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The SCF^{COI1} Ubiquitin-Ligase Complexes Are Required for Jasmonate Response in Arabidopsis

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Xie and colleagues previously isolated the Arabidopsis *COI1* gene that is required for response to jasmonates (JAs), which regulate root growth, pollen fertility, wound healing, and defense against insects and pathogens. In this study, we demonstrate that COI1 associates physically with AtCUL1, AtRbx1, and either of the Arabidopsis Skp1-like proteins ASK1 or ASK2 to assemble ubiquitin-ligase complexes, which we have designated SCF^{COI1}. COI1$_{E22A}$, a single amino acid substitution in the F-box motif of COI1, abolishes the formation of the SCF^{COI1} complexes and results in loss of the JA response. *AtRbx1* double-stranded RNA-mediated genetic interference reduces AtRbx1 expression and affects JA-inducible gene expression. Furthermore, we show that the AtCUL1 component of SCF^{COI1} complexes is modified in planta, where mutations in *AXR1* decrease the abundance of the modified AtCUL1 of SCF^{COI1} and lead to a reduction in JA response. Finally, we demonstrate that the *axr1* and *coi1* mutations display a synergistic genetic interaction in the double mutant. These results suggest that the *COI1*-mediated JA response is dependent on the SCF^{COI1} complexes in Arabidopsis and that the *AXR1*-dependent modification of the AtCUL1 subunit of SCF^{COI1} complexes is important for JA signaling.

INTRODUCTION

Plant hormones influence many diverse developmental processes, ranging from seed germination to root, leaf, shoot, and flower formation. Jasmonic acid and its cyclopentanous derivatives (collectively referred to here as jasmonates [JAs]) are a new class of plant hormones that modulate the expression of numerous genes (Reymond et al., 2000) and regulate plant developmental processes, including root growth, pollen formation, tuberization, and fruit ripening. They also mediate responses to stress, wounding, insect attack, and pathogen infection (for reviews, see Staswick, 1992; Sembdner and Parthier, 1993; Blechert et al., 1995; Creelman and Mullet, 1997; Wasternack and Parthier, 1997; Reymond and Farmer, 1998; Zhao and Ma, 2000; Berger, 2001; Farmer, 2001).

The effects of JA on a variety of plant species were discovered initially through the application of exogenous JA to plants (for review, see Creelman and Mullet, 1997). Furthermore, plant responses to JA were defined mainly through genetic analysis of mutants, including *fad3/fad7/fad8* (McConn and Browse, 1996), *opr3* (Sanders et al., 2000; Stintzi and Browse, 2000; Stintzi et al., 2001), *jar1* (Staswick et al., 1992, 1998), *coi1* (Feys et al., 1994), *jin1* and *jin4* (Berger et al., 1996), *cev1* (Ellis and Turner, 2001), *cet* (Hilpert et al., 2001), *cex1* (Xu et al., 2001), and others (Howe et al., 1996; Petersen et al., 2000). Although the effect of JA on plants is well characterized, little is known about the mechanisms whereby JAs exert their biological functions.

The Arabidopsis mutants *jar1*, *coi1*, *jin1*, and *jin4* have been used to study the signal transduction pathway that modulates JA action. The *jar1*, *jin1*, and *jin4* mutants exhibit decreased sensitivity to JA, as assayed for the inhibition of root growth and for the inducible expression of the Arabidopsis vegetative storage protein (AtVSP) in response to JA

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treatment. The *coi1-1* mutant is male sterile, insensitive to JA, and lacks the expression of JA-induced proteins, including AtVSP and the plant defense–related proteins Thi2.1 and PDF1.2 (Feys et al., 1994; Benedetti et al., 1995; Penninckx et al., 1998; Xie et al., 1998). The *COI1* gene was found to be required for all JA-regulated plant fertility and defense processes (Feys et al., 1994; Xie et al., 1998) and is suggested to act upstream of *JIN1* and *JAR1*/*JIN4* in the JA signal transduction chain (Berger et al., 1996; Rojo et al., 1998; Berger, 2001).

Molecular analysis of the *COI1* sequence indicated that *COI1* might encode a protein containing Leu-rich repeats and a degenerate F-box motif (Xie et al., 1998). These structural features are characteristic of F-box proteins that function in ubiquitin-ligase complexes for the ubiquitylation of substrate proteins targeted for degradation. The F-box motif is present in many yeast and mammalian proteins that interact with Skp1 and Cdc53 (cullin) to assemble SCF ubiquitin-ligase complexes (Skp1-Cdc53-F-box protein) (for reviews, see Hershko and Ciechanover, 1998; Deshaies, 1999). In the ubiquitin-dependent proteolytic pathway, ubiquitin is linked to substrates through a well-organized process involving the sequential action of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The ubiquitin-related proteins RUB/NEDD8 also are conjugated to target proteins by a mechanism similar to the ubiquitin conjugation process involving the action of a RUB-activating enzyme and a RUB-conjugating enzyme.

One target for RUB conjugation is the Cdc53/cullin component of SCF complexes (Lammer et al., 1998; del Pozo and Estelle, 1999; Gray et al., 1999), and this RUB modification of Cdc53/cullin is important in regulating the activity of SCF ubiquitin ligases (del Pozo and Estelle, 1999; Osaka et al., 2000; Podust et al., 2000; Lyapina et al., 2001). In Arabidopsis, the Cdc53-like protein AtCUL1 (del Pozo and Estelle, 1999) and the Skp1-like proteins ASK1 and ASK2 interact with F-box proteins such as TIR1 to assemble SCF complexes (Gray et al., 1999, 2001; del Pozo and Estelle, 2000; Schwechheimer et al., 2001). AtCUL1 is modified by the ubiquitin-related protein RUB1 in vivo (del Pozo and Estelle, 1999). RUB1 is activated for conjugation to AtCUL1 by a RUB-activating enzyme that is composed of AXR1 and ECR1 and the RUB-conjugating enzyme RCE1 (del Pozo et al., 1998, 2002; del Pozo and Estelle, 1999).

In this study, we demonstrate that the Arabidopsis F-box protein COI1 associates with AtCUL1, AtRbx1, and the Skp1-like proteins ASK1 and ASK2 to assemble SCF^{COI1} ubiquitin-ligase complexes in planta. $COI1_{E22A}$, a single amino acid substitution in the F-box motif of COI1, abrogates the formation of SCF^{COI1} complexes and causes defects in JA response. Double-stranded *AtRbx1* RNA-mediated genetic interference reduces AtRbx1 expression and affects the inducible accumulation of JA-inducible gene expression.

Furthermore, we find that the AtCUL1 component of SCF^{COI1} complexes is modified in planta, where mutations in *AXR1* decrease the abundance of the modified AtCUL1 of SCF^{COI1} and lead to a reduction in JA response. Finally, we demonstrate that the *axr1* and *coi1* mutations display a synergistic interaction in double mutants. Together, these results indicate that the SCF^{COI1} complexes are required for JA response in Arabidopsis and that the *AXR1*-dependent modification of AtCUL1 is important for JA signaling.

RESULTS

Two-Hybrid Screen for COI1-Interacting Proteins

To identify Arabidopsis proteins with which COI1 can interact in the yeast two-hybrid system, we constructed two COI1 "baits" with in-frame fusions to either the GAL4 or LexA DNA binding domains in pGBKT7 or pLexA, resulting in pGBKT7-COI1 and pLexA-COI1, respectively. pGBKT7-COI1 and pLexA-COI1 were used to exhaustively screen the corresponding GAL4- or LexA-based Arabidopsis cDNA libraries, leading to the identification of 137 positive colonies. Cross-hybridization analysis and restriction enzyme digestion of the PCR-amplified cDNA inserts of the 137 positive colonies indicated that they belonged to four distinct classes.

Representative cDNAs from each class were sequenced. Two of the four genes were identical to the Arabidopsis Skp1-like genes *ASK1* and *ASK2*, represented by 37 and 24 colonies, respectively (Porat et al., 1998; Gray et al., 1999; Samach et al., 1999; Yang et al., 1999). ASK1 and ASK2 share strong similarity with each other and with the SKP1 proteins from human and yeast (Bai et al., 1996; Connelly and Hieter, 1996; Gray et al., 1999; Samach et al., 1999). Figure 1 shows that COI1 specifically interacted with ASK1 and ASK2 in yeast. The interactions of COI1 with ASK1 and ASK2 were further characterized in planta using coimmunoprecipitation assays.

COI1 Forms SCF Complexes with ASK1, ASK2, AtRbx1, and AtCUL1 in Arabidopsis

Many yeast and mammalian F-box proteins interact with Skp1 and Cdc53 (cullin) to assemble SCF ubiquitin-ligase complexes (for reviews, see Hershko and Ciechanover, 1998; Deshaies, 1999). Recent studies have identified a small RING finger protein (Roc1, Rbx1, or Hrt1) that is a new subunit of SCF complexes (Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999; Tan et al., 1999). In Arabidopsis, a DNA database search identified two Arabidopsis Rbx1-like proteins (AAL13435 and CAB87200, referred to here as AtRbx1a and AtRbx1b) that have strong identities to each other and to mammalian Rbx1/ROC1 (Figure 2). The Arabidopsis Skp1-like proteins ASK1 and ASK2 have been shown to associate with AtCUL1 and the F-box protein TIR1

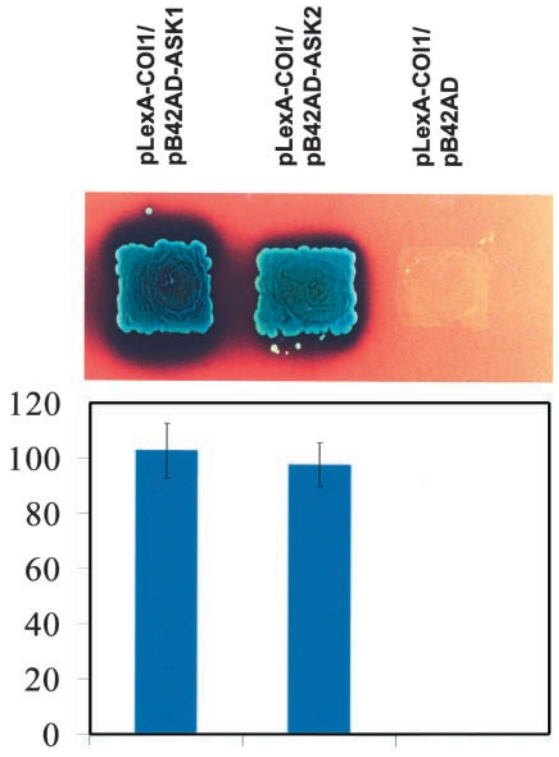

Figure 1. COI1 Interacts with ASK1 and ASK2 in the Yeast Two-Hybrid System.

COI1 was fused to the LexA DNA binding domain in pLexA and examined for interaction with ASK1 and ASK2 fused in frame to the activating domain in pB42AD. The two-hybrid reporter strain EGY48 coexpressing the indicated fusion proteins was grown on 2% Gal/1% raffinose/SD/-Ura/-His/-Trp/-Leu/X-β-Gal (top). Interactions of the indicated constructs were quantified by routine β-galactosidase assay and are expressed in Miller units (bottom).

in planta (Gray et al., 1999, 2001). Having demonstrated the interaction of COI1 with ASK1 and ASK2 in yeast, we examined whether COI1 interacts with ASK1 and ASK2 in planta and further explored the possible association of COI1 with AtCUL1 and AtRbx1.

As a prelude to determining whether COI1 interacts with ASK1, ASK2, AtRbx1, and AtCUL1 using coimmunoprecipitation assays, we examined the specificity of polyclonal antibodies against ASK1, ASK2, AtCUL1, and COI1 and the possible recognition of AtRbx1 by the polyclonal antibody against human Rbx1. The results are shown in Figure 3. The anti- (α-)ASK1 antiserum detected an ~21-kD band in the wild type but not in the mutant *ask1-1* (Yang et al., 1999), suggesting that the α-ASK1 antiserum specifically recognized ASK1. This result was confirmed by protein gel blot analysis of transgenic plants expressing Myc-tagged ASK1 (Figure 3A). The α-ASK2 polyclonal antibody was shown previously to cross-react with ASK1 and to detect another larger band (~23 kD) that presumably was the ASK2 protein (Gray et al., 1999).

By protein gel blot analysis of Myc-tagged ASK2 transgenic plants and the *ask1-1* mutant, we further demonstrated that α-ASK2 antiserum was able to detect both ASK2 and ASK1 (Figure 3B). The molecular masses of ASK1 and ASK2 detected in immunoblots were larger than their calculated masses, indicating that ASK1 and ASK2 might be modified in Arabidopsis, as SKP1 was modified by glycosylation in *Dictyostelium* (Teng-umnuay et al., 1998, 1999). The anti-Rbx1 antiserum, raised against the C-terminal region of the human Rbx1, detected an ~18-kD band that presumably was AtRbx1a and/or AtRbx1b in Arabidopsis (Figure 3C).

The anti-AtCUL1 antibody used in this study was raised against the N-terminal 20–amino acid peptide of AtCUL1 and was found to specifically recognize two predominant bands (Shen et al., 2002), corresponding to AtCUL1 and the RUB-conjugated AtCUL1, as reported previously (Gray et al., 1998; del Pozo and Estelle, 1999). We also confirmed the specificity of this antibody by competition assays with the peptide (E. Lechner and P. Genschik, unpublished data) and through protein gel blot analysis of transgenic plants expressing Myc-tagged AtCUL1 (data not shown).

The α-COI1 antiserum detected a 65-kD band plus a slightly smaller band in protein extracts from the wild type, and the 65-kD band was absent from the null mutant allele (*coi1-1*) of *COI1* (Figure 3D), indicating that the 65-kD band corresponds to the COI1 protein and the smaller band is a nonspecific protein cross-reacted with the α-COI1 antiserum. To avoid immunoprecipitation of nonspecific proteins by the α-COI1 antiserum, we generated transgenic plants expressing the epitope-tagged versions of COI1 for coimmunoprecipitation assays. The epitope-tagged versions of COI1 were expressed in *coi1-1* mutant plants and detected specifically by the α-COI1 antiserum (Figure 3D).

We used the α-Flag monoclonal antibody for immunoprecipitation from protein extracts of transgenic plants expressing Flag-tagged COI1, and the resulting immunoprecipitates were immunoblotted with α-COI1, α-ASK1, α-ASK2, α-Rbx1, and α-AtCUL1 antisera. The results are shown in Figure 4A. The Flag-tagged COI1 was coimmunoprecipitated with ASK1, ASK2, AtRbx1, and AtCUL1, suggesting that COI1 associates with ASK1, ASK2, AtRbx1, and AtCUL1 to assemble SCF^{COI1} complexes in Arabidopsis. In the α-Flag immune complexes, the α-AtCUL1 antiserum detected both AtCUL1 and a slightly larger band that presumably was the RUB-modified AtCUL1 (Gray et al., 1998; del Pozo and Estelle, 1999). These results suggest that the AtCUL1 component of SCF^{COI1} complexes was modified in planta, probably by RUB conjugation.

To exclude the possibility that the Flag tag may affect the

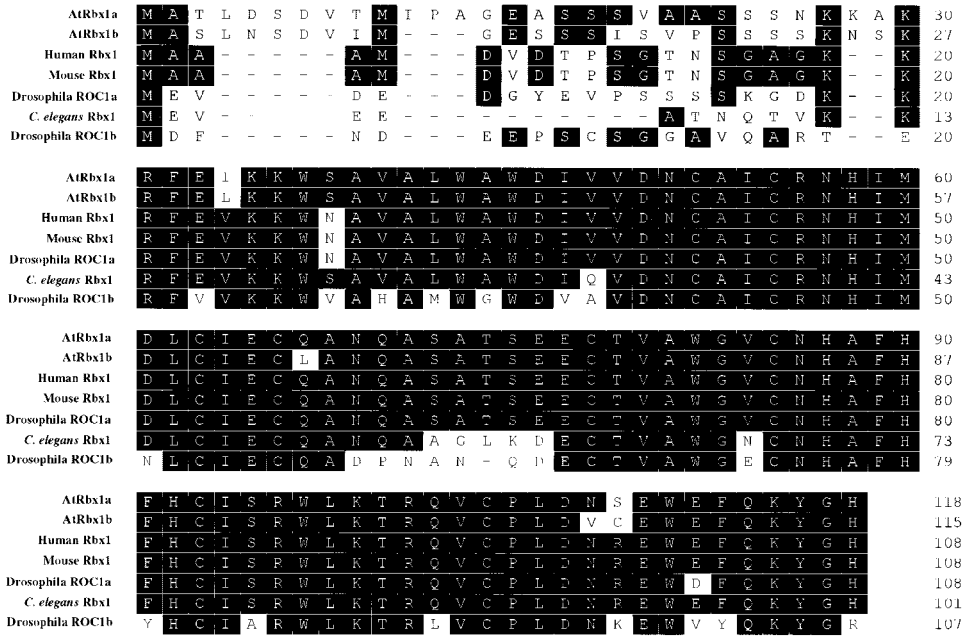

Figure 2. Alignment of Rbx1 Amino Acid Sequences of Arabidopsis, *C. elegans*, Drosophila, Mouse, and Human.

Identical amino acid residues are shaded in black. Dashes denote gaps introduced by the alignment program. Protein names are indicated at left, and amino acid residue positions are indicated at right.

overall folding of COI1, which in turn may affect the association of COI1 with AtCUL1 and ASK1, we also used the α-COI1 antiserum to immunoprecipitate endogenous COI1-containing complexes from wild-type Arabidopsis and found that AtCUL1, ASK1, and ASK2 were present in the α-COI1 immune complexes (data not shown). These results are consistent with the data generated from epitope-tagged COI1 transgenic plants.

COI1 Forms Two Complexes Separately with ASK1 or ASK2

Because the Arabidopsis SKP1-like proteins ASK1 and ASK2 were present in the immunoprecipitated SCF^{COI1} complex, we investigated whether COI1 assembled two complexes separately with ASK1 or ASK2. We made transgenic plants expressing Myc-tagged versions of ASK1 or ASK2, the α-Myc antiserum was used for immunoprecipitation from the transgenic plants, and the immunoprecipitated complexes were analyzed with α-COI1, α-Myc, and α-ASK2 or α-ASK1 antisera.

The results shown in Figure 4B indicate that Myc-tagged ASK2 was coimmunoprecipitated with COI1 but not with ASK1. Conversely, Myc-tagged ASK1 was coimmunoprecipitated with COI1 but not with ASK2 (data not shown). The observed association of COI1 separately with Myc-tagged ASK1 and ASK2 demonstrates that COI1 assembles two separate SCF^{COI1} complexes with ASK1 and ASK2 in Arabidopsis. It remains unclear whether the two SCF^{COI1} complexes have redundant functions.

A Single Amino Acid Replacement of Glu-22 with Ala in COI1 Reveals Essential Roles for SCF^{COI1}

To investigate whether the formation of the SCF^{COI1} complexes correlated with function in planta and to elucidate the consequences of the disruption of the SCF^{COI1} complexes, we introduced a single amino acid substitution from Glu to Ala at amino acid 22 ($COI1_{E22A}$) in the COI1 F-box motif that is required for its binding to Skp1-like proteins. $COI1_{E22A}$ was Myc tagged and expressed subsequently in the null mutant *coi1-1* (referred as to *coi1*::Myc-$COI1_{E22A}$). The *coi1-1* plants transgenic for the Myc-tagged COI1 (*coi1*::Myc-COI1) or Myc vector (*coi1*::Myc) were generated as controls.

Protein gel blot analysis revealed that the Myc-tagged $COI1_{E22A}$ was expressed in *coi1-1* at a similar level to the control, the Myc-tagged COI1. As expected, the Myc-tagged COI1 was coimmunoprecipitated with ASK1, ASK2,

and AtCUL1 (Figure 4C), consistent with the results obtained from Flag-tagged COI1 transgenic plants (Figure 4A). The Myc-COI1$_{E22A}$ protein failed to associate with AtCUL1, ASK1, and ASK2 (Figure 4C), indicating that the E22A mutation in COI1 abrogates the SCF^{COI1} complexes.

Functional analysis demonstrated that the COI1$_{E22A}$ mutation abolished JA response. The results shown in Figure 5 indicate that *coi1*::Myc-COI1$_{E22A}$ plants were resistant to JA, deficient in the production of viable pollen grains and seeds, and failed to express JA-inducible genes such as *PDF1.2*. In contrast, *coi1*::Myc-COI1 plants were fertile and exhibited normal induction of JA-inducible gene expression. The

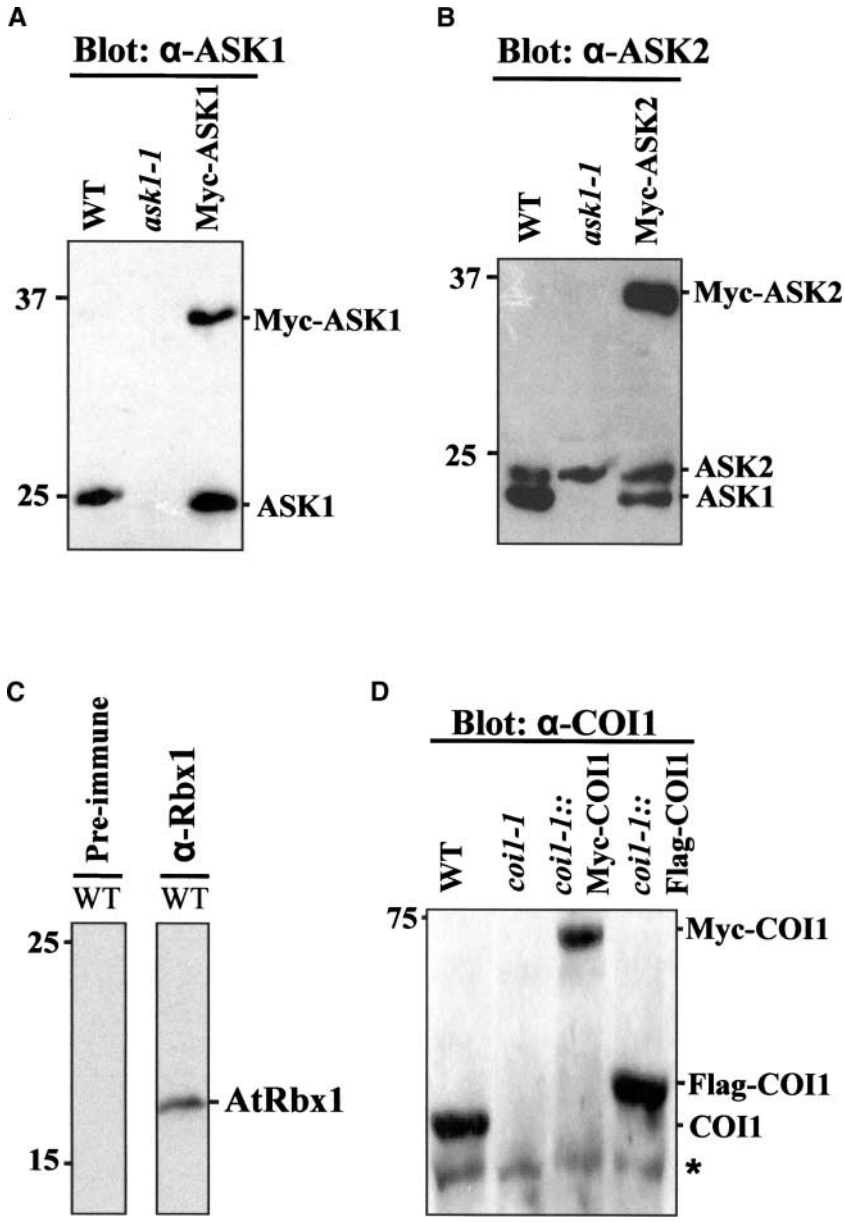

Figure 3. Protein Gel Blot Analysis of Extracts Prepared from the Indicated Arabidopsis Plants.

Blots were probed with polyclonal antisera against ASK1 **(A)**, ASK2 **(B)**, Rbx1 **(C)**, or COI1 **(D)**. The bands corresponding to each protein are indicated. The asterisk indicates the position of a nonspecific immunoreacting band. Molecular mass standards are given in kD (left). WT, wild type.

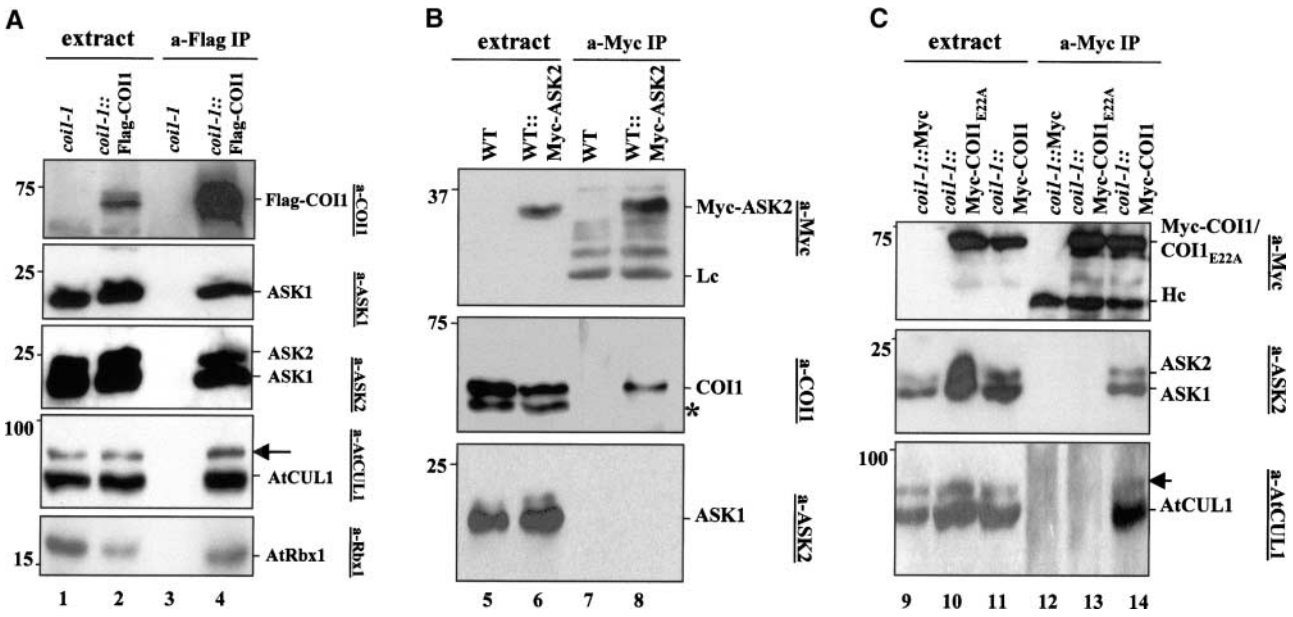

Figure 4. Coimmunoprecipitation Assays.

(A) The Flag-tagged COI1 forms an SCF complex in Arabidopsis. Protein extracts from *coi1-1* (lane 1) and transgenic *coi1-1* expressing Flag-tagged COI1 (*coi1-1*::Flag-COI1; lane 2) were immunoprecipitated with α-Flag antibody (lanes 3 and 4).

(B) ASK2 associates physically with COI1 but not with ASK1 in planta. Protein extracts from a control wild-type plant (lane 5) and a transgenic plant expressing the Myc-tagged ASK2 (WT::Myc-ASK2; lane 6) were immunoprecipitated with α-Myc antibody (lanes 7 and 8).

(C) $COI1_{E22A}$ abrogates SCF^{COI1}. Protein extracts from *coi1-1* plants transgenic for vector (*coi1-1*::Myc; lane 9), Myc-tagged $COI1_{E22A}$ (*coi1-1*::Myc-$COI1_{E22A}$; lane 10), or Myc-tagged COI1 (*coi1-1*::Myc-COI1; lane 11) were immunoprecipitated with α-Myc antibody (lanes 12 to 14).

The resulting immunoprecipitates were resolved by SDS-PAGE and detected with the indicated antibodies. Molecular mass standards are given in kD. Arrows in **(A)** and **(C)** indicate the modified version of AtCUL1. The asterisk in **(B)** indicates the position of a nonspecific immunoreacting band. Hc, immunoglobulin heavy chain; IP, immunoprecipitate; Lc, immunoglobulin light chain; WT, wild type.

$COI1_{E22A}$ mutation disrupted the SCF^{COI1} complexes and simultaneously abolished JA response, suggesting an essential role for the SCF^{COI1} complexes in JA signaling.

AtRbx1 Is Required for the Normal Induction of JA-Inducible Gene Expression

To investigate JA response mediated by the AtCUL1, ASK1, and ASK2 components of SCF^{COI1} complexes, T-DNA knockout mutants of *AtCUL1* and *ASK2* (W. Peng and D. Xie, unpublished data) and the Ds transposon mutant *ask1-1* were examined. The null mutation in *AtCUL1* caused early arrest during embryogenesis, and the homozygous mutant was lethal and unavailable for analysis of JA response (Shen et al., 2002; W. Peng and D. Xie, unpublished data). The *ask1-1* or *ask2* mutation did not obviously alter JA-inhibitory root growth and JA-induced gene expression (W. Peng and D. Xie, unpublished data), probably resulting from functional overlapping between ASK1 and ASK2.

We investigated the possible function of AtRbx1 in JA response through analysis of *AtRbx1* double-stranded RNA interference (*RNAi*) mutant plants, generated by Genschik and colleagues (E. Lechner and P. Genschick, unpublished data), which contained a glucocorticoid-inducible *AtRbx1* double-stranded RNA construct (Aoyama and Chua, 1997; Chuang and Meyerowitz, 2000; E. Lechner and P. Genschick, unpublished data).

Figure 6A demonstrates that *AtRbx1* double-stranded RNA–mediated genetic interference reduced AtRbx1 expression in *AtRbx1*(*RNAi*) mutant line A2. Dexamethasone (DEX; a strong synthetic glucocorticoid) treatment caused a decrease in *AtRbx1* mRNA accumulation in glucocorticoid-inducible *AtRbx1*(*RNAi*) mutant plants. Protein gel blot analysis demonstrated that the decrease of *AtRbx1* mRNA was correlated with a reduction of the AtRbx1 protein in DEX-treated *AtRbx1*(*RNAi*) mutant plants. As expected, AtRbx1 expression in control plants transgenic for a glucocorticoid-inducible vector was not affected by DEX treatment.

The appearance of noninduced *AtRbx1*(*RNAi*) mutant plants (without DEX treatment) is normal. However, when germinated and grown continuously on medium containing DEX, *AtRbx1*(*RNAi*) mutant plants exhibited severe defects of growth and development (data not shown), which makes

it difficult to examine the phenotype of JA-inhibitory root growth and JA-regulated fertility in these DEX-induced *AtRbx1*(*RNAi*) mutant plants. Therefore, we focused on analysis of the possible effect of *AtRbx1* double-stranded RNA interference on JA-inducible gene expression.

AtRbx1(*RNAi*) mutant and empty vector control plants were grown for 3 weeks without DEX treatment. These 3-week-old plants then were treated with DEX (24 h) or JA (9 h) or both. Total RNAs were isolated from these plants, and JA-inducible genes, except for defense-related genes such as *PDF1.2*, induced by DEX in some transgenic plants containing a glucocorticoid-inducible vector (Kang et al., 1999), were used as probes in RNA gel blot analysis.

Results from *AtRbx1*(*RNAi*) mutant line A2 are shown in Figure 6B. Induction of *AtVSP* and allene oxide synthase (*AOS*) (Laudert and Weiler, 1998) by JA was normal in vector control and *AtRbx1*(*RNAi*) mutant plants not treated with DEX. However, in DEX-treated *AtRbx1*(*RNAi*) mutant plants, although these transcripts were still induced by JA, their accumulation declined in response to JA. Similar expression profiles were obtained for other JA-inducible genes, including lipoxygenase 2 (Bell and Mullet, 1993) (data not shown).

RNA gel blot analysis with *AtRbx1* probe confirmed that *AtRbx1* mRNA accumulation was decreased in DEX-treated *AtRbx1*(*RNAi*) mutant plants, which correlated with the observation that the accumulation of JA-inducible genes was reduced in these mutant plants. These results demonstrated that the reduction of AtRbx1 expression in DEX-induced *AtRbx1*(*RNAi*) mutants led to a severe decrease of JA-inducible gene expression, indicating a role for the AtRbx1 subunit of SCF^{COI1} in JA response.

Mutations in *AXR1* Affect Modification of the AtCUL1 Subunit of SCF^{COI1} and Result in Reduced JA Response

The Arabidopsis cullin AtCUL1 is modified by the ubiquitin-related protein RUB1, and the formation of RUB-AtCUL1 is dependent on AXR1 and ECR1 in vivo (del Pozo et al., 1998, 2002; del Pozo and Estelle, 1999). Because we found that the AtCUL1 component of SCF^{COI1} complexes was modified in vivo (Figure 4), we further demonstrated that the AtCUL1 subunit of SCF^{COI1} was modified via *AXR1* and that this modification was important for JA signaling.

Two mutant alleles, *axr1-3* and *axr1-12*, of *AXR1* were identified previously through a genetic screen for auxin-resistant mutants (Lincoln et al., 1990). To determine whether mutations in *AXR1* affected modification of the AtCUL1 subunit of SCF^{COI1}, we generated transgenic *axr1* plants expressing the Flag-tagged COI1 (*axr1*::Flag-COI1) and used the α-Flag antibody for immunoprecipitation. The α-Flag immunoprecipitates then were probed with α-AtCUL1, α-ASK1, and α-COI1 antisera.

The results in Figure 7 show that a relatively low level of the modified AtCUL1 was detected in total proteins extracted from *axr1-3* and *axr1-3*::Flag-COI1 plants (Figure 7,

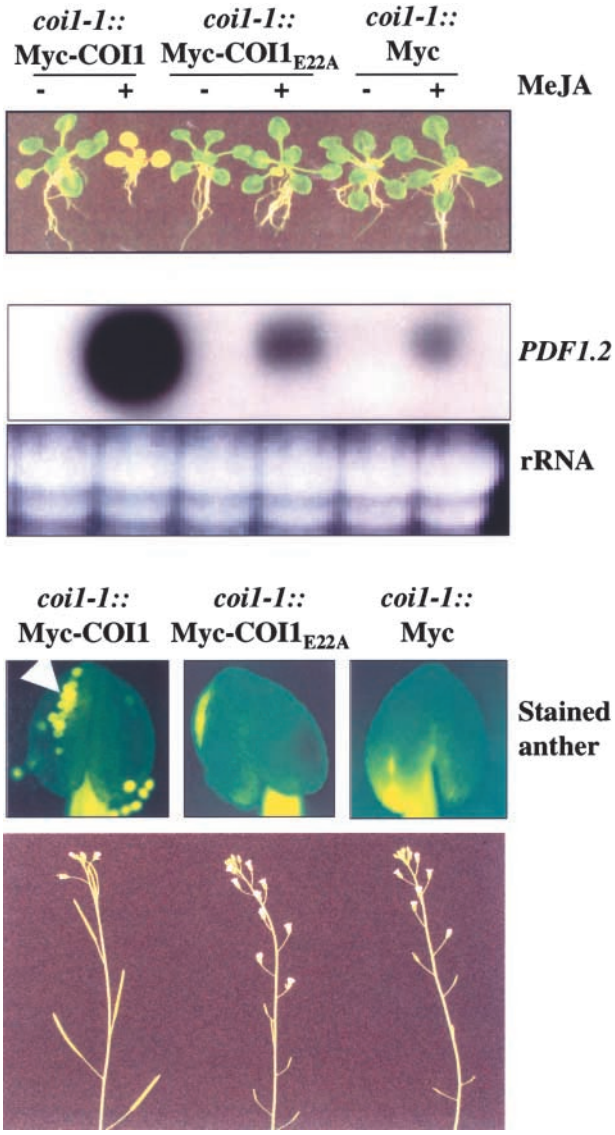

Figure 5. *COI1$_{E22A}$* Abolishes JA Response.

coi1-1 plants transgenic for Myc-tagged COI1$_{E22A}$, Myc-tagged *COI1*, or vector (see Figure 4C) were examined for JA-inhibitory root growth (top), JA-inducible expression of the defense gene *PDF1.2* (middle), and JA-regulated pollen development and plant fertility (bottom). Seedlings were grown on Murashige and Skoog (1962) (MS) medium with (+) or without (−) 10 μM methyl jasmonate (MeJA; top). Total RNA was stained with ethidium bromide as an equal loading control (middle). The white arrow indicates the viable pollen grains in anther, which were stained with 1% fluorescein diacetate (catalog No. F-7378; Sigma) and visualized by fluorescence microscopy. The inflorescence was photographed in 6-week-old soil-grown plants (bottom).

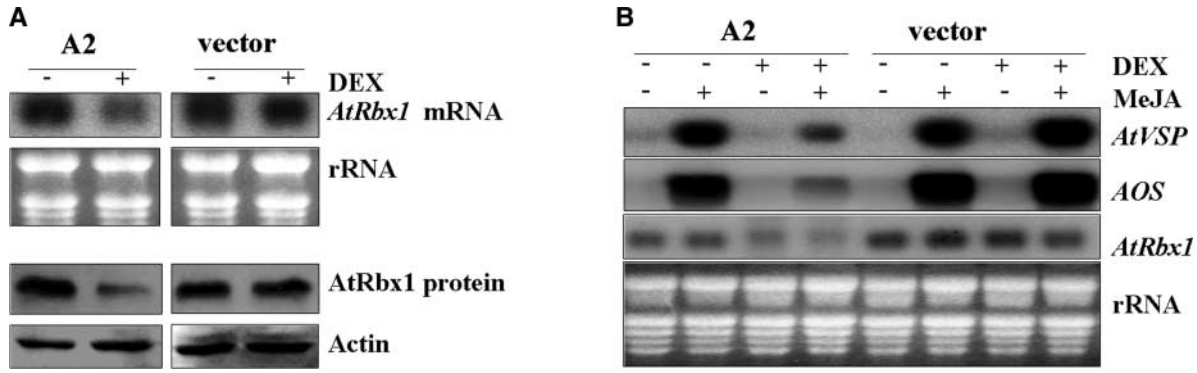

Figure 6. Analysis of Glucocorticoid-Inducible *AtRbx1*(*RNAi*) Mutant Line A2.

(A) DEX treatment resulted in a decrease of both *AtRbx1* mRNA and AtRbx1 protein in the glucocorticoid-inducible *AtRbx1*(*RNAi*) mutant line A2. Arabidopsis plants transgenic for the glucocorticoid-inducible *AtRbx1*(*RNAi*) construct (line A2) or vector were grown on MS medium for 3 weeks and treated with 0.5 μM DEX (+) (catalog No. D-1756; Sigma) or with water as a control (−) for 24 h. The plants were harvested for RNA gel blot analysis (top) and protein gel blot analysis (bottom). Total RNAs were stained with ethidium bromide as an equal loading control. The immunoblot was detected with α-actin antibody as a protein loading control.

(B) DEX treatment led to a reduction of JA-inducible accumulation of *AtVSP* and *AOS* transcripts. Three-week-old Arabidopsis plants were mock treated (−) or treated with 1 μM DEX for 24 h and then followed by mock treatment (−) or treatment with MeJA (+) for 9 h. The RNA gel blot was hybridized with digoxigenin-labeled *AtVSP* and *AOS* probes. Total RNAs were stained with ethidium bromide as an equal loading control. Consistent with the results shown in **(A)**, the blot was probed with digoxigenin-labeled *AtRbx1* to demonstrate that the DEX treatment led to a decrease of *AtRbx1* expression in these glucocorticoid-inducible *AtRbx1*(*RNAi*) mutant plants.

lanes 1 and 2) compared with that from *AXR1*::Flag-COI1 plants (Figure 7, lane 3). Quantitative analysis using a scanning densitometer showed that the abundance of the modified AtCUL1, normalized to the level of unmodified AtCUL1, in *axr1-3* and *axr1-3*::Flag-COI1 was approximately one-third of that in *AXR1*::Flag-COI1 (data not shown). Figure 7 also demonstrates that Flag-COI1 was coimmunoprecipitated with ASK1 and AtCUL1 to assemble SCF^{COI1} complexes in both *axr1-3*::Flag-COI1 and *AXR1*::Flag-COI1 (Figure 7, lanes 5 and 6). However, the modified AtCUL1 in the α-Flag immunoprecipitates from *axr1-3*::Flag-COI1 plants was reduced significantly compared with that from *AXR1*::Flag-COI1 plants (Figure 7, lanes 5 and 6).

Previous studies have confirmed that RUB1 is activated by AXR1 and ECR1 for conjugation to AtCUL1 in vivo (del Pozo et al., 1998, 2002; del Pozo and Estelle, 1999). The observation that the abundance of the modified AtCUL1 of SCF^{COI1} was decreased severely in *axr1*::Flag-COI1 demonstrates that the AtCUL1 subunit of SCF^{COI1} was modified via *AXR1*, probably by RUB modification. A similar conclusion was reached from the results generated with transgenic *axr1-12* plants expressing Flag-tagged COI1 (data not shown).

To further explore the possible role of *AXR1*-dependent modification of the AtCUL1 component of SCF^{COI1} in JA signaling, we examined JA response in *axr1-3* and *axr1-12* compared with wild-type and JA-resistant mutant *coi1-1* plants. The auxin transport inhibitor response mutant *tir1-1*, a mutant allele of *TIR1* encoding an F-box protein required for auxin response (Gray et al., 1999), also was included as a control. The results shown in Figure 8 demonstrate that both *axr1-3* and *axr1-12* seedlings were moderately resistant to JA, and the JA inhibition rate for *axr1-3* or *axr1-12* was between that of the wild-type and that of *coi1-1*.

When seedlings were grown on MS medium containing 25 μM MeJA, ~50% inhibition of root elongation was observed for *axr1-3* and *axr1-12*, whereas ~70% inhibition was observed for both the wild type and *tir1-1* and no inhibition was observed for *coi1-1*. Furthermore, the induction of *AtVSP*, *Thi2.1*, and *PDF1.2* by JA in the *axr1-3* and *axr1-12* mutants was reduced compared with that in the wild type and *tir1*. Collectively, the evidence that mutations in *AXR1* reduced the abundance of the modified AtCUL1 subunit of SCF^{COI1} and led to a reduction of JA response indicates that the *AXR1*-dependent modification of the AtCUL1 component of SCF^{COI1} is important for JA signaling.

axr1 and *coi1* Display a Synergistic Genetic Interaction

To conveniently explore the possible genetic interaction between *AXR1* and *COI1*, we identified two leaky mutant alleles of *COI1* (Figure 9): *coi1-2*, with missense mutation L245F, and *coi1-8*, with G543L, which exhibited reduced JA insensitivity and partial fertility. The null mutant *coi1-1* was completely male sterile and resistant to JA. We generated

coi1 axr1 double mutants homozygous for both *coi1-2* and *axr1-3* or *axr1-12*. The results shown in Figure 10 indicate that the *coi1-2 axr1-3* double mutants displayed a significant reduction of JA response compared with their parent mutants *coi1-2* and *axr1-3*.

The *coi1-2 axr1-3* seedlings were fully resistant to JA, and no inhibition of root elongation was observed at 25 μM MeJA, whereas the single mutants *coi1-2* and *axr1-3* were partially resistant to JA: ~20 and 50% inhibition on root elongation by 25 μM MeJA was observed for *coi1-2* and *axr1-3*, respectively (Figures 10A and 10B). These results suggest that *axr1-3* and *coi1-2* display a synergistic genetic interaction in double mutants.

A similar conclusion was reached when the *coi1-2 axr1-3* double mutants were examined for other JA responses, including JA-regulated fertility and inducible gene expression in response to JA. RNA gel blot analysis demonstrated that the inducible accumulation of *AtVSP*, *PDF1.2*, and *Thi2.1* by JA was reduced significantly in the *coi1 axr1* double mutants compared with that in the single mutants *coi1-2* and *axr1-3* (Figure 10D). Furthermore, a dramatic reduction in plant fertility was observed in the double mutants. The *coi1-2 axr1-3* double mutation resulted in male sterility, whereas the single mutant *axr1-3* produced normal seeds and *coi1-2* was partially fertile (Figure 10C). The examination of double mutants homozygous for *coi1-2* and *axr1-12* reached the same conclusion, that the *coi1 axr1* double mutation resulted in a more severe JA response defect than either of the single mutations (data not shown).

DISCUSSION

Xie et al. (1998) previously isolated the *COI1* gene that is required for normal JA response, including JA-regulated plant

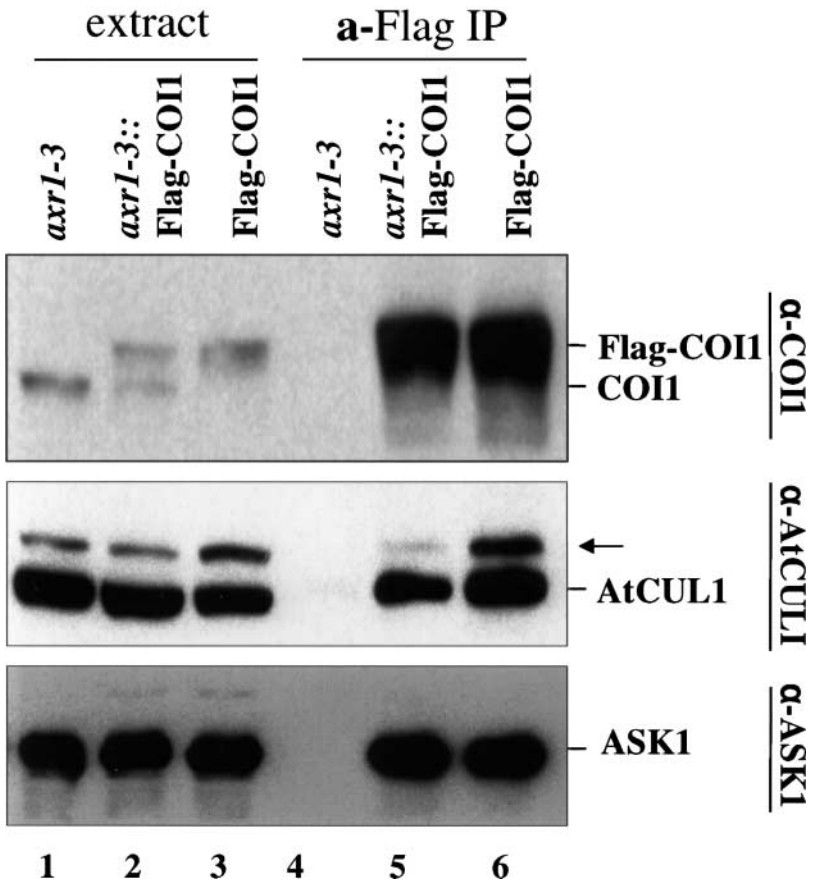

Figure 7. The Abundance of the Modified AtCUL1 of SCF^{COI1} Was Reduced in the *axr1-3* Mutant.

Protein extracts from *axr1-3* (lane 1), *axr1-3* expressing Flag-tagged COI1 (*axr1-3*::Flag-COI1) (lane 2), and wild-type *AXR1* plants expressing Flag-tagged COI1 (*AXR1*::Flag-COI1) (lane 3) were immunoprecipitated with α-Flag antibody (lanes 4 to 6). The α-Flag immune complexes were probed with α-COI1, α-AtCUL1, or α-ASK1 antisera. The arrow indicates the modified version of AtCUL1.

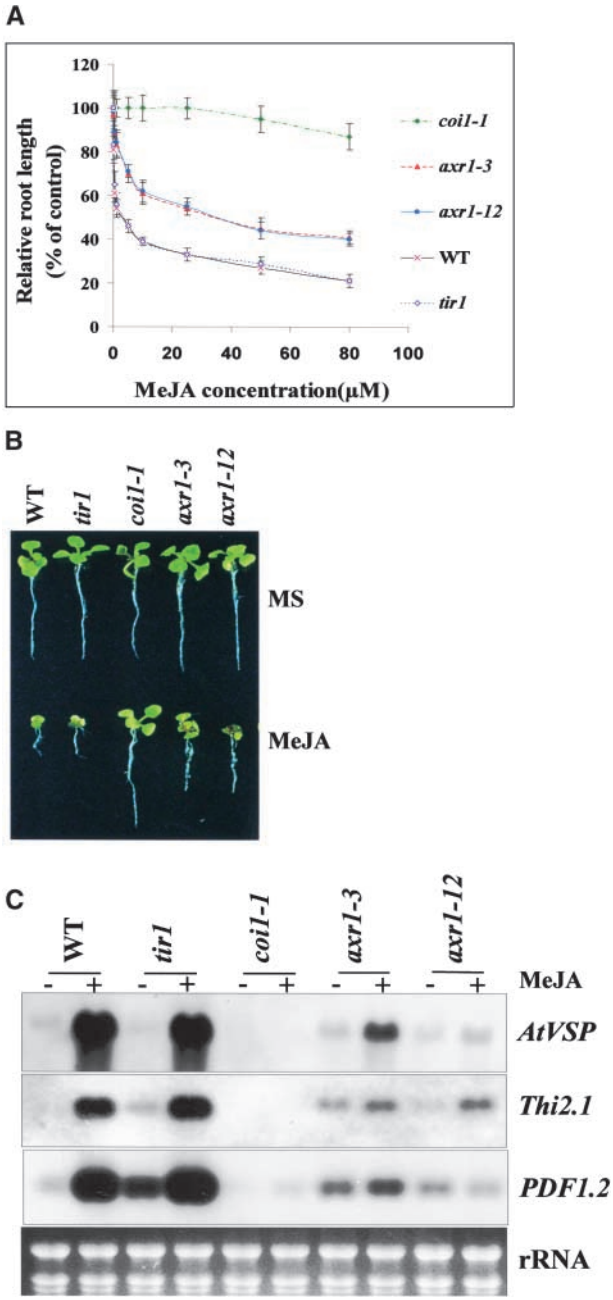

Figure 8. *axr1-3* and *axr1-12* Exhibited Reduced JA Response.

(A) MeJA dose–response curve of root growth. *axr1-3*, *axr1-12*, *tir1-1*, *coi1-1*, and wild-type (WT) plants were grown for 1 week on MS medium containing various concentrations of MeJA. Root length of the seedlings grown on MS containing MeJA (0.1, 0.5, 1, 5, 10, 25, 50, or 80 μM) was expressed as a percentage of root length on MS medium. Each data point is the mean of >15 samples. The experiment was repeated four times, and the results were consistent. Error bars represent SD ($n > 15$).

(B) Phenotype of 10-day-old seedlings grown on MS medium or MS with 25 μM MeJA.

(C) RNA gel blot analysis of the JA-inducible expression of *PDF1.2*, *Thi2.1*, and *AtVSP*. Ethidium bromide staining of rRNAs shown at bottom indicates the equal loading amount of total RNA on the gel.

fertility and defense. It was speculated that COI1 might be an F-box protein that recruits regulators of defense response and pollen development for modification by ubiquitylation (Xie et al., 1998). In this study, we showed biochemical and genetic evidence to demonstrate that COI1 associates physically with Arabidopsis AtCUL1, AtRbx1, and either of the SKP1 orthologs, ASK1 or ASK2, to assemble SCF^{COI1} complexes in Arabidopsis.

The E22A mutation in the F-box motif of COI1 disrupted SCF^{COI1} complexes and abolished the JA response. Downregulation of AtRbx1 led to a reduction of JA response in transgenic plants, as assayed for JA-inducible gene expression. In addition, we demonstrated that mutations in *AXR1* decreased the abundance of the modified AtCUL1 component of SCF^{COI1} and reduced JA response in planta. Furthermore, we found that the *coi1 axr1* double mutations resulted in a severe defect of JA response. These results strongly suggest that the SCF^{COI1} complexes are required for JA response and that the *AXR1*-dependent modification of the AtCUL1 subunit of SCF^{COI1} is important for JA signaling in Arabidopsis.

Formation of the SCF Complex

The SCF ubiquitin-ligase complex is conserved structurally in yeast, *Caenorhabditis elegans*, fruit fly, and human, and the conserved Skp1, cullin (Cdc53), and Rbx1 (ROC1/Hrt1) proteins serve in multiple degradation pathways via the assembly of various SCF complexes involving different F-box proteins that recognize distinct target proteins and determine the specificity of protein ubiquitylation (Bai et al., 1996; Connelly and Hieter, 1996; Li and Johnston, 1997; Skowyra et al., 1997; Patton et al., 1998; Deshaies, 1999). In Arabidopsis, the cDNA and genome sequencing project revealed many putative F-box proteins (Xiao and Jang, 2000). Several F-box proteins, including FKF1/ZEITLUPE (Nelson et al., 2000; Somers et al., 2000), ORE9 (Woo et al., 2001), TIR1, COI1, and UFO have been shown to play major roles in various crucial biological processes.

AtCUL1 and the Skp1-like proteins ASK1 and ASK2 have been shown to interact with the F-box protein TIR1 to assemble the SCF^{TIR1} ubiquitin-ligase complex required for auxin response (Gray et al., 1999). ASK1 and ASK2 also have been shown, in the yeast two-hybrid system, to associate with UFO, which is required for normal patterning and growth in the floral meristem (Samach et al., 1999; Zhao et al., 1999). In this study, we demonstrate that AtCUL1,

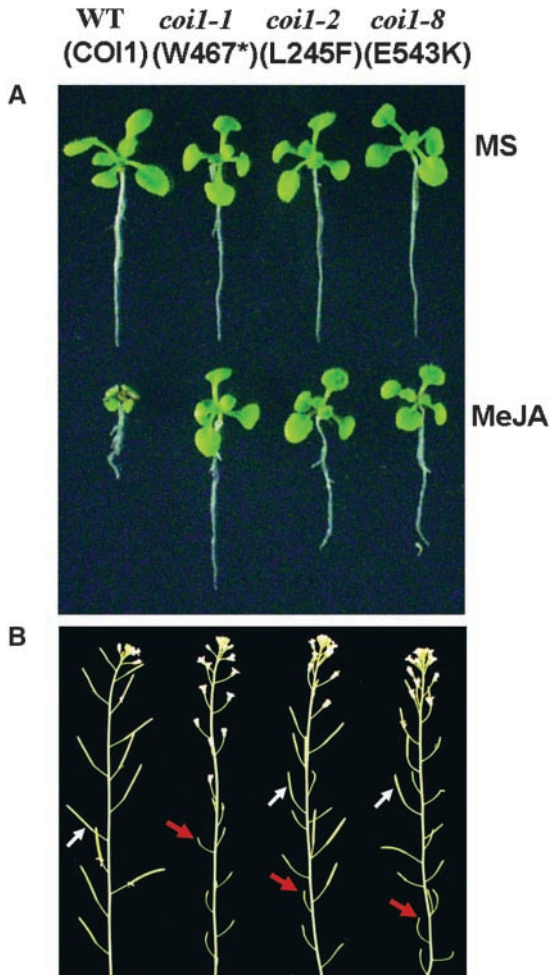

Figure 9. Identification of Leaky Mutant Alleles *coi1-2* and *coi1-8* of *COI1*.

(A) *coi1-2* and *coi1-8* exhibit insensitivity to JA-inhibitory root elongation compared with the wild type (WT), but it is less severe than that in *coi1-1*.

(B) *coi1-2* and *coi1-8* display a partial fertility phenotype, whereas the wild type is fertile and *coi1-1* is male sterile. Shown are inflorescences of 8-week-old plants grown in soil under 16-h-light (21–23°C)/8-h-dark (16–19°C) growth conditions. The white arrows indicate fertile siliques, and the red arrows indicate sterile flowers.

AtRbx1, ASK1, and ASK2 interact with COI1 in planta and that COI1 assembles two SCF complexes containing either ASK1 or ASK2. The two SCF^{COI1} complexes may have different but overlapping functions in the JA signaling pathway.

Some F-box proteins, such as the yeast F-box proteins Pop1 and Pop2, form homodimers and heterodimers (Kominami et al., 1998). In this study, we found that COI1 functions as a monomer in the SCF complex. The results shown in Figure 7 (lane 5) demonstrated that COI1 was not present in Flag-COI1–containing SCF complexes that were coimmunoprecipitated by α-Flag antibody from the plants expressing both COI1 and the Flag-tagged COI1.

In F-box proteins, the F-box motif has been identified as the SKP1-interacting domain, and their variable protein–protein interaction domains (such as the Leu-rich repeat or WD40) function as substrate recognition domains (Bai et al., 1996; Connelly and Hieter, 1996; Li and Johnston, 1997; Schulman et al., 2000). Consistent with these conclusions, our results suggest that the F-box domain of COI1 is required for its association with AtCUL1 and the Skp1-like proteins. A single amino acid substitution of Glu-22 to Ala in the F-box domain of COI1 resulted in its dissociation with ASK1, ASK2, and AtCUL1 and abolished all JA response.

Function of SCF Components

It is envisaged that the Arabidopsis AtCUL1, AtRbx1, and ASK proteins are conserved subunits of various SCF complexes containing different F-box proteins, including COI1, TIR1, UFO, and others, that recruit different substrates and confer specificity on SCF complex function. It is speculated that mutations in one F-box protein would not affect the function mediated by other F-box proteins. However, mutations in these conserved components, including AtCUL1, AtRbx1, and ASK, would affect diverse phenotypes mediated by various SCF complexes. Because these SCF complexes may play crucial roles in plant growth and development, complete disruption of these SCF conserved components could result in a lethal phenotype or severe defects of plant growth and development.

Indeed, mutation in the F-box protein TIR1 led to a defect in the auxin response pathway (Ruegger et al., 1998) but not in the JA response pathway (Figure 8). Likewise, mutations in COI1 result in a defective JA response but have no effect on the TIR1-mediated auxin response (L. Xu and D. Xie, unpublished data).

In support of the speculation that mutations in the conserved components of SCF complexes would cause diverse and severe phenotypes, we found that mutation in *AtCUL1* led to early arrest of embryogenesis (Shen et al., 2002; W. Peng and D. Xie, unpublished data). The mutation in ASK1, which has been shown to interact with UFO in the yeast two-hybrid system (Samach et al., 1999) and with TIR1 (Gray et al., 1999) and COI1 (Figure 4) in planta, caused diverse phenotypes. The *ask1-1* mutation caused abnormality in floral morphology analogous to that caused by *ufo* (Zhao et al., 1999) and was resistant to auxin, like *tir1* (Gray et al., 1999).

ask1-1 was male sterile and defective in male meiosis and failed to produce viable pollen grains (Yang et al., 1999), and the *coi1-1* mutant also was male sterile (Feys et al., 1994; Xie et al., 1998) and failed to produce viable pollen grains. Surprisingly, the *ask1* or *ask2* mutation did not obviously alter JA response, as assayed for JA-inhibitory root growth and inducible gene expression (W. Peng and D. Xie, unpublished data). This finding might result from the functional redundancy

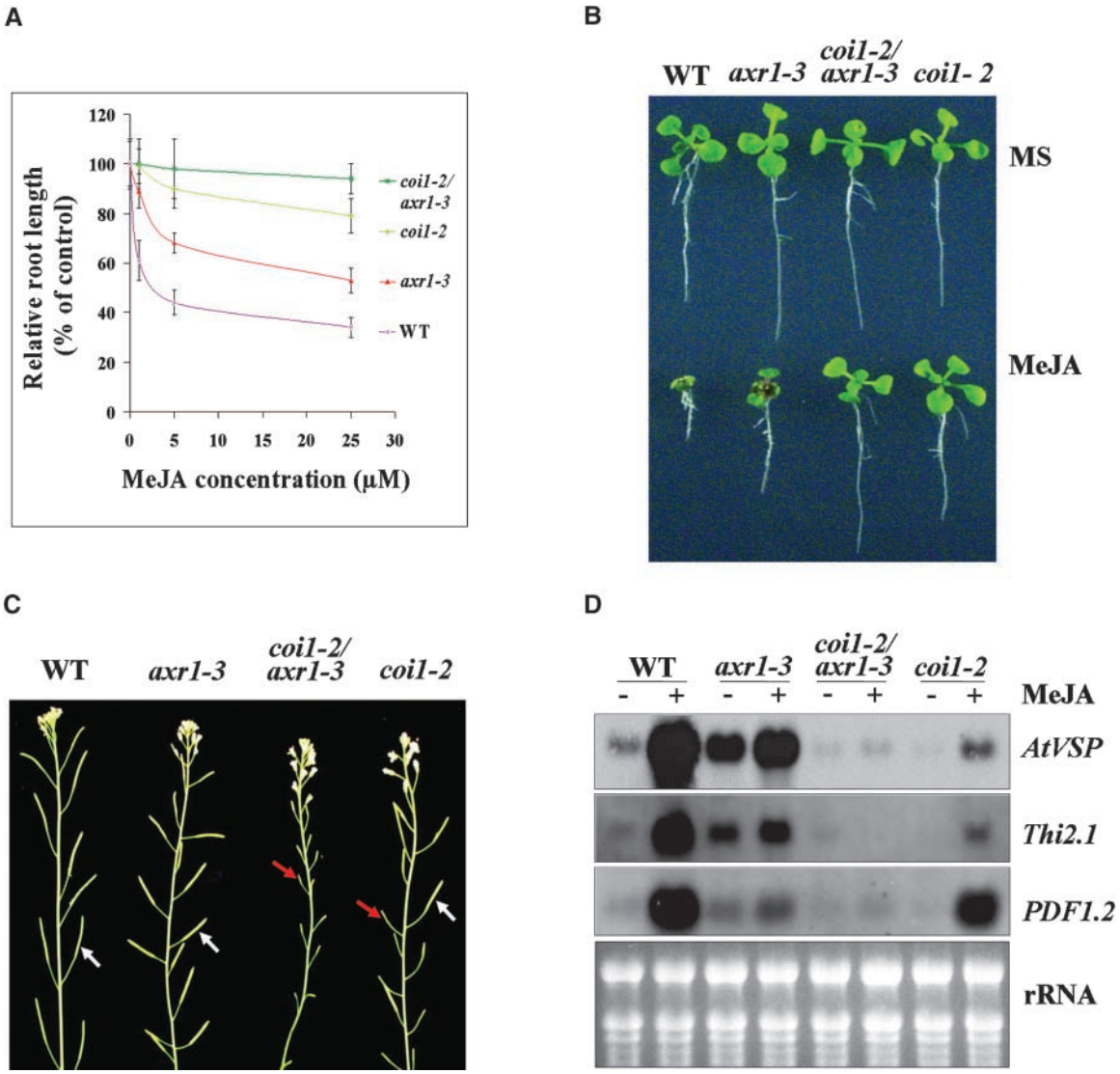

Figure 10. The *coi1-2 axr1-3* Double Mutant Exhibited a Severe Defect in JA Response.

(A) MeJA dose–response curve of root growth. Root length of 1-week-old seedlings grown on MS medium containing 1, 5, or 25 μM MeJA is expressed as a percentage of root length on MS. Each data point represents the mean of >15 samples. The experiment was repeated three times. Error bars represent SD ($n > 15$).

(B) Phenotype of 8-day-old seedlings grown on MS or MS with 25 μM MeJA.

(C) The *coi1-2 axr1-3* double mutation resulted in male sterility. Shown are inflorescences of 8-week-old plants. The white arrows indicate fertile siliques, and the red arrows indicate sterile flowers.

(D) The *coi1-2 axr1-3* double mutation led to a significant reduction of JA-inducible accumulation of *AtVSP*, *PDF1.2*, and *Thi2.1* transcripts. Ethidium bromide staining of rRNAs shown at bottom indicates the equal loading amount of total RNA on the gel.

WT, wild type.

of ASK1 and ASK2, correlated with our result that COI1 forms two SCF^{COI1} complexes containing either ASK1 or ASK2. It would be interesting to examine the phenotype of *ask1 ask2* double mutants.

Arabidopsis harbors two related *AtRbx1* genes, *AtRbx1a* and *AtRbx1b*, that are highly identical to mammalian Rbx1/ROC1, particularly within internal and C-terminal regions (Figure 2). The α-Rbx1 antibody against the C-terminal region of human Rbx1 recognized Arabidopsis AtRbx1 but could not distinguish AtRbx1a and AtRbx1b. We demon-

strated the association of COI1 with AtRbx1, but it remains unclear whether COI1 forms SCF^{COI1} complexes with both or either AtRbx1a or AtRbx1b and whether both have distinctive or similar functions. Because of the high similarity between their sequences, *AtRbx1* double-stranded RNA–mediated genetic interference probably would reduce the expression of both AtRbx1a and AtRbx1b.

Functional analysis of the glucocorticoid-inducible *AtRbx1-(RNAi)* mutant showed that *AtRbx1* double-stranded RNA interference decreased AtRbx1 expression, and declining AtRbx1 expression is correlated with a reduction of JA-inducible gene expression, suggesting a role for AtRbx1 in JA signaling. The alteration of AtRbx1 expression also is correlated with other phenotypes, such as a decrease of some auxin-related phenotypes (E. Lechner and P. Genschik, unpublished results), indicating the possible association of AtRbx1 with other signaling complexes, including SCF^{TIR1}.

AXR1-Dependent Modification of the AtCUL1 Subunit of SCF^{COI1} Complexes

The ubiquitin-related proteins, including mammalian NEDD8 or the NEDD8 homologs of yeast and Arabidopsis (called RUB) (Kamitani et al., 1997; del Pozo et al., 1998; Lammer et al., 1998; Liakopoulos et al., 1998), and mammalian SUMO (Okura et al., 1996) and yeast Smt3 (Meluh and Koshland, 1995), were shown to modify other cellular proteins. In Arabidopsis, RUB1 is activated for conjugation to AtCUL1 via AXR1 and ECR1; this modification of AtCUL1 is required for the normal activity of SCF^{TIR1}, and mutations in *AXR1* lead to a defect of auxin response (del Pozo et al., 1998, 2002; del Pozo and Estelle, 1999; Gray and Estelle, 2000). The essentially antagonistic steps of RUB1 conjugation mediated by AXR1 and deconjugation promoted by the COP9 signalosome both are required for proper auxin response (Schwechheimer et al., 2001).

In this study, we demonstrate that the AtCUL1 subunit of SCF^{COI1} complexes is modified and that this modification is required for a normal JA response. Mutations in *AXR1* decreased the abundance of the modified AtCUL1 subunit of SCF^{COI1} and led to a reduced JA response. The *axr1* mutant is not completely deficient in RUB-AtCUL1 (del Pozo et al., 2002), because we also found that the relatively low level of the AtCUL1 subunit of SCF^{COI1} still is modified in *axr1* mutant plants (Figure 7, lane 5). The existing modification of the AtCUL1 subunit of SCF^{COI1} in *axr1* mutant plants may be caused by RUB conjugation independent of AXR1 and/or by conjugation of other Arabidopsis ubiquitin-related proteins. The low level of the modified AtCUL1 subunit of SCF^{COI1} detected in the *axr1* mutant may maintain moderate JA response.

Alternatively, the role of the RUB modification is to enhance the activity of SCF^{COI1}, as the NEDD8 pathway enhances E3 activity (Morimoto et al., 2000; Wu et al., 2000). Consistent with this suggestion, mutations in AXR1 were found to moderately but not severely reduce the SCF^{COI1}-mediated JA response, whereas the *coi1-2 arx1-3* and *coi1-2 axr1-12* double mutations significantly decreased the SCF^{COI1}-mediated JA response (Figure 10). It would be interesting to investigate whether other RUB modification–required components, such as ECR1 and RCE1 (del Pozo and Estelle, 1999), are involved in JA signaling.

Substrates of SCF Complexes

F-box proteins are the substrate recognition components of SCF complexes: they bind substrates through variable protein–protein interaction domains such as WD40 or Leu-rich repeats. In Arabidopsis, substrates for the SCF^{TIR1} complex were identified recently (Gray et al., 2001; Schwechheimer et al., 2001). SCF^{TIR1} recruits substrate auxin/indoleacetic acid proteins for degradation through the ubiquitin-proteasome pathway (Gray et al., 2001). SCF^{TIR1} also interacts with the COP9 signalosome, a multiple-protein complex that acts as a negative regulator of photomorphogenesis in Arabidopsis and that is required for efficient degradation of the target protein PSIAA6 (Schwechheimer et al., 2001).

The substrates for SCF^{COI1} are unknown. Recently, several mutants (*cev*, *cet*, and *cex*) that confer constant expression of JA-inducible genes or that continuously activate the JA pathway were identified (Ellis and Turner, 2001; Hilpert et al., 2001; Xu et al., 2001). Some of these genes might encode negative regulators that are potential candidate substrates for SCF^{COI1}. The diverse phenotypes associated with *coi1-1* suggest that SCF^{COI1} might have multiple substrates. We propose that these multiple substrates could act as putative repressors to negatively regulate the expression of their downstream target genes that are involved in the JA response.

In response to JA, environmental, or developmental cues (such as wounding and flowering), the JA signal might be activated to modify some or all of the substrates, conceivably through phosphorylation or dephosphorylation. COI1 could recruit the modified substrates to SCF^{COI1} for ubiquitylation. Subsequent degradation of the ubiquitylated substrates by the 26S proteasome would result in removal of the putative repressors, leading to the expression of the downstream genes that mediate the JA response. Identification and functional analysis of these substrates and the other potential factors involved in this signaling pathway will be essential to understanding the molecular mechanism by which JA regulates plant growth, development, and defense.

METHODS

Yeast Two-Hybrid Screen

Yeast (*Saccharomyces cerevisiae*) host strain AH109 (catalog No. K1612-1; Clontech, Palo Alto, CA) was cotransformed with the bait

construct pGBKT7-COI1 (a *Trp1* marker), and the GAL4-based *Arabidopsis thaliana* cDNA library was made from mRNAs isolated from Arabidopsis suspension cells, young seedlings, flowers, stems, roots, and leaves (in pGADT7, a *Leu2* marker; a gift from N.H. Chua, Rockefeller University, New York, NY). Approximately 5×10^6 yeast transformants were screened on the selective medium Synthetic Dropout (SD)/-Ade/-His/-Leu/-Trp/X-α-Gal according to the manufacturer's instructions.

For the two-hybrid screen using pLexA-COI1 as bait, a LexA-based Arabidopsis cDNA library constructed from mRNAs isolated from methyl jasmonate-treated plants (in pB42AD; a gift of D.F. Huang) was transformed into yeast strain EGY48 according to the manufacturer's instructions (catalog No. K1609-1; Clontech). A total of 8×10^7 yeast transformants were selected on 2% Gal/1% raffinose/SD/-Ura/-His/-Trp/-Leu /X-β-Gal.

β-Galactosidase Assay

β-Galactosidase activities were measured using a modified method based on Samach et al. (1999). Yeast strains in the LexA-based two-hybrid system were grown in 2% Gal/1% raffinose/SD/-Ura/-His/-Trp. Yeast cells cultured in 2% Glc/SD/-Ura/-His/-Trp were used as a control.

Plasmid Constructs, Arabidopsis Transgenic Lines, and *coi1* Mutant Alleles

The $COI1_{E22A}$ mutation was generated using the overlap PCR extension strategy (Vallejo et al., 1995) by introducing the desired amino acid substitution (from Glu to Ala at amino acid 22) into the specially designed mutagenizing complementary primers E22A-forward (5′-TTGATGATGTCATCGCGCAAGTCATGACCT-3′) and E22A-reverse (5′-AGGTCATGACTTGCGCGATGACATCATCAA-3′).

Six copies of the Myc epitope (MEQKLISEEDLNE) (Rupp et al., 1994; Turner and Weintraub, 1994) were cloned at the BamHI and SmaI sites into plasmid pROK2, which contains the 35S promoter of *Cauliflower mosaic virus* in the binary vector pBIN19, resulting in pMYC2. $COI1_{E22A}$ amplified by Pfu DNA polymerase (catalog No. 600,135; Stratagene) was fused in frame to the Myc epitope at the SmaI site in pMYC2, resulting in pMyc-$COI1_{E22A}$. The coding region of *ASK1*, *ASK2*, or *COI1* was amplified by PCR and fused in frame to the Myc epitope at the SmaI site in pMYC2, resulting in pMyc-ASK1, pMyc-ASK2, or pMyc-COI1.

To make Flag-tagged COI1, the Flag epitope was introduced into the specially designed primer with the Flag sequence (underlined) fused in frame to the *COI1* 5′ sequence starting from the ATG codon (5′-ATGGATTACAAGGATGATGATGATAAGATGGAGGATCCTGATATCAAGAGGT-3′). The full length of *COI1* was Flag tagged by PCR amplification and cloned into pROK2 at the SmaI site, resulting in pFlag-COI1.

The constructs described above were sequence verified and transferred into Arabidopsis plants by in planta *Agrobacterium tumefaciens*–mediated vacuum infiltration (Bechtold et al., 1993; Bent, 2000). Transgenic plants expressing epitope-tagged proteins at levels similar to those of their corresponding endogenous proteins were identified and used for further experiments.

AtRbx1 double-stranded RNA interference (*RNAi*) mutant plants were generated by E. Lechner and P. Genschick. A PCR-amplified BamHI-SpeI green fluorescent protein (*GFP*) fragment was first introduced into the pBluescript KS+ vector (Stratagene). The *AtRbx1a* coding region then was introduced at XhoI and EcoRI restriction sites in the antisense orientation upstream of *GFP* after PCR amplification with primers 5′-ATCTAGCTCGAGTGCGGTGGTAACCAAATGAAC-3′ and 5′-ACTAGTGAATTCGCGACACTAGACTCCGACGT-3′ and at SpeI and SacI restriction sites in the sense orientation downstream of *GFP* after PCR amplification with primers 5′-TGATCATCTAGAGCGACACTAGACTCCGACGT-3′ and 5′-TACGTTGAGCTCACTAGTTGCGGTGGTAACCAAATGAAC-3′. Finally, the XhoI-SpeI DNA fragment containing the *AtRbx1* double-stranded *RNAi* was subcloned into the glucocorticoid-inducible vector pTA7002 (Aoyama and Chua, 1997).

The leaky mutant alleles *coi1-2* and *coi1-8* of *COI1* were identified from ethyl methanesulfonate–mutagenized Arabidopsis ecotype Columbia M2 seeds (stock No. M2E-02-03; Lehle Seeds, Round Rock, TX) using the method described by Staswick et al. (1992). Sequencing results revealed the missense mutations L245F in *coi1-2* and G543L in *coi1-8*.

Antibodies

Polyclonal antibodies against the full length of COI1 and ASK1 were generated in rabbits. The antibodies against ASK2 and AtCUL1 have been described previously (Gray et al., 1999; Shen et al., 2002). The crude antisera were used at a dilution of 1:1000 except for anti-(α-)ASK2 antiserum, which was used at a dilution of 1:4000. The rabbit α-Rbx1 polyclonal antibody, raised against the synthetic peptide Cys-PLDNREWEFQKYGH, corresponding to the C terminus of human Rbx1, was purchased from BioSource (Camarillo, CA; catalog No. AHO0402).

The α-actin goat polyclonal antibody was purchased from Santa Cruz Biotechnology (catalog No. SC-1615; Santa Cruz, CA). The α-Myc monoclonal mouse antibody (9E10) and α-Myc affinity matrix were purchased from Berkeley Antibody Company (catalog No. MMS-150P; AFC-150P; Richmond, CA). The α-Flag monoclonal mouse antibody (M2) and the α-Flag affinity matrix were purchased from Sigma (catalog Nos. F3165 and A1205). The secondary antibodies (goat anti-rabbit IgG–horseradish peroxidase [HRP], rabbit anti-goat IgG-HRP, and goat anti-mouse IgG-HRP) were purchased from Pierce. The commercial antibodies were used as recommended by the suppliers.

Protein Gel Blot Analysis

Protein extracts were prepared by homogenizing Arabidopsis tissues in ice-cold extraction buffer (50 mM sodium phosphate, pH 7.0, 200 mM NaCl, 10 mM $MgCl_2$, 0.2% β-mercaptoethanol, and 10% glycerol) supplemented with the protease inhibitor cocktail (catalog No. 1836170; Roche, Mannheim, Germany). Proteins were separated on SDS-PAGE and transferred onto Hybond enhanced chemiluminescence membranes (catalog No. RPN303D; Amersham). The blots were blocked in PBST (PBS plus 0.05% Tween 20) containing 5% nonfat milk for 2 h and incubated for an additional 2 h with primary antibody (the α-Rbx1 antibody was prepared in PBST with 2.5% nonfat milk). The blots were washed five times with PBST and then incubated with secondary antibody for 1 h. Immunoblotting bands were detected using the enhanced chemiluminescence system (catalog No. CK52563; Pierce).

Coimmunoprecipitation Assay

Extracts (1 mL) containing 5 to 10 mg of total proteins prepared from young seedlings (2 weeks old) and mature plants (flowers and leaves) were incubated with 50 to 200 μL of α-Flag or α-Myc affinity matrix for 4 h at 4°C with gentle rocking. The matrix was washed five times with the extraction buffer. The α-Flag matrix then was eluted with 0.1 M Gly, pH 3.0, and the α-Myc matrix was suspended in SDS-PAGE sample buffer and heated for 5 min at 100°C. The immunoprecipitates then were subjected to protein gel blot analysis with various antibodies.

RNA Gel Blot Analysis

The specific primers were designed based on their DNA sequences to PCR amplify RNA gel blot probes *PDF1.2*, *Thi2.1*, *AtVSP*, *AOS*, *AtRbx1*, and 18S rDNA. The fragments were labeled with the PCR digoxigenin probe synthesis kit according to the manufacturer's instructions (catalog No. 1-636-090; Roche). Plant RNA was isolated using Trizol regent (catalog No.15596; Gibco BRL). Ten to 20 μg of RNAs was separated on 1.5% formaldehyde agarose gel and transferred onto Hybond N^+ membranes (catalog No. RNP203B; Amersham). Hybridization and detection were performed according to procedures that are standard for the digoxigenin system, as advised by the manufacturer (Roche).

Plant Growth Conditions and Treatment

Seeds were surface-sterilized, chilled at 4°C for 3 days, and then germinated and grown in a growth room under a 16-h-light (21–23°C)/8-h-dark (16–19°C) photoperiod. For jasmonate treatment experiments in RNA gel blotting, seedlings were grown on Murashige and Skoog (1962) medium with 2% Suc for 2 to 3 weeks and then treated with 100 μM methyl jasmonate (catalog No. 39270-7; Aldrich) or water for 8 h in daytime. For root length measurement experiments, seedlings were grown on Murashige and Skoog (1962) medium supplemented with various concentrations of methyl jasmonate for 1 week before measurement. To test plant fertility, seedlings were transferred into soil and grown under a 16-h-light (21–23°C)/8-h-dark (16–19°C) photoperiod.

Upon request, all novel material described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

Accession Numbers

The GenBank accession numbers for the sequences shown in Figure 2 are AAL13435 (AtRbx1a), CAB87200 (AtRbx1b), BAB83695 (*C. elegans* Rbx1), T13388 (Drosophila ROC1a), AAF32313 (Drosophila ROC1b), NP_062686 (mouse Rbx1), and NP_055063 (human Rbx1). Accession numbers of the sequences used as probes in RNA gel blot analysis are T04323 (*PDF1.2*), L41344 (*Thi2.1*), AY072506 (*AtVSP*), X92510 (*AOS*), AY052401 (*AtRbx1*), and X16077 (18S rDNA).

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