**Cullins 3a and 3b Assemble with Members of the Broad Complex/Tramtrack/Bric-a-Brac (BTB) Protein Family to Form Essential Ubiquitin-Protein Ligases (E3s) in Arabidopsis**

Selective modification of proteins by ubiquitination is directed by diverse families of ubiquitin-protein ligases (or E3s). A large collection of E3s use Cullins (CULs) as scaffolds to form multisubunit E3 complexes in which the CUL binds a target recognition subcomplex and the RBX1 docking protein, which delivers the activated ubiquitin moiety. Arabidopsis and rice contain a large collection of CUL isoforms, indicating that multiple CUL-based E3s exist in plants. Here we show that Arabidopsis CUL3a and CUL3b associate with RBX1 and members of the broad complex/tramtrack/bric-a-brac (BTB) protein family to form BTB E3s. Eighty genes encoding BTB domain-containing proteins were identified in the Arabidopsis genome, indicating that a diverse array of BTB E3s is possible. In addition to the BTB domain, the encoded proteins also contain various other interaction motifs that likely serve as target recognition elements. DNA microarray analyses show that BTB genes are expressed widely in the plant and that tissue-specific and isoform-specific patterns exist. Arabidopsis defective in both CUL3a and CUL3b are embryo-lethal, indicating that BTB E3s are essential for plant development.

The covalent attachment of ubiquitin (Ub) to specific proteins controls numerous cellular processes in eukaryotes, including chromatin remodeling, gene expression, endocytosis and vesicular trafficking, and selective protein breakdown (1, 2). This ubiquitination is directed by an ATP-dependent conjugation cascade involving the sequential action of Ub-activating (E1), Ub-conjugating (E2), and Ub-protein ligase (E3) enzyme families. As the last components in the cascade, E3s control the specificity of ubiquitination by identifying appropriate targets for Ub transfer. The Ub moiety is connected via an isopeptide bond between the C-terminal Gly of Ub and one or more accessible lysyl ε-amino groups in the target. In many cases, chains of poly-Ub are assembled by reiterative conjugation cycles using Lys residues within previously attached Ubs as the binding sites. The complexity of ubiquitination is illustrated by the fact that almost 1,300 distinct genes encoding E3 components have been identified thus far in the Arabidopsis thaliana genome whose protein products potentially modify an equally large number of targets (2).

Studies with yeast and animals have identified four broad families of E3s based on subunit composition and mechanism of action. These include the real interesting new gene (RING) E3s and their structural relatives the U-box E3s, the homologous to S. cerevisiae HECT E3s, the anaphase-promoting complex (APC), and the Cullin (CUL)-based E3s (1, 2). A fourth subunit, RBX1 (or ROC1 and HRT1) was subsequently identified as integral to the complex. Biochemical and structural analyses indicate that the RBX1 subunit recruits the Ub-E2 intermediate and presumably align the two for optimal Ub transfer. The best characterized CUL-based E3 is the multisubunit SCF complex, named after its founding member from yeast, which contains SKP1, the CUL CDC53 (or CUL1), and the F-box protein CDC4 (3). A fourth subunit, RBX1 (or ROC1 and HRT1) was subsequently identified as integral to the complex. Biochemical and structural analyses indicate that the RBX1 subunit recruits the Ub-E2 intermediate and the SKP1/F-box protein pair serves as a target recognition module. The F-box protein binds the target directly with SKP1 linking the F-box protein to CUL1 (3, 4). Other CUL-based E3s include the von-Hippel Lindau/Elongin B/Elongin C (VBC) E3s that use CUL2 and -5 to assemble suppressor of cytokine signaling; TAZ, transcriptional adapter zinc finger; TPR, tetratricopeptide repeat; UFO, unusual floral organs; VBC, von-Hippel Lindau (VHL)/Elongin B/Elongin C; Y2H, yeast two-hybrid; COP, constitutive photomorphogenic; ARIA, armadillo repeat protein interacting with ABP2; BOPI, blade-on-petiole; NPR1, nonexpressor of PR genes; NPH1, nonphototropic hypocotyl; RPT2, root phototropism; RT, reverse transcription; BT, BTB-TAZ; HECT, homologous to E6-AP C terminus; RUB, related to Ub; At, A. thaliana; Os, O. sativa; Hs, Homo sapiens; Sp, S. pombe; Ce, C. elegans.

**From the Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706, Department of Biology, Indiana University, Bloomington, Indiana 47405, Institute for Biology and Applied Genetics, Albrecht-Thaer Weg 6, Freie Universität Berlin, 14195 Berlin, Germany, and Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520-8104**

Derek J. Gingerich‡, Jennifer M. Gagne‡, Donald W. Salter‡‡, Hanjo Hellmann¶¶, Mark Estelle¶, Ligeng Ma‡‡, and Richard D. Vierstra***

*This work was supported by grants from the National Science Foundation Arabidopsis 2010 Program (Grant MCB-0115870 to R. D. V. and M. E.), the Research Division of the University of Wisconsin College of Agriculture and Life Sciences (to R. D. V.), a National Institutes of Health postdoctoral fellowship (Grant F32-GM68361 to D. J. G.), and a National Science Foundation Research Opportunity Award fellowship (to D. W. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**To whom correspondence should be addressed: Dept. of Genetics, 425-G Henry Mall, University of Wisconsin, Madison, WI 53706. Tel.: 608-262-8215; Fax: 608-262-2976; E-mail: vierstra@wisc.edu.

§ Present address: Dept. of Environmental and Biological Sciences, 201C Bibb Graves, University of West Alabama, Livingston, AL 35470.

¶ To whom correspondence should be addressed: Dept. of Genetics, 425-G Henry Mall, University of Wisconsin, Madison, WI 53706. Tel.: 608-262-8215; Fax: 608-262-2976; E-mail: vierstra@wisc.edu.

1 The abbreviations used are: Ub, ubiquitin; APC, anaphase-promoting complex; ASK, Arabidopsis SKP-like; BTB, broad complex/tramtrack/bric-a-brac; COP, constitutive photomorphogenic; ARIA, armadillo repeat protein interacting with ABP2; BOPI, blade-on-petiole; NPR1, nonexpressor of PR genes; NPH1, nonphototropic hypocotyl; RPT2, root phototropism; RT, reverse transcription; BT, BTB-TAZ; HECT, homologous to E6-AP C terminus; RUB, related to Ub; At, A. thaliana; Os, O. sativa; Hs, Homo sapiens; Sp, S. pombe; Ce, C. elegans.

**This paper is available on line at http://www.jbc.org**

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 280, No. 19, Issue of May 13, pp. 18810–18821, 2005

Printed in U.S.A.
RBX1 with a target recognition module composed of a family of SOCS box recognition factors and the Elongin B and C adaptor proteins (5), and the damaged DNA-binding protein (DBB) 1 E3 complex that uses CUL4 to bind RBX1 and a target recognition module containing DDB1 and -2, the de-etrilation protein (DET) 1, and constitutive photomorphogenic (COP) 1 (6). The >10-subunit APC complex also contains a CUL-like protein (APC2) that presumably performs a similar scaffolding function (7).

Searches of various plant genomes indicate that plants utilize E3 types similar to those found in yeast and animals (8–12). The only apparent exception is the VHL E3 complex where potential orthologs of the Elongin B and C and SOCS box proteins are not evident. Remarkably Arabidopsis encodes almost 700 F-box proteins, 21 SKPs (designated ASKs in Arabidopsis), and two CUL1 orthologs (CUL1 and -2a), indicating that a large array of SCF E3s is assembled in plants (11, 13).

Sequence and/or functional orthologs of yeast CUL4 and APC2 that should associate with the DDB1/DETI/COP1 and the APC E3 complexes, respectively, are evident in various plant genomes, indicating that these E3 types are present as well (9, 12). However, analysis of the Arabidopsis genome has uncovered at least seven more CUL-type proteins (12, 14), some of which may be specific to this kingdom, suggesting that more CUL-based ligases are possible in plants.

Recently a new type of CUL-based multisubunit E3 was discovered in Caenorhabditis elegans, Schizosaccharomyces pombe, and humans (15–19). It includes the CUL3 isoform, RBX1, and members of the broad complex/ramtrack/birc-a-brac (BTB) (or prokivirus and zinc finger (POZ)) protein family (20, 21). These proteins share a degenerate BTB(POZ) domain of ~140 amino acids that appears to assume a three-dimensional structure similar to the CUL1/2 interaction domains in the SKP1 and Elongin C adaptor proteins (4, 22, 23). Either N- or C-terminal to the BTB domain is one of several interaction motifs, suggesting that the full-length proteins function as SKP1/F-box protein hybrids that deliver targets to CUL3.

The best studied example is the C. elegans E3 complex containing the BTB-protein MEL-26. MEL-26 in association with CUL3 promotes the ubiquitination and subsequent turnover of MEI-1, a subunit of the katanin-like microtubule-severing heterodimer MEI-1/MEI-2 (15, 17, 18).

BTB proteins have been identified in a number of eukaryotes. As examples, the Saccharomyces cerevisiae, S. pombe, C. elegans, Drosophila melanogaster, and human genomes are predicted to encode 3, 105, 141, and 208 BTB domain-containing proteins, respectively (15, 24). In searches of various plant protein sequence databases, we and others detected likely CUL3 orthologs, suggesting that similar BTB E3 complexes also exist in plants (12, 14). Wang et al. (49) provided further support with the discovery that the Arabidopsis ethylene overproducer (ETO)-1 protein contains a BTB domain that binds CUL3a. Furthermore eto1 mutants show abnormally high levels of the amincyclopropane synthase 5, implying that amincyclopropane synthase 5 is the ubiquitination target of the BTBETO1 complex.

Here we provide a description of the CUL3/BTB E3s in Arabidopsis. Through reiterative BLAST searches of the near complete genomic sequence, 80 proteins were identified that contain the BTB consensus sequence that could be divided into 10 subfamilies. Yeast two-hybrid (Y2H) interaction studies confirmed that members of the BTB family assemble with CUL3a and CUL3b to form E3 complexes. Many, but not all, of the BTB subfamilies are expressed in most Arabidopsis tissues, implying a pervasive action in plant growth and development. The importance of the BTB E3s to plant biology was supported by analysis of a cul3a cul3b double mutant. Loss of both CUL3s generated an embryo-lethal phenotype in which embryogenesis arrested at variable stages. Several members of the Arabidopsis BTB protein family have been previously described as proteins of unknown function that participate in specific cellular events. In addition to ethylene biosynthesis, the list includes disease resistance (genexpressor of PR genes (NPR) 1), phototropism (non-phototropic hypocotyl (NPH) 3 and root phototropism (RPT) 2), hormone perception (armadillo repeat protein interacting with ABF2 (ARIA)), and leaf morphogenesis (blade-on-geteio1 (BOP1)) (25–29). Their possible roles in selective ubiquitination by BTB E3s may now help explain their seemingly disparate cellular actions.

MATERIALS AND METHODS

Identification of Arabidopsis Genes Encoding BTB Domain Proteins—The SMART database (smart.embl-heidelberg.de) was used to locate the BTB(POZ) domain in 37 BTB proteins from a variety of organisms (C. elegans, S. pombe, D. melanogaster, Schizosaccharomyces pombe, and humans). These sequences were used as queries in NCBI BLASTP searches (final search concluded on 11/12/2003) of the near complete A. thaliana ecotype Columbia (Col)-0 genome sequence available in The Arabidopsis Information Resource (www.arabidopsis.org/Blast). These queries recovered 48 non-redundant sequences based on an E-value cutoff of 0.02. This cutoff value was sufficient to eliminate random sequence and was equally or more stringent than similar domain-based searches in Arabidopsis, including those for F-box proteins (see Refs. 11 and 30). BLASTP searches were repeated using the SMART- and PFAM-predicted BTB domains from all 48 proteins as queries; this search recovered 29 additional loci with sequence scores beneath the 0.02 cutoff. This process was repeated a third time with these additional sequences and recovered two more loci. Finally eight other BTB(POZ) domain E3s were recovered from the six-frame translated Arabidopsis genome. These searches recovered one additional locus (At1g22104), which was subsequently predicted by hand analysis to encode a BTB domain. Additional BLAST searches against the Arabidopsis protein database with all 80 predicted BTB domains recovered no additional sequences beneath the cutoff score. The annotation of At2g19850 was revised to include an additional exon and intron at the 5′-end based on sequence alignments with its close sequence relative At1g50280. For At5g19000, alignment of the genomic sequence with that of a corresponding cDNA recovered by a Y2H screen necessitated changes in The Arabidopsis Information Resource-predicted sequence that combined into one intron: 23 bp upstream of the second intron, the second intron, the third exon, and the third intron. For At2g0440, the annotation was revised based on sequence alignments with the closest relative At2g04450, resulting in extension of the second predicted exon to a stop codon and removal of the predicted third and fourth exons. At3g03510 was revised based on sequence similarity to At5g17850, which altered annotation of the first predicted intron. See Supplemental Data Set 5 for the revised sequences.

The SMART/PFAM data bases predicted BTB domains in 79 of the 80 proteins. The outer limits of the domain were refined by hand analysis based on sequence alignments. These adjusted domains were used in the final alignments. For At2g13860, the BTB domain was predicted by sequence alignment and hand analysis. For the three genes (At1g04390, At2g04074, and At2g30600) that encoded proteins with two separate BTB domains, the BTB domain with the lowest SMART or PFAM E-value was used.

Alignment and Phylogenetic Analysis—Using previously identified animal and Arabidopsis CUL sequences as queries, CUL and CUL-like proteins were identified by BLASTP of rice (Oryza sativa) using the Gramene comparative genome database (www.gramene.org/db/searches/blast). This search recovered 13 non-redundant rice sequences with E-values ≤ 0.000058.

Full-length sequences, BTB domains, and putative SKP1/BTB/DBD-binding regions were aligned using ClustalX PC version 1.81 (31). Unrooted phylogenetic trees were generated in MEGA2.1 (32) by the neighbor-joining method, using the Poisson distance method and pairwise deletion, and a 1,000× bootstrap replicate. Similar trees were obtained by phylogenetic analysis with ClustalX (31). Amino acid sequence alignments were calculated and displayed using MacBoxshade version 2.15 (Institute of Animal Health, Pirbright, UK). Additional domains were identified from the SMART and PFAM databases,
BLAST searches, sequence alignments, and the PROF (33) and COILS algorithms (www.ebi.ac.uk/Tools/coils_form.html). The J subfamily was compared with the NPH3 family.3

RT-PCR and DNA Gel Blot Analysis—For RT-PCR, total RNA isolated from 10-day-old seedlings by the TRIzol reagent was used as the template. Each first strand cDNA reaction was performed using gene-specific primers; CUL3a (primer B or D), CUL3b (primer E), ETO1 (primer H), EOL1 (primer J), or EOL2 (primer L or N), a PAE2-specific primer, 1 µg of RNA, and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Primers for CUL3a are: A, GCCAACGAGCTGCTACATCC; B, CCACAGGTGGTCAAGCTGA; C, GAGGGGTGTTAGCATGCTG; and D, GTGAGGCGTTTAAGCATCGAGTCG; for CUL3b are: E, ATGAGTAACTCAGAAGAGGAGAAATTTCC and ACAATCACAAGACTTGGT; for ETOL1 are: F, GTCCAAGCTCATGAACATGAG; and H, CAAAGTAGGGTCAAACTCGGT; primers for EOL2 are: K, GCCCAATCTCAACTTCATTTGA; L, CTACGCAAGGAAGAATATCCG; M, CTCTTGTTGCTTCAAGCTT; and N, CCATGTCAAGATCTTCTTGGG; the position of each is identified in Fig. 8C. The PAE2 primers are as described previously (10). RT-PCR products were confirmed by sequencing.

For RNA gel blot analysis, total RNA was isolated from Arabidopsis seedlings (10-day-old) by the extraction foliation buffer (20) in combination with either a left border (Lba1, TG-TGTTACATGAGCGGTAAAG) or a right border (CGTTCTAGTGACACCTGCGAGCA) T-DNA-specific primer. The cul3b-1 mutation was followed by PCR using the gene-specific primers GACCTCCCAAACAGTAATCTGGTTTATGC and TTGCCAAGATGCTTGGCAGC in combination with the border primer JL202 (CATTTTATAAACCAGCTCGAGATTGAC). For phenotypic analyses of the cul3a-1 and cul3b-1 mutants, seeds were surface-sterilized in 50% bleach, placed on half-strength Murashige and Skoog medium (Invitrogen)-containing agar and incubated at 4 °C for 2 days. The plates were then incubated at 21 °C in a 16-h light/8-h dark photoperiod. After 2 weeks the seedlings were genotyped and transferred to soil. Siliques of different stages of maturity were harvested, opened, and fixed for 15 min at room temperature in neutral buffered formalin (Histofix). They were stored in 70% ethanol with 5% gum arabic, 100 g of chloral hydrate, and 5 mL of glycerol in 30 mL of water. Cleared wild-type and mutant embryos were examined by differential interference contrast microscopy using a Leica DMLB microscope (Leica Microsystems AG, Wetzlar, Germany).

For phenotypic analyses of the eto1-1, eto1-2, eto1-12 mutants, wild-type and mutant seeds were surface-sterilized in 50% bleach, placed on half-strength Murashige and Skoog medium (minus sugar)-containing agar, incubated at 4 °C for 4 days, exposed to light for 1 h, and then moved to the dark at 23 °C for 3 days. Hypocotyl lengths were measured from microscope images using NIH Image (version 1.61).

Microarray Analysis of BTB Superfamily Gene Expression—Expression patterns of the Arabidopsis BTB gene family was determined by using Agilent Arabidopsis total RNA microarray for the full repertoire of gene-specific oligonucleotides for the near complete gene list of A. thaliana Col-0 (36). Each slide included a total of 26,090 oligonucleotide features and 192 mismatch negative control features. Wild-type Col-0 RNA was isolated from the Qiagen RNeasy plant miniprep kit (Valencia, CA) from roots of 6-day-old seedlings, seeds, rosette leaves from 3-week-old plants, cauline leaves and stem from 4-week-old plants, floral organs at flowering, and siliques 3 or 8 days after pollination. At least two independent biological RNA samples were used for each tissue/stage. Fluorescent labeling of probe, slide hybridization, washing and scanning, and data analysis were as described previously (36). Two to four high quality replicate data sets (three replicates for most) were obtained for each experiment with at least one quality data set from each independent RNaseA sample. A negative control cutoff value for determining genes that were "expressed" versus "not expressed" for each hybridization was obtained by ranking the values of the 192 negative control mismatch oligonucleotide features from low to high and using the 90% percentile spot (number 173) as the cutoff value.

RESULTS

Sequence Analysis of Arabidopsis and Rice CUL3 Proteins—Sequence alignments indicate that plants encode a highly diverse set of CULs (12, 14), but the functions of only a few have been described thus far. To help assign functions, we thoroughly characterized the Arabidopsis CUL family and identified CUL and CUL-like proteins in the near complete genome of the monocot rice (O. sativa). CULs are defined by the
E3s based on the three-dimensional structure of human SCF rice and the length of each is listed on the right. E2s are shown as ubiquitination sites in the target or RUB-binding sites in CULs. The amino acid organization of the CUL protein family. The positions of one or more signature domains important for their scaffolding functions. These include two α-helical domains (helices II and V) near the N terminus that bind specific adaptor domains (e.g., SKP and Elongin B) and a CUL homology domain, a portion of which binds RBX1 (4, 23) (Fig. 1B). Many CULs also share a positionally conserved Lys for RUB1/NEDD8 attachment whose reversible recruitment appears to play an important role in resetting CUL-type E3 complexes during their ubiquitination cycles (37).

In our exhaustive search of the Arabidopsis genomic database, no additional loci were evident beyond the 11 described previously (12, 14) (Fig. 1B). In rice, 13 genes predicted to encode CUL proteins were recovered (Supplemental Fig. 1). Phylogenetic analysis with representative CULs from other eukaryotes allowed us to cluster most into six separate clades that potentially reflect their functional specificity (Fig. 1C). Like CUL1 orthologs in other eukaryotes (38–42), AtCUL1 directly interacts with RBX1 and SKP1/F-box target recognition modules to form SCF E3s (11, 34, 43). AtCUL2a has been suggested to interact with SKP1/F-box target recognition modules by Y2H analysis (14). A third CUL1-like locus also exists, designated AtCUL2b (At1g43140); it contains a frameshift in the coding region that should direct the synthesis of a truncated protein missing the CUL homology domain and downstream sequence (14). Although AtCUL2b expression was not apparent from the EST database (www.Arabidopsis.org) or the Massively Parallel Signature Sequencing databases (mpss.udel.edu/at/java.html (44)), we detected possible low level expression in leaves and sepals by DNA microarray analysis (data not shown). Rice encodes three canonical CULs that cluster within the plant CUL1/2 group (GRMP00000100454, GRMP00000100813, and GRMP0000039783), which we predict form SCF-type E3s and thus were named OsCUL1a, OsCUL1b, and OsCUL1c, respectively (Supplemental Fig. 1).

Members of the CUL2 and CUL5 groups can be found in mammals, Drosophila, and C. elegans but not in the yeasts S. cerevisiae and S. pombe. No Arabidopsis or rice orthologs of CUL2/5, Elongin B, Elongin C, or proteins bearing a SOCS box motif were evident in the near complete genomic databases, strongly suggesting that plants do not utilize this E3 subtype. Arabidopsis and rice each encode one CUL isoform that clustered within the CUL4 group (At5g46210 and GRMP0000027181, designated AtCUL4 and OsCUL4, respectively) (Fig. 1C). A single CUL-related protein was detected in Arabidopsis and rice (GRMP0000143862) that is similar to the APC2 subunit of the animal APC E3 complex (7).

Many of the remaining Arabidopsis and rice CUL and CUL-like proteins are substantially smaller in size than the canonical CULs. Although they are missing obvious CUL homology domains and the RUB1/NEDD8 attachment sites, these truncated forms still contain many of the N-terminal features found in the canonical CULs, including a predicted series of N-terminal α-helices II and V near the N terminus that bind specific adaptor domains (e.g., SKP and Elongin B) and a CUL homology domain, a portion of which binds RBX1 (4, 23) (Fig. 1B). Many CULs also share a positionally conserved Lys for RUB1/NEDD8 attachment whose reversible recruitment appears to play an important role in resetting CUL-type E3 complexes during their ubiquitination cycles (37).

In our exhaustive search of the Arabidopsis genomic database, no additional loci were evident beyond the 11 described previously (12, 14) (Fig. 1B). In rice, 13 genes predicted to encode CUL proteins were recovered (Supplemental Fig. 1). Phylogenetic analysis with representative CULs from other eukaryotes allowed us to cluster most into six separate clades that potentially reflect their functional specificity (Fig. 1C). Like CUL1 orthologs in other eukaryotes (38–42), AtCUL1 directly interacts with RBX1 and SKP1/F-box target recognition modules to form SCF E3s (11, 34, 43). AtCUL2a has been suggested to interact with SKP1/F-box target recognition modules by Y2H analysis (14). A third CUL1-like locus also exists, designated AtCUL2b (At1g43140); it contains a frameshift in the coding region that should direct the synthesis of a truncated protein missing the CUL homology domain and downstream sequence (14). Although AtCUL2b expression was not apparent from the EST database (www.Arabidopsis.org) or the Massively Parallel Signature Sequencing databases (mpss.udel.edu/at/java.html (44)), we detected possible low level expression in leaves and sepals by DNA microarray analysis (data not shown). Rice encodes three canonical CULs that cluster within the plant CUL1/2 group (GRMP00000100454, GRMP00000100813, and GRMP0000039783), which we predict form SCF-type E3s and thus were named OsCUL1a, OsCUL1b, and OsCUL1c, respectively (Supplemental Fig. 1).

Members of the CUL2 and CUL5 groups can be found in mammals, Drosophila, and C. elegans but not in the yeasts S. cerevisiae and S. pombe. No Arabidopsis or rice orthologs of CUL2/5, Elongin B, Elongin C, or proteins bearing a SOCS box motif were evident in the near complete genomic databases, strongly suggesting that plants do not utilize this E3 subtype. Arabidopsis and rice each encode one CUL isoform that clustered within the CUL4 group (At5g46210 and GRMP0000027181, designated AtCUL4 and OsCUL4, respectively) (Fig. 1C). A single CUL-related protein was detected in Arabidopsis and rice (GRMP0000143862) that is similar to the APC2 subunit of the animal APC E3 complex (7).

Many of the remaining Arabidopsis and rice CUL and CUL-like proteins are substantially smaller in size than the canonical CULs. Although they are missing obvious CUL homology domains and the RUB1/NEDD8 attachment sites, these truncated forms still contain many of the N-terminal features found in the canonical CULs, including a predicted series of N-terminal α-helices II and V near the N terminus that bind specific adaptor domains (e.g., SKP and Elongin B) and a CUL homology domain, a portion of which binds RBX1 (4, 23) (Fig. 1B). Many CULs also share a positionally conserved Lys for RUB1/NEDD8 attachment whose reversible recruitment appears to play an important role in resetting CUL-type E3 complexes during their ubiquitination cycles (37).

Arabidopsis BTB Ubiquitin Ligases

Fig. 1. Structure of CUL-based E3s and organization of the rice and Arabidopsis CUL family. A, organization of the CUL-based E3s based on the three-dimensional structure of human SCFTrc, S2C2, and VBCVHL E3 complexes (4, 23, 73). Binding of the substrate to the target recognition factor triggers Ub transfer from a Ub-E2 intermediate bound to the RBX1-CUL subcomplex. K, lysines that serve as ubiquitination sites in the target or RUB-binding sites in CULs. B, protein organization of the Arabidopsis CUL protein family. The positions of the various signature domains are indicated. The amino acid length of each is listed on the right. C, unrooted phylogenetic tree of the
Os sequences (Fig. 1) clustered together in a distinct clade with other eukaryotic CUL3 genes. The two Arabidopsis isoforms, AtCUL3a (At1g26830) and AtCUL3b (At1g69670), are 88% identical to each other and share between 35 and 51% sequence identity and 49 and 63% similarity to their animal counterparts. These two proteins along with three rice sequences (GRMP0000153145, GRMP0000147914, and GRMP0000096442, which we have named OsCUL3a, OsCUL3b, and OsCUL3c, respectively) and one more distantly related and shorter rice sequence (GRMP0000147911) clustered together in a distinct clade with other eukaryotic CUL3 sequences (Fig. 1C). Included in this group are CeCUL3, HsCUL3, and SpCUL3, which were demonstrated previously to interact with BTB domain-containing proteins (15–19). Sequence alignments of representative CUL3 proteins show that the plant versions are strongly related to their animal and fungal counterparts especially around the helix II and V regions (Fig. 1D). In particular, the conserved Leu-47, Ser-48, Phe-49, and Glu-50, residues that are predicted to be critical for the interaction of C. elegans SKP1 and DDB1-type adaptors, respectively.

Both Arabidopsis and rice encode potential orthologs of animal and S. pombe CUL3. The two Arabidopsis isoforms, AtCUL3a (At1g26830) and AtCUL3b (At1g69670), are 88% identical to each other and share between 35 and 51% sequence identity and 49 and 63% similarity to their animal counterparts. These two proteins along with three rice sequences (GRMP0000153145, GRMP0000147914, and GRMP0000096442, which we have named OsCUL3a, OsCUL3b, and OsCUL3c, respectively) and one more distantly related and shorter rice sequence (GRMP0000147911) clustered together in a distinct clade with other eukaryotic CUL3 sequences (Fig. 1C). Included in this group are CeCUL3, HsCUL3, and SpCUL3, which were demonstrated previously to interact with BTB domain-containing proteins (15–19). Sequence alignments of representative CUL3 proteins show that the plant versions are strongly related to their animal and fungal counterparts especially around the helix II and V regions (Fig. 1D). In particular, the conserved Leu-47, Ser-48, Phe-49, and Glu-50, residues that are predicted to be critical contact points for the interaction of C. elegans and S. pombe CUL3 with their respective BTB proteins MEL-26 and BTB3 (16–18) are conserved in the Arabidopsis and rice CUL3s but not in the other CUL isoforms (Fig. 1, D–F).

CUL3a and CUL3b are Essential for Arabidopsis Embryo Development—To define the roles for CUL3a and CUL3b in Arabidopsis development, we identified T-DNA lines containing insertions in the coding regions of these two genes (Fig. 2A). The cul3a-1 allele (SALK_SO46638) in the Col-0 ecotype contains a T-DNA insert within the second exon with the left border junction at nucleotide 1801 (from the translation start site). The right border junction is at nucleotide 1815, implying that the insertion caused a 14-base pair deletion. The cul3b-1 allele, obtained from the University of Wisconsin T-DNA collection in the Wassilewskija ecotype, contains a T-DNA insertion near the end of the first exon with the left border junction occurring at nucleotide 152 of the coding region. We failed to detect the CUL3a or CUL3b transcripts in the corresponding homozygous cul3b-1 and cul3b-1 seedlings by RT-PCR or the CUL3b mRNA in the cul3b-1 seedlings by RNA gel blot analysis (Fig. 2, B and C). As a consequence, it is likely that that these mutants represent null alleles.

Plants homozygous for either the cul3a-1 or cul3b-1 mutations appear to germinate and develop normally under standard growth conditions, indicating that neither locus is essential by itself (data not shown). To test for functional redundancy, we attempted to generate individuals homozygous for both mutations. Double heterozygous cul3a-1 cul3b-1 F1 plants from a cross of single homozygous plants also grew normally. We then analyzed the F2 progeny generated by self-pollination. In a population of 97 F2 seedlings, we identified all expected allelic combinations by PCR genotyping except individuals that were homozygous for insertions in both loci, strongly suggesting that at least one intact CUL3 locus is required for Arabidopsis embryogenesis. To confirm this possibility, we identified by PCR individuals that were homozygous for one mutation and heterozygous for the other. These cul3a-1/CUL3a cul3b-1/CUL3b and cul3a-1 cul3b-1/CUL3b plants appeared indistinguishable from wild-type plants and flowered normally, indicating that at least one wild-type allele of CUL3 is sufficient (Fig. 3A). PCR genotyping of 59 progeny from self-pollination of these plants also failed to identify any individuals homozygous for insertions at both loci, confirming the importance of the CUL3 loci.

Upon close examination of the siliques following self-pollination of both sets of cul3a1/cul3b homozygous/heterozygous plants, an obvious defect in embryogenesis was evident. In addition to seeds that developed normally, a class of defective seeds emerged that likely represented double homozygous individuals. Initially these defective seeds expanded normally but instead of turning green as they matured, these seeds remained white and later became brown and shriveled (Fig. 3B). In addition to these, a smaller percentage of the developing seeds failed to expand and remained as small nubs attached to the funiculus, possibly representing embryos that underwent very early arrest (Fig. 3C). We also found empty spaces in the siliques that were the either the result of reabsorption of early arrested seeds or possibly failed female gametogenesis. We were unable to accurately count the number of nubs and empty spaces. For the homozygous cul3a-1 parent, 17.5% of the seeds were defective versus 12.5% for the homozygous cul3b-1 mutant parent when we counted just the white/shriveled seeds (Table 1). Whether this difference means that more embryos from parents containing an intact CUL3b locus proceeded further in development and thus were counted by us as aborted seeds is not yet known.

To determine the stage of embryo arrest of the white-colored abnormal seeds, immature siliques from selfed cul3a-1/CUL3a cul3b-1/cul3b-1 and cul3a-1 cul3a-1/CUL3a cul3b-1/CUL3b plants were opened, fixed, and cleared by Hoyer’s solution, and the seeds were visualized by differential interference contrast mi-
Arabidopsis BTB Ubiquitin Ligases

As can be seen in Fig. 3D, these seeds contained embryos displaying the hallmarks of embryo arrest. Wild-type seeds from siliques of a similar developmental age and green seeds of the same siliques bearing the mutant seeds contained embryos at various late stages of embryogenesis, including torpedo and walking stick (45). In older siliques, fully mature embryos were found. In contrast, the white (and likely double homozygous mutant) seeds arrested at much earlier stages, including globular and heart stages, and never proceeded further. In a few mutant seeds, it appeared that the cotyledons began torpedo stage elongation, but hypocotyl elongation was not observed (Fig. 3D). The stage of embryo arrest was variable even within the same siliques, suggesting that this defect was not primarily caused by a block in a specific developmental checkpoint but more likely the result of a general growth inhibition of the embryo.

Arabidopsis CUL3a and CUL3b Interact with BTB Domain Proteins—To help define why Arabidopsis cul3a/b mutants are embryo-lethal, we exploited a Y2H assay to identify potential binding partners. To avoid biasing the screen, we used full-length CUL3a as bait to search two random cDNA libraries generated from Arabidopsis RNA. The two libraries, totaling ~1.8 x 10⁷ clones, were created from RNA derived from 3-day-old etiolated seedlings and 3-week-old seedlings subjected to an array of environmental stresses. These screens identified three clones that tested positive by growth on histidine dropout medium and by β-galactosidase assays. One prey was a predicted full-length cDNA derived from locus At4g08455, whereas the other two were partial cDNAs that encoded amino acids 126 through 326 of locus At1g21780 or amino acids 176 through 407 of locus At5g19000.

Subsequent directed Y2H interactions showed that the three prey specifically bound full-length versions of both CUL3a and CUL3b (Fig. 4A). By comparing their derived amino acid sequences, we discovered that the three prey contained a conserved region predicted by the SMART and PFAM databases to be a BTB domain (46, 47) (Fig. 4B). Although scans of the full-length protein sequences of At4g08455 and At1g21780 failed to predict additional protein motifs, a meprin and TRAF homology (MATH) motif was evident N-terminal to the BTB domain in At5g19000 (Fig. 4B). In C. elegans, humans, and plants, similar pairings of MATH and BTB domains have been observed with the proteins confirmed as CUL3 interactors (15, 17, 18). Both the truncated (without MATH domain) and full-length forms of At5g19000 interacted with CUL3a and CUL3b, indicating that the MATH domain is not essential for CUL3 binding and that the BTB domain alone mediates the CUL3 interaction (Fig. 4A).

The S. pombe and animal CUL3s also associate with RBX1 to form BTB E3 complexes (15, 16). To confirm that binding of the three BTB proteins was specific for CUL3 and that the CUL3-BTB complex would in turn bind RBX1 to form BTB E3s, we tested by Y2H assay various combinations of CUL3a, CUL3b, and CUL1 with RBX1, ASK1, and the representative F-box protein UFO. As can be seen in Fig. 4A, both CUL3s along with CUL1 interacted with the common RBX1 factor (Fig. 4A).

**TABLE I**

|            | WT (green) | Mutant (white/brown) | Total | Percent mutant |
|------------|------------|----------------------|-------|---------------|
| cul3a-1/cul3a-1 cul3b-1/CUL3b | 534        | 113                  | 647   | 17.5          |
| cul3a-1/CUL3a cul3b-1/cul3b-1  | 1,312      | 187                  | 1,499 | 12.5          |
| CUL3a/CUL3a CUL3b/CUL3b        | 361        | 2                    | 363   | 0.55          |
CUL3 failed to bind ASK1, and CUL1 failed to bind any of the three BTB proteins. In contrast, CUL1 bound to ASK1, and ASK1 in turn interacted with UFO with the four-subunit complex (UFO-ASK1-CUL3-RBX1) likely forming the SCF complex (Fig. 4A).

**Arabidopsis Encodes 80 Putative BTB Domain-Containing Proteins**—Given the strong possibility that plant BTB proteins, like their animal and yeast counterparts, function in CUL3-based Ub ligases, we attempted to define the full complement in *Arabidopsis*. By reiterative NCBI BLASTP searches of the complete *Arabidopsis* genome using animal and yeast BTB sequences as queries and an empirically determined E-value cutoff of 0.02, we identified 80 loci encoding one or more BTB motifs. Preliminary SMART searches of the rice genome indicated that over 112 potential BTB proteins are present in this species as well. For several of the *Arabidopsis* loci, available ESTs predicted the presence of multiple splice variants. In three cases (At1g01640, At2g30600, and At3g05675), this alternative splicing changed the 5′-untranslated regions without affecting the protein-coding regions, whereas in two cases (At5g16000 and At5g55000), this alternative splicing changed the sequence of the C-terminal 4 and 17 residues, respectively. The corresponding BTB genes are spread throughout the *Arabidopsis* genome. Nearly all are singletons with just one example of apparent tandem duplication (At2g04040 and At2g04050).

Analysis of the amino acid sequences both up- and downstream of the BTB domain identified other protein-protein interaction motifs in members of the *Arabidopsis* BTB family, including ankyrin, armadillo, pentapeptide, and tetra-tricopeptide (TPR) repeats and transcriptional adapter zinc finger (TAZ), MATH, and NPH3 domains (Fig. 6B). Like BTB proteins in other eukaryotes (48), the signature BTB domain is typically near the N terminus. Interestingly several proteins in the *Arabidopsis* BTB superfamily have been described previously, including NPH3, ETO1, NPRI, POZ-BTB protein 1, RPT2, ARIA, BOP1, and the BTB-TAZ (BT) 1–5 family (see below). With the exception of ETO1 (49), the biochemical functions of these proteins are unknown.

As can be seen from an alignment of the total collection (Supplemental Fig. 2) and representative members (Fig. 5), a consensus BTB motif emerged that contains three conserved regions separated by short variable sequences. Importantly, the residues in the BTB domains of CcMEL-26 and SpBTP3, previously identified to be important for binding CUL3, are in these conserved stretches (16, 17), particularly Asp-2, His-20, Ile-60, Asp-62, Asp/Glu-120, Tyr-122, and Ile/Leu-125 (Fig. 5). Several *Arabidopsis* BTB domains appear to contain amino acid insertions of variable sizes and locations (Supplemental Fig. 2 and Fig. 5). Although such insertions have been noted in the BTB domains of other eukaryotes (48), their functional significance awaits the three-dimensional structure of this region.

Using the BTB domain alone, a phylogenetic tree of the complete *Arabidopsis* family was generated using MEGA2.1 and a bootstrap value of 1,000 (Fig. 6A). The tree clustered the family into 10 families containing from two to 31 members (designated A–J) with two families split further into two subfamilies (A1 and A2 and D1 and D2). The four *Arabidopsis* BTB proteins demonstrated by Y2H assay to bind AtCUL3 (Fig. 4 and Ref. 49) were found in four separate clades (A1, C, D2, and E), implying that other proteins within these same clades also interact with CUL3a/b. Weber et al. (50) recently supported this notion by showing that another member of the MATH domain-containing A1 family also binds CUL3a/b. For the remaining clades, representative BTB proteins were tested for binding with CUL3a/b by Y2H assay, but interactions were not apparent when compared with negative controls (data not shown). However, the fact that many of the residues predicted to be important for CUL3 binding in other organisms, including Asp-2, His-20, Ile-60, Asp-62, and Tyr-122 (16, 17), are also conserved in these clades suggests that they form a similar interface (Fig. 5). We note that some of the predicted BTB proteins lack one or more of these conserved residues. This list includes ETO1, which can bind AtCUL3a (49), indicating that only a subset of these residues may be necessary for BTB-CUL3 interaction.

When the BTB tree in Fig. 6A was color-coded for the presence of other motifs predicted by SMART, a similar clustering was evident, thus providing independent support for the groupings. For example, all six MATH domain-containing, all five TAZ motif-containing, and all 30 of the NPH3 domain-containing BTB proteins were grouped together in the separate A1, E, and J clades, respectively. The only exception was the ankyrin repeat protein At2g04740, which failed to group with the other ankyrin-containing members within the G family. At2g04740 has a significantly different structure than the six members of the G family, suggesting it is not evolutionarily related to these ankyrin-containing proteins. The eight BTB proteins in the D1 clade are substantially shorter (165–282 residues in length) and contain only the BTB domain. Similar BTB-alone proteins exist in *S. pombe* and *C. elegans* and where tested were found to bind CUL3 (16, 17). The absence of obvious target-binding elements implies either a novel role or that a second target recognition component is required, much like the SKP1/F-box hybrid in SCF E3s.

Like most *Arabidopsis* F-box proteins (11), few in the BTB
collection appear to have obvious sequence orthologs in other eukaryotes. For example, no yeast and animal BTB proteins have been found to be associated with armadillo, TPR, and NPH3 domains (48). Of the 32 previously identified NPH3-containing proteins in *Arabidopsis* (26), 30 also contain a BTB domain and cluster in the J family. In addition, 24 members of FIG. 5.

Sequence alignment of representative *Arabidopsis* BTB domains. The ~120-amino acid core BTB sequences from bric-a-brac, tramtrack, and MEL-26 were aligned with representatives for each of the 12 *Arabidopsis* BTB protein groups by ClustalX and displayed with MacBoxshade using a threshold of 55%. Conserved and similar amino acids are shown in black and gray boxes, respectively. Dashes denote gaps. Designations on the left identify the BTB family (see Fig. 6) and *Arabidopsis* Genome Initiative number for each protein. Members shown to interact with CUL3a or CUL3b by Y2H assay (Fig. 4 and Refs. 49 and 50) are underlined. Asterisks mark the amino acid positions important for the CUL3-BTB interactions between *C. elegans* and *S. pombe* CUL3 and MEL-26/BTB3 (16, 17). Predicted α helices and β sheets are indicated by the boxes and bold lines, respectively. A full alignment of all 80 BTB motifs from *Arabidopsis* can be found in Supplemental Fig. 2.

FIG. 6.

Phylogenic tree of the complete BTB protein superfamily from *Arabidopsis*. A, the ~120-amino acid BTB domains from all 80 possible BTB proteins were aligned by ClustalX. The alignment was used to generate a non-rooted phylogenetic tree with MEGA2.1 using the Poisson distance method and a bootstrap value of 1,000. The 10 subfamilies identified from the phylogenic analysis are marked on the bottom. Individual members of the tree are color-coded by the nature of the domain(s) either N- or C-terminal to the BTB domain. Where possible, designations for proteins previously identified by other methods were used. Asterisks indicate members that interact with CUL3a or CUL3b by Y2H assay. An expanded version of the tree bearing the *Arabidopsis* Genome Initiative numbers for each locus can be found in Supplemental Fig. 3.

Ank, ankyrin; Arm, armadillo; CC, coiled coil; Pent, pentapeptide; CaM BD, calmodulin-binding domain. "A2," "D2," "F," and "H" identify sequences/motifs not yet annotated that are common to members of the respective clades. B, grouping of the BTB subfamilies based on the nature of additional motifs outside of the BTB domain along with a protein diagram of representative members. aa, amino acids.
the J family contain a C-terminal coiled-coil motif, as predicted by SMART and/or COILS, that may serve as another protein-protein interaction site (26). One lone exception in the J group (At3g49990) was not predicted by PFAM to have an NPH3 domain and does not contain many of the conserved residues that define this motif. For several clades (A2, D2, F, and H), no additional motifs were predicted by SMART or PFAM. However, hand alignments identified one or more conserved regions that appear to be plant-specific and may be important for target recognition (Fig. 6B). For example, the five members of the H family all share a ~300-amino acid region of 32–63% similarity C-terminal to the BTB domain. Finally three members of the Arabidopsis BTB superfamily were predicted to have two separate BTB domains (At2g04740 and At1g04390 (I family) and At2g30600 (F family)).

The Arabidopsis BTB Superfamily Is Widely Expressed—Evidence in the literature supports the involvement of BTB proteins in a wide range of processes in Arabidopsis (see below), suggesting that the corresponding genes are expressed in many cell types. To examine this, we used a 70-mer oligonucleotide microarray to profile the expression of 75 of the 80 BTB genes in 12 RNA populations isolated from various tissues at one or more developmental stages (Fig. 7). We considered a gene to be expressed if the signal exceeded a calculated negative control cutoff value (see “Materials and Methods”). Not expressed genes are indicated on the graph by the absence of a value. BTB genes not represented on the chip are indicated by the asterisks. An expanded view of the data along with Arabidopsis Genome Initiative numbers for each gene can be found in Supplemental Fig. 4.

Overall the microarray data showed that mRNAs for most BTB genes are detectable in most tissues tested with some individual mRNAs accumulating to high levels (Fig. 7). Typically members from the same clade displayed similar expression patterns. For instance, the genes in the A1, E, and F families were nearly all highly expressed, whereas genes within the D1 subfamily failed to display expression in most tissues beyond non-expressed controls. In some cases, strong tissue/development-specific expression was evident even when compared with other members within the same family. For example, At1g30440 expression in pistils was induced ~4-fold following pollination to a level at least 2-fold higher than any other J family member in that tissue. Two members of the E family (At3g48360 and At15g63160) were highly expressed in rosette leaves, whereas another member of the E family (At5g67480) was highly expressed in stamens (Fig. 7). Other BTB genes showing very high level expression include At5g64330 and At2g30600 in petals, At2g46260 in stamens, and At5g45110 in roots. Transcripts for all five of the confirmed CUL3 interactors (this report and Refs. 49 and 50) were detected: mRNA abundance for ETO1, At1g21780, and At2g39760 was generally high, particularly in petals and roots, whereas the mRNA abundance for At5g19000 and At4g08455 was generally low with best expression detected in petals and roots, respectively.

Members of the TPR Subfamily of BTB Proteins Have Differing Functions—Members of many of the BTB subfamilies are substantially similar in amino acid sequence to each other, raising the possibility that BTB proteins within the subgroups share similar or overlapping functions. To test this possibility, we isolated 5’ and 3’ exonic T-DNA insertion mutants in the genes encoding each of the three members of the TPR (“C”) subfamily that includes ETO1 (At5g51770), EOL1 (At4g02680), and EOL2 (At5g58550) (Fig. 8C). These three BTB proteins have been implicated in the regulation of ethylene synthesis through the recognition and presumed ubiquitination of aminoacylpropane synthase 5 and related enzymes (49). The EOL1 and -2 proteins are 47 and 52.6% identical to ETO1, respectively, with all three displaying substantially overlapping patterns of expression (Fig. 7). Because null eol1 mutants generate a constitutive ethylene phenotype (49), we tested whether similar phenotype(s) could be generated by eol1 and eol2 mutants, presumably through stabilization of aminoacylpropane synthase 5 or its relatives.

As shown in Fig. 8B, T-DNA insertion mutants eol1, eol1, and eol2 were identified that dramatically block mRNA accumulation (as determined by RT-PCR). Consistent with previous studies (49, 52), the three homozygous alleles of eol1 (eol1-11 to -13) generated a constitutive ethylene “triple response” in etiolated seedlings, consisting of a shortening and swelling of the hypocotyl and an exaggerated apical hook, a phenotype that was presumably induced by ethylene overproduction (Fig. 8, C and D). In contrast, a similar triple response phenotype was not apparent for the eol1 and eol2 mutants. The single eol1-1 mutant and the three eol2 mutants (eol1-1 to -3) grew like wild type. Thus, despite their similar sequence, the three members of the C subfamily may not share identical functions in targeting aminoacylpropane synthase 5-type proteins for degradation.

Discussion
Recent interaction analyses of CUL3s from yeast and animals have led to the identification of the BTB-CUL3-RBX complex as another major class of E3s directing protein ubiquitination. In the complex, CUL3 serves to bring together the Ub-E2 docking protein RBX1 with a target recognition module composed of one of many BTB proteins that associate with CUL3 through its BTB domain. A reconstituted BTB<sup>Med-26</sup> E3, consisting of the BTB protein MEL-26, CUL3, and RBX1, has been shown to ubiquitinate a target (MEI-1) in vitro, thus confirming the Ub ligase activity of this complex (15). In this capacity, the single BTB proteins perform the same functions as the SKP1/F-box protein hybrid in SCF E3 complexes. Pre-
further support by showing that another Arabidopsis BTB protein containing a MATH domain interacts with CUL3a/b. Residues important for BTB docking by CUL3 and for CUL3 docking by the BTB domain in the yeast and animal versions (15–18) are strongly conserved in the Arabidopsis and rice counterparts, strongly suggesting that a similar interaction lattice is used to bind the plant BTB proteins to the CUL3a/b-RBX1 subcomplex. Although predicted to be similar to the mechanism used by CUL1 and -2 to bind the adapter proteins SKP1 in the SCF E3s (4, 22), sufficient differences within the docking interface must be present to prevent inappropriate interactions of CUL3 with SKP1 and CUL1/2 with BTB proteins.

By acting as recognition factors, BTB proteins target a diverse array of proteins for ubiquitination (and likely degradation), including those needed for mitotic spindle degradation in C. elegans (MEI-1) (15, 17, 24), oxidative and electrophilic stress gene expression in humans (NRF2) (19), and ethylene biosynthesis in Arabidopsis (aminocyclopropane synthase 5) (49), suggesting that CUL3-based E3s regulate a wide range of processes in different organisms. Their importance has been borne out by analysis of cul3 mutants. Although knock-outs of CUL3 in S. cerevisiae have no obvious phenotypes (53), RNA interference knock-downs of CeCUL3 disrupt microtubule-mediated processes (54), whereas deletion of the single CUL3 genes in S. pombe and mice caused abnormal cell elongation and slow growth (55) and misregulation of the cell cycle at S phase and embryo lethality, respectively (56). We show here that loss of both CUL3a and CUL3b in Arabidopsis generates an embryo-lethal phenotype. Although AtCUL1 knock-out mutants also result in embryo lethality, embryo arrest in these mutants consistently occurs very early in embryogenesis prior to the first cell divisions following fertilization (12), implying that the Ub ligases formed by CUL1 and CUL3a/b have distinct roles in Arabidopsis embryogenesis. That the CUL3a/cul3a-1 cul3b-1 cul3b-1 mutants and cul3a-1cul3a-1 CUL3b cul3b-1 seedlings are phenotypically similar to those found in animals (e.g. MATH/BTB proteins), a majority have sequences and domain structures that appear to be plant-specific. Absent from the Arabidopsis superfamily are two BTB protein types prominent in the animal kingdom, the actin-binding Kelch/BTB proteins and the DNA-binding zinc finger BTBs (restricted to vertebrates and arthropods) (48). Interestingly 97 members of the Arabidopsis F-box superfamily contain Kelch repeats, raising the possibility that the corresponding SCF E3 ligases have assumed the role(s) played by the missing Kelch-containing BTB E3s in plants. Also missing from the Arabidopsis superfamily is the BTB-like T1 tetramerization domain present in potassium channels (48). Taken together, this diversity among BTB proteins from different eukaryotes implies that the BTB domain has become connected to a variety of protein interaction domains during evolution.

Prior to our phylogenetic analysis, genetic studies linked individual BTB proteins to several processes in plants. For example, NPH3 and RPT2 were both discovered as proteins required for phototropism toward blue light where they interact with and act downstream of the PHOT1 and PHOT2 photoreceptors (26, 57). NPR1 regulates pathogen-response gene expression in the salicylic acid-mediated systemic acquired resistance defense pathway as well as regulating the jasmonic

---

**FIG. 8.** Description and phenotype of TPR (C) BTB subfamily mutants in Arabidopsis. A, diagram of the ETO1, EOL1, and EOL2 genes. Boxes and lines denote exons and introns, respectively. The positions of the T-DNAS are indicated by the arrowheads. The locations of the primers used for RT-PCR in B are indicated by the horizontal arrows. B, RT-PCR analysis of mRNA isolated from wild-type Col-0 and eto1-13, eto1-12, and eto1-11 mutants in Arabidopsis. Each primer is shown as a bar represents the average (±S.E.) of 10–14 seedlings. WT, wild type.
Arabidopsis BTB Ubiquitin Ligases

acid-mediated defense pathway (25). ARIA acts as a positive regulator of ascorbic acid responses possibly via direct interaction with the ascorbic acid response transcription factor AFB2 (28). BOP1 is involved in leaf morphogenesis and represses expression of class 1 KNOX genes, which regulate meristem cell activity (29). ETO1 was identified from a screen for Arabidopsis mutants in ethylene perception; it binds CUL3a and likely forms a BTBETO1 E3 that targets aminocyclopropane synthase 5 for degradation (49).

For other Arabidopsis BTB proteins, possible functions have been inferred biochemically. For example, Du and Poovaiah (51) have recently described the five TAZ members of the F family as calmodulin-binding proteins and designated them BT1–5. A conserved 24- amino acid region near their C termini contains the calmodulin-binding site (51). At2g13690 was first annotated as interacting with the WD protein PRL1, a protein that has pleiotropic effects on Arabidopsis hormone and sugar responses and development (58). PRL1 interacts with SNF1 protein kinases and with the SCF components ASK1 and CUL1 and a 26 S proteasome subunit (13), suggesting that PRL1 has a central role in Ub-mediated events. At5g55000, which is unique in the superfamily because it contains C-terminal pentapeptide repeats (Fig. 6), was found by Y2H analysis to interact with Arabidopsis formin homology 1 (59), a protein required for polar pollen tube extension by inducing actin cable assembly (60). Based on sequence homology, members of the F family are potential orthologs of a barley BTB protein, GMPOZ, isolated by its interaction with the gibberellin-inducible transcription factor GAMYB. Reductions in GMPOZ alter regulation of gibberellin- and abscisic acid-responsive genes in aleurone cells (61). One member of the F family named POZ-BTB protein 1 (At3g61600) was also identified in a screen for Arabidopsis cDNAs that could activate the glucose repression pathway in yeast (62).

Thus far, only a small number of the 80 Arabidopsis BTB proteins have been confirmed as CUL3-binding proteins (this report and Refs. 49 and 50); thus, it remains possible that some BTB proteins perform functions outside of ubiquitination. For example, certain animal BTB proteins also use their BTB domains for homo- and heterodimerization and appear to have functions that may not be reconciled solely with assembly into a BTB E3 complex. The DNA-binding C2H2 zinc finger domain (48) of class of proteins present in vertebrates and Drosophila acts as transcriptional regulators by using the BTB domain to assist in interactions with various co-repressor proteins and histone deacetylase I (63–65) as well as mediate homodimerization (66, 67) and heterodimerization (20). One member of this group, promyelocytic leukemia zinc finger, has been shown to interact with HsCUL3 via its BTB domain (15), thus raising the possibility that a single BTB protein can use its BTB domain to bind CUL3 itself, and other proteins. The BTB domains of the actin-associated Kelch repeat/BTB proteins of animals and poxviruses (including Keap1, Mayven, and Kelch) may also display a similar set of diverse interactions. Like promyelocytic leukemia zinc finger, the BTB domain in members of this group mediate dimerization (68–70), and one (Keap1) can interact with CUL3 to target breakdown of the transcription factor NrF2 (15, 19). In a similar fashion, our preliminary data with some members of the Arabidopsis BTB family indicate that they can homodimerize and heterodimerize with close family members, whereas those of Inada et al. (71) suggest that the BTB domain of BPT2 interacts with NPH3. Finally it is possible that some BTB proteins bind CUL3 not for the purpose of bringing targets to the E3 complex but so that they are ubiquitinated themselves (16). Such “self”-ubiquitination could play a role in regulating BTB protein levels similar to that reported for some F-box and SOCS box proteins (71, 72).

The discovery of CUL3-based BTB E3s further expands the diversity of Ub ligases in plants and brings the estimated number well beyond 1,300 in Arabidopsis when all types and isoforms are included (HECT, RING/U-box, APC, SCF, and BTB). However, because the five truncated CUL-type proteins from Arabidopsis await assignment, it is possible that the actual collection of E3s is considerably larger than this current prediction. The unusual organization of these shorter forms raises the intriguing possibility that plants contain other CUL-based E3s that are kingdom-specific. With 80 potential members, the BTB E3 family has the capacity to selectively ubiquitinate an equally large number of proteins. In fact, preliminary genetic analysis of just one of the BTB subfamilies encoding ETO1, EOL1, and EOL2 suggests that even closely related BTB proteins have non-redundant functions. Determining the roles of BTB proteins in Arabidopsis biology will be aided by reverse genetic analysis of the individual members as well as by biochemical/interaction studies directed toward identifying potential targets/interacting factors. Using ETO1, NPR1, NPH3, BT1–5, ARIA, and BOP1, which are involved in hormone biosynthesis, defense signaling, photoreception, Ca2+ signaling, hormone perception, and leaf morphogenesis as examples, BTB proteins clearly have the potential to play pervasive and diverse roles in plant biology.

Acknowledgments—We thank the Arabidopsis Biological Resource Center for providing the EST clones and SALK T-DNA lines, Eileen Maher and the University of Wisconsin-Madison Molecular Interaction Facility for work with the Y2H library screens, Ben Harrison and Dr. Donna Fernandez for assistance with the embryo microscopy and interpretation, and Dr. Xing-Wang Deng for access to microarray analyses.

Note Added in Proof—A complementary study of CUL3a and the BTB gene family in Arabidopsis was recently published by Dieterle et al. (Plant J. 41, 386–399). It demonstrates several BTB-CUL3 interactions by Y2H analysis, including one with At5g13060 that has not been described elsewhere.

REFERENCES

1. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
2. Smalle, J., and Vierstra, R. D. (2004) Annu. Rev. Plant Physiol. Plant Mol. Biol. 55, 555–590
3. Deshaies, R. J. (1999) Annu. Rev. Cell Dev. Biol. 15, 435–467
4. Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Poganas, S., Conaway, R. C., Conaway, J. W., Harper, J. W., and Pavletich, N. P. (2002) Nature 416, 703–709
5. Kile, B. T., Schulman, B. A., Alexander, W. S., Nicola, N. A., Martin, H. M., and Hilton, D. J. (2002) Trends Biochem. Sci. 27, 235–241
6. Wertz, I. E., O’Rourke, K. M., Zhang, Z., Dornan, D., Arnot, D., Deshaies, R. J., and Dixit, V. M. (2004) Science 303, 1371–1374
7. Capron, A., Serralho, O., Fulop, K., Frugier, F., Farinetti, Y., Dong, A., Lecureuil, A., Guerche, P., Kondorosi, E., Scheres, B., and Genschik, P. (2005) Plant Cell 17, 2370–2382
8. Azevedo, C., Santos-Rosa, M. J., and Shirasu, K. (2001) Trends Plant Sci. 6, 354–358
9. Capron, A., Okresz, L., and Genschik, P. (2003) Trends Plant Sci. 8, 83–89
10. Downes, B. P., Stupar, R. M., Gingerich, D. J., and Vierstra, R. D. (2003) Plant J. 35, 729–742
11. Gagne, J. M., Onimura, A., Basu, S. H., Durski, A. M., and Vierstra, R. D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11519–11524
12. Shibahara, T., Parmentier, V., Hellmann, H., Lechner, E., Dong, A., Masson, J., Granier, F., Lepinec, L., Estelle, M., and Genschik, P. (2002) Mol. Biol. Cell 13, 1916–1928
13. Farrar, R., Ferrando, A., Jazik, J., Kleinhov, T., Okresz, L., Tubiric, A., Salchert, K., del Pozo, C., Schell, J., and Ronce, C. (2001) EMBO J. 20, 2742–2756
14. Rissewuk, R. P., Daskaloudh, T. E., Banks, T. W., Liu, E., Cotelesage, J., Hellmann, H., Estelle, M., Somers, D. E., and Crosby, W. L. (2003) Plant J. 34, 753–767
15. Furukawa, M., He, Y. J., Borchers, C., and Xiong, Y. (2003) Nat. Cell Biol. 5, 1001–1007
16. Geyer, R., Wee, S., Anderson, S., Yates, J., and Wolf, D. A. (2003) Mol. Cell 12, 783–790
17. Xu, L., Wei, Y., Rebold, J., Vaglio, P., Shin, T. H., Vidal, M., Elledge, S. J., and Harper, J. W. (2005) Nature 435, 316–321
18. Pintard, L., Willis, J. H., Willems, A., Johnson, J. L., Shrayo, M., Kurz, T., Glaser, S., Mains, P. E., Tyers, M., Bowerman, B., and Peter, M. (2003) Nature 425, 311–316
