Arabidopsis AtCUL3a and AtCUL3b Form Complexes with Members of the BTB/POZ-MATH Protein Family[1]

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The ubiquitin proteasome pathway in plants has been shown to be important for many developmental processes. The E3 ubiquitin-protein ligases facilitate transfer of the ubiquitin moiety to substrate proteins. Many E3 ligases contain cullin proteins as core subunits. Here, we show that Arabidopsis (*Arabidopsis thaliana*) AtCUL3 proteins interact in yeast two-hybrid and in vitro pull-down assays with proteins containing a BTB/POZ (broad complex, tramtrack, bric-a-brac/pox virus and zinc finger) motif. By changing specific amino acid residues within the proteins, critical parts of the cullin and BTB/POZ proteins are defined that are required for these kinds of interactions. In addition, we show that AtCUL3 proteins assemble with the RING-finger protein AtRBX1 and are targets for the RUB-conjugation pathway. The analysis of AtCUL3a and AtCUL3b expression as well as several *BTB/POZ-MATH* genes indicates that these genes are expressed in all parts of the plant. The results presented here provide strong evidence that AtCUL3a and AtCUL3b can assemble in Arabidopsis with BTB/POZ-MATH and AtRBX1 proteins to form functional E3 ligases.

The ubiquitin proteasome pathway participates in a broad variety of physiologically and developmentally controlled processes in plants (for an overview, see Smalle and Vierstra, 2004). A critical step involves E3 ubiquitin ligases that facilitate the transfer of ubiquitin moieties to a substrate protein, leading to degradation via the 26S proteasome.

In Arabidopsis (*Arabidopsis thaliana*), the best-characterized E3s are the SCF (Skp1-cullin-F-box) complexes that consist of at least four subunits: a cullin protein (AtCUL1), an ASK (Arabidopsis Skp1 ortholog) protein, a RING finger protein (RBX1), and an F-box protein (Gray et al., 1999, 2001; Lechner et al., 2002; Shen et al., 2002). The cullin is a scaffolding subunit for the SCF and binds the ASK-F-box and RBX1 subunits within NH_2- and COOH-terminal domains, respectively (Zheng et al., 2002). F-box proteins are substrate adaptors that confer specificity to the assembled SCF complexes (Gagne et al., 2002). AtCUL1-dependent E3s are crucial regulators for phytohormone responses, flowering, embryo development, and other processes (Smalle and Vierstra, 2004).

At least 11 cullins are encoded in the Arabidopsis genome (Shen et al., 2002). However, only six members contain the conserved RUB (related to ubiquitin)-modification site characteristic of cullins that assemble into an SCF or related E3 complex (del Pozo and Estelle, 1999; Querido et al., 2001; del Pozo et al., 2002). Based on homology to cullins in other organisms, these six Arabidopsis cullins can be classified into three groups. The first group is the CUL1 family, which includes AtCUL1 (At4g02570), AtCUL2 (At1g02980), and AtCUL5 (At1g43140). It is likely that all three members participate in an SCF complex (Shen et al., 2002; Risseeuw et al., 2003). The second group comprises the CUL3 family with AtCUL3a (At1g26830) and AtCUL3b (At1g69670). These two cullins are approximately 88% identical to each other and represent potential Arabidopsis orthologs of *Caenorhabditis elegans* and human CUL3 proteins (Pintard et al., 2003). Finally, AtCUL4 (At5g46210) is distinct from the other Arabidopsis cullins and more closely resembles human and mouse CUL-4a (Shen et al., 2002), here referred to as the CUL4 family. For the Arabidopsis CUL3 family and for CUL4, the nature of the E3 complexes that they contribute to is unknown.

Recently, it was demonstrated that *C. elegans* and human cullin 3, CeCUL3 and HsCUL3, respectively, assemble with proteins containing a BTB/POZ (broad complex, tramtrack, bric-a-brac/pox virus and zinc finger) domain defining a new class of E3 ligases (Furukawa et al., 2003; Pintard et al., 2003; Xu et al., 2003). The BTB/POZ domain, found in eukaryotes and some viruses, is a highly conserved protein motif with a length of around 116 amino acids that folds into 5 β-sheets and 6 α-helices (Zollman et al., 1994; Ahmad et al., 1998). The domain has been shown to be involved in homophilic and heterophilic interactions

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(Bardwell and Treisman, 1994). Many BTB/POZ proteins are transcriptional regulators containing a C_2H_2 domain for DNA binding (Tsukiyama et al., 1994; Dong et al., 1996; Huynh and Bardwell, 1998). The BTB/POZ domain, however, can also be found in combination with other protein-protein interaction motifs, such as ANKYRIN, ARMADILLO, or MATH (meprin and TRAF [tumor necrosis factor receptor-associated factor] homolog), indicating its involvement in diverse biological processes (David et al., 1998; Csankovszki et al., 2001). Interestingly, CUL3 proteins appear to have specific interactions across a broad range of proteins. For example, Furukawa et al. (2003) reported interaction of HsCUL3 with more than a dozen different BTB/POZ proteins containing secondary domains such as KELCH, zinc finger, or MATH.

Similar to CUL1-based E3 ligases, interaction with the BTB/POZ proteins occurs within an NH_2-terminal region of the CUL3 proteins (Pintard et al., 2003). It is noteworthy that SKP1 and related proteins also contain a motif structurally resembling the BTB/POZ fold that is required for interaction with CUL1 (Schulman et al., 2000). A comparable situation has been described for elongin C, a cullin-interacting subunit of the VBC (von-Hippel-Lindau-Elongin B-Elongin C) E3 complex (Zheng et al., 2002).

Although these findings indicate a general requirement for NH_2-terminal cullin protein interactions, complex assembly appears to be highly specific among the different cullin families. For example, CeCUL3 does not interact with members of the SKP1 family, and cullins of SCF and VBC complexes do not interact with the BTB/POZ proteins that bind CeCUL3 and HsCUL3 (Furukawa et al., 2003; Pintard et al., 2003; Xu et al., 2003). In Arabidopsis, it is also reported that AtCUL3a and AtCUL3b do not interact with members of the Arabidopsis SKP1 family (Risseeuw et al., 2003). One of the main differences between the SCF and VBC E3 ligases and the BTB/POZ-binding E3s is the mechanism of substrate binding to the E3 complex. Whereas binding of the substrate in SCF and VBC E3 ligases requires secondary adaptors (Jackson et al., 2000), BTB/POZ proteins interact directly with both the cullin and the substrate (Pintard et al., 2003). This has been demonstrated for the BTB/POZ-MATH protein CeMel-26 from *C. elegans*, which interacts with CeCUL3 via its BTB/POZ domain and also with the substrate protein CeMEI-1 via its secondary MATH domain (Furukawa et al., 2003; Pintard et al., 2003; Xu et al., 2003).

Because of the characteristics of BTB/POZ domains and their conservation between human and *C. elegans*, it is likely that Arabidopsis proteins possessing these motifs interact with Arabidopsis cullins of the CUL3 family to assemble into functional E3 ligases. Using yeast two-hybrid (Y2H) and in vitro assays, we show that Arabidopsis AtCUL3a and AtCUL3b interact with members of the BTB/POZ-MATH protein family. As in *C. elegans*, interaction between the cullins and BTB/POZ proteins requires cullins with an intact NH_2-terminal domain. In addition, the BTB/POZ-MATH proteins form homodimers and heterodimers through their BTB/POZ domain. We also show that AtRBX1 assembles in Y2H and in vitro assays with the cullins, and that both proteins are targets for modification by the ubiquitin-related protein RUB1. In summary, our results provide strong evidence for the presence of functional AtCUL3-BTB/POZ E3 ligases in plants.

RESULTS

The BTB/POZ-MATH Protein Family in Arabidopsis

Recent findings in *C. elegans* and humans showed that CUL3 proteins can broadly interact with proteins carrying a BTB/POZ domain (Furukawa et al., 2003; Pintard et al., 2003). Some of these proteins, such as MEL-26 and the nuclear speckle-type POZ (Spop) protein, have a secondary MATH domain. In *C. elegans* and humans, Spop proteins have been implicated in transcriptional control, chromatin folding, and actin binding (Takahashi et al., 2002; La et al., 2004). We decided to focus on related BTB/POZ-MATH proteins from Arabidopsis. There are six such proteins encoded in the Arabidopsis, all of which show closest homology to the Spop class of proteins with approximately 30% amino acid sequence identity (Nagai et al., 1997). Based on the presence of the BTB-POZ and MATH domains, the six Arabidopsis genes were named *AtBPM1* (*At5g19000*), *AtBPM2* (*At3g06190*), *AtBPM3* (*At2g39760*), *AtBPM4* (*At3g03740*), *AtBPM5* (*At5g21010*), and *AtBPM6* (*At3g43700*) for *BTB/POZ-MATH*. All members of the AtBPM family are highly conserved within their domains and range in size from 410 to 421 amino acids with a predicted molecular mass of 44 to 50 kD. As in CeMEL-26 and Spop, the MATH domains are located within the NH_2-terminal half of the protein, whereas BTB/POZ domains are in the COOH-terminal region (Fig. 1). Phylogenetic analysis showed that the six AtBPM proteins can be classified into four subgroups with AtBPM1 and AtBPM2 in one group, AtBPM5 and AtBPM6 in a second, and AtBPM3 and AtBPM4 each in a separate group (Fig. 1B).

AtCUL3a and AtCUL3b Proteins Display Specific Interactions with Members of the AtBPM Family

To analyze interactions with AtCUL3a and AtCUL3b, cDNAs of four *AtBPM* (*AtBPM1*, *AtBPM3*, *AtBPM5*, and *AtBPM6*) genes and the two AtCUL3 cullins were cloned into Y2H vectors. As shown in Figure 2A, both cullins interacted equally well with AtBPM1. By contrast, AtCUL3a clearly showed less interaction with AtBPM3 compared to AtCUL3b. Interestingly, neither of the cullins interacted with AtBPM5 and AtBPM6. To verify interaction with AtBPM1, AtCUL3a and AtCUL3b were synthesized in a coupled transcription-translation reaction and

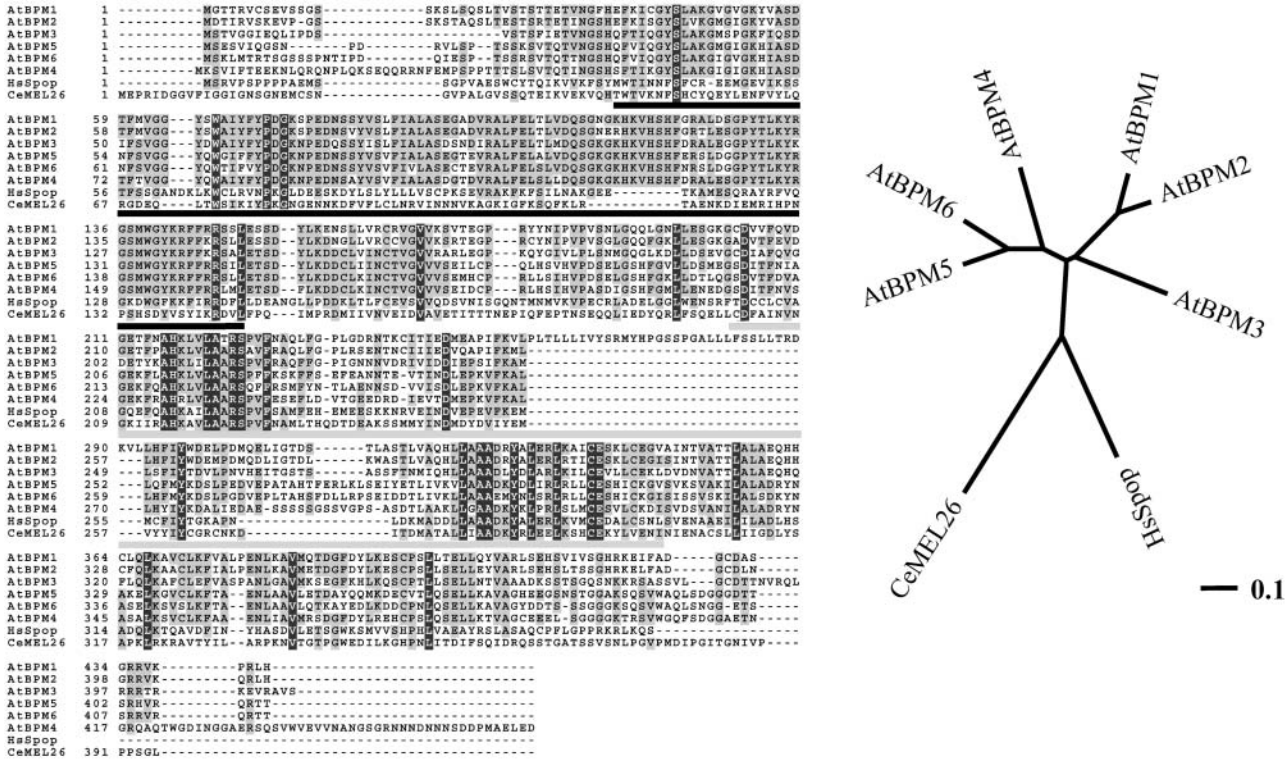

Figure 1. Protein alignment (left) and unrooted phylogenetic dendrogram (right) of the AtBPM family, CeMEL26 (NP_492449), and HsSpop (AAH03385). Underlined in black is the MATH domain, whereas light gray indicates the position of the BTB/POZ domain. Identical residues are in black and conserved amino acid residues are in gray, respectively. Line in the lower right of the dendrogram gives the distance between each branch (percentage divergence/100). Graphics were done on the Workbench Web site (http://workbench.sdsc.edu) using ClustalW, PHYLIP, and Boxshade version 3.3.1 programs. Trees were generated using the neighbor-joining method (Saitou and Nei, 1987); protein grouping was verified by bootstrapping (1,000 replicates, 111 random seed number; Felsenstein, 1985).

used for pull-down assays with bacterial expressed and purified GST (glutathione *S*-transferase) or GST::AtBPM1 proteins. In agreement with the Y2H data, interaction was only detectable with GST::AtBPM1, not with GST alone (Fig. 2C). As a further control for specificity of interactions, the closely related AtCUL4 protein was tested for interaction with AtBPM1 using Y2H and pull-down assays, and no interaction was observed between the two proteins (Fig. 2, A and C). In addition, the AtCUL3 proteins did not interact with members of the ASK family (data not shown). These findings are in agreement with both Pintard et al. (2003), who demonstrated specific assembly of *C. elegans* CeCUL3 with BTB/POZ proteins, and with Risseeuw et al. (2003), who also found that AtCUL3 proteins did not interact with members of the ASK protein family.

AtBPM Proteins Assemble to Homodimers and Heterodimers

The BTB/POZ domain has been reported to allow assembly of homodimers and heterodimers with other members of the BTB/POZ protein family (Bardwell and Treisman, 1994). To investigate whether this is true for the AtBPM family, combinatorial interaction studies of AtBPM1 with the other AtBPM proteins were performed. As shown in Figure 2B, AtBPM1 and AtBPM3 assemble into homodimers but are also capable of interacting with the other three AtBPM proteins to form heterodimers, although the AtBPM3-AtBPM6 interaction was weak. This is especially interesting since AtBPM5 and AtBPM6 did not appear to interact with either of the two AtCUL3 proteins. As in the case of AtCUL3a and AtCUL3b, the Y2H result for AtBPM1 was verified by using in vitro translated AtBPM1 protein for pull-down assays with GST or GST::AtBPM1 (Fig. 2C). Consistent with results from the Y2H assays, interaction was only detectable for GST::AtBPM1 proteins. Taken together, these findings indicate that interactions within the AtBPM family appear to be less specific compared to their assembly with cullin proteins.

AtCUL3a and AtCUL3b Interact with AtRBX1 and Are Targets for a RUB Modification

It has been shown in a variety of systems that E3 ligases that include members of the CUL1, CUL3, and CUL4 families also contain the RING finger protein

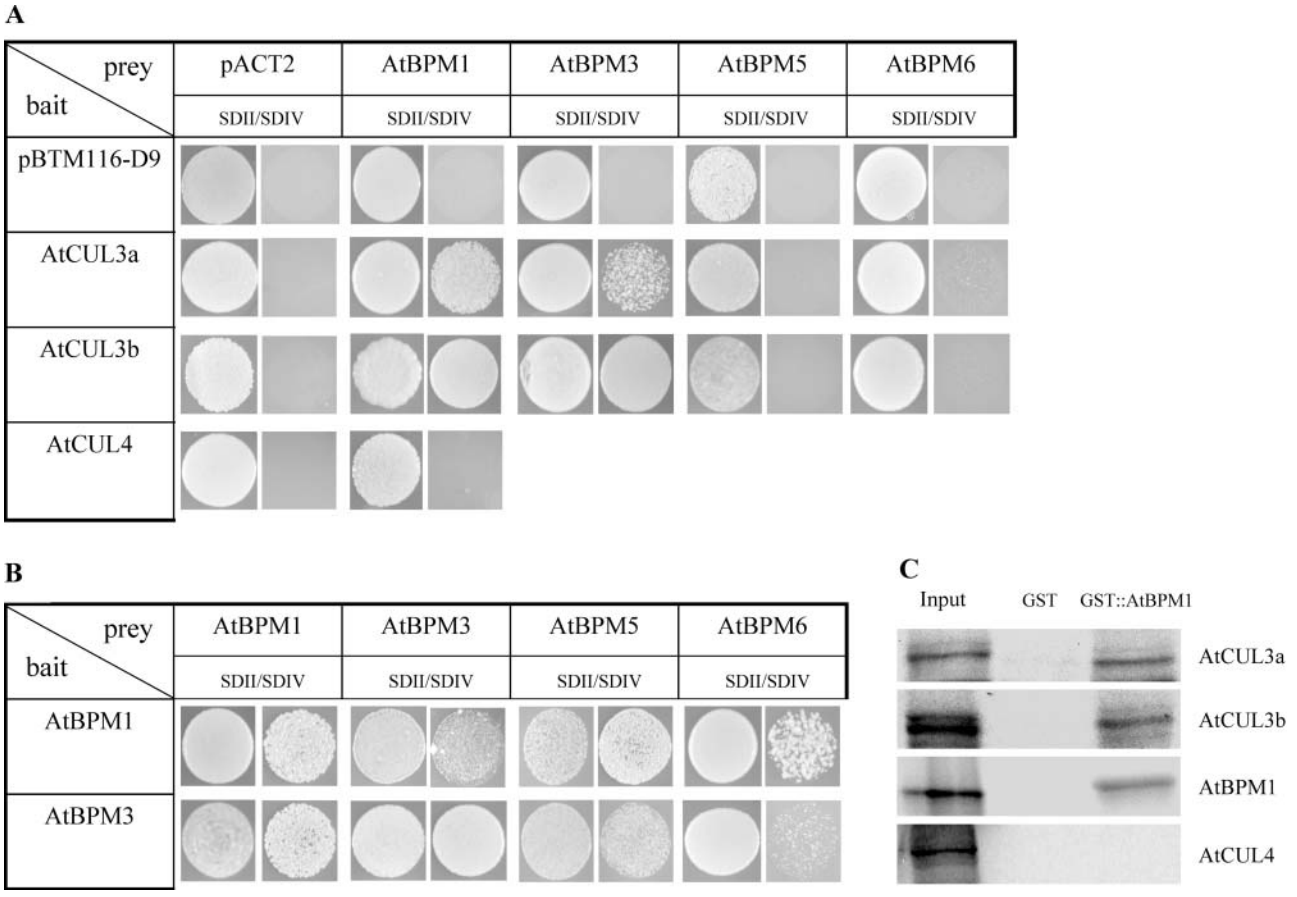

Figure 2. Interaction studies of AtCUL3a, AtCUL3b, AtCUL4, and selected AtBPM proteins. A, AtCUL3a and AtCUL3b can both interact in Y2H assays with AtBPM1 and AtBPM3 but not with AtBPM5 and AtBPM6. AtCUL4 was used only with AtBPM1 and did not show any interaction. B, Y2H interaction studies show that AtBPM1 and AtBPM3 assemble to homodimers and heterodimers with the other tested AtBPM proteins. C, Results from the Y2H studies were confirmed for AtCUL3a, AtCUL3b, AtCUL4, and AtBPM1 by pull-down assays with bacterially expressed and purified GST or GST::AtBPM1 proteins. First lane of each assay shows 1 μL of [^{35}S]Met-labeled protein used for pull downs. SDII, Selection medium for transformation with bait (*pBTM112-D9*) and prey (*pACT2*) plasmids supplemented with uracil and His; SDIV, selection medium for interaction studies without uracil and His supplements. Photos were taken from single spots.

RBX1 (Wirbelauer et al., 2000; Gray et al., 2002; Lechner et al., 2002; Furukawa et al., 2003). In Arabidopsis, AtRBX1 has been shown to interact with AtCUL1 and AtCUL4 (Gray et al., 2002; Lechner et al., 2002). To investigate this possibility with AtCUL3a and AtCUL3b, Arabidopsis AtRBX1 (Lechner et al., 2002) was cloned into the two-hybrid vector pACT2 and used for Y2H assays with both cullins. As shown in Figure 3A, AtRBX1 interacted equally well with each of the two cullins. This was further confirmed by in vitro studies in which AtCUL3a translated in rabbit reticulocyte lysate was incubated with GST::AtRBX1 synthesized in *Escherichia coli*. Pull-down assays led to recovery of AtCUL3a, further underlining true interaction between AtCUL3 and AtRBX1 proteins (Fig. 3B).

Activity of cullin-based E3 ubiquitin ligases depends on modification of the cullin subunit by the ubiquitin-related protein Nedd8/RUB1. For example, it has been demonstrated that Arabidopsis AtCUL1 is a target for RUB1 modification and that this modification is required for proper SCF functioning (del Pozo and Estelle, 1999; Gray et al., 2001; Schwechheimer et al., 2001). The RUB1 attachment site is a Lys residue located within a highly conserved motif (VRIMK) at the COOH-terminal region of AtCUL1 (del Pozo and Estelle, 1999). Since AtCUL3a and AtCUL3b contain the conserved motif with the Lys residue at position 678 (Fig. 4A), we asked whether Arabidopsis AtCUL3 proteins are also targets for RUB1 modification. To answer this question, both AtCUL3 cullins were in vitro translated and incubated with a purified GST::RUB1 fusion protein. RUB1 modification of both cullins was indicated by a shift in their mobility on SDS-PAGE (Fig. 3C). The specificity of the modification was confirmed by changing the conserved Lys residues to Met (AtCUL3a^{K678M} and AtCUL3b^{K678M}, respectively). Neither mutant protein was modified by RUB1 (Fig.

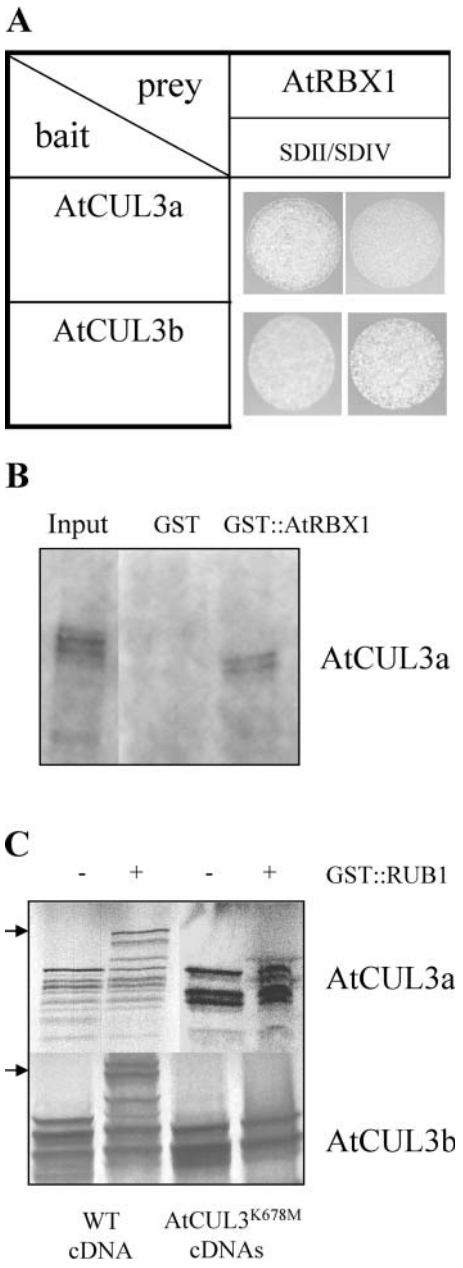

Figure 3. AtCUL3 proteins interact with AtRBX1 and can be modified by RUB1. A, In Y2H assays, AtRBX1 shows interaction with both AtCUL3 proteins. B, Interaction was confirmed for AtCUL3a by using purified GST or GST::AtRBX1 protein in pull-down assays. Most outer lane shows 1 μL of in vitro translated AtCUL3a protein used for pull downs. C, Left half, GST::RUB1 modification of AtCUL3a and AtCUL3b (indicated by arrows); right half, mutagenesis of a conserved Lys to Met at position 678 (AtCUL3^{K678M}) resulted in loss of AtCUL3 modification by GST::RUB1.

3C). These results demonstrate that both cullins are potential targets for a RUB-modification pathway in planta. Together with the AtRBX1 and AtBPM interaction studies, our results give strong evidence that AtCUL3a and AtCUL3b can assemble into functional E3 ligases in Arabidopsis.

AtCUL3 Proteins Require Conserved Amino Acid Residues at Their NH_2 Terminus for Interaction with AtBPM Proteins

To characterize critical amino acid residues required for interaction between AtCUL3 and AtBPM proteins, a mutagenesis approach was used with a focus on AtCUL3a and AtBPM1. Conserved Ser and Phe residues that are present in the CUL3 family have been reported to be crucial for assembly with BTB/POZ proteins (Pintard et al., 2003). Corresponding residues are located at positions 50 and 51 in both AtCUL3a and AtCUL3b. Interestingly, sequence comparison of AtCUL3s, CeCUL3 and HsCUL3 with AtCUL1 and AtCUL4, showed that the corresponding residues are absent in the two latter cullins (Fig. 4A). Mutagenesis of the two residues to Ala (AtCUL3a^{S50AF51A}) resulted in total loss of AtCUL3a interaction with AtBPM1 (Fig. 4B). By contrast, the AtCUL3a^{K678M} protein showed normal interaction with AtBPM1 (Fig. 4B). Furthermore, assembly with AtRBX1 was not affected by any of the amino acid substitutions (AtCUL3a^{S50AF51A}, AtCUL3a^{K678M}), indicating that these substitutions probably do not result in total loss of AtCUL3a's native protein folding. Most importantly, however, these findings describe the NH_2-terminal part of AtCUL3a as the functional interacting region with BTB/POZ proteins.

AtBPM1 Requires Its BTB/POZ Domain for Interactions with Both AtCUL3a and Other AtBPM Proteins

To underscore the relevance of the BTB/POZ motif for assembly with AtCUL3 and other AtBPM proteins, a stop codon was introduced at position 189 (AtBPM1^{L189Stop}) between the MATH and BTB/POZ domains of AtBPM1 (Fig. 5A). As shown in Figure 5, B and C, loss of the BTB/POZ domain prevented all interaction in Y2H and in vitro pull-down studies, respectively, with both AtCUL3a and AtBPM1. Additionally, a highly conserved Asp residue that is known to be critical for dimerization with BTB/POZ proteins (Ahmad et al., 1998) can be found within the first α-helices and second β-sheet of the BTB/POZ motif at position 204 in AtBPM1. When this residue was changed to Ala (AtBPM1^{D204A}), we observed total loss of interaction with AtCUL3a and AtBPM1 in Y2H assays (Fig. 5B). However, using in vitro pull downs, a weak interaction was still detectable, indicating that the mutation has not fully disrupted the protein conformation and ligand binding ability (Fig. 5C). To further investigate the nature of the interaction, the Asp residue was also changed to an Asn (AtBPM1^{D204N}), and analysis showed that this mutant interacted with AtCUL3a and AtBPM1 in the Y2H system (Fig. 5B). Based on the crystal structure of SCF complexes and the BTB/POZ protein PLZF, several additional residues in the BTB/POZ motif may be relevant for interaction with CUL3 proteins (Ahmad et al., 1998; Schulman et al., 2000; Pintard et al., 2004).

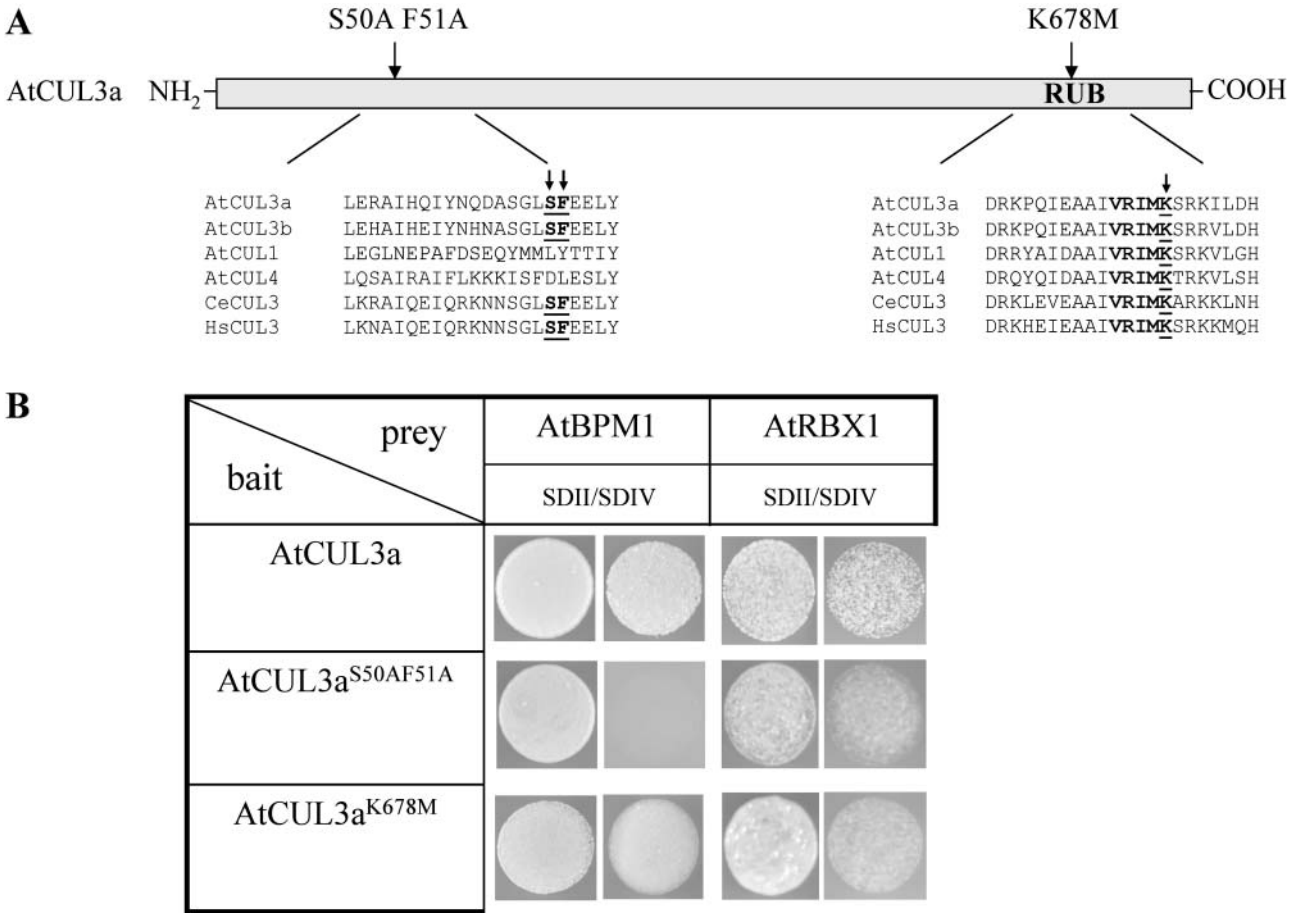

Figure 4. Mutagenesis of conserved amino acid residues in AtCUL3a affects interaction with AtBPM proteins. A, Schematic overview of AtCUL3a and locations of mutagenized amino acid residues (underlined and pointed out by arrows). B, Changing two conserved residues at the NH_2 terminus, a Ser-50 and a Phe-51 to Ala, disrupted interaction with AtBPM1 but not with AtRBX1. By contrast, mutagenesis of Lys-678 to Met had no influence on AtCUL3a-AtBPM1 assembly.

Phe-328, Tyr-330, and Leu-332 are all located within the fifth α-helices of the BTB/POZ domain of AtBPM1 (Fig. 5A). Substitution of Phe-328 or Tyr-330 with Ala reduced the interaction with AtCUL3a (Fig. 5B). By contrast, a similar substitution of Leu-332 had no effect on interaction with AtCUL3a. However, all three substitutions affected the interaction with AtBPM1, indicating that Leu-332 is more important for assembly with AtBPM proteins (Fig. 5B). In summary, these findings show that the BTB/POZ domain is required for interaction with both AtCUL3 and other AtBPM proteins.

Expression Pattern of AtCUL3a, AtCUL3b, and AtBPM Proteins

To gain information on which tissues AtCUL3-based E3 ligases might be active, reverse transcription (RT)-PCR analysis was performed for *AtCUL3* and the four *AtBPM* genes. Figure 6A shows that *AtCUL3a* and *AtCUL3b* are expressed in flowers, leaves, and shoots, and the different *AtBPM* genes mainly varied in the level of overall expression. Strongest expression was found for AtBPM3, whereas AtBPM6 was barely detectable but was present in all tissues examined (Fig. 6A). Analysis of transgenic *AtCUL3a* promoter::GUS (*pAtCUL3a*::*GUS*) plants showed GUS (β-glucuronidase) expression in root tips of lateral roots (Fig. 6, D and E). Even at very early stages of lateral root development, GUS staining was detectable in these lines. GUS staining was also visible in vascular tissue of leaves (Fig. 6, B and C). Interestingly, expression in flowers was age dependent; in young flower primordia, no GUS expression was observed. However, as flower development progressed, expression spread to all parts of the young flower (Fig. 6, F and G). By contrast, expression in older and fully differentiated flowers was only noticeable in the area of the pistil (Fig. 6, H and I). Expression in young flowers was confirmed by in situ hybridization (Fig. 6, J and K). pAtCUL3b::GUS lines also showed strong expression in all parts of the shoot and root (Fig. 6, L–Q), consistent with the RT-PCR data.

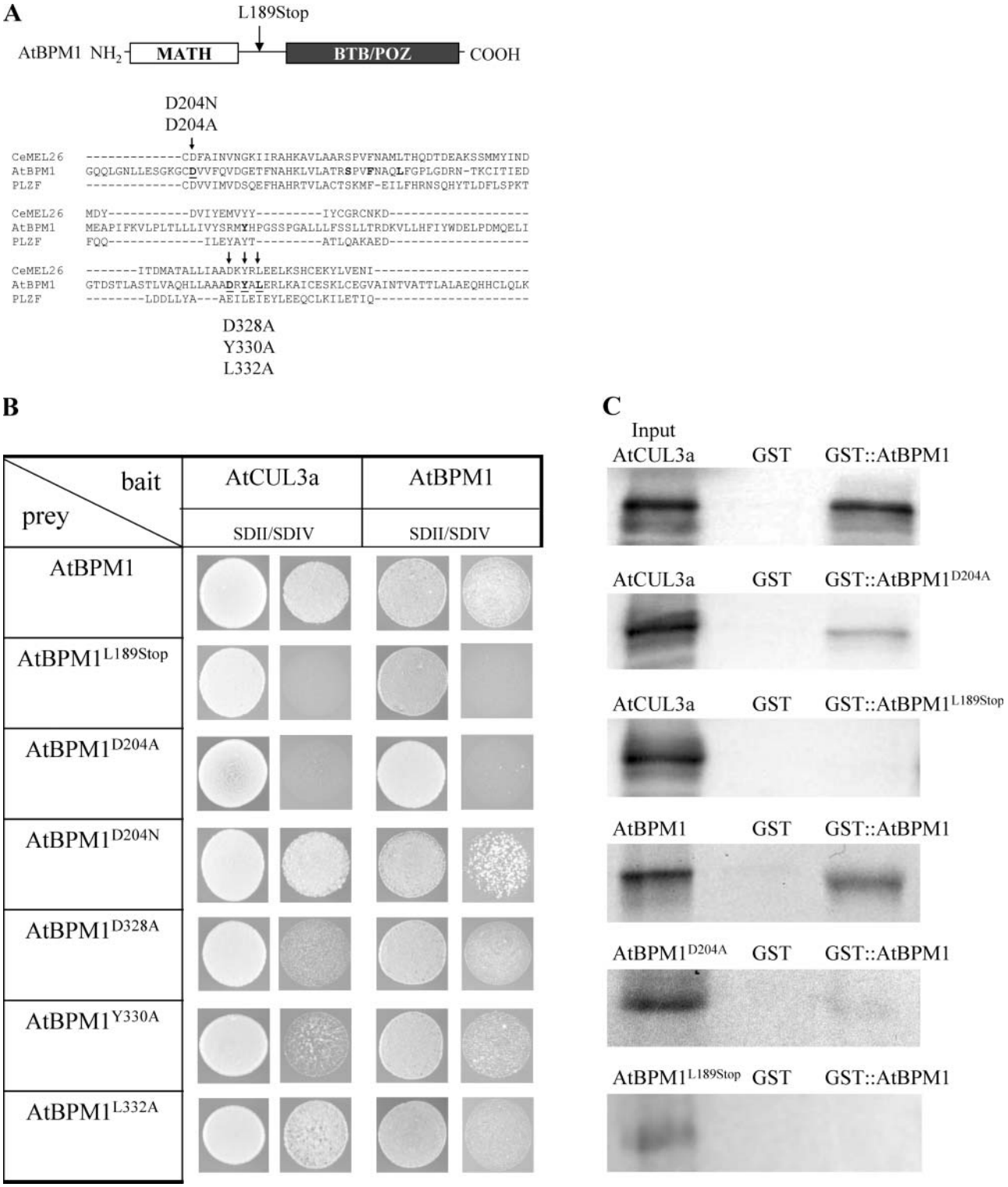

Figure 5. Mutagenesis of conserved residues in AtBPM1 affects assembly with AtCUL3 and AtBPM1 proteins. A, Schematic overview of AtBPM1 and locations of mutagenized amino acid residues (underlined and pointed out by arrows). B, In contrast with AtBPM1 and AtBPM1^{D204N}, AtBPM1^{L189Stop} and AtBPM1^{D204A} did not assemble with AtCUL3a and AtBPM1 proteins in Y2H assays. In addition, AtBPM1^{D328A} and AtBPM1^{Y330A} strongly reduced interaction with AtCUL3a and AtBPM1. The introduction of a point mutation at position 332 (AtBPM1^{L332A}) only affected interaction with AtBPM1. C, Results from the Y2H studies were verified for AtBPM1^{D204A} and AtBPM1^{L189STOP} by pull-down assays with in vitro translated AtCUL3a, AtBPM1 and mutated AtBPM1 versions, and bacterially expressed and purified GST, GST::AtBPM1, GST::AtBPM1^{D204A}, or AtBPM1^{L189STOP} fusion proteins.

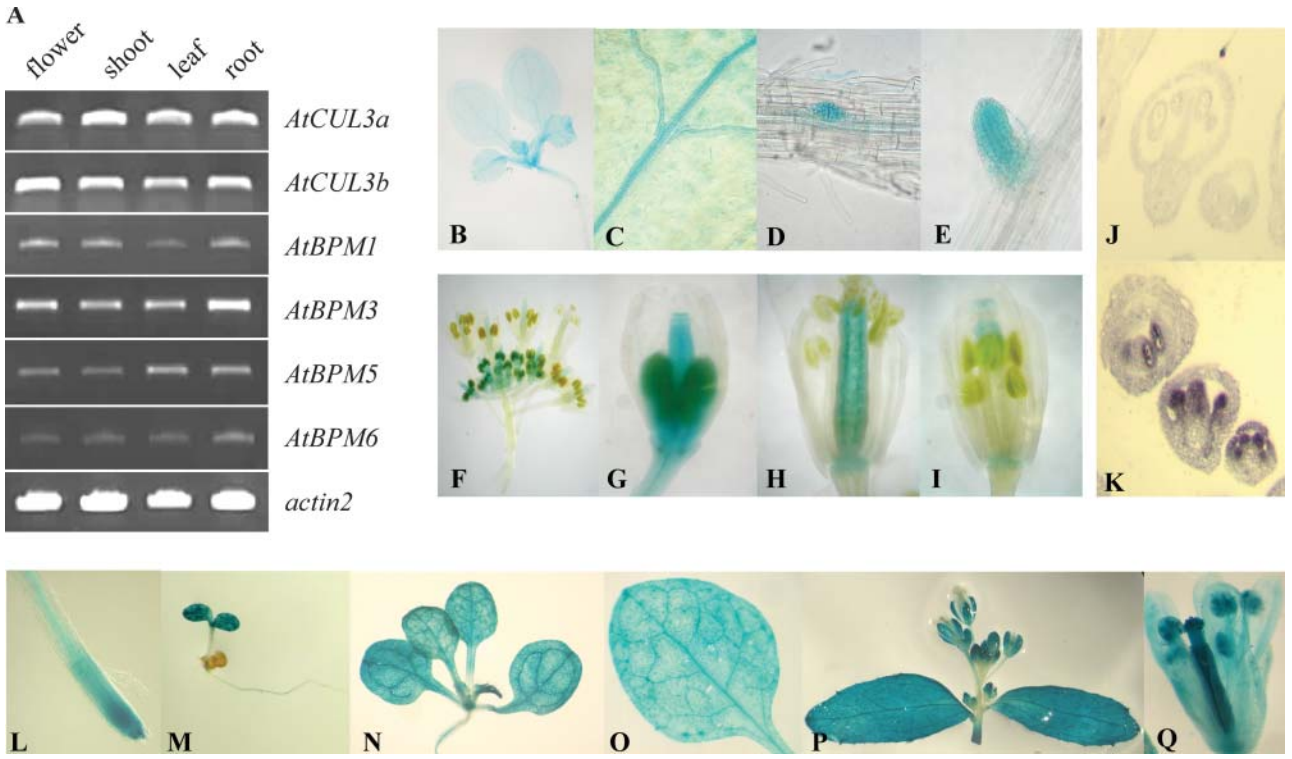

Figure 6. Expression pattern of *AtCUL3a, AtCUL3b,* and the four tested *AtBPM* genes. A, Semiquantitative RT-PCR shows expression of all tested genes in root, leaf, shoot, and flower. B to I, Analysis of *AtCUL3a* promoter GUS lines showed GUS expression in all organs (B, seedling; C, close up to vascular tissue; D and E, lateral roots; F–I, inflorescence). J and K, GUS expression was confirmed for flower tissue by in situ hybridization. Expression was detectable in pistil and anthers (J, sense; K, antisense). L to Q, Analyses of *AtCUL3b* promoter GUS lines showed expression similar to *pAtCUL3a*::*GUS* lines in all tested tissues (L, root; M, seedling; N, rosette leafs; O, leaf close up; P, inflorescence with two cauline leafs; Q, flower).

DISCUSSION

Recent studies by Pintard et al. (2003) and others have described a new class of E3 ubiquitin ligases that is based on interaction with BTB/POZ proteins. Here, we have demonstrated that Arabidopsis AtCUL3a and AtCUL3b interact with proteins containing a BTB/POZ motif (called AtBPM proteins). In addition, we show that AtBPM proteins form heterodimers and homodimers (Fig. 7). Similar to other cullins, both AtCUL3 proteins interact with AtRBX1 and are potential targets for RUB modification (Fig. 7). These results provide strong evidence that CUL3-dependent E3 ligases may be active in plants.

Mutagenesis of AtCUL3a clearly defines the NH_2-terminal part of the cullin to be required for interaction with the AtBPM proteins. As in CeCUL3, we show that adjacent Ser and Phe residues near the NH_2 terminus are essential for this interaction (Pintard et al., 2003), suggesting a conserved mechanism for assembly of CUL3 with BTB/POZ proteins in animals and plants. Our studies of the AtBPM1 protein indicate that the BTB/POZ domain is required for interaction with both BTB/POZ and AtCUL3 proteins. In the PLZF protein, the highly conserved Asp residue at position 204 is important for BTB/POZ-BTB/POZ interactions (Ahmad et al., 1998), while Asp-328, Tyr-330, and Leu-332 are required for interaction with cullin proteins (Pintard et al., 2004). In our studies, changing Asp-204 to Ala disrupted or strongly reduced interaction with both AtCUL3 and AtBPM1 proteins, demonstrating this residue to be highly important for both kinds of interactions. Surprisingly, changing the Asp to Asn had no effect on interaction. This may be due to the similar side chains allowing hydrogen bonds to build up in a comparable manner so that protein folding and ligand binding is hardly affected. Similarly, $AtBPM1^{D328A}$ and $AtBPM1^{Y33A}$ appeared to interact normally with both AtCUL3 and as homodimers. These findings indicate that the fifth α-helix is equally required for interactions with cullins and AtBPM proteins. It remains open why the mutagenesis of Leu-332 to Ala has a greater impact on AtBPM1-AtBPM1 assembly than on AtCUL3a-AtBPM1. However, so far our results do not provide any evidence that Asp-204 or the fifth α-helix is specifically required for interaction with AtCUL3 or AtBPM1 proteins, but demonstrate that both parts of the BTB/POZ domain are necessary for dimerizations.

The capability of AtBPM proteins to assemble with AtCUL3a and AtCUL3b as well as with other AtBPM proteins opens the possibilities for formation of diverse AtCUL3-based E3 complexes. It is of importance,

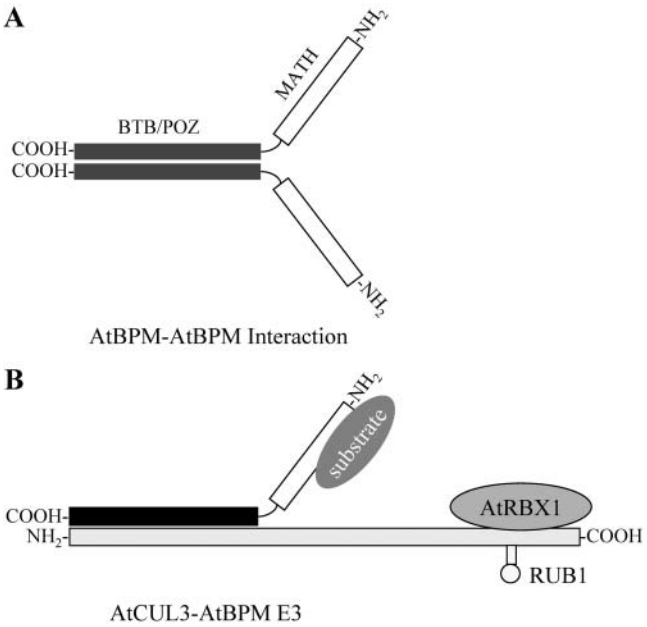

Figure 7. Schematic model of AtBPM and AtCUL3 assembly. A, AtBPM proteins assemble with other AtBPM proteins by using their BTB/POZ domain (black bar). B, AtCUL3 proteins interact at their NH_2-terminal part with the BTB/POZ domain of AtBPM proteins and within their COOH-terminal region with AtRBX1. The AtCUL3 proteins can be modified by the ubiquitin-related protein RUB1. As potential substrate adaptors, the AtBPM proteins contact substrates via their MATH domains (white bar).

however, that the two AtCUL3 cullins did not assemble with all tested AtBPM proteins. This interaction appears to be restricted to AtBPM1 and AtBPM3, although the molecular nature of this behavior remains unclear. However, this result significantly reduces the potential number of active E3 ligase complexes. By contrast, each of the AtBPM proteins we tested was capable of forming homodimers and heterodimers with all other members of the family. This indicates that the BTB/POZ domain might facilitate interaction with many other BTB/POZ proteins. The BTB/POZ motif can be found in more than 60 proteins encoded by the Arabidopsis genome (http://www.sanger.ac.uk/Software/Pfam). Taking into consideration the possibility of BTB/POZ homodimerization and heterodimerization, there is potential for a very large number of distinct E3s, rivaling the number of SFC-type E3s (Gagne et al., 2002).

Recently, Wang and co-workers showed that the BTB/POZ protein ETO1 (ethylene overproducer 1) interacts in vitro and in planta with the ethylene biosynthesis protein ACS5 and negatively affects ethylene biosynthesis. In addition, ETO1 interacts with AtCUL3a, suggesting that these proteins form an $AtCUL3a^{ETO1}$ E3 ligase in planta (Wang et al., 2004). These exciting findings further support our results that AtCUL3 proteins might indeed interact with BTB/POZ proteins in planta to form functional E3 ligases. Thus, major tasks for the future will be the description of AtCUL3-AtBPM E3 ligase activities in planta and the identification of possible substrate proteins. The broad and overlapping expression patterns of *AtCUL3*s and *AtBPM1* and *AtBPM3* genes suggest that corresponding E3 activities are likely to have an impact on biological processes in most organs of Arabidopsis. Substrate recognition by AtBPM proteins most likely depends on their MATH motif, as shown for *C. elegans* Mel-26 and its substrate MEI-1 (Pintard et al., 2003). The MATH motif comprises around 150 amino acids forming eight β-sheets. Like the BTB/POZ motif, it was found primarily in eukaryotes (Sunnerhagen et al., 2002). The domain was only recently noted based on homology of a COOH-terminal region of meprins A and B and the TRAF-C domain (Uren and Vaux, 1996). Although the specific function of the MATH domain is still unknown, Sunnerhagen et al. (2002) have suggested that the domain is an independent folding motif that participates in various modular arrangements based on other multimerization domains linked to it. The name for the MATH domain is composed of meprins and TRAFs, which participate in a broad variety of cellular processes, such as cell growth signaling and apoptosis (Bond and Beynon, 1995; Baker and Reddy, 1996; Arch et al., 1998; Bauvois, 2001; Bertenshaw et al., 2001). However, meprins and TRAFs are protein families that appear to be absent in Arabidopsis (our search; Zapata et al., 2001). Since AtBPM proteins show highest homology to the Spop protein (AAH03385) from human, it is possible that AtBPM proteins participate in related processes described for Spop proteins. In mammals, Spop interacts in Y2H assays and in vivo via its TRAF-C domain with the macrohistone H2A1.2 (macroH2a) (Takahashi et al., 2002). MacroH2a has been implicated in X chromosome inactivation in mammals (Costanzi and Pehrson, 2001; Csankovszki et al., 2001). In addition, activity of the transcription factor PDX1 (pancreatic-duodenal homeobox 1) from human is repressed by the Spop-related protein PCIF-1 (PDX1 C-terminal interacting factor; Liu et al., 2004). PDX1 belongs to a family of evolutionarily conserved homeodomain-containing transcription factors that influence body architecture during early embryonic development and control transcription of a variety of important transporter genes for Glc or insulin (Galant and Carroll, 2002; Ronshaugen et al., 2002). Repression of PDX1 activity is mediated by interaction with PCIF-1, but it remains unclear which part of PCIF-1 is required for this kind of assembly (Liu et al., 2004). Although sequence comparison of macrohistone and PDX1 with the Arabidopsis database did not yield in any comparable protein, it is still likely given the high level of conversation between Spop and AtBPM proteins that both families function in related pathways. This opens the possibility that identified substrate proteins of $AtCUL3^{AtBPM}$ E3 ligases can be involved in related processes in plants.

MATERIAL AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) plants were of the *Col-0* ecotype. Plants were grown on soil in a greenhouse at 20°C under long-day conditions (16 h light, 8 h dark).

Cloning, Plant Vectors, and Plant Transformation

Promoters (P) and cDNAs (D) from *AtCUL3*s and *BTB/POZ-MATH* genes were amplified with specific primers (PAtCUL3aFW, accaatgaaggatggatgat; PAtCUL3aRW, attttgaaacctgaatccaa; PAtCUL3bFW, aaaaagcaggctatgttctgtatcaaaacgatgcatatc; PAtCUL3bRW, agaaagctgggttgttgaaaagtaaactg; DAtCUL3aFW, atgagtaatcagaagaagag; DAtCUL3aRW, ttaggctagatagcggtaaa; DAtCUL3bFW, atgagtaatcagaagaagagaaatttcc; DAtCUL3bRW, ctaacaatcacaagactcaataaac; DAtBPM1FW, atgggcacaactagggtctgc; DAtBPM1RW, tcagtgcaaccggggcttcac; DAtBPM3FW, atgagtaccgtcggaggtatag; DAtBPM3RW, ctaagacactgctcgcacttc; DAtBPM5FW, atgtcagaatcagtgattcag; DAtBPM5W, ctaggtggttcgttgtctaac; DAtBPM6FW, atgtcaaagctaatgaccag; DAtBPM6RW, ctaagtggttcgctgcctgac) from genomic DNA or cDNA libraries, respectively (Minet et al., 1992), using Bio-X-Act polymerase (Bioline, Randolph, MA). The *AtCUL3b* promoter was cloned using GATEWAY BP and LR reactions (Invitrogen, Carlsbad, CA) into the binary vector *pBGWFS7* (Karimi et al., 2002), whereas the *AtCUL3a* promoter was cloned into *Spe*I restriction sites of the binary vector *pCB308* (Xiang et al., 1999). Binary vectors were introduced by electroporation into Agrobacterium and subsequently used in plant transformation, according to the method of Clough and Bent (1998). cDNAs were cloned into *pCR2.1* (Invitrogen) for sequencing and use for further subclonings.

Yeast Two-Hybrid Assays and Mutagenesis

A lexA-based two-hybrid system was used containing *pBTM116-D9* (kindly provided by Dr. Erich Wanker) as bait plasmid and *pACT2* (CLONTECH, Palo Alto, CA; GenBank accession no. U29899) as prey plasmid, together with the yeast reporter strain L40ccU3 [MATa, *his3-200*, *trp1-901*, *leu2-3*, *112ade2 LYS2*::(*lexAop*)*4-HIS3*, *URA*::(*lexAop*)*8-lacZ*, *GAL4*, *gal80*]. Both vectors were modified with a GATEWAY cassette (Invitrogen). Additionally, *pBTM116-D9* plasmid was also modified with a tetracycline resistance. cDNAs of cullins and *BTB/POZ* genes were cloned into *pDONR221*, and *AtRBX1* was cloned into *pDONR201* (Invitrogen) and subsequently introduced into two-hybrid plasmids by GATEWAY reactions (Invitrogen). cDNAs of *AtCUL3a* in *pBTM116-D9* and *At5g19000* in *pACT2* were directly used for introduction of point mutations by a Stratagene (La Jolla, CA) mutagenesis kit (AtCUL3aS50AF51A, gatgctagcggtctcgctgccgaagaactttacag; cAtCUL3aS50AF51A, ctgtaaagttcttcggcagcgagaccgctagcatc; AtCUL3aK678M, catcgtaaggatcatgatgtccaggaaaatactag; cAtCUL3aK678M, ctagtattttcctggacatcatgatccttacgatg; AtBPM1L189Stop, gccagtttctaactagggacaacagttggg; cAtBPM1L189Stop, cccaactgttgtccctagttagaaactggc; AtBPM1D204N, gtgggaaaggctgtaatgttgttttccaagttg; cAtBPM1D204N, caacttggaaaacaacattacagcctttcccac; AtBPM1D204A, gtgggaaaggctgtgctgttgttttccaagttg; cAtBPM1D204A, caacttggaaaacaacagcacagcctttcccac; AtBPM1D328A, ctagcagcggcagcccgttatgctcttgag; cAtBPM1D328A, ctcaagagcataacgggctgccgctgctag; AtBPM1Y330A, cagcggcagaccgtgctgctcttgagcggcttaaag; cAtBPM1Y330A, ctttaagccgctcaagagcagcacggtctgccgctg; AtBPM1L332A, gcagaccgttatgctgctgagcggcttaaagc; cAtBPM1L332A, gctttaagccgctcagcagcataacggtctgc). For Y2H assays, yeast cells were subsequently transformed with bait and prey plasmid constructs using a standard lithium acetate technique. Cells were grown for 3 d at 30°C on synthetic dextrose (SD) minimal medium supplemented with Leu and His (SDII), as described by Sambrook and Russell (2001). Selected colonies were diluted 1:2,000 in autoclaved distilled water before 50 μL were dotted on SD minimal medium without supplements (SDIV) for control of interaction. Photographs of single dots were taken 3d after transfer.

Pull-Down and RUB-Modification Assays

In vitro translated cullins, AtBPM1, and mutagenized proteins were synthesized with the TNT-reticulocyte lysate system (Promega, Madison, WI) using [^{35}S] trans-labeled Met (Amersham, Buckinghamshire, UK). GST-RBX1, -AtBPM1, -AtBPM1^{D204A}, and -AtBPM1^{Stop} fusion proteins and sole GST proteins were generated in *Escherichia coli* DH5α from *pGEX-2TK* plasmid (Pharmacia, Piscataway, NJ). For pull downs, proteins were incubated at 4°C for 2 h before incubation for 1 h with equal amounts of GST or GST fusion proteins. Proteins were recovered by adding glutathione beads (Sigma, St. Louis) and incubating for 1 h. Binding assays and washing were done in standard buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.2% Nonidet P-40). For RUB-modification assays, in vitro translated *AtCUL3a*, *AtCUL3b*, and mutant cDNAs were incubated for 15 min at 25°C with purified GST::RUB1 in the presence of 3 mM ATP, 0.1 mM dithiothreitol, 5 mM $MgCl_2$, and 10 units/mL inorganic pyrophosphatase (Roche, Basel). Reactions were stopped by adding 4× SDS/dithiothreitol loading buffer and boiling for 10 min. Proteins were resolved on SDS-PAGE. Products were detected by autoradiography.

RT-PCR Analysis

For expression analysis, different tissues (flower, shoot, leaf, and root) were harvested from 4-week-old soil-grown plants and directly frozen in liquid nitrogen. Total RNA extraction and DNase I digestion were done using a NucleoSpin RNA Plant kit (Macherey-Nagel, Duren, Germany). RT-PCR was done using a One-Step RT-PCR kit (Qiagen, Valencia, CA). A total of 90 ng of total RNA was used for each RT-PCR reaction. Reactions were done according to the Qiagen manual with 25 cycles, 60°C annealing temperature, and 1 min of elongation time, using the same cDNA-specific *AtBPM* primers mentioned above. *AtCUL3a* and *AtCUL3b* were amplified using the following primers: CUL3aRTPCRFW, gaactttacagaaatgcgta; CUL3aRTPCRRW, gagtgtttagaagccttgtatgt; CUL3bRTPCRFW, gagctttacagaaacgcata; and CUL3bRTPCRRW, gcgtattcaatagcctagtctga. actin2 was amplified using the following primers: actin2FW, tacaacgagcttcgtgttgc; and actin2RW, gattgatcctccgatccaga.

In Situ Hybridization and GUS Staining

Flower tissue from 28-d-old soil-grown plants was fixed in freshly prepared 4% paraformaldehyde before embedding in paraffin. Ten-micrometer sections were prepared for in situ hybridization according to Jackson (1991). As template for RNA synthesis, a 650-bp fragment from the 5′ end of *AtCUL3a* was used. Labeling of sense and antisense RNA was done with a DIG-RNA labeling kit (Roche). Hybridization was followed according to Jackson (1991). For detection of GUS expression, different tissues were incubated up to 24 h in GUS-staining solution according to Jefferson (1989).

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