Recently in yeast and animal cells, one particular class of ubiquitin ligase (E3), called the SCF, was demonstrated to catalyze diverse processes involving cell cycle and development. In plants SCF-dependent proteolysis is also involved in different developmental and hormonal regulations. To further investigate the function of SCF, we characterized at the molecular level the Arabidopsis RING-H2 finger protein AtRbx1. We demonstrated that the plant gene is able to functionally complement a yeast knockout mutant strain and showed that AtRbx1 protein interacts physically with at least two members of the Arabidopsis cullin family (AtCul1 and AtCul4). AtRbx1 also associates with AtCul1 and the Arabidopsis SKP1-related proteins in planta, indicating that it is part of plant SCF complexes. AtRbx1 mRNA accumulates in various tissues of the plant, but at higher levels in tissues containing actively dividing cells. Finally to study the function of the gene in planta, we either overexpressed AtRbx1 or reduced its expression by a dsRNA strategy. Down-regulation of AtRbx1 im paired seedling growth and development, indicating that the gene is essential in plants. Furthermore, the AtRbx1-silenced plants showed a reduced level of AtCul1 protein, but accumulated higher level of cyclin D3.

The SCF complex(es) (named after the three original yeast protein subunits: Skp1, Cdc53 (cullin), and F-box proteins) belong to a class of ubiquitin ligase (ubiquitin-protein isopeptide ligase (E3)) required for the degradation of key regulatory proteins involved in cell cycle progression, development, and signal transduction (reviewed in Refs. 1–3). The F-box proteins are thought to be the adapter subunits that recruit specifically signaling proteins. The F-box protein SKP2 is involved in the turnover of the transcription factor E2F-1 (5) and the cyclindependent kinase inhibitor p27 (6), whereas hCDC4/Fbw7/Ago are involved in cyclin E protein degradation (7–9). β-TRCP is an F-box protein that interacts with phosphorylated IκBα and β-catenin proteins, thereby playing an important role in β-catenin/Tcf and nuclear factor κB-dependent signaling (10, 11).

The SCF complexes are also constituted by a fourth essential stoichiometric subunit named in yeast and animal cells Hrt1p/Rbx1p/Roc1p (12–15) and hereafter called Rbx1. The Rbx1 protein belongs to the ring finger domain RING-H2 protein family and plays a key function in stimulating the ubiquitylation reaction (reviewed in Ref. 16). The structure of the human quaternary SCF complex (Cul1-Rbx1-Skp1-F-box) has recently been achieved (17). A model has been proposed in which Cul1 is positioning the substrate via the F-box protein SKP2 in close proximity to the catalytic module consisting of Rbx1 and the ubiquitin-conjugating enzyme. Because Rbx1 interacts both with cullin and an ubiquitin-conjugating enzyme, it may thus serve as an allosteric activator of the later. Cullin proteins are also covalently modified by an ubiquitin-related protein called Nedd8/Rub1 (18, 19), and it has been shown that Rbx1 may be involved in this process (20). Mutation of Rbx1 gene in budding yeast results in a cell cycle arrest before DNA replication, and the mutant is defective in degradation of Sic1 and the G1 cyclin Cln2 (12, 14). Developmental and/or physiological consequences of Rbx1 overexpression or depletion are poorly understood in higher eukaryotes.

In plants genetic studies have highlighted the important function of F-box proteins in a number of developmental and physiological processes. Both auxin and jasmonate perception are controlled, at least in part, by SCF-like complexes involving the F-box proteins Tir1 and Coi1, respectively (Refs. 21 and 22; reviewed in Refs. 23–25). Indeed, recently it was demonstrated that auxin stimulates the binding of the SCFTyr 1 to the AUX/ IAA proteins leading to their degradation (26). Furthermore mutants in the Arabidopsis F-box genes UFO (27); ZTL, FKFP, and LKP2 (28–30); EID1 (31); ORE9 (32); and SON1 (33)
suggest that SCF-dependent ubiquitylation reactions are also involved in floral patterning, the regulation of circadian rhythm, phytochrome A-specific light signaling, natural and hormone-induced senescence processes, and defense responses to pathogen, respectively. Arabidopsis genome encodes almost 700 possible F-box proteins (34), suggesting that SCF-dependent proteolysis is a fundamental mechanism in plants regulating various cellular and cell wall processes. To further investigate the function of SCF in plants, we characterized at the molecular level atRbx1 from Arabidopsis. We found that the AtRbx1 gene is able to functionally complement a yeast mutant strain. Furthermore, we demonstrate that AtRbx1 associates physically with at least two members of the Arabidopsis cullin family (AtCul1 and AtCul4) and two SKP1-like proteins (ASK1 and ASK2). RNA gel blots were performed to study the expression of the AtRbx1 genes in various tissues of plants and after different stimuli. Finally, we used both inducible overexpression and dsRNA strategies to investigate the function of the gene in planta.

EXPERIMENTAL PROCEDURES

Unless stated otherwise, all procedures for manipulating DNA and RNA were carried out according to Refs. 33, 37, and 56. Chemicals—Dex (Sigma) was dissolved in ethanol and kept at a concentration of 30 mm.

Construction of Plasmids—Construct pTA::GFP is described in Ref. 37, and construct pTA::GUS was a gift from L. Lepiniec. To construct pTA::Rbx1, we used PCR-based site-directed mutagenesis to introduce XhoI and BamHI restriction sites upstream and downstream of the coding region of AtRbx1;1 using oligonucleotide 5'-CGGGTACCTCGAGAATGCGGACACTAGCTGGATTAATCATGATTCC-3' and oligonucleotide 5'-GACTAGTGACCATATTTCTGAAACTCCC-3', respectively. For gene silencing, AtRbx1 antisense and sense coding regions were separated by a spacer sequence consisting of the GFP coding sequence. To construct pTA::dsRNA-Rbx1, a BamHI-SpeI GFP fragment, obtained by PCR, was first introduced in the pBlueScript vector (Stratagene) resultating in plasmid pKS-GFP. AtRbx1 coding region was introduced in antisense orientation upstream from the GFP by PCR amplification using oligonucleotide 5'-ATCTAGCTGGATGTCGA-3' and oligonucleotide 5'-ACTAGTGAATTCGCGACATACTAGCTGG-3', and using the XhoI and EcoRI restriction sites. To introduce AtRbx1 coding region in sense orientation downstream the GFP, AtRbx1 sequence was PCR-amplified using oligonucleotide 5'-TGTACTCACTAGGGACAGCTGACGTCG-3' and oligonucleotide 5'-TACGTGGACTGTCGTTGCTAGTACCAATACGGAC-3', digested by XhoI and SacI, and introduced into SpeI and SacI restriction sites, resulting in construct pKS::dsRNA-Rbx1. The XhoI-SpeI DNA fragment, encoding the dsRNA-Rbx1 construct, was subcloned into the Des-inducible vector pTA7002 (38), resulting in plasmid pTA::dsRNA-Rbx1.

To make myc-tagged ASK1 or ASK2, full-length ASK1 (At1g79590) or ASK2 (At5g42190) was PCR-amplified and fused in frame to the six his tag using a yeast mutant strain. Furthermore, we demonstrate that AtRbx1 associates physically with at least two members of the Arabidopsis cullin family (AtCul1 and AtCul4) and two SKP1-like proteins (ASK1 and ASK2). RNA gel blots were performed to study the expression of the AtRbx1 genes in various tissues of plants and after different stimuli. Finally, we used both inducible overexpression and dsRNA strategies to investigate the function of the gene in planta.

Molecular Characterization of Arabidopsis Rbx1

Two putative Arabidopsis genes, At1g07920 and At5g42190, were PCR-amplified and fused in frame to the six his tag using a yeast mutant strain. Furthermore, we demonstrate that AtRbx1 associates physically with at least two members of the Arabidopsis cullin family (AtCul1 and AtCul4) and two SKP1-like proteins (ASK1 and ASK2). RNA gel blots were performed to study the expression of the AtRbx1 genes in various tissues of plants and after different stimuli. Finally, we used both inducible overexpression and dsRNA strategies to investigate the function of the gene in planta.

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**Molecular Characterization of Arabidopsis Rbx1**

**RESULTS**

Arabidopsis thaliana Genome Encodes Two Proteins Structurally Related to Yeast and Human Rbx1—A. thaliana data base searches led to the identification of two different genes called AtRbx1;1 and AtRbx1;2, located on chromosome 5 (At5g20570) and chromosome 3 (At3g42830), respectively. The sequence of the longest EST for AtRbx1;1 was determined, and it encodes an 118-amino acid open reading frame, which is complete at its N-terminal end as indicated by an in-frame stop codon upstream of the initiation codon. AtRbx1;1 and the predicted AtRbx1;2 protein sequences exhibit 87% sequence identity. Both proteins are closely related to human Rbx1 protein (86% identity and over 91% similarity between AtRbx1;1 and HsRbx1 sequences). The plant sequences were aligned with yeast and different animal Rbx1 protein sequences (see Fig. 1). All the residues proposed to form the zinc binding sites (50) are perfectly conserved. Interestingly, both Arabidopsis proteins belong to the Roc1/Rbx1 subgroup and are more distant from the Roc2/Rbx2 proteins.

**AtRbx1 Complements the Lethality of Yeast**

MCY557 rbox1 Deletion Strain—rbox1 is an essential gene in yeast, and its inactivation arrests the cell cycle at the G1/S transition and results in the stabilization of different SCF substrates, like Sic1p and Cln2p (12, 14). The diploid rbox1 deletion strain MCY557 (12), which produces only two viable spores from each tetrad, was transformed with a plasmid expressing either the plant or the human Rbx1 gene. After tetrad dissection, all four spores were viable on YPD and SD-uracil media, indicating that both plant (Fig. 2) and mammalian (Refs. 12 and 14 and data not shown) Rbx1 genes were able to rescue the viability defect of the yeast mutant strain.

**AtRbx1 Interacts with AtCul1 and AtCul4 in a Yeast Two-hybrid Assay and Is Part of the SCF Complex Containing Both AtCul1 and ASK Proteins**—We next determined whether AtRbx1 interacts with plant culins (Fig. 3). AtRbx1 was cloned into the pGBK7 bait plasmid to produce a fusion protein with Gal4 DNA binding domain. Another small Arabidopsis RingH2 protein (49) not related to the primary protein sequence of AtRbx1 was also introduced into pGBK7 plasmid. AtCul1 (At4g02570) and AtCul4 (At4g46210) were cloned into the prey pGADT7 vector to produce fusion proteins with the Gal4 activation domain. Only AtRbx1 and not the other small RingH2 protein interacted with the culin proteins by the two-hybrid assay (Fig. 3A). β-Galactosidase activities, used to quantify the protein interactions, indicated that the AtRbx1 interacts similarly with both AtCul1 and AtCul4 proteins (Fig. 3B).

To further demonstrate the physical interaction between AtRbx1 and the AtCul1 protein, we performed pull-down assays. AtRbx1;1 was expressed in Escherichia coli as a translational fusion protein with GST. The purified GST-Rbx1 fusion protein (or GST alone as a control) was incubated with Arabidopsis Rbx1 (AtRbx1) and AtCul1 (AtCul1) COOH-terminal deletions were immunoprecipitated from transgenic plant extracts expressing either the myc-tagged ASK1 or the myc-tagged ASK2 proteins and analyzed for the presence of AtRbx1 and AtCul1. Both AtRbx1 and AtCul1 proteins co-immunoprecipitated with either myc-tagged ASK1.
It is worth noting that the interaction between AtRbx1 and the ASK proteins is indirect and most likely mediated by the AtCul1 protein. Thus, our data clearly demonstrate the ASK1/2, AtCul1, and AtRbx1 proteins form complexes in planta.

Expression of AtRbx1 in Plants and in Response to External Stimuli—Based on the available EST sequences, it appears that AtRbx1;1 is expressed at a higher level than AtRbx1;2; in the current data bases, six different ESTs are found for AtRbx1;1, but none for AtRbx1;2. To determine whether the AtRbx1;2 gene is expressed, we performed semiquantitative RT-PCR with specific primers designed from the 3'-untrans-

Modulation of AtRbx1 Protein Level in Arabidopsis—To investigate the function of AtRbx1 in planta, we designed con-
structs to either overexpress or silence the gene in *Arabidopsis* (see Fig. 5). Because *Rbx1* is an essential gene in budding yeast and is involved in SCF-dependent ubiquitylation and degradation of cell cycle regulatory proteins (12, 14, 15), we investigated its function in plants by setting up inducible PTGS and overexpression assays. We also engineered different control plants: either empty vector transgenic plants or plants expressing GFP or GUS under the control of the inducible vector. GFP and GUS expression were confirmed in some of the plants. Seeds of the T1 generation from more than 25 different transgenic lines for each construct were sown *in vitro* in the presence or absence of Dex (1 μM) and were analyzed phenotypically and at the molecular level.

Accordingly to the results published in Ref. 54, but to a much lesser extent, 2 control lines (pTA::4 and pTA::GFP6) of 39 lines analyzed (24 empty vector, 10 pTA::GUS, and 5 pTA::GFP lines), showed alteration in seedling development. Line pTA::GFP6 had a low yield of germination, whereas line pTA::4 showed shrink-shaped cotyledons (see below).

As expected higher levels of *AtRbx1* mRNA and protein were found in the pTA::Rbx1-overexpressing lines (called sense lines) (Fig. 6 (A and B) and data not shown). The antibody used was specific, as demonstrated by the competition assays (Fig. 6C). 19 out of 27 sense lines analyzed showed a shrink cotyledon shape (Fig. 7, A and B). However, a similar phenotype was found in control line pTA::4 (data not shown), exhibiting strong expression of the chimeric GVG transcription factor (38) and the induction of the defensin gene PDF1.2 (55) (Fig. 6A). Thus we did not consider the shrinkage of the cotyledons as a phenotype because of *Rbx1* overexpression, but rather as side effects of the vector. Furthermore in all sense lines and control lines, the development of the leaves was not disturbed and

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**Fig. 3.** AtRbx1;1 protein interacts with *Arabidopsis* cullin and SKP1-related proteins. **A**, yeast diploid AH109/Y187 cells co-expressing the indicated proteins were plated onto media lacking tryptophan and leucine (SD/-Trp-Leu) to check for the presence of both bait and prey plasmids or onto media lacking adenine, histidine, tryptophan, and leucine (SD/- Ade−His−Trp−Leu) to assay for the interaction between bait and prey proteins. **B**, yeast diploid AH109/Y187 cells co-expressing the indicated proteins were tested for the β-galactosidase activities. As a control we used pGBK7-53 and pGADT7-T control vectors (Clontech), which give strong protein interaction. The galactosidase activity is given as the mean ± S.D. of five independent yeast lines for each combination of constructs. **C**, pull-down assays with either GST or Rbx1-GST were probed with the specific *Arabidopsis* anti-Cullin1 antibody (6AtCul1). The asterisk indicates the Rub1-modified form of AtCul1. The competition experiment was performed with 1 μM antigen peptide (shown on the right side of the panel). **D**, co-immunoprecipitation assay. Protein extracts from wild type (WT) (lane 1) and transgenic plants expressing the myc-tagged ASK2 (WT::myc-ASK2, lane 2) were immunoprecipitated with anti-myc antibody (lanes 3 and 4). The resulting immunoprecipitate (IP) was resolved on SDS-PAGE and detected with the indicated antibodies. The asterisk indicates the Rub1-modified form of AtCul1.
Plants grew normally on the Dex-containing medium, at least during the early vegetative growth phase. Similar to the sense lines, most of the pTA::dsRNA lines (called dsRNA lines) (21/28 lines, 75%) also showed the shrunk-shaped cotyledon phenotype (data not shown). AtRbx1 mRNA and protein levels were found reduced in those lines (Fig. 8A, lanes 3 and 4). Interestingly, 83% of the dsRNA lines grown for more than 30 days in the presence of Dex developed small green bushy plants (5–30%/plate), as illustrated for line dsRNA-84 (Fig. 7C) with reduced AtRbx1 RNA and protein.
levels (Fig. 8A, lane 5). When the 3-week-old in vitro bushy plants were transferred to soil, either they remained dwarf but produced few flowers (Fig. 7D, inset) or they developed plants with reduced apical dominance (data not shown). Because SCF pathway is involved in auxin signaling (reviewed in Ref. 53), we expected to find auxin-related phenotypes. Indeed, ~10% of the T0 dsRNA lines already showed reduced apical dominance, probably as a result of the leaky expression of the promoter. Similarly, plants exhibiting a reduction in apical dominance with a strong increase in the number of lateral branches were observed in the progeny of several dsRNA lines, and those plants always exhibited a reduction of AtRbx1 mRNA levels (as illustrated for line dsRNA-76; Fig. 7, E and inset). A more detailed analysis of auxin responses in these lines is under way.

Interestingly, in three dsRNA lines (dsRNA-2, dsRNA-20, and dsRNA-33), the development of the seedlings was severely impaired (as shown for line dsRNA-2; Fig. 7, F and G). In those plants, and in contrast to the control or non-induced lines, cotyledons did not develop at all (remaining white) and strong leaf growth retardation and leaf expansion effects were observed. At 10 days after germination, the average length of cotyledons for the dsRNA-2 transgenic plants was 0.82 mm (± 0.05 mm, n = 20) compared with 2.48 mm (± 0.12 mm, n = 23) for cotyledons of Dex-treated wild type plants. At this stage, no difference could be found in cotyledon vascularization patterns (data not shown), overall tissue organization, or cellular size (Fig. 7, H and I). Only the plastid content was found to be different, exhibiting swollen plastids phenotypically identical to amyloplasts. High starch content of the latter was confirmed by Lugol staining (data not shown). Nevertheless at a latter stage (3 weeks after germination), both the size and shape of cotyledon epidermal cells were different. Whereas in the non-induced dsRNA-2 line the average area of cotyledon epidermal cells was 8259 μm² (± 3948 μm², n = 115), this value was reduced by a factor of 10 in the AtRbx1-silenced plants (average area of the cells was 801 μm² (± 338 μm², n = 115)).

AtRbx1 mRNA (Fig. 8B, lane 3) and protein (Fig. 9) levels were strongly reduced in those lines, but still detectable. We believe that the strong developmental alteration found in those

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**Fig. 6.** Northern and Western blot analysis of AtRbx1-overexpressing and control lines. The identity of the lines is indicated: WS (non-transformed Arabidopsis Wassilewskija ecotype), AtRbx1-overexpressing lines Sense-66 and Sense-94, and control line pTA::4. A, total RNA was isolated from 10-day-old Arabidopsis seedlings germinated in presence or absence of Dex as indicated. The RNA gel blot analysis was performed by successive hybridization with different probes, as indicated. B, total proteins were prepared from the same samples as indicated in A. Western blots were performed with the antibodies against AtRbx1 (@AtRbx1) and the conserved CDK kinase motif PSTAIRE (@PSTAIRE). C, competition experiment realized with 1 μM AtRbx1;1 peptide (right panel) or without peptide (left panel) on the same extracts from line Sense-94, as indicated in B. The asterisk indicates a nonspecific cross-reacting protein band.
dsRNA lines is caused by the Rbx1 depletion and not by the chimeric GVG transcription factor expression (or other effects) because this phenotype was never found in control or Rbx1-overexpressing lines. It is also worth noting that the intermedial RNA can be detected by both AtRbx1 and GFP probes and that at least some of the spacer-GFP RNA always accumulates in the Dex-induced plants (Fig. 8B). More than 30 plants of line dsRNA-2 (at the stage indicated in Fig. 7F) were transferred to plates with or without Dex. One week later, only the plants on the Dex-free medium recovered, developing normal leaves and re-accumulating the AtRbx1 transcripts (see Fig. 8B, lane 4). Nevertheless, some of the plants became bushy (data not shown). Thus, in contrast to a systemic PTGS reaction (reviewed in Refs. 56 and 57), silencing occurring in the dsRNA lines was reversible.

To confirm that AtRbx1 mRNA decay was the result of dsRNA-induced PTGS, we assayed for the presence of 21–23-nucleotide AtRbx1 mRNAs 4 days after Dex treatment, specifically in the dsRNA line (Fig. 8C). 21–23-nucleotide species were readily detected in the low molecular weight RNA fractions of the Dex-treated pTA::dsRNA plants (Fig. 8D), but not in the GFP-expressing control lines or wild type plants (data not shown). The small RNA fragments appeared 24 h after the treatment and reached the highest accumulation at 96 h.

To understand the biochemical basis of the severe growth defects observed in some of the dsRNA lines, protein extracts from dsRNA-2 line and control line were blotted with different antibodies (Fig. 9). Interestingly, we noted that AtRbx1-depleted seedlings do not only show a strong reduction of AtRbx1 protein level, but also a decreased AtCul1 protein level. In fact, both Rub1-modified and unmodified AtCul1 protein bands were weaker. To check whether the reductions of AtRbx1 and, to a lesser extent, AtCul1 would affect the pattern of ubiquitin conjugates, the blot was probed with an anti-ubiquitin antibody in the green house were sprayed with a solution of 10 μM Dex and harvested at different times. Semiquantitative RT-PCR analysis of the plants indicated a strong reduction of AtRbx1 mRNAs 4 days after Dex treatment, specifically in the dsRNA line (Fig. 8C). 21–23-nucleotide species were readily detected in the low molecular weight RNA fractions of the Dex-treated pTA::dsRNA plants (Fig. 8D), but not in the GFP-expressing control lines or wild type plants (data not shown). The small RNA fragments appeared 24 h after the treatment and reached the highest accumulation at 96 h.

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Fig. 8. **Molecular analysis of AtRbx1 dsRNA and control lines.** The identity of the lines is indicated: WS (non-transformed Arabidopsis Wassilewskija ecotype), GUS (pTA-GUS-transformed Arabidopsis line), and dsRNA lines 2, 64 and 84. **A**, AtRbx1 mRNA and protein accumulation in dsRNA and control lines. Total RNA was isolated from 10-day-old Arabidopsis control seedlings (lanes 1 and 2) or from line dsRNA-64 (lanes 3 and 4) germinated in the absence or presence of Dex. Lane 5 corresponds to the in vitro bushy plants produced after longer periods of growth on Dex medium (here line dsRNA-84, shown on Fig. 7C). The asterisk indicates the intermediary RNA. Western blots were performed with the antibodies against AtRbx1 (@AtRbx1) and the conserved CDK kinase motif PSTAIRE (@PSTAIRE). **B**, induction and reversion of PTGS in the dsRNA lines. Total RNA was isolated from 10-day-old Arabidopsis seedlings germinated in absence or presence of Dex (lanes 2 and 3, respectively). Total RNA was also isolated from transgenic seedlings first grown on Dex medium for 12 days, then transferred to a Dex-free medium for 7 days (lane 4). The RNA gel blot analysis was performed by successive hybridization with different probes, as indicated. Asterisks indicate the intermediary RNA. **C**, RT-PCR analysis of dsRNA (line dsRNA-2) and control lines after spraying with a solution of 10 μM Dex and harvested at different times after the treatment. For controls, RT-PCR reactions were performed with an oligonucleotide set for AtCul1. **D**, Northern hybridization analysis of equal amounts of low molecular weight RNA from the Dex-sprayed dsRNA plants (dsRNA-2), at different times after the treatment. The blot was hybridized with AtRbx1 in vitro-transcribed 32P-labeled RNA probe. The lane on the left contains 5′-32P-labeled oligomers as size markers.
that recognizes both free and conjugated ubiquitin. Indeed, we noted that several protein bands either disappeared or were strongly reduced in the Dex-induced dsRNA line. The level of free ubiquitin also was reduced in those plants. However, the pattern of most ubiquitin conjugates were still similar between the non-induced and the induced line. This may not be surprising because Arabidopsis counts many other E3s aside the SCF (58).

Mammalian D-type cyclins are highly unstable proteins, and several experiments substantiate the involvement of SCF complexes in their degradation (59, 60). Similarly to the animal D-type cyclins, the expression of Arabidopsis cyclin D3 was found regulated by growth signals and the protein shown to be very unstable (47, 61, 62). Interestingly, in the Rbx1-depleted plants, we found a significant higher level of cyclin D3 protein (Fig. 9) whereas cyclin D3 mRNAs accumulated at a similar level than in the Dex non-induced or control plants (data not shown). However, this protein band is detected at ~46 kDa, whereas cyclin D3 detected in roots is ~55 kDa (47). Because the antibody was raised against the C-terminal peptide of cyclin D3 (47), the 46-kDa form of the protein must represent the loss of an N-terminal fragment or could be an alternative splice form or a modified form of the protein. Whether cyclin D3 is a target of plant SCF complexes remains to be demonstrated.

**DISCUSSION**

SCF is a highly conserved ubiquitin protein ligase that is constituted by at least four subunits: SKP1 (called ASK1 in Arabidopsis), Cul1, F-box proteins, and the Ring-H2 finger protein Rbx1 (reviewed in Ref. 16). In plants the functions of the SCF have been highlighted by the characterization of different loss-of-function mutants in genes encoding F-box proteins (see Introduction). The F-box protein is the specific adapter interacting directly with the substrate(s) and allowing its ubiquitylation. The best understood SCF complex in plants is probably SCFTir1, in which the F-box protein Tir1 interacts with a subdomain of the AUX/IAA protein, leading to their degradation after auxin stimulation (see Ref. 26 and references therein). In addition to the F-box proteins, Arabidopsis genome sequence also revealed the presence of SKP1- and cullin-related genes. Whereas there is only a single SKP1 protein identified so far in humans, there are 19 SKP1 orthologs present in Arabidopsis genome (34). The function of only one of the plant SKP1-related gene, called ASK1, has been investigated so far. Mutation of the ASK1 gene produces diverse phenotypes including abnormal floral morphology (63), male sterility (64), and auxin resistance (21, 65). Nevertheless, the homozygous mutant is still viable, suggesting some functional redundancy with other SKP1-related genes. Cullins also belong to a multigenic gene family, and, in Arabidopsis, there are 11 cullin-related genes. Recently, we investigated the function of one of these genes, AtCul1, which is closely related to yeast CDC53 and human Cul1 and Cul2 (45). The homozygous mutant displayed an early arrest in embryogenesis (at the first cell divisions after fertilization of the zygote), indicating that the gene and, as a consequence, SCF-dependent proteolysis are essential in Arabidopsis.
Molecular Characterization of Arabidopsis Rbx1

In the present work, we characterized in Arabidopsis the fourth component of the SCF complex: the Ring-H2 finger protein AtRbx1. Arabidopsis encodes two Rbx1-related genes: AtRbx1;1 and AtRbx1;2. Accordingly to the essential function of SCF-dependent ubiquitylation in all eukaryotes, AtRbx1;1 was found expressed in all plant organs. AtRbx1;2 may only be expressed in particular cell types or at a very low level or not at all. Interestingly, higher AtRbx1 mRNA accumulation was found in tissues containing actively dividing cells, suggesting that, as in yeast and animal cells (reviewed in Ref. 2), SCF-dependent ubiquitylation in plants may also be involved in the turnover of key cell cycle regulatory proteins. Interestingly most of AtRbx1 transcripts (as well as AtCul1 transcripts; data not shown) disappeared after heat shock, suggesting that SCF-dependent ubiquitylation may not play a major role in the proteolytic events following heat stress in plants.

Both our genetic and biochemical data identified AtRbx1 as the plant ortholog of Hrt1/ Rbx1/Roc1 in yeast and animal cells. The Arabidopsis gene was able to rescue the viability defect of the yeast Rbx1-deletion mutant strain. Furthermore, we demonstrated that AtRbx1 protein physically interacted with AtCul1 and is part of a plant SCF complex. Analysis of our dsRNA lines also supported the notion that AtRbx1 is part of the fourth component of the SCF complex: the Ring-H2 finger protein Rub1. In contrast, our Dex-induced Sense lines did not show auxin-related phenotypes and examination of AtCul1 protein did not reveal Rub1-hypermodification (data not shown). The reasons of these discrepancies are not clear, but it is possible that the overexpression of AtRbx1 in the Dextreated plants was not maintained long enough to reveal those phenotypes. In addition, reduction of AtRbx1 expression in our dsRNA lines led also to a decreased jasmonate response (67). Thus, deregulation of AtRbx1 protein level leads to phenotypes similar to those observed in the aux1 (68), tir1 (21), and coi1 (22) mutants and substantiates the role of AtRbx1 as a component of the SCF<sup>Tir1</sup> and SCF<sup>Coi1</sup> complexes. Indeed, AtRbx1 co-immunoprecipitated with Coi1 (67) and a GST-AtRbx1 recombinant protein was able to pull down the Tir1 protein (66).

Nevertheless, depending on transgenic lines, more severe phenotypes were observed. Thus, in at least three independent dsRNA lines, seedling development was totally blocked. Consistent with plant survival of those lines, we never found a complete suppression of the AtRbx1 mRNA and protein. Introduction of an antisense AtRbx1 construct into Arabidopsis plants also resulted in the death of young seedlings or caused severe dwarf phenotypes (66). Interestingly, in our dsRNA lines we observed a significant reduction of AtCul1 protein level, suggesting that AtRbx1 protein is necessary to accumulate a stable SCF complex. Thus, SCF-dependent ubiquitylation, not only is essential for Arabidopsis embryogenesis (45), but also seems to be required for post-embryonic development (Ref. 66 and this work). The reasons for the developmental arrest in the AtRbx1-depleted seedlings are unknown, but the stabilization of key cell cycle regulatory proteins may be the cause of this arrest. In fungi and animal cells, many cell cycle proteins, particularly at the G1 to S transition, are subjected to SCF-dependent ubiquitylation (reviewed in Refs. 2, 16, and 69). Among them are the G1-type cyclins, cyclin E, cyclin-dependent kinase inhibitors, and the E2F-1 transcription factor. Although many of these proteins are conserved in plants (reviewed in Ref. 70), how they are regulated at a post-transcriptional level is poorly understood. Interestingly, in the AtRbx1-silenced plants, we found a higher accumulation of cyclin D3, which like animal d-type cyclins is an unstable protein (47). Whether cyclin D3 is a substrate of a plant SCF complex remains to be demonstrated. Nevertheless, based on the huge number of F-box proteins in Arabidopsis (34), it is expected many regulatory proteins are stabilized in the dsRNA-arrested seedlings.

However, the phenotypes observed in the dsRNA transgenic plants may not only arise from functional alteration of Cul1/SCF complexes. In animal cells, it was shown that Rbx1 protein interacts with several other members of the cullin family (13). Accordingly, the essential residues found in the C-terminal globular a/b domain of the Cull1 protein, which are involved in interaction with Rbx1, are also conserved in the other cullin members (17). The function of Cull1-related proteins is only poorly understood, and it is not known whether all of them are part of SCF-like complexes. Cul2 has been shown to assemble a ubiquitin-protease complex, including Rbx1, elongin C, and the SOCS-box family protein VHL (reviewed in Ref. 71). In the yeast two-hybrid assay, we demonstrated that AtRbx1 is also able to interact with at least another member of the cullin family, AtCul4, which is functionally distinct from AtCul1 (45). Thus, more experiments will be required to elucidate the function of Rbx1-containing SCF complexes and to understand how they eventually participate in ubiquitin-dependent proteolysis.

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Molecular Characterization of Arabidopsis Rbx1

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