MUBs, a Family of Ubiquitin-fold Proteins That Are Plasma Membrane-anchored by Prenylation

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Ubiquitin (Ub)-fold proteins are rapidly emerging as an important class of eukaryotic modifiers, which often exert their influence by post-translational addition to other intracellular proteins. Despite assuming a common β-grasp threedimensional structure, their functions are highly diverse because of distinct surface features and targets and include tagging proteins for selective breakdown, nuclear import, autophagic recycling, vesicular trafficking, polarized morphogenesis, and the stress response. Here we describe a novel family of Membrane-anchored Ub-fold (MUB) proteins that are present in animals, filamentous fungi, and plants. Extending from the C terminus of the Ub-fold is typically a cysteine-containing CAAX (where A indicates aliphatic amino acid) sequence that can direct the attachment of either a 15-carbon farnesyl or a 20-carbon geranylgeranyl moiety in vitro. Modified forms of several MUBs were detected in transgenic Arabidopsis thaliana, suggesting that these MUBs are prenylated in vivo. Both cell fractionation and confocal microscopic analyses of Arabidopsis plants expressing GFP-MUB fusions showed that the modified forms are membrane-anchored with a significant enrichment on the plasma membrane. This plasma membrane location was blocked in vivo in prenyltransferase mutants and by mevinolin, which inhibits the synthesis of prenyl groups. In addition to the five MUBs with CAAX boxes, Arabidopsis has one MUB variant with a cysteine-rich C terminus distinct from the CAAX box that is also membrane-anchored, possibly through the attachment of a long chain acyl group. Although the physiological role(s) of MUBs remain unknown, the discovery of these prenylated forms further expands the diversity and potential functions of Ub-fold proteins in eukaryotic biology.

The superfAMILY of ubiquitin (Ub)4-type proteins has emerged over the last decade as an influential set of post-translational modifiers in eukaryotes. These small proteins (~75–180 amino acids) contain a signature β-grasp (or Ub-fold) core domain, created by an α-helix transversing a groove created by an arched four-strand β-sheet (1). Extending from the core is a short flexible C-terminal extension that enables their attachment to other molecules through the terminal carboxyl group. The first and best-known member of this family is Ub (2, 3). It functions by becoming ligated to other proteins through an isopeptide bond between the C-terminal glycine of Ub and free lysine ε-amino groups in the target. The main role of Ub is to direct selective proteolysis by assembling chains of Ub monomers linked internally through Lys-48 onto various target proteins to promote their recognition by the 26 S proteasome. Others include roles in DNA repair via attachment of Lys-63-linked chains and endomembrane trafficking, transcription, and chromatin remodeling via attachment of Ub monomers (2, 3).

Since the discovery of Ub, a number of related modifiers have been found, including Related to Ub-1 (RUB1 or NEDD8), Small-Ub-like Modifier (SUMO), Autophagy (ATG)-8 and -12, Homology to Ub (HUB), Ub-Fold-Modifier (UFM), and Ub-Related Modifier (URM) (1, 4, 5). Despite sometimes showing little relatedness at the amino acid sequence level, the three-dimensional structures of the mature proteins share the β-grasp architecture followed by a C-terminal extension that often ends in a glycine (6–13). The notable exception is HUB that terminates in other amino acids (14). In most cases, the targets are proteins, using an enzymatic cascade analogous but distinct from ubiquitination for isopeptide linkage. However, their functions are often nonproteolytic. For example, RUB1/NEDD8 becomes reversibly conjugated to the Cullin subunit of SCF (Skip-Cullin-F-Box) and BTB (Bric-a-Brac-Tramtrack-Broad) complexes to promote protein ubiquitination by these ligases (15), ATG8 and -12 assist in the encapsulation of cytoplasm during autophagy (16), and SUMO, among other functions, can antagonize Ub by binding to the same lysine on a target and thus protect the target from subsequent ubiquitination (17).

In addition to modifying proteins, recent studies have shown that nonproteinaceous molecules can be conjugated to Ub-fold

4 The abbreviations used are: Ub, ubiquitin; A, aliphatic amino acid; GFP, green fluorescent protein; CaMV, cauliflower mosaic virus; PBS, phosphate-buffered saline; RFP, red fluorescent protein; PFT, protein farnesyltransferase; EST, expressed sequence tag.
protein-anchored Ub-fold proteins have important roles in the budding process within the MUB family suggests that these membrane proteins use a long chain acyl moiety for membrane association. Consequently, the prenyltransferases contain a common site located at the C terminus of the target. A protein farnesyltransferase (PFT) or geranylgeranyltransferase (PGGT I) couples via a thioether linkage the prenyl group to the cysteine, a C protein (21, 22). The modified cysteine is part of a consensus motif that is modified by prenylation. Despite low sequence similarity, these lipidated viral forms remain unclear.

Another type of membrane-anchoring system involves prenylation, the covalent attachment of 15 (farnesyl)- or 20 (geranylgeranyl)-carbon isoprene groups to a cysteine residue in the protein (21, 22). The modified cysteine is part of a consensus motif. Most Lipidated Ub-Related Genes in Arabidopsis representatives assume an obvious AAX (cysteine, aliphatic, aliphatic, any amino acid) box located at the C terminus of the target. A protein farnesyltransferase (PFT) or geranylgeranyltransferase (PGGT I) couples via a thioether linkage the prenyl group to the cysteine, a CAXX tripeptide, and then a prenyl-cysteine methyltransferase methylates the terminal carboxyl group. The prenyltransferases contain a common α subunit and one of two different β subunits that confer specificity for either farnesyl or geranylgeranyl addition. Although the prenyl group typically anchors the protein to the plasma membrane, recent studies showed that it can also act as a “greasy finger” to enhance protein-protein interactions (23). Scans of various eukaryotic genomes indicate that many proteins are potential prenylation targets (>100 in Arabidopsis thaliana (24)). The best studied example is the human oncprotein Ras, a GTP-binding protein that associates with the plasma membrane upon addition of a farnesyl moiety (25). Surprisingly, neither PFT nor PGGT I activities are essential in Arabidopsis, but their genetic inactivation has pleiotropic consequences, including defects in leaf phyllotaxy and floral homeosis and signaling by the hormones auxin and abscisic acid (26–30).

Here we describe a new family of membrane-anchored Ub-fold proteins (or MUBs) in plants, animals, and filamentous fungi that is modified by prenylation. Despite low sequence homology to other members of the Ub-fold superfamily, an Arabidopsis representative assumes an obvious β-grasp structure followed by a flexible C-terminal extension (31). Most MUBs terminate in a CAXX box, which can be prenylated in vitro with either a farnesyl or geranylgeranyl moiety. In Arabidopsis, this modification directs the in vivo association of MUBs with the plasma membrane. Arabidopsis also contains a plasma membrane-anchored MUB variant ending in a cysteine-rich sequence distinct from the CAXX box, which may use a long chain acyl moiety for membrane association. Conservation within the MUB family suggests that these membrane-anchored Ub-fold proteins have important role(s) in the cell biology of multicellular eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Sequence Alignments and Phylogenetic Analysis—Arabidopsis**

MUB1 was identified by visual inspection of Ub-fold containing proteins in the Protein Data Bank using the PyMol Molecular Graphics System (DeLano Scientific LLC, San Carlos, CA). The AtMUB1 (At3g01050) amino acid sequence was then used as a query to identify by BLAST relatives in the Arabidopsis genomic data base and other eukaryotes in the GenBank™ and TIGR Gene Indices data bases. Protein sequences were aligned using ClustalX MAC version 1.83 and displayed with MacBoxshade version 2.15 (Institute of Animal Health, Pirbright, UK). Phylogenetic analysis was performed with MEGA3 (32) by the neighbor-joining method using the Poisson distance correction and pairwise deletion. The GenBank™ accession number for each MUB can be found in the Supplementary Material.

**Recombinant Protein Production, Immunoblot Analyses, and in Vitro Prenylation and Palmitoylation Assays—Full-length cDNAs for A. thaliana ecotype Columbia (Col)-0 MUB2-4 and -6 were obtained from the Arabidopsis Biological Resource Center (Ohio State University).** The AtMUB1 cDNA was a gift from Dr. John Markley, and the AtMUB5 cDNA was obtained by reverse-transcribed PCR amplification of total RNA isolated from flowers. Animal and fungal MUB cDNAs were obtained through the American Type Culture Collection (Manassas, VA) with the exception of Drosophila melanogaster MUB, which was amplified by PCR from a cDNA library. The full coding sequence of each MUB was PCR-amplified with primers designed to introduce Ndel and Xhol sites on the 5′ and 3′ ends, respectively, and the products were digested with Ndel and Xhol and inserted into similarly cut pET28b (Novagen, Madison, WI) to express the proteins with an N-terminal His tag. The corresponding SAAX mutants were created by PCR with 3′ primers designed to convert the cysteine codon to that for serine. The various cysteine mutants of AtMUB2 were generated in a similar fashion using PCR primers that introduced the desired codon changes. All constructions were confirmed as correct via dye-deoxy sequencing.

The His-MUB proteins were expressed in BL21 Escherichia coli at 37 °C following a 3-h induction with 1 mM isopropyl β-D-thiogalactoside. Cells were lysed in PBS (150 mM NaCl and 50 mM phosphate (pH 7.0)) containing 6 mM guanidine-HCl, and the recombinant proteins were isolated by nickel-chelate affinity chromatography (nickel-nitrilotriacetic acid-agarose; Qiagen, Valencia, CA). The AtMUB1 amino acid sequence was then used as a query to identify by BLAST relatives in the Arabidopsis genomic data base and other eukaryotes in the GenBank™ and TIGR Gene Indices data bases. Protein sequences were aligned using ClustalX MAC version 1.83 and displayed with MacBoxshade version 2.15 (Institute of Animal Health, Pirbright, UK). Phylogenetic analysis was performed with MEGA3 (32) by the neighbor-joining method using the Poisson distance correction and pairwise deletion. The GenBank™ accession number for each MUB can be found in the Supplementary Material.

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lated from nonprenylated isoforms. Here the electrophoretic times were extended to increase separation.

In vitro protein prenylation was performed essentially as described (35). The reactions contained 10 μg of purified recombinant His₆-AtMUB, 37 μg of protein from an Arabidopsis cell culture as the source of protein prenyltransferase activities, 20 mM MgCl₂, 5 mM dithiothreitol, 50 mM Hepes (pH 7.5), and 0.01 μCi μl⁻¹ of either [³H]farnesyl pyrophosphate (16.1 Ci mmol⁻¹) or [³H]geranyleranyl pyrophosphate (23 Ci mmol⁻¹; PerkinElmer Life Sciences) in a final volume of 62.5 μl. Recombinant human Ras with CAIM, CAIL, or SVLS C-terminal sequences were used as control substrates (35). Reactions were incubated for 60 min at 30 °C, resolved by SDS-PAGE, and analyzed by fluorography using the Amplify fluorographic reagent (Amersham Biosciences).

In vitro palmitoylation was performed essentially as described (36). The reactions contained 5 μg of purified recombinant His₆-AtMUB2 and 0.25 μCi of [¹⁴C]palmitoyl-coenzyne A (55 mCi mmol⁻¹, PerkinElmer Life Sciences) in PBS. Reactions were incubated for 60 min at 25 °C, resolved by SDS-PAGE, and analyzed by fluorography as described above. 2-Bromopalmitic acid (Sigma) was added to 1 mm.

Expression of AtMUBs in Arabidopsis and E. coli—For Myc-tagged MUBs, the Arabidopsis MUB coding regions were altered by PCR to contain XbaI sites at both ends, digested with XbaI, and inserted into similarly cut pGSVE9 plasmid that would express the protein with an N-terminal 3xMyc tag, all under the control of the cauliflower mosaic virus (CaMV) 35S promoter. To create the pGSVE9 plasmid with an N-terminal Myc tag, a PCR product containing a 3xMyc tag was amplified from pMPY-3xMYC (37) with primers that introduced NheI and XbaI sites on the 5’ and 3’ ends, respectively. This product was cut with Nhel and XbaI and then cloned into XbaI-digested pGSVE9. The resulting plasmids were introduced into Agrobacterium tumefaciens strain GV3101 and transformed into wild-type (Col-0) Arabidopsis by the floral dip method (38). Hygromycin-resistant T₃ seedlings were screened by immunoblot analysis with anti-Myc antibodies to identify individuals expressing the tagged proteins. The identical Myc-tagged proteins were expressed in E. coli, by introducing Asel and Xhol sites at the 5’ and 3’ ends and cloning PCR-produced fragments from the above plasmids into Nhel- and Xhol-digested pET21b for expression in BL21 E. coli cells. For GFP-tagged AtMUBs, the coding regions were modified by PCR to contain EcoRI and Xhol sites on the 5’ and 3’ ends, respectively, digested with EcoRI and Xhol, and cloned into a similarly cut pEGAD vector (39). This insertion appended in-frame a GFP coding region to the N terminus of each AtMUB, all of which was preceded by the CaMV 35S promoter. The plasmids were introduced into Arabidopsis ecotype Col-0 as described above, except that the transgenic plants were selected by resistance to 25 μg/ml glufosinate ammonium (Crescent Chemical Co., Islandia, NY).

Confocal Microscopic Localization of MUBs in Arabidopsis—Stable transformed lines expressing GFP-AtMUBs were analyzed by fluorescence microscopy using a Zeiss 510-Meta scanning laser confocal microscope and the GFP filter set. For transient expression in Arabidopsis protoplasts, mutant and wild-type GFP-AtMUBs and an RFP-H⁻-ATPase fusion were placed under the control of the CaMV 35S promoter in a pUC vector (40). Purified plasmids were introduced into Arabidopsis ecotypes Col-0 and Landsberg erecta (Ler) and the era1-4 and ggb-2 mutant leaf tissue protoplasts by polyethylene glycol-mediated transformation according to Lee et al. (41). GFP/RFP expression was monitored 24 h after transformation using the BP505–530 (excitation 488 nm, emission 505–530 nm), Chs 560–615 (excitation 543 nm, emission 560–615 nm), and Chs 650–670 (excitation 633 nm, emission 650–670 nm) filter sets to detect GFP, RFP, and chlorophyll autofluorescence, respectively.

For the mevinolin inhibitor studies, the drug or an equivalent volume of buffer (10 μl) was added to Col-0 protoplasts 1 h after transfection, and the protoplasts were then incubated for 8 or 32 h at 24 °C before confocal microscopy. Only protoplasts that displayed adequate fluorescence were included in the quantifications. A 10 mM stock solution of mevinolin (Sigma) was prepared by dissolving the inhibitor in 15% ethanol and 10 mM NaOH, heating the solution for 1 h at 60 °C, and then adjusting the pH to 6.0 with potassium phosphate.

Membrane Isolation—Wild-type Col-0 and Ler, Myc-AtMUB, or GFP-AtMUB expressing Arabidopsis plants and ggb-2 mutants were grown for 7 days, whereas the slower growing plp1-1 and era1-4 mutants were grown for 10 days in Gamborg’s B5 liquid growth medium (GM; Sigma) containing 2% sucrose at 22 °C under continuous light. Whole seedling homogenates were separated at 0–4 °C into the soluble and membrane fractions as described (42). Tissue was chopped in 1 ml/g of extraction buffer (400 mM sucrose, 1 mM Na₃EDTA, 100 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, and 1× Complete protease inhibitors (Roche Applied Science)) with a razor blade, filtered through Miracloth (EMD Biosciences, San Diego), and then clarified at 500 × g. The supernatants were centrifuged at 100,000 × g for 60 min at 4 °C to separate the cytosolic and membrane fractions. The pellets were resuspended in an equal volume of extraction buffer without or with 1% Triton X-114, 1 M NaCl, or 0.1 M sodium carbonate (pH 11), incubated at 0–4 °C for 1 h, and then recentrifuged at 100,000 × g for 1 h. The plp1-1, era1-4, and ggb-2 mutants were described previously (26, 28, 30).

RESULTS

Identification of the MUB Family—In an attempt to define the breadth of Ub-fold proteins in plants, we searched the various genomic and proteomic data bases of Arabidopsis for related structures. Upon visual inspection of the Protein Data Bank for proteins similar in size to Ub, we discovered the recently released three-dimensional NMR structure ISE9 for a 117-amino acid protein derived from the Arabidopsis locus At3g01050 (31). Despite being only 20/33% identical/similar in amino acid sequence to Ub, the tertiary structure of At3g01050 (called here A. thaliana Membrane-anchored Ub-fold-1 or AtMUB1) clearly has the signature β-grasp fold with a four-strand β-sheet cradling a long diagonal α-helix and capped by a second shorter α-helix (Fig. 1B). When PyMol was used to align
the AtMUB1 and Ub structures, a C-α root mean square deviation of only 1.5 Å was calculated between the two structures, indicative of strong three-dimensional similarity. However, AtMUB1 is distinct from Ub by the presence of a seven-residue N-terminal extension, several insertions that generate pronounced external loops in the tertiary structure, and a 20-residue C-terminal tail.
BLAST searches using the AtMUB1 sequence as a query identified five additional MUB genes in the Arabidopsis genome (At5g15460, At4g24990, At3g26980, At1g77870, and At1g22050 were designated AtMUB2–6, respectively) with the encoded proteins ranging in size from 119 to 124 amino acids and showing 73 to 41% identity to AtMUB1. Expanded searches detected homologs in a variety of other eukaryotes for which sufficient genomic or expressed sequence tag (EST) sequence data were available. The list includes other plants (e.g. oilseed rape, soybean, Medicago, cotton, tomato, wheat, poplar, rice, maize, tobacco, sorghum, and pine), animals (e.g. humans, mice, frogs, zebrafish, D. melanogaster, Caenorhabditis elegans, and dogs), and various filamentous fungi (e.g. Aspergillus nidulans and Neurospora crassa) (Fig. 1A and Supplemental Table 1). Literature reviews also revealed a brief description of D. melanogaster MUB (previously called UBL3), which suggested that it has a Ub-type fold (43). MUB gene sequences were not evident in any prokaryotic genomes nor detectable in the genomes of the budding and fission yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, respectively, suggesting that MUBs are restricted to multicellular eukaryotes. Rice, like Arabidopsis, appears to express a small family of four MUBs. In contrast, most of the animal and fungal genomes were predicted to contain a single MUB gene (the exception is the partially complete zebrafish genome that has at least two MUB genes). The fungal representatives are distinct in containing long variable N-terminal extensions (112–130 residues in length), which were not included in the alignment in Fig. 1A. These long extensions are weakly similar to each other but bear no resemblance to any other protein sequences/motifs in the GenBank™ data base. The most similar are the MUB extensions from Gibberella zeae and Magneportha grisea that share 50/35% similarity/identity over the first ~120 amino acids.

Phylogenetic analysis of the MUB family combined with representatives of other Ub-fold proteins/domains (1) and the E. coli ThiS and MoaD polypeptides (considered to be the progenitors of this protein family (44, 45)) showed that MUBs cluster on separate clades from the other Ub-fold proteins (Fig. 1A). Three main MUB clades were apparent that separated the animal, plant, and fungal forms. We found that the Arabidopsis MUBs clustered along with their rice orthologs into three subclades containing AtMUB1 and -2, AtMUB3 and -4, and AtMUB5 and -6 isoforms. Consistent with this pairing, AtMUB1/2 and AtMUB5/6 genes are in predicted synteny blocks within the Arabidopsis genome (46), suggesting that each pair arose by a large-scale chromosomal duplication.

cDNAs are available for many plant, animal, and fungal MUBs, indicating that the corresponding genes are transcriptionally active. Expression patterns of the Arabidopsis MUB genes presented in the Genevestigator DNA microarray (47), EST, and Massively Parallel Signature Sequences data bases revealed that all six loci are expressed with AtMUB3 being the most active. There are 3, 8, 18, 12, 1, and 9 ESTs available for AtMUB1–6, respectively. Genevestigator data indicated that the expression patterns of AtMUB2–6 strongly overlap with the relative abundance of each transcript varying only slightly among all tissues examined. (Expression data for AtMUB1 were not available in this data base.) Two exceptions were AtMUB2, which had a 3-fold higher transcript level in stamens, and AtMUB3, which had a 3-fold higher transcript level in senescing leaves. Examining the mRNA abundance for AtMUB2–6 in response to various stimuli did not reveal any substantial changes in expression, except AtMUB4, whose mRNA levels rose in response to cycloheximide and ozone treatment and infection by the pathogens A. tumefaciens and Myzus persicae. The significance of these changes awaits confirmation by other methods.

Protein sequence alignments of the collection with other members of the Ub-fold superfamily (Ub, RUB1/NEED8, HUB1, and ATG8) revealed a contiguous region of homology encompassing the Ub-fold. A number of conserved residues are apparent, including His-89 (numbering refers to the residues in AtMUB1) and several lysines (Lys-35 and Lys-63 in plant and fungal MUBs and Lys at position 84 in animal MUBs). The corresponding lysines (Lys-27, Lys-48, and Lys-63 in Ub) are important in Ub by serving as internal polymerization sites during Ub chain formation (48). The most striking feature of the MUBs was the presence of a canonical four-amino acid CAAX box (cysteine, aliphatic, aliphatic, any amino acid) instead of the C-terminal glycine domain found in most other Ub-fold proteins. CAAX boxes often signal the addition of prenyl groups (21, 22), suggesting that members of the MUB family are not bound covalently to proteins but are modified with these long chain hydrophobic groups. Similar to other proteins that are efficient substrates for prenylation, the MUB CAAX boxes are preceded by a series of basic residues. The only MUB of the entire collection that did not contain an obvious CAAX box was AtMUB2; it terminates in a cysteine-rich CVCLCFGARC motif that could instead be the thioester acceptor site for acylation, possibly by the saturated 16-carbon lipid palmitic acid (36, 49).

**MUB Proteins Can Be Prenylated in Vitro**—To demonstrate that members of the MUB family can be prenylated, we tested recombinant versions from a variety of eukaryotes in vitro using an extract from Arabidopsis cultured cells as a source of protein prenyltransferase activities and [3H]farnesyl pyrophosphate or [3H]geranylgeranyl pyrophosphate as isoprenoid donors. As shown using various human Ras derivatives (Fig. 2, A and B), this preparation has both PFT and PGGT I activities that conform to the CAAX sequence specificity of these enzymes, i.e. PFT favors proteins where “C” is either serine, cysteine, methionine, glutamine, or alanine, whereas PGGT I favors proteins where X is leucine (35). We found that Ras, which had the CAAX box cysteine substituted for serine (SVLS), was not modified, whereas Ras with CAIM or CAIL C termini were preferentially modified with farnesyl or geranylgeranyl moieties, respectively.

When the six Arabidopsis MUBs and an assortment of animal MUBs were tested similarly, almost all could be prenylated in reactions dependent on the CAAX sequence. Following a 1-h incubation, we detected farnesyl or geranylgeranyl derivatives for all the animal MUBs and five of the six Arabidopsis MUBs when wild-type proteins were used but not when mutant proteins in which the CAAX box cysteine was converted to serine (SAX) were used. The human, mouse, Xenopus, Drosophila, and zebrafish MUBs were preferentially modified with geranylgeranyl moieties in accord with the preference of PGGT I for
a leucine C-terminal amino acid (Fig. 2, A and B). Likewise, *Arabidopsis* MUB3, -5, and -6 with C-terminal leucine residues were preferentially modified with geranylgeranyl moieties (Fig. 2, C and D). AtMUB1 and -4, on the other hand, were preferentially modified with farnesyl moieties in accord with the preference of PFT for a methionine C-terminal residue. The only exception was *Arabidopsis* MUB2. Consistent with the absence of an obvious C-terminal CAAX box, the recombinant AtMUB2 protein was poorly modified with either prenyl group (Fig. 2, C and D).

*Arabidopsis* MUBs Are Modified in Vivo—Both the presence of a C-terminal CAAX box and the *in vitro* assays strongly suggested that most members of the MUB family are naturally prenylated. To detect indirectly this modification *in vivo*, we exploited the observation that addition of either farnesyl or geranylgeranyl groups in conjunction with removal of the “AAX” sequence increases the electrophoretic mobility of the modified proteins (50). Here we expressed Myc- and GFP-tagged versions of AtMUB1 and -6, which are presumed targets of PFT and PGGT I activities, respectively, with or without the essential cysteine (CAAX and SAAAX) in *Arabidopsis* and assayed for the prenylated forms by faster migration during SDS-PAGE. As can be seen from the immunoblots in Fig. 3A, the Myc-tagged version of AtMUB1 with its CAAX box had the same apparent molecular mass as a variant with an SAAAX box when both were expressed in *E. coli* (which lacks prenyltransferase activities). However, when the two were expressed in *Arabidopsis*, the migration of the CAAX version was noticeably faster. Similarly, plants expressing either GFP-AtMUB1 or GFP-AtMUB6 contained a faster migrating form relative to plants expressing the SAAAX mutant counterparts, a size difference that was clearly evident by mixing the extracts prior to electrophoresis (Fig. 3B).

For *Arabidopsis* MUB2, the cysteine-rich C terminus raised the possibility that this isoform is acylated and not prenylated (49). In support, we found that recombinant AtMUB2 could be modified with palmitic acid when incubated with [14C]palmitoyl-coenzyme A *in vitro* (Fig. 3C). This acylation was sensitive to the palmitoylation inhibitor 2-bromopalmitate (36) when added simultaneously to the reaction. Because acyl addition can decrease the SDS-PAGE mobility of the target protein (36, 51, 52), we tested for this retardation by comparing the SDS-PAGE mobility of AtMUB2 expressed in *E. coli* versus *Arabidopsis*. As can be seen in Fig. 3D, a Myc-tagged version of AtMUB2 had a significantly higher apparent molecular mass when expressed in *Arabidopsis* relative to its recombinant counterpart, indicating that AtMUB2 is modified *in planta*, possibly by acylation.

*Arabidopsis* MUB Proteins Associate with Membranes—The addition of prenyl or long chain acyl groups (e.g. palmitic acid) often serves to anchor the modified proteins to membranes (21, 22, 49). To provide support for this intracellular location, we examined the membrane and soluble distribution of *Arabidopsis* MUB1, -2, or -6. The source of the crude cell lysates was either wild-type seedlings or transgenic lines expressing Myc- or GFP-tagged versions using the comparable SAAAX mutants for AtMUB1 and -6 as nonprenylated controls. Plants were homogenized under conditions that help preserve the integrity of the various cell compartments, separated into the soluble

**FIGURE 2**. MUB proteins can be prenylated *in vitro*. Prenylation reactions were performed *in vitro* with recombinant His6-tagged MUBs from animals (A and B) and *Arabidopsis* (C and D) using either [3H]farnesylpyrophosphate or [3H]geranylgeranyl pyrophosphate as the isoprenoid donors and an *Arabidopsis* protein extract as a source of prenyltransferase activity. The reaction products were subjected to SDS-PAGE and detected by fluorography. For each MUB containing a wild-type CAAX C-terminal sequence, a site-directed mutant was also tested in which the cysteine residue was changed to a serine (SAAAX). For AtMUB2, a cysteine 115 to serine mutant (C115S) was tested. Recombinant human Ras with CAIM, CAIL, and SVLS C-terminal sequences were included to demonstrate the PFT and PGGT I specificities of the prenyltransferase preparation. Coomassie Blue-stained gels of the reaction mixtures are shown below each fluorograph to confirm equal loading of the MUB and Ras proteins. The MUB and Ras proteins are identified by the arrowheads with the farnesylated and geranylgeranylated forms denoted with F and GG, respectively. Species abbreviations can be found in the legend of Fig. 1.
and membrane-bound fractions by ultracentrifugation, and the fractions were then assayed for the presence of MUB proteins by immunoblot analysis. For wild-type plants, the MUB proteins were detected with antibodies generated against Arabidopsis MUB1. These antibodies recognize AtMUB1 well and

FIGURE 3. Modified forms of Arabidopsis MUB proteins are present in vivo. Myc- or GFP-tagged versions of AtMUB1, -2, and/or -6 were either expressed in Arabidopsis (At) or E. coli (Ec). Crude extracts were subjected to SDS-PAGE and immunoblot analysis with anti-Myc (A and C) or anti-GFP antibodies (B). A, Myc-tagged AtMUB1 bearing either wild-type CAAX or mutant SAAX C termini were expressed in Arabidopsis seedlings or E. coli. B, GFP-tagged wild-type CAAX or mutant SAAX versions of AtMUB1 and -6 were expressed in Arabidopsis seedlings and either electrophoresed separately or as an equal mixture (Mix). C, modification of AtMUB2 with palmitic acid (PA). Recombinant AtMUB2 was incubated in vitro with [14C]palmitoyl-coenzyme A in the absence or presence of 1 mM 2-bromopalmitate (BPA), and the reaction was separated by SDS-PAGE and stained for protein (bottom panel) or subjected to fluorography (top panel). Position of [14C]palmitic acid-AtMUB2, [14C]palmitic acid, and AtMUB2 are indicated by the arrowheads. D, wild-type AtMUB2 was expressed with a Myc tag in Arabidopsis seedlings or E. coli. The unmodified (non) and potentially prenylated (prenyl) forms of the AtMUB1 and -6 are indicated in A and B. The asterisk in B identifies an unknown immuno-cross-reacting species.

FIGURE 4. MUB proteins are membrane-associated in Arabidopsis. A, specificity of the anti-AtMUB1 antibody. Equal amounts (25 ng) of His6-tagged recombinant AtMUB proteins were subjected to SDS-PAGE and immunoblot analysis with either anti-AtMUB1 or anti-His6 antibodies. B, total crude extracts (T) from 7-day-old, liquid-grown wild-type Col-0 seedlings or seedlings expressing GFP alone or as fusions to AtMUB1, -2, and -6 were separated into the soluble (S) and pelleted membrane (P) fractions, and the fractions were subjected to SDS-PAGE and immunoblot analysis. For each AtMUB, the localization of the authentic protein (CAAX for AtMUB1 and 6) was compared with a C-terminal cysteine to serine mutant (SAAX for AtMUB1 and -6 and C115S for AtMUB2). Extracts from wild-type plants were probed with anti-MUB1 antibodies, those from Myc-AtMUB1 plants were probed with an anti-Myc monoclonal antibody, and those from GFP-AtMUB1 and -6 plants were probed with anti-GFP antibodies. C, solubilization of the AtMUB1 with detergent. The total membrane fraction from plants expressing Myc-AtMUB1 was resuspended in buffer (Buf) without or with 1 M NaCl, 0.1 M sodium carbonate (Na2CO3) (pH 11), or 0.1% Triton X-114 (TX114). The samples were incubated on ice for 1 h and then recentrifuged to collect the membranes. The soluble and membrane fractions were subjected to SDS-PAGE and immunoblot analysis with anti-AtMUB1 antibodies. D, effect of Arabidopsis prenylation mutants on the membrane association of AtMUB1. Total crude extracts prepared from homozygous plp-1, era1-4, and ggb-2 mutant seedlings were separated into the soluble (S) and pelleted (P) membrane fractions and subjected to SDS-PAGE and immunoblot analysis with anti-AtMUB1 antibodies. Wild-type Ler extracts were included as the control for the era1-4 mutation.
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show weak cross-reactivity with AtMUB2 and AtMUB4 but poorly recognize the other three isoforms (Fig. 4A).

Under our extraction conditions, free GFP was present entirely in the soluble fraction (Fig. 4B), which is consistent with its typical diffuse distribution within the cytoplasm and nucleus when expressed in plants (39, 53). In contrast, nearly all of the signal for the MUB proteins in wild-type plants or plants expressing Myc-AtMUB1, GFP-AtMUB1, or GFP-AtMUB6 was recovered in the membrane-enriched fraction (Fig. 4B). For AtMUB1 and -6, this distribution was dependent on the CaAXA sequence as the comparable SAAAX mutant proteins were found exclusively in the soluble fraction. In agreement with a possible modification by acylation, GFP-MUB2 was almost entirely membrane-associated as well. In an attempt to block the modification and subsequent membrane association of AtMUB2, we expressed a cysteine to serine mutant at position 115, one of the four C-terminal cysteines that could theoretically be acceptor sites for thioester acylation (see Fig. 1). However, the membrane association of this C115S mutant was identical to the wild-type AtMUB2, indicating that this residue is not essential for its membrane affinity (Fig. 4B).

Insertion of the prenyl group into the lipid bilayer confers a tight association of the protein with membrane(s), often requiring detergents for effective solubilization. To confirm this tight binding, we tested several conditions known to dissociate either loosely bound (1 M NaCl and 0.1 M sodium carbonate) or integral membrane proteins (1% Triton X-114). Here the membrane-enriched fraction from plants expressing Myc-AtMUB1 was either resuspended in buffer, NaCl, sodium carbonate, or Triton X-114, incubated for 1 h, and then centrifuged to collect the membrane fraction. Although buffer and NaCl were ineffective and sodium carbonate only partially effective, complete solubilization of Myc-AtMUB1 and wild-type AtMUB1 was possible with Triton X-114, consistent with a tight association of AtMUB1 with membranes (Fig. 4C and data not shown).

MUB Proteins Are Localized to the Plasma Membrane in Arabidopsis—Given the observations that GFP-AtMUB1 is membrane-bound in Arabidopsis like its nonderivatized counterpart (Fig. 4B), we used similarly tagged versions to help define the intracellular location of the entire Arabidopsis family by confocal fluorescence microscopy. In most cases, both stably transformed intact plants and transfected protoplasts were analyzed, which gave identical results. For each AtMUB, the distribution of the wild-type version was compared with either SAAAX mutants for GFP-AtMUB1, AtMUB3-6, or a collection of C-terminal Cys mutants for GFP-AtMUB2 to reveal whether prenylation or acylation of these cysteines were required for normal protein targeting.

From the analysis of roots of stably transformed lines, we found that free GFP was present in the cytoplasm and nucleus (Fig. 5A), consistent with previous reports of GFP localization (39, 53). In contrast, roots expressing GFP fused to each of the AtMUB sequences displayed little cytoplasmic or nuclear fluorescence with most of the signal concentrated at the periphery of the cell, a distribution suggestive of plasma membrane binding (Fig. 5A and data not shown). Consistent with the SAAAX mutants preventing both prenylation in vitro and the association of AtMUB1 and -6 with the membrane fraction (Figs. 2 and 4), the SAAAX mutants for GFP-AtMUB1 and -6 were detected in the nucleus and throughout the cytoplasm, a distribution similar to that of free GFP (Fig. 5A). Similar results were also obtained with GFP-AtMUB3–5, the CaAXA versions were plasma membrane-localized, whereas the SAAAX versions had soluble distributions (data not shown). Conversely, the C115S mutant for GFP-AtMUB2 remained localized to the plasma membrane, in agreement with the retention of this mutant in the membrane fraction (Fig. 5A).

To demonstrate that the GFP-AtMUBs were specifically associated with the plasma membrane and not the cell wall, we exposed root cells of GFP-AtMUB1 seedlings to high osmotic conditions in an attempt to separate the two structures by plasmolysis. During this process, we could clearly see the GFP fluorescence moving away from the wall in the GFP-AtMUB1 plants and remaining with the plasma membrane, indicating that this membrane is the main target (Fig. 5B). In fact, fluorescent strands could be detected during plasmolysis that likely represented adhesion points of the plasma membrane to the plasmodesmal connections between cells (Fig. 5B). In contrast, both free GFP and the SAAAX version of AtMUB1 remained in the cytosolic and nuclear compartments during plasmolysis with no indications of a plasma membrane location.

To further confirm a plasma membrane location, we transiently co-expressed GFP-AtMUB1 and -2 with the plasma membrane marker H+-ATPase (encoded by the AHA1 gene) tagged with RFP in protoplasts and then visualized both markers simultaneously by confocal fluorescence microscopy. Chlorophyll autofluorescence was also captured as an indirect marker for pockets of cytoplasm. As can be seen in Fig. 5C, both wild-type GFP-AtMUB reporters were localized to the periphery of the cell, a pattern that was coincident with the RFP-H+-ATPase and clearly distinct from chlorophyll autofluorescence. This pattern for GFP-AtMUB1 changed dramatically to a cytoplasmic and nuclear distribution when the corresponding SAAAX mutant was used (Fig. 5C).

Acylated proteins typically use one or more C-terminal cysteines as acceptor sites for thioester addition of the acyl moiety (49). To test whether the four terminal cysteines in AtMUB2 (residues 115, 117, 119, and 124 (Fig. 5D)) are required for sub-cellular targeting, we converted all four in the GFP-AtMUB2 reporter to serines and examined the location of the mutant protein by transient expression. As shown in Fig. 5E, this 4C-S mutant acquired a cytoplasmic/nuclear distribution. To identify which of the four cysteines are important for targeting, we then tested the four residues individually. Whereas the C115S and C117S mutants remained bound to the plasma membrane like wild-type AtMUB2, the C119S and C124S mutants were mainly in the cytoplasm/nucleus, indicating that these two more C-terminal cysteines are essential for membrane association, presumably via acylation (Fig. 5E).

Mevinolin Inhibits the Plasma Membrane Association of Arabidopsis MUBs—As further support for the prenylation of AtMUB1 and AtMUB3-6 in vivo, we examined the effect of the HMG-CoA reductase inhibitor mevinolin (or lovastatin) on the distribution of GFP-AtMUB1 and -2 in Arabidopsis protoplasts. By inhibiting HMG-CoA reductase, mevinolin blocks
the accumulation of the five-carbon precursor mevalonic acid, which is used to generate long chain prenyl moieties by successive condensation reactions (54). Prior studies confirmed that mevinolin is effective in plant suspension cultures and will reduce the availability of farnesyl and geranylgeranyl pyrophosphate in vivo (35, 55). Following transfection, the protoplasts...
were treated for 8 or 32 h with the drug before microscopic analysis.

As can be seen in Fig. 6, nearly all protoplasts (e.g. 93% after 8 h) transfected with GFP-AtMUB1 in the absence of mevinolin displayed a fluorescent pattern that was exclusively associated with the plasma membrane. Only a small percent (1% after 8 h) showed cytosolic and nuclear fluorescence with the remaining protoplasts (6% after 8 h) still exhibiting a strong plasma membrane labeling along with weaker pockets of fluorescent cytoplasm (see Mix in Fig. 6A). However, after exposure to 10 μM mevinolin, a substantial relocalization of GFP-AtMUB1 was seen with a majority of the protoplasts now displaying strong GFP fluorescence in the cytoplasm and nucleus (52% after 8 h) and only a few retaining fluorescence solely on the plasma membrane (Fig. 6, A and B). In contrast, the near exclusive plasma membrane distribution of GFP-AtMUB2 was unaffected by the presence of mevinolin, with almost all of the transfected protoplasts showing strong fluorescence at this membrane even after 32 h of incubation with the drug (data not shown). In addition to indicating that the change in GFP-AtMUB1 distribution induced by mevinolin was not the result of general toxicity, the data for AtMUB2 further support the notion that this isoform is membrane-anchored by a mechanism other than prenylation.

MUB Localization Is Disrupted in Prenylation-defective Arabidopsis Mutants—To further confirm that prenyl addition anchors MUBs to membranes, we examined the membrane association of Arabidopsis MUB1 in several previously described prenyltransferase mutants. The plp-1 mutant lacks the common α subunit of prenyltransferases (26), whereas the era1-4 (29, 30) and ggb-2 (28) mutants are defective in the β subunits specific for either the PFT or PGGT-1 activities, respectively. Whole seedling extracts from the mutants along with their comparable wild-types (Col-0 for plp-1 and ggb-2 and
Ler for era1-4) were separated into the soluble and membrane fractions as above and analyzed by SDS-PAGE and immunoblotting with anti-AtMUB1 antibodies. Similar to wild-type Col-0 (Fig. 4B), nearly all of AtMUB1 was membrane-bound in the wild-type Ler background (Fig. 4D). However, as would be expected for a prenylated protein, most of AtMUB1 was in the soluble fraction of extracts from era1-4 plants (lacking PFT) (Fig. 4D). Unlike the distribution seen with the SAAAX mutant, a small amount of AtMUB1 remained membrane-bound in the era1-4 and plp-1 mutants. This residual pool could represent the addition of a geranylgeranyl moiety to AtMUB1 in the absence of PFT activity or the modification of AtMUB1 with other membrane anchors (e.g. long chain acyl groups).

As further evidence for in vivo prenylation of AtMUBs, we examined the localization of GFP-AtMUB1 and GFP-AtMUB6 in protoplasts derived from wild-type and era1-4 and ggb-2 mutants. (Cells from the plp-1 mutant plants were not sufficiently hardy to survive efficient protoplast formation.) As seen in Fig. 6, C and D, the majority of GFP-AtMUB1 signal was localized to the plasma membrane of cells in wild-type and ggb-2 protoplasts but was found in mainly the cytoplasm and nucleus of the PFT-deficient era1-4 protoplasts (Fig. 6, C and D), in agreement with our in vitro assays (Fig. 2, C and D) and the membrane association of AtMUB1 in prenylation-deficient mutants (Fig. 4D). Conversely, the bulk of the GFP-AtMUB6 signal was confined to the plasma membrane of wild-type and era1-4 protoplasts but was mainly cytoplasmic and nuclear localized in PGGT I-deficient ggb-2 protoplasts. Both GFP-AtMUB1 and GFP-AtMUB6 localized to the plasma membrane in the majority of wild-type Col-0 and Ler protoplasts.

**DISCUSSION**

It is now apparent that the superfamily of Ub-fold proteins encompasses an expanded set of modifiers with highly divergent targets and functions (1, 4, 5). In addition to becoming conjugated to other proteins (e.g. Ub, SUMO, ATG12, ISG15, URM, and HUB), the recent characterization of ATG8 revealed that lipids can also be attached. Here we add the MUB family to the Ub-fold pantheon with the unique feature that they become modified by prenylation and/or possibly acylation. We could detect MUB representatives in all eukaryotes for which sufficient genomic sequence was available, with the exception of unicellular budding and fission yeasts, suggesting that they are restricted to species with multicellular development. Of potential significance is that another Ub-fold protein, Ufm1, displays a similar distribution, being present in plants, filamentous fungi, and animals but absent in budding and fission yeasts (56). Like others in the Ub-fold group, Arabidopsis MUB1 adopts a β-grasp core with a protruding C-terminal tail. Consistent with the extreme structural stability of the β-grasp domain, the Arabidopsis MUBs, like Ub and SUMO, are heat-stable and remained soluble at temperatures exceeding 90 °C (data not shown). Like Ub (57), the plant and animal MUBs tested here likely refold easily, because they could still be prenylated even after exposure to strong denaturing conditions (8 M urea (see Fig. 2)). Most of the MUBs we identified were 117–124 amino acids in length. The only exceptions were orthologs from various filamentous fungi, all of which have long N-terminal extensions of 110–130 residues. These extensions bear no resemblance to known protein motifs, thus leaving their function(s) unclear.

In most cases, MUBs terminate in a C-terminal CAAX box that can direct prenylation in vitro. The preferences for farnesylation or geranylgeranylation vary among different species, e.g. human and yeast Ras (62, 63)). Such dual membrane anchors are likely created to increase the requirements for membrane targeting, with the most likely candidate for AtMUB1 being farnesylation. Fifth, this membrane association can be disrupted for one representative MUB (AtMUB1) in Arabidopsis mutants defective in prenyltransferase activity. And sixth, the plasma membrane localization of GFP-AtMUB1 and GFP-AtMUB6 can be disrupted in era1-4 and ggb-2 protoplasts lacking PFT and PGGT-I activities, respectively. These preferential effects of the mutants further confirm in vivo the specificity of Arabidopsis prenylation and CAAX box enzymes for the terminal amino acid of the CAAX box.

Although we present strong evidence that MUBs are prenylated in vivo, we cannot rule out the possibility that they are acylated as well, as some prenylated proteins have been shown to be initially prenylated and then palmitoylated on nearby cysteine residues (e.g. human and yeast Ras (62, 63)). Such dual modification may create a stronger membrane anchor than is possible by prenylation alone (64). It is noteworthy that almost all MUBs contain a conserved cysteine residue one or two
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amino acids upstream of the CAXX box that could serve as an acyl acceptor.

In addition to the more typical versions, Arabidopsis contains a variant (MUB2) that does not end in a CAXX box but with an extended sequence rich in cysteine residues. We have been unable to detect AtMUB2 orthologs in any other organism, suggesting that this isoform may be restricted to Arabidopsis. Although AtMUB2 was poorly prenylated in vitro, in vivo localization studies with a GFP fusion showed that it is also membrane-anchored and more specifically to the plasma membrane. How this soluble protein becomes membrane-localized has not been conclusively determined, but a likely mechanism is the post-translation addition of a long chain acyl moiety such as palmitic acid (49). Protein acylation does occur in plants (65). In fact, Lavy et al. (36) recently showed that a subfamily of membrane-bound type II RAC proteins in Arabidopsis are not prenylated but modified with palmitic acid. For AtRAC8, this modification slows the electrophoretic mobility of the protein much the same way that AtMUB2 expressed in vivo has a slower mobility than its recombinant counterpart. Here we found that recombinant AtMUB2 can be modified with palmitate at least in vitro, but the site of addition is not yet known. Mutating potential acyl acceptor cysteines in the C terminus of MUB2 causes its mislocalization from the plasma membrane. Assuming that AtMUB2 is acylated, one obvious consequence is that Arabidopsis will have at least one MUB isoform bound to the plasma membrane even in the absence of either prenyl- or acyl-transferase activities.

At present, the physiological functions of MUBs are unclear. Their widespread distribution among most eukaryotes implies that they participate in a general metabolic/developmental process. The proposed prenyl/acyl modifications at their C termini preclude them from becoming attached to proteins and thus functioning like many other members of the Ub-fold superfamily. Notably, MUBs have several of the lysines used by Ub for subsequent polymerization of Ub chains, suggesting that MUBs could be substrates for other members of the Ub-fold family. However, immunoblot analysis with anti–AtMUB1 antibodies failed to consistently detect higher molecular mass forms in Arabidopsis (data not shown), indicating that if these conjugates do exist, they are present at low levels. Of potential importance are our observations that both GFP- and Myc-tagged versions of AtMUB1 and -6 bearing a CAXX C terminus, which would a priori not be prenylated, failed to elicit any abnormal phenotypes when expressed to high levels in Arabidopsis. Although the lack of effect may indicate that N-terminal extensions are detrimental to AtMUB function or disrupt any AtMUB-binding partner interactions, it is also possible that the action(s) of CAXX box-containing MUBs are not affected dominantly by the presence of free nonprenylated forms. Given the pleiotropic consequences for the Arabidopsis plp, era, and ggb mutants missing PFT and/or PGGT-I activities (26, 28–30), it is apparent that prenylation is critical for normal growth and development in plants. Although the underlying reason(s) for most of these defects remains unclear, it is possible that one or more is associated with a mis-localization of MUBs.

Whatever the function(s) of MUBs, it is likely that plants have expanded their actions. Although animal and fungal species appear to express only a single MUB isoform, multiple MUBs are present in higher plants, with six evident in Arabidopsis. DNA microarray expression studies detected relatively consistent levels of AtMUB2–6 transcripts in all Arabidopsis tissues examined (AtMUB1 was not included in these studies), implying that MUBs are required for a physiological process that is universal in all plant tissue and cell types. There is also little evidence from current microarray data that the expression patterns of individual AtMUB genes are regulated by external factors. We have recently identified transfer-DNA insertion mutants for AtMUB2 and -3. The homozygous plants were indistinguishable from wild type under standard growth conditions, suggesting that functional redundancy within the family exists (data not shown). Consequently, it may be best to genetically dissect MUB function in species with only one MULB gene.

One possible function for MUBs is that they serve as docking sites to facilitate the association of other proteins to the plasma membrane. A model for such a role may be the Ub-fold protein ATG8 (18). Upon modification with phosphatidylethanolamine, ATG8 associates with autophagosomes to help promote the delivery of these vesicles to the vacuole/lysosome during autophagic recycling (18, 66). Recently, ATG8 has been shown to interact with microtubules, thus providing a potential mechanism by which the lipidated form of ATG8 directs the transport of autophagic vesicles (67, 68). In a similar fashion, Ub participates in protein sorting and vesicular trafficking by becoming associated with membrane-localized proteins containing UIM and UBA domains after its conjugation to other membrane-bound proteins (69–71). Given the plethora of proteins with binding sites for Ub, it is conceivable that analogous binding proteins exist for MUBs. Clearly, to define the physiological functions of MUBs, it is now essential that we identify other cellular factors that associate with these membrane-anchored Ub-fold proteins.

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