanes 1 and 2) but is present in immunoprecipitations and present in a dex-dependent fashion (Figure 3C, lanes 3 to 6). These data confirm that in addition to 3HA-RUB1, 3HA-RUB2 attaches to CUL1 in plant extracts.

Figure 2. All Ovules Are Fertilized, but Some Arrest at the Two-Cell Stage.

(A) Fully developed siliques from RUB1 rub2(left), RUB1/rub1-1 rub2 (middle), and rub1 RUB2/rub2 (right).

(B) Close up of two ovules from the same silique with the genotype RUB1/rub1-1 rub2 representing the large (left) and small (right) ovules observed. The e indicates location of the embryo, and n indicates location of the endosperm. Bars = 50 μm.

(C) Close up of two ovules from the same silique with the genotype rub1-1 RUB2/ rub2 representing the large (left) and small (right) ovules observed. The e indicates location of the embryo, and n indicates location of the endosperm. Bars = 50 μm.
Expression of dsrub in Arabidopsis Reduces RUB1 and RUB2 mRNA and RUB1/2 Protein Levels and Decreases the Amount of Conjugated CUL1

Given that elimination of all RUB1 and RUB2 proteins from Arabidopsis was lethal, an approach was taken to reduce, but not eliminate, endogenous Rub expression. A transgene, called dsrub, designed to express an mRNA containing the RUB1 coding region in both a sense and antisense direction separated by an intron, was introduced into Arabidopsis (Figure 1A, dsrub). When expressed in planta, this mRNA has the potential to form a double-stranded RNA capable of eliciting PTGS (Waterhouse et al., 2001). RUB1 and RUB2 share 85% nucleotide identity in the Rub coding region, so both mRNAs are likely to be affected by RUB1-elicited PTGS. Arabidopsis contains a third gene, UBQ16, encoding a ubiquitin-like protein (RUB3) with 16 amino acid substitutions from RUB1 (Rao-Naik et al., 1998). The corresponding nucleotide sequence has lower identity to RUB1 (74%), and the dsrub construct is not expected to decrease the amount of UBQ16 mRNA. In addition, there is no stretch of nucleotide identity of >21 nucleotides, the minimum required to elicit PTGS.

One hundred and fifty-six antibiotic resistant T1 seedlings, each representing an independent transformation event, were characterized phenotypically at the T1 generation. Twenty-three percent of the 156 plants died soon after transplanting before producing any seed, often before the emergence of true leaves. Fifteen percent had severe changes in their general morphology compared with wild-type plants, but grew to maturity. The remaining 62% showed little or no differences in morphology or development from the progenitor, Col. From the 156 T1 individuals, 30 with phenotypic differences from the wild type were propagated; the phenotype or antibiotic resistance of progeny of some lines was unstable. Of the 12 lines with a strong phenotype that was maintained in subsequent generations, three independent lines produced sufficient seeds to identify homozygous lines for biochemical studies: dsrub-1, dsrub-2, and dsrub-3. dsrub-con, a kanamycin-resistant line generated at the same time with the same transgene and not differing phenotypically from wild type, was propagated for use as a transgenic negative control. These four transgenic lines were used in subsequent studies, and their phenotypes are representative of other lines that were analyzed less extensively.

To characterize the efficacy of induction of PTGS, the levels of mRNA and protein for RUB1 and RUB2 were analyzed in transgenic lines dsrub-1, dsrub-2, dsrub-3, dsrub-con, and Col. The mRNA levels for RUB1 and RUB2 were amplified from cDNA with CUL1 antibodies creating a band that comigrated with the slowest band from extracts (lanes 1 and 2).

**Figure 3.** 3HA-RUB1 and 3HA-RUB2 Attach to the Same Proteins.

(A) Dex-induced expression of 3HA-RUB1 and 3HA-RUB2 in seedlings has the same conjugation pattern and is unchanged by auxin treatment. Total plant extract (80 μg) from seedlings expressing 3HA-RUB1 (lanes 2 and 3) or 3HA-RUB2 (lanes 4 and 5) treated with 10 μM 2,4-D (lanes 2 and 4) or mock treated (lanes 3 and 5) for 30 min was reacted with anti-HA antibodies. Background bands (marked with asterisks) are determined by electrophoresis of extract from untreated Col seedlings (lane 1). The numbers indicate size markers in kilodaltons.

(B) Expression of 3HA-RUB1 and 3HA-RUB2 at high levels leads to CUL1 existing in three forms. Immunoblot (IB) with anti-CUL1 antibodies of extracts from seedlings treated with dex to express 3HA-RUB1 (lane 1) or 3HA-RUB2 (lane 2) for 17 h illustrates the unmodified (fastest band), RUBx-modified (middle band), and 3HA-RUBx-modified (slowest band) forms of CUL1. Samples eluted from anti-HA antibody–conjugated beads after incubation in extracts from seedlings dex- or mock-treated for 2 h maintain a CUL1 band, in a dex-dependent manner. The conjugate pattern of CUL1 in the lines expressing the 3HA-RUBx dex-induced for 2 h is limited to only two bands (lanes 1 and 2).
gene-specific primers (Rao-Naik et al., 1998). Primers amplifying cDNA for the polyubiquitin gene UBQ10 were used as a control for cDNA levels. Control experiments indicate that the reactions were analyzed in the linear range of amplification (data not shown). RNA from Col and dsrub-con gave comparable levels of amplified DNA for all three genes (Figure 1C, lanes 1 and 5). The sizes of the PCR products were smaller than that obtained using genomic DNA as a template (Figure 1C, lane 7) and are identical to the predicted sizes of spliced mRNAs and PCR products observed previously (Rao-Naik et al., 1998). Two of the dsrub transgenic lines, dsrub-1 and dsrub-3, showed approximately one-tenth of the level of RUB1 mRNA and 30 and 50%, respectively, of the level of RUB2 mRNA compared with control lines (Figure 1C, lanes 2 and 4). In dsrub-2, mRNA for RUB1 was moderately reduced to 70% of the control level, whereas RUB2 mRNA level was roughly equivalent to the controls (Figure 1C, lane 3). In conclusion, all three dsrub lines showed significant decreases in RUB1 mRNA, and two lines showed significant reduction in RUB2 mRNA levels compared with the wild type.

To determine if the RUB1 and RUB2 protein levels were also decreased in the dsrub lines, RUB1/2-specific antibodies were developed and used. Because RUB1 and RUB2 only differ by one amino acid, it is extremely difficult to create antibodies that distinguish between these two Rub family members. Instead, antibodies were raised and affinity purified against a peptide sequence identical between RUB1 and RUB2 but sufficiently diverged from RUB3 and ubiquitin to prevent cross-reactivity. The specificity of the affinity-purified antibodies was tested on purified glutathione S-transferase (GST) fusion proteins using immunoblots. The antibodies reacted specifically with GST-RUB1 and not GST alone, GST-RUB3, or GST ubiquitin even when the latter was present at 200 × higher concentration (Figure 4A, bottom panel).

These antibodies were also tested for their ability to specifically recognize endogenous RUB1 and RUB2 in plant protein extracts. The anti-RUB1/2 antibodies reacted with purified recombinant RUB1 and a comigrating band in Col extracts but did not recognize purified ubiquitin (Figure 4B, top panel). To confirm that the anti-RUB1/2 immunoreactive band visualized in Col extracts was RUB1/2, Col extract was enriched with purified ubiquitin before electrophoresis, and the intensity of the band was unchanged (Figure 4B, lanes 3 and 4). The same extracts were probed with anti-ubiquitin antibodies to demonstrate the inclusion of ubiquitin in this sample (Figure 4B, bottom panel). This result demonstrates that the RUB1/2 antibodies are visualizing endogenous RUB1/2 and not ubiquitin.

Anti-RUB1/2 antibodies were used in immunoblots against protein extracts from the dsrub transgenic lines. Protein extracts from Col and dsrub-con had detectable levels of RUB1/2 (Figure 4C, lanes 2, 3, and 7), whereas dsrub-1 and dsrub-3 had RUB1/2 levels below the level of detection (Figure 4C, lanes 8 and 4). RUB1/2 protein was faintly visible in some immunoblots containing extracts from dsrub-2, suggesting higher levels of RUB1/2 protein in this line compared with dsrub-1 and -3 (data not shown). axr1-13, an AXR1 null line, extracts contained unconjugated RUB1/2 levels equivalent to that seen in Col (Figure 4C, lane 5). These data indicated that the reduction in RUB1 and RUB2 mRNAs resulted in a significant decrease in the total amount of RUB1 and/or RUB2 proteins in dsrub lines.

Rub proteins are found in the two states in the cell: the monomer and the conjugated state. It is believed that the Rub functions through its conjugation to cullin proteins. After confirming that both RUB1 and RUB2 attach to CUL1 and that the dsrub lines have a decrease in RUB1/2 monomer levels, it was important to see if the conjugation state of CUL1 was affected in these lines. As described above, CUL1 exists as a doublet, with the slower migrating band identified as a RUBx-CUL1 complex. This doublet was visible with both Col and dsrub-con extract (Figure 4D, lanes 3 and 2). It has been shown previously that the conjugation state of CUL1 is changed in the axr1-12 line, such that there is an increase in the total amount of unmodified CUL1 and no change in modified form, decreasing the ratio (del Pozo and Estelle, 1999). This decrease in the proportion of conjugated form of CUL1 was also found in axr1-13 and in the three dsrub lines (Figure 4D, lane 6 compared with lanes 1, 4, and 5). These data correlate the phenotype of the dsrub lines with a decrease in the ratio of Rub-conjugated CUL1 to un conjugated CUL1.

**Downregulation of RUB1/2 Correlates with Dwarfed Plants**

Figure 5 shows representative phenotypes of the lines whose molecular characterization was shown in Figures 1 and 4 (dsrub-1, dsrub-2, and dsrub-3), plus the phenotypes of additional independent lines that did not produce sufficient tissue for thorough molecular analyses (dsrub-4, dsrub-5, and dsrub-6). All of these dsrub lines grew slower as seedlings and produced smaller plants. After transplanting and 3 weeks in soil, Col seedlings had green cotyledons with one pair of fully expanded leaves and a second emergent pair (Figure 5A). By contrast, dsrub lines grown under the same condition had produced either smaller cotyledons with no visible true leaves (dsrub-4, Figure 5B) or smaller cotyledons and true leaves at a slightly slower rate (dsrub-5, Figure 5C). However, dsrub lines flowered with the same number of leaves as control lines (data not shown). Seedlings with a slight purple color were regularly seen (Figure 5C), indicating anthocyanin production, which suggests induction of a stress response (Dixon and Palva, 1995). By 5 weeks, the difference in rosette size between Col and the dsrub lines was even more evident (Figures 5D to 5F).

During reproductive growth, Col plants extend multiple racemes with some branching (Figure 5J, left). For the three dsrub lines characterized molecularly, the racemes were short (Figures 5G to 5I). Some lines did not extend a raceme at all, producing just a few siliques right out of the rosette, such as dsrub-3 (Figure 5G). Although with different severity, all of the transgenic partial loss-of-function RUB1/2 lines had similar attributes, suggesting disruption of the same pathways in multiple lines.

The activating enzyme for RUB1 in Arabidopsis is an AXR1/ ECR1 heterodimer (del Pozo et al., 1998). axr1-13 has short inflorescences with increased branching on the primary inflorescence and a greater number of secondary inflorescences than the wild type, resulting in a bushy appearance (Figure 5J, right). The dsrub lines were more severely dwarfed than axr1-13 (Figure 5J, center and right), and in lines with elongating inflorescences,
there were fewer than for axr1-13 (Figures 5H to 5J, right). These differences suggest that although axr1-13 and the transgenic lines are both smaller than the wild type, the dsrub lines have a dwarfed phenotype distinct from that seen in axr1-13 lines.

dsrub Seedlings Are Altered in Auxin Responses

Because AXR1 is part of a RUB1 activating enzyme (Leyser et al., 1993), the expectation was that dsrub lines and axr1-13 plants would have similar phenotypes. This was not observed in mature plants where dsrub lines were wild type with respect to branching, whereas axr1-13 plants were highly branched and dsrub lines were more severely dwarfed than axr1-13 (Figure 5J). This led us to determine whether the auxin resistance observed in axr1-12 roots (Lincoln et al., 1990) was also present in dsrub roots. Low levels of auxin are stimulatory to primary root growth, but inhibitory at higher concentrations, whereas lateral root initiation is stimulated by higher levels of auxin (Taiz and Zeiger, 1998). The effect of exogenous auxin on primary root growth and lateral root initiation of light-grown dsrub lines was determined (Figure 6). Growth of Col primary roots in the presence of 0.1 μM 2,4-D were inhibited >90%, whereas axr1-13 was only weakly inhibited with <10% growth inhibition (Figure 6A). The three dsrub lines showed significantly lower levels of inhibition than Col, but more than axr1-13 at 45 to 75% (Figure 6A). Auxin treatment increased lateral root initiation in Col, but this increase was completely lost in axr1-13 (Figure 6B). The phenotypically most affected line with the lowest amount of RUB1/2 mRNA, dsrub-3, also showed a complete loss of sensitivity to auxin. By contrast, the least severe line, dsrub-2, exhibited a wild-type response, producing as many lateral roots per millimeter as Col. A correlation between the magnitude of the decrease in RUB1/2 mRNAs and insensitivity to auxin is illustrated in both root assays. Like AXR1, RUB1 and/or RUB2 are involved in auxin response in roots.

Figure 4. RUB1/2 Protein Levels Are Decreased in dsrub Lines.

(A) RUB1/2 affinity-purified antibodies specifically react with GST-RUB1. Immunoblot (IB) of purified GST (lane 1) and GST fusion proteins: GST-RUB1 (lane 2), GST-RUB3 (lane 3), GST-UBQ (lane 4) probed with affinity-purified anti-RUB1/2 antibodies (bottom panel). The antibodies were also tested on 100× (lane 5) and 200× (lane 6) GST-UBQ. An anti-GST IB (top panel) verifies protein levels (lane 6 has 400× GST-UBQ). (B) Affinity-purified anti-RUB1/2 antibodies show specificity against whole plant extract. Immunoblot analysis of purified ubiquitin (UBQ) (lane 1), purified RUB1 (lane 2), Col protein extract (lane 3), and Col protein extract enriched with purified ubiquitin (lane 4). The anti-RUB1/2 antibodies (top panel) detect endogenous RUB1/2 protein, and the anti-ubiquitin antibodies (bottom panel) verify the presence of purified ubiquitin, as well as visualizing endogenous ubiquitin in Col extract (lane 3). (C) Immunoblot with anti-RUB1/2 antibodies on 200 μg of total protein extracted from Col (lanes 2 and 7), dsrub-con (lane 3), dsrub-3 (lane 4), axr1-13 (lane 5), and dsrub-1 (lane 8). Purified RUB1 is used as a positive control (lanes 1, 6, and 9). Lane 7 contains half the protein as lane 8. (D) The dsrub lines have a decreased ratio of unmodified CUL1 to modified CUL1. Immunoblot with anti-CUL1 antibodies of extracts from seedlings of control lines, Col (lane 3) and dsrub-con (lane 2), dsrub-1 (lane 1), dsrub-2 (lane 4), dsrub-3 (lane 5), and axr1-13 (lane 6). Coomassie blue stain of identically loaded samples serves as a loading control (bottom panel).
Figure 5. Growth of dsrub Plants Is Significantly Slower and Overall Size Is Severely Reduced.

(A) to (C) Seedlings at 3 weeks: Col (A), dsrub-4 (B), and dsrub-5 (C). Bar = 0.5 cm.
(D) to (F) Plants at 5 weeks: Col (D), dsrub-6 (E), and dsrub-5 (F). Bar = 1.0 cm.
(G) to (I) Plants at 8 weeks: dsrub-3 (G), dsrub-1 (H), and dsrub-2 (I). Bar = 1.0 cm.
(J) Col (left), dsrub-3 (middle), and axr1-13 (right) at 8 weeks. Bar = 2.0 cm.
hook with slightly expanded cotyledons (Figures 7B to 7D and 7I). These differences were also observed in additional dsrub lines with a similar light growth phenotype as dsrub-1, indicating that it was characteristic of this category of dsrub lines (data not shown). The shorter, thicker hypocotyls and the exaggerated hook were reminiscent of the triple response that is seen in dark-grown, wild-type seedlings exposed to the gaseous hormone ethylene or its immediate precursor ACC. Exposure of Col seedlings to ACC (Figure 7E) resulted in a swollen hypocotyl, an exaggerated hook, and reduced hypocotyl and root growth (Schaller and Kieber, 2002). The dsrub lines had all of these characteristics, except their roots were still elongated, illustrating a partial triple response phenotype.

The constitutive triple response could result from a lesion in the ethylene response pathway, leading to constitutive activation of the pathway, or from a lesion in regulation of ethylene production leading to increased synthesis of the hormone. To distinguish between these possibilities in the dsrub lines, dsrub-1 seedlings were grown in the presence of Ag⁺, an ethylene receptor inhibitor that blocks perception of ethylene (Beyer, 1976). Growth in the presence of Ag⁺ completely abolished the triple response phenotype of dsrub-1 seedlings (Figure 7F); the hypocotyl was long and narrow and the hook straightened. This indicated that the hypocotyls of dsrub seedlings have a functional ethylene response pathway and established a role for RUB1/2 upstream of the ethylene receptor.

To determine whether increased ethylene production was leading to the partial triple response in dsrub lines, dsrub-1 seedlings were treated with aminoethoxyvinyl glycine hydrochloride (AVG), an inhibitor of ACS whose activity typically limits in vivo ethylene production (Capitani et al., 2002). This treatment completely abolished the triple response (Figure 7G), strongly suggesting that the dsrub dark-grown phenotype resulted from increased ethylene production.

Because AXR1 functions to activate RUB1 for attachment to cullins, the dark-grown phenotype of axr1-13 seedlings was compared with that of the dsrub lines. Strikingly, dark-grown axr1-13 seedlings did not exhibit the partial triple response seen in the dsrub lines. Their hypocotyl length was longer, identical to Col, and as previously observed, the hypocotyls were hookless (Lehman et al., 1996) (Figure 7H).

dsrub Lines Overproduce the Hormone Ethylene

The constitutive partial triple response phenotype and its loss in the presence of AVG strongly suggested that the dsrub dark-grown seedlings overproduced ethylene. To test this directly, ethylene released by dark-grown dsrub seedlings over 4 d was measured by gas chromatography (GC) and compared with Col. The dsrub lines produced three to five times more ethylene than Col (Figure 8). These measurements confirmed that the dsrub seedlings were overproducing ethylene when grown in the dark. By contrast, the amount of ethylene released from the axr1-13 line was less than from Col (Figure 8). These results implicate AXR1 in enhancing, and RUB1/2 in the opposite, suppressing ethylene levels in dark-grown seedlings, although that may be an oversimplified model.

dsrub Seedlings Exhibit Altered Growth Morphology When Grown in the Dark, Suggesting Alterations in the Ethylene Pathway

Dark-grown dsrub seedlings had obvious morphological differences from the wild type. Relative to light-grown seedlings, dark-grown Col seedlings develop an elongated hypocotyl, arrested chloroplast development, unexpanded cotyledons, and an apical hook, collectively referred to as skotomorphogenesis (Figures 7A and 7J). Whereas the transgenic control line dsrub-con exhibits normal skotomorphogenesis (data not shown), when grown in the dark dsrub-1, dsrub-2, and dsrub-3 seedlings have shorter, thicker hypocotyls and an exaggerated

Figure 6. dsrub Seedlings Exhibit Insensitivity to Exogenous Auxin.

(A) Inhibition of primary root growth by 0.1 μM 2,4-D. The primary root of Col, dsrub-1, dsrub-2, dsrub-3, and axr1-13 seedlings grown on 2,4-D for 10 d is presented as a percentage of the growth of seedlings on germination media (GM) for the same length of time.

(B) Increase in the number of lateral roots by 0.1 μM 2,4-D. The number of lateral roots on the primary root of Col, dsrub-1, dsrub-2, dsrub-3, and axr1-13 seedlings grown on GM for 10 d is subtracted from the number on the same primary root grown on 2,4-D or KOH alone for an additional 6 d. Each bar represents the average ± se of two experiments with a minimum of 10 seedlings each. Single asterisks represent lines that are statistically different from Col with a P < 0.0001, and double asterisks represent lines that are statistically different from Col with a P < 0.02.
DISCUSSION

Previous work on the Rub pathway in Arabidopsis came from analyses of mutant lines affected in expression of enzymes required for activation of the Rub proteins. Initial and pioneering knowledge was determined through analysis of the null allele of AXR1, axr1-12 (Lincoln et al., 1990); AXR1 is a subunit of the heterodimeric complex that activates RUB1 in vitro (Leyser et al., 1993; del Pozo et al., 1998). axr1-12 plants are viable, but dwarfed and affected in multiple hormone signaling pathways (Lincoln et al., 1990; Timpte et al., 1995). Combining the axr1-12 mutation with partial mutants in other Rub conjugation enzymes had an additive effect, but the most severe effect was seedling lethality. Because some RUB1 attachment to target proteins remains in axr1-12 and axr1-12 rce1 plants and may be attributed to AXR1 and RCE1 homologs (del Pozo et al., 2002; Dharmasiri et al., 2003), the phenotype of these lines may not give a full picture of the role of the Rub pathway in Arabidopsis.

Null mutations in RUB1 and RUB2 have allowed us to address the question of the requirement for the Rub pathway. These studies have shown that RUB1 and RUB2 are functionally redundant, and the presence of at least one functional RUB-encoding gene is required. Whereas single RUB1 or RUB2 mutant plants are viable and have a wild-type phenotype, no double mutant seedling was recovered after PCR genotyping of more than 300 progeny. The ability of rub1 rub2 female and male gametes to produce seed when fertilized by either RUB1 RUB2 or single mutant gametes is reduced. This suggests either reduced viability of double null gametes or a lethal effect at fertilization or the first zygotic division that cannot be rescued by wild-type genes.

This essentiality of the Rub pathway in plants is consistent with data from genetic studies of the Rub pathway in organisms from other kingdoms. S. pombe cells with disruptions in the Rub/Nedd8 pathway arrest after a few divisions, and in mouse and...
C. elegans, cells downregulated in Rub/Nedd8 conjugation exhibit embryonic defects (Parry and Estelle, 2004). S. cerevisiae is currently the only organism that remains viable in the absence of Rub1p or its conjugation pathway (Lammer et al., 1998).

In vitro studies with mammalian SCF complexes demonstrated that conjugation of Rub/Nedd8 to CUL1 is required for robust polyubiquitylation of target proteins. In Arabidopsis, CUL1 has been shown to be a component of multiple SCF complexes required for auxin signaling, jasmonic acid response, and floral development (Hellmann and Estelle, 2002; Wang et al., 2003). We have demonstrated that both 3HA-RUB1 and 3HA-RUB2 attach to CUL1 in vivo. Based on these data, we hypothesize that RUB1/2 are essential in Arabidopsis because their attachment to CUL1 is required for the function of this component of SCF complexes. Consistent with this, the rub1 rub2 mutants described here have the same phenotype as cul1 null lines. cul1 null lines are embryonic lethal, and the cul1 null allele is inherited at a reduced rate from both parents (Shen et al., 2002; Hellmann et al., 2003). Also in support of this hypothesis, a decrease in the ratio of Rub-modified CUL1 to unmodified CUL1 and altered phenotype, although this comparison is limited. The modified to unmodified CUL1 ratio in mutants with only one or two functional RUB1/2-encoding genes is similar to the wild type (data not shown), and these plants have a wild-type phenotype. In the dsrub lines, the amount of unmodified CUL1 is increased, whereas the amount of Rub-modified CUL1 is unchanged, and these plants have a severe phenotype (Figure 4). These modifications in ratio have been observed previously in axr1-12 and axr1-12 rce1 lines with altered phenotypes (Dharmasiri et al., 2003) and may be attributed to the increased stability of CUL1 when it is unmodified (Morimoto et al., 2003).

There are additional cullin proteins in Arabidopsis, and Rub/Nedd8 has been shown to conjugate to multiple cullins in mammals (Hori et al., 1999) and S. cerevisiae (Lammer et al., 1998; Laplaza et al., 2004). Although not yet demonstrated, it remains possible that other cullins are modified by RUB1/2 in Arabidopsis, and changes in modification of these cullins could contribute to the phenotypes observed, as will be discussed below.

Because rub1 rub2 null lines are lethal and the lines with only a single functional RUB-encoding gene had a wild-type phenotype, further characterization was not possible with the T-DNA lines. In an attempt to understand the function of RUB1 and RUB2 in Arabidopsis, dsrub lines were created that are strongly downregulated in RUB1/2 protein. Analysis of the dsrub lines revealed roles for RUB1/2 that had not been observed previously. When attempting to produce transgenic lines downregulated in RUB1/2 expression, 23% of the initial transformants revealed phenotypes derived from eto1 and eto2 plants (Chae et al., 2003). These data complement work showing that the BTB domain-containing protein ETO1 is required for appropriate degradation of ACS5 (Chae et al., 2003). It is unclear if this is the pathway for degradation of other ACS family members, although a subset, ACS5, 9, 8, and 4, do have a conserved C-terminal motif required for degradation (Chae et al., 2003). It is evident that both the Rub and ubiquitin pathways are required for maintaining low ethylene levels; however, the molecular linkage between the two pathways is currently unknown and work is in progress to determine the molecular basis for the requirement for Rub in this regulation.

The mechanism by which alterations in Rub expression affect ethylene synthesis is unknown. However, there are several potential targets for ubiquitin-mediated proteolysis. The synthesis of ACC, catalyzed by ACS, is generally considered to limit ethylene production, and some ACS isozymes have been demonstrated to be short lived (Kim and Yang, 1992; Chae et al., 2003). Additional or other potential sites of control for ethylene synthesis are possible, including ACO abundance or regulation of the abundance of transcription factors that control production of mRNAs required for ethylene synthesis. Alternatively, the release of ACC from conjugated forms can increase flux through the pathway, as can increased availability of S-adenosyl Met.

The mechanism by which dsrub lines overproduce ethylene is currently under investigation, although recent publications have given two different hypotheses. Larsen and Cancel (2004) have recently characterized an rce1-2 recessive line, which overproduces ethylene. In vitro activity measurements detected an increase in ACO, not ACS activity. Because ACO activity increases in the presence of ethylene (Petruzelli et al., 2000), the increase in ACO activity may not be derived directly from the mutation in the Rub pathway. By contrast, Wang et al. (2004) recently published data implicating an SCF-like complex in regulating ACS5 protein levels. Through protein–protein interaction studies and genetic analyses, this study bridged a gap between the newly defined SCF-like, CUL3-containing, ubiquitin-ligase complex (Pan et al., 2004) and the ethylene overproduction phenotypes derived from eto1 and eto2 plants (Chae et al., 2003). These data complement work showing that the BTB domain-containing protein ETO1 is required for appropriate degradation of ACS5 (Chae et al., 2003). It is unclear if this is the pathway for degradation of other ACS family members, although a subset, ACS5, 9, 8, and 4, do have a conserved C-terminal motif required for degradation (Chae et al., 2003). It is evident that both the Rub and ubiquitin pathways are required for maintaining low ethylene levels; however, the molecular linkage between the two pathways is currently unknown and work is in progress to determine the molecular basis for the requirement for Rub in this regulation.

The difference between ethylene productions in axr1-13 versus the dsrub lines was surprising and could have several separate or combined etiologies. Direct measurements proved that dark-grown axr1-13 produced less, rather than more, ethylene compared with Col. If AXR1 and RUB1/2 function in the same pathway, then ethylene overproduction in axr1-13 is expected. However, the morphology in dark-grown axr1-13
seedlings suggests one possible explanation for its reduced ethylene production below the wild type. axr1-13 seedlings are hookless with expanded cotyledons, suggesting some activation of the photomorphogenic pathway in the dark. Ethylene synthesis is negatively regulated by red light (Finlayson et al., 1999), so its production could be suppressed in axr1-13 because the photomorphogenic pathway is activated to a greater extent in these seedlings relative to the wild type. This suppression of ethylene production would have to be dominant over the misregulation of ethylene synthesis caused by mutation in the ethylene production gene integral to the T-DNA. The photomorphogenic program seen in axr1-13 is not as developed in the dsrub lines, even when ethylene production is lowered with AVG treatment (data not shown), and this developmental difference could affect regulation of ethylene production. Alternatively, because an ethylene overproduction phenotype is not seen in axr1-13, RUB1/2 could participate in an AXR1-independent process that affects ethylene synthesis. If AXL1 functioned as the activating enzyme for this process, it would have wild-type activity in axr1-13 plants but be limited in the dsrub lines as a result of lack of RUB1/2, although ax1 null lines do not have a triple response phenotype (data not shown).

The activity of the third RUB family member, RUB3, is unknown. RUB3 is more diverged in amino acid sequence from RUB1 and RUB2, has a unique gene morphology, and is expressed at a lower level in a more limited fashion (Rao-Naik et al., 1998). It is clear that RUB3 is unable to compensate for the complete loss of RUB1 and RUB2 because a rub1 rub2 plant is inviable. To date, it has not been determined if the UBO16 transcript, already low, is decreased in the dsrub lines. It is possible that the phenotype of these lines is derived from a loss of RUB3 protein.

METHODS

Plant Lines

rub1-1, a T-DNA insertion in At1g31340, was acquired from SALK (SALK 52941) and was confirmed by PCR and sequencing. rub1-2, a T-DNA insertion in At2g35635, was acquired from the University of Wisconsin Biotecnology Center Arabidopsis Knockout Facility and was confirmed by PCR, DNA gel blot, and DNA sequencing. rub2-1 and rub2-2 were GARLIC lines acquired from Syngenta (587 A09.b1a.Lb3Fa and 379 D02.b.1a.Lb3Fa) and were confirmed by PCR and DNA sequencing.

The RUB1 open reading frame was amplified from the ABRC (Arabidopsis Biological Resource Center) cDNA clone, p25022, with primers 5’-GCCGGCTGACTGACTGACATGATTAGTGAAGC-3’ and 5’-GCCGGGATCCTGCTCAGAGAAGGACAC-3’. The PCR product was cloned into pRNA69 at two different sites in reverse orientation (Qui et al., 2002). The expression cassette was moved into pBIN19 (Bevan, 1984) and the derivative plasmid transformed into Agrobacterium tumefaciens strain AGL1. Col plants were transformed by floral dipping (Clough and Bent, 1998). Seed from the dipped plants were screened on plates with kanamycin to identify plants carrying the dsrub DNA and segregating 3:1 for kanamycin resistance, which were then propagated to obtain homozygous seed.

RT-PCR

Total RNA was extracted from 7-d-old seedlings using TriZol (Invitrogen, Carlsbad, CA) following the protocol supplied. cDNA was produced using the Invitrogen SuperScript first-strand synthesis kit and a poly-T primer. The primers used to amplify RUB1 and RUB2 were previously described. The primers used to amplify UBO10 were 5’-TCAATCTCTCAGCTGATCAAAG-3’ and 5’-TTCCATCAAACTGGAGTTG-3’. PCR was confirmed to be in the linear range by removing samples at 27, 29, and 31 cycles, and products were fractionated on agarose gels, visualized with Kodak 1D 3.0 software (Rochester, NY), and quantified with ImageQuant 4.0 software. RUB1 and RUB2 PCR fragments were normalized to the PCR products from UBQ10 and to the average from control lines Col and dsrub-con. PCR on T-DNA lines, rub1-1, rub1-2, rub2-1, rub2-2, and control lines were performed for 35 to 40 cycles to increase sensitivity.

Genotyping

Some plants were genotyped by PCR at the locus of interest for the wild-type allele and/or the T-DNA allele with gene and T-DNA specific primers. The majority of seedlings were genotyped by the antibiotic resistance gene integral to the T-DNA. rub1-2 carries the NPTII gene conferring kanamycin resistance. Both rub2-1 and rub2-2 carry the BAR gene conferring BASTA resistance. All crosses using the rub1-1 allele were genotyped by PCR; the NPTII gene was silenced in this line. Select individuals genotyped by antibiotic resistance were subsequently genotyped by PCR as indicated to confirm the scoring.

Microscopy

Siliques were dissected with two, 30-gauge needles, cleared overnight with Hoyer’s solution (chloral hydrate, 50% glycerol), and visualized by a Zeiss Axioskopt 2+ (Jena, Germany), using differential interference contrast imaging and a 40 × objective lens. Pictures were taken using AxioVision 4.0 software.

Production of Transgenic dsrub Lines

The RUB1 open reading frame was amplified from the UBQ15 ABRC (Arabidopsis Biological Resource Center) cDNA clone, p25022, with primers 5’-GCCGGCTGACTGACTGACATGATTAGTGAAGC-3’ and 5’-GCCGGGATCCTGCTCAGAGAAGGACAC-3’. The PCR product was cloned into pRNA69 at two different sites in reverse orientation (Qui et al., 2002). The expression cassette was moved into pBIN19 (Bevan, 1984) and the derivative plasmid transformed into Agrobacterium tumefaciens strain AGL1. Col plants were transformed by floral dipping (Clough and Bent, 1998). Seed from the dipped plants were screened on plates with kanamycin to identify plants carrying the dsrub DNA and segregating 3:1 for kanamycin resistance, which were then propagated to obtain homozygous seed.

Growth Conditions

For growth measurements, seeds were surface sterilized with 30% bleach and grown on 0.8% agar, 1 × MS (Sigma, St. Louis, MO), and 1% sucrose under continuous dark. Seedlings were measured after removal from plate, scanned using an AGFA Arcus II scanner (Septestaat, Germany), and quantified by MacBas 2.0 software. For treatments, chemicals were spread on plates. ACC (Sigma) and AVG (Sigma) were dissolved in 1 × MS plus 1% sucrose and used at final concentrations of 50 and 5 μM, respectively. Silver nitrate (Sigma) was dissolved in water and used at a final concentration of 100 μM. 2,4-D (Sigma) was dissolved in 0.1 M KOH at a concentration of 20 mM and diluted in 1 × MS plus 1% sucrose and used at a final concentration of 10 μM.

Auxin Sensitivity Assays

Primary root growth assays were performed by germinating seeds on GM, growing them horizontally for 3 d, transferring seedlings to vertical plates with 0.1 μM 2,4-D or KOH alone, and marking the root position.
seedlings were grown for 7 d, and the root length was measured. For the lateral root assay, seedlings were germinated on growth media with 0.6% agar and grown horizontally for 10 d. The number of lateral roots was counted, and the seedlings were transferred to vertical plates with 0.1 μM 2,4-D or KOH alone, the primary root position was marked, and the seedlings were grown for an additional 6 d. The plates were scanned using a tablettop AGFA Arcus II scanner, root length was quantified by MacBas 2.0 software, and the number of lateral roots was counted.

Immunoblot Analysis

The anti-RUB1/2 antibodies were raised against a peptide common to the two proteins, but divergent from ubiquitin and RUB3, Acetyl-EIEIDIEDTDT-C (US Biological, Swampsco, MA). The peptide was conjugated to keyhole limpet hemocyanin through the C-terminal Cys and injected into a rabbit. The antibodies were affinity purified with the same peptide conjugated to biotin immobilized on an avidin agarose matrix (Pierce, Rockford, IL). The eluent was tested against purified Escherichia coli expressed GST fusion proteins of ubiquitin, RUB1, and RUB3, as well as GST alone. Protein loading was tested with a GST immunoblot using anti-GST antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5000).

For immunoblots using affinity-purified, anti-RUB1/2 antibodies, anti-ubiquitin antibodies (Worley, 1997), horseradish peroxidase (HRP)-conjugated anti-HA antibodies (Roche, Indianapolis, IN), and anti-CUL1 antibodies with plant extracts, seedlings were grown for 7 d in constant light on plates as described above, harvested, ground in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% [v/v] Nonidet P-40, 0.05% [w/v] deoxycholic acid, 0.1% [w/v] SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail [Roche]), and spun at 14,000 rpm for 20 min. Purified ubiquitin (Sigma), 24 ng of purified RUB1 (Rao-Naik et al., 1998), soluble Col protein extract, and Col protein extract enriched with 200 ng of ubiquitin were fractionated on a high percentage Tris-Tricine gel. The proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA), autoclaved for 20 min to redenature the proteins, and probed with affinity-purified RUB1/2 antibodies (1:500) or ubiquitin antibodies (1:20000). HA immunoblots were performed with 80 μg of total soluble protein, at a 1:500 dilution, and extracts were run on a 20% gradient Tris-Glycine gel (Invitrogen). CUL1 immunoblots were performed with 50 μg of protein as described (del Pozo and Estelle, 1999), and extracts were generally run on an 8% Tris-Glycine gel. The blots were developed with ECL (Amersham-Pharmacia, Piscataway, NJ) and visualized with a Storm 680 PhosphorImager and ImageQuant 4.0 software (Molecular Dynamics, Piscataway, NJ).

Immunoprecipitations

Seven-day-old seedlings were grown in constant light on GM and were chemically treated as indicated. Dex (Sigma) was diluted in ethanol at a concentration of 3 mM and diluted in 1× MS plus 1% sucrose and used at a final concentration of 30 μM. Seedlings were ground in Plant IP Buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 20 mM EDTA, 10% glycerol, 0.15% Nonidet P-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM 1,10-o-phenanthroline [Sigma], and 1× protease inhibitor cocktail), spun for 20 min at 14,000 rpm, and tested for protein concentration with Bradford reagent. Samples were reserved, to be run as extract. Sepharose beads conjugated with HA antibody (Sigma) were mixed with 3 to 5 mg of total soluble protein and incubated at 4°C for 2 h. Beads were spun down and washed five times with 1 mL of PBS. Protein was eluted with 5× Laemmli sample buffer and boiled for 5 min before electrophoresis.

Ethylene Measurement

Seedlings were germinated and grown for 4 d in complete darkness in GC vials with 6 mL of 0.8% agar containing 1× MS, 1% sucrose, and 100 4-mm glass beads, leaving a head room of 15 mL. One-milliliter samples of the head volume from a single vial were removed and injected into a custom-packed Supelco gas chromatograph column, 4-foot 8% NaCl on Alumina F1 with 80/100 mesh with a flame detector in triplicate. Standards of 75 ppb, 1.77 ppm, and 7.4 ppm ethylene were used to verify retention time and quantify the peaks produced by the seedlings. Background values were calculated as the ethylene produced from vials with GM alone and subtracted from each sample.

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