ARABIDOPSIS MEMBRANE-ANCHORED UBIQUITIN-FOLD (MUB) PROTEINS LOCALIZE A SPECIFIC SUBSET OF UBIQUITIN-CONJUGATING (E2) ENZYMES TO THE PLASMA MEMBRANE

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The covalent attachment of ubiquitin (Ub) to various intracellular proteins plays important roles in altering the function, localization, processing, and degradation of the modified target. A minimal ubiquitylation pathway uses a three enzyme cascade – E1, E2, and E3 – to activate Ub and select target proteins for modification. While diverse E3 families provide much of the target specificity, several factors have emerged recently that coordinate the subcellular localization of the ubiquitylation machinery. Here we show that the family of Membrane-anchored Ubiquitin-fold (MUB) proteins recruit and dock specific E2s to the plasma membrane. Protein interaction screens with Arabidopsis MUBs reveal that interacting E2s are limited to a well-defined subgroup that is phylogenetically related to human UbcH5 and yeast Ubc4/5 families. MUBs appear to interact non-covalently with an E2 surface opposite the active site that forms a covalent linkage with Ub. Bimolecular fluorescence complementation (BiFC) demonstrates that MUBs bind simultaneously to the plasma membrane via a prenyl tail and to the E2 in planta. These findings suggest that MUBs contribute subcellular specificity to ubiquitylation by docking the conjugation machinery to the plasma membrane.

Protein ubiquitylation is a reversible, post-translational modification regulating target protein activity, localization, and degradation in diverse signaling pathways (1,2). The fate of a Ubiquitin (Ub)-targeted protein ultimately depends on the length and linkage of the attached Ub molecules (3). Ub Lys48-linked chains, for instance, typically direct target proteins into the Ub/26S Proteasome System (UPS) for degradation. Ubiquitylation at the plasma membrane often differs from the canonical UPS through reversible monoubiquitylation, protein recycling between the membrane and endomembrane system, the assembly of Lys63-linked chains, and terminal lysosomal targeting (4). For example, monoubiquitylation of plasma membrane proteins triggers their endocytosis (1,3,5,6).

Ub and related proteins are part of the β-grasp protein structure family that is characterized by a transverse α-helix cradled in a five-stranded β-sheet. The C-terminal diglycine of Ub is necessary for activation by an E1 enzyme, transfer to the active site of a Ub-Conjugating enzyme (E2 or UBC), and covalent attachment to a target protein guided by an E3 enzyme. Ub-related proteins should be considered either Ub-like when they are used in a covalent protein conjugation reaction, or, if not, simply Ub-fold. Functional variation in protein conjugation is attributed to a growing list of Ub-like proteins including SUMO, RUB (NEDD8), UFM, and others (7). Alternatively, Ub-fold proteins, which lack a C-terminus for protein conjugation, use the β-grasp for protein interactions that guide subcellular organization of ubiquitylation. For instance, RAD23 directs ubiquitylated cargoes to the 26S proteasome, and phosphatidylethanolamine-conjugated ATG8 marks membranes in developing autophagosomes (8,9).

Throughout the UPS, the Ub-fold is recognized by various Ub Binding Domains (UBD) from the initial E1 enzyme (10) to the Ub-conjugate docking 26S Proteasome subunits RPN1, 10, and 13 (11). An Ile44-centered hydrophobic surface on the exterior of the Ub β-sheet is most commonly recognized by UBDs. Certain E2s can also interact with this surface without encroaching on their active site. Specifically, solution-based 2D-NMR studies detected Ub bound non-covalently near Ser22 on...
human (Hs)UbcH5c (12), which has been confirmed by activity assays (13,14) and crystallography for HsUbcH5b (14). Non-covalent Ub-E2 interaction is proposed to aid assembly of Ub-E2 polymeric complexes for enhanced target protein polyubiquitylation (12,14). Ubiquitin conjugating Enzyme Variants (UEVs) resemble E2s but lack an active site cysteine thus precluding covalent conjugation to Ub-like proteins. Nevertheless, UEVs can diversify E2 activities (15-17). In fact, a non-covalent interaction between Ub and the UEV Mms2 promotes the UEV/E2 heterodimer Mms2/Ubc13 to form Lys63-linked poly-Ub chains (16). In yeast, a related non-covalent interaction between SUMO and its E2, Ubc9, also stimulates poly-SUMO chain formation (18-23).

Membrane-anchored Ub-fold (MUB) proteins are recent additions to the β-grasp structure family. When compared to Ub, Arabidopsis MUB1 (AtMUB1) and human MUB (HsMUB2, previously known as UBL3) show strong 3-dimensional similarity in the β-grasp, but longer N- and C-termini and extended loops create MUB-unique surfaces (24-26). MUBs are distinguished from other Ub-fold proteins by a C-terminal CAAX box that is modified through protein prenylation with a hydrophobic membrane anchor. Prenylation and membrane localization preclude attachment of MUBs to target proteins. MUBs from nematode, insect, fish, and mammals, including humans, are prenylated in vitro. In planta, AtMUB membrane localization is prenylation-dependent where mutation of the prenyl-attachment CAAX cysteine to a serine (SAAX) prevents processing and causes accumulation of non-membrane localized MUB. Likewise, Arabidopsis prenyltransferase mutants block membrane localization of endogenous AtMUB1 (26). The six MUB genes of Arabidopsis are divided by sequence homology into three subgroups – MUB1/2, MUB3/4, and MUB5/6 – suggesting functional diversification of plant MUBs. In contrast, multicellular fungi and animals have only a single MUB gene, and no MUB is evident in yeast (26). While the structure and the post-translational prenyl modifications of MUBs have been well characterized, the function of these Ub-fold proteins is unknown.

In this work, we demonstrate that Arabidopsis MUB proteins interact with a specific subgroup of E2s. We provide evidence that MUBs directly interact with an E2 non-covalent binding surface in vitro and validate the interaction in planta by co-immunoprecipitation (co-IP) and bimolecular fluorescence complementation (BiFC). From these data, we propose that MUB proteins contribute subcellular specificity to the ubiquitylation system by recruiting specific E2s to the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Phylogenetic Sequence and Structure Analyses**-E2 amino acid sequences were trimmed to core domains as identified previously (27). Initial pairwise alignment of the sequences was done in ClustalX2 (28) using the PAM350 protein weight matrix permitting gap opening and extension penalties of 35.0 and 0.75. Final alignment (Supplemental Fig. 1) was manually performed with Se-Al version 2.0a11 (29). The reported phylogenetic tree is the strict consensus of three possible trees generated by a maximum parsimony full heuristic search in PAUP* version 4.0b10 (30) with Arabidopsis UBC1 (E2 for UFM1) as an outgroup. Bootstrap support was determined with 1000 pseudoreplicates by the TBR branch swapping algorithm and mapped onto the strict consensus. Alignments were annotated for identity and conservation using JalView version 2.5 (31,32). Protein structures for AtMUB1 (1SE9) and Ub-UbcH5c (2FUH) from NCBI Molecular Modeling Structure Database were annotated using PyMOL Viewer (http://www.pymol.org/).

**Vectors and Plasmid Construction**-All Gateway entry and ligation cloned vectors were sequence verified. Primer sequences for entry vectors and mutagenesis are listed in Supplemental Table I. For Gateway™ cloning (Invitrogen), genes within entry vectors were recombined into destination vectors by LR Clonase II reactions and confirmed by restriction mapping. All Gateway-cloned constructs are summarized in Supplemental Table II. Amino acid substitutions in AtMUBs and AtUBCs were made following the QuickChange Site-Directed Mutagenesis protocol (Stratagene). Arabidopsis E2 sequences, excluding AtUBC7 and AtUBC23, were obtained from the ABRC (Columbus, OH) as cDNA clones in pDONR201 (27) (Supplemental Table II). Construction of
plasmids pDEST-GBK7 (33), pACT2.2gtwy (Addgene plasmid 11346), pEarleyGate (pEG) 100, pEG104, pEG201, pEG202 (34), pGGWA, pHGWA (35), pET28a-UbcH5c, pET28a-UbcH5c S22R (Addgene plasmids 12643 and 12644) (12), and pET28b-AtMUB1 through AtMUB6 (26) were previously described. The pSAT1-CAMBIA-nEFYP-C1 and pSAT1-CAMBIA-cEFYP-C1 N-terminal split-YFP BiFC transformation vectors were constructed with expression cassettes from pSAT1-nEYFP-C1 and pSAT1-cEYFP-C1 (36) inserted into pCAMBIA0380 (37).

To create Gateway entry vectors, AtMUB3 and AtMUB4 were PCR amplified from published pET28 vectors (26) with forward primers including a 5' CACC sequence and reverse primers designating 3' CAAX or SAAX; products were directionally cloned into pENTR/D-TOPO (Invitrogen). AtCOP10 and AtUEV1B were amplified from Columbia-0 cDNA, HsMUB from Sk-Hep cDNA (38), and similarly cloned into pENTR/D-TOPO. The AtCOP10 splice variant At3g13550.1 was gel excised prior to D-TOPO cloning. For the FLAG-GUS control vector, PCR-modified pENTR-GUS (Invitrogen) was recombined into pEG202 (Supplemental Table II).

The mCerulean plant nuclear marker was created by PCR amplifying the N-terminal nuclear localization signal (NLS) of AtZFP11 (At2g42410) (39) from Columbia-0 genomic DNA, primers adding a 5' CACC and a 3' Xhol site, and inserted into pENTR/D-TOPO. The mCerulean (mCER) sequence was PCR amplified from pmCerulean-C1 (40) to include a C-terminal stop codon (*) and flanking Xhol sites, ligated into similarly cut pENTR/D-TOPO+ZFP11(NLS), sequence confirmed, and recombined into pEG100 (34) to create the binary vector pEG100+ZFP11(NLS):mCER*.

Using conventional cloning methods, AtMUB1, 2, 3, 6 (CAAX and SAAX forms), and Ub were PCR amplified with flanking 5' NdeI and 3' PstI sites and cloned into pGBK7 yeast two-hybrid vector (Clontech). AtMUB4 and AtMUB5 were PCR amplified with 5' EcoRI and 3' BamHI sites and cloned into pGBK7. AtMUB3, AtMUB4, AtUBC8, and AtUBC9 were PCR amplified from entry vectors with 5' Xhol and 3' XbaI sites and cloned into similarly cut pSAT1-CAMBIA BiFC vectors.

Yeast Two-Hybrid Analyses-The yeast two-hybrid cDNA library screen was performed by the Molecular Interaction Facility (Madison, WI) following standard protocols (41). Haploid yeast mating strains PJ694A (for MUBs) and PJ694a (for UBCs and AtUEVs) (42) were transformed using the Frozen-EZ Yeast Transformation II method (Zymo Research). Yeast were grown at 30°C on YEPD or synthetic drop-out (SD) media lacking amino acids as appropriate for mating and plasmid selection. Sterile 3-amino-1,2,4-triazole (3-AT) at 0.5, 1, 2, or 4 mM added stringency on SD media lacking histidine. Interaction data are reported only in the absence of growth of empty vector controls. Results were confirmed with standard β-gal filter lift assays (data not shown).

Protein Purification and In vitro Interaction Studies-Co-expression of AtMUB3 SAAX and AtUBC8 was carried out in BL21 E. coli. All other proteins were expressed in Rosetta2 E. coli (Novagen). Expression of 6xHis-tagged AtMUB3 was induced with 0.2 mM IPTG (β-D-1-thiogalactopyranoside) for 12 hr at 19°C. Cells were extracted by ultrasonication in PBS (20 mM phosphate, 200 mM NaCl, pH 6.8) and clarified by centrifugation. PMSF was maintained at 1 mM throughout purification. Extract was incubated with TALON metal affinity resin (Clontech) for 30 min followed by three washes in 10 mM imidazole PBS, three washes in 50 mM imidazole PBS, and elution with 250 mM imidazole PBS. Expression of GST-tagged AtUBC8 and AtUBC8 S22R was induced as above, soluble protein extracts incubated with immobilized GSH resin (Thermo Scientific) for 30 min followed by three washes with PBS. GST-tagged proteins were eluted in 15 mM reduced GSH PBS. For pull-down assays, GST-tagged AtUBC8 or AtUBC8 S22R was immobilized on GSH resin and mixed with purified 6xHis-tagged AtMUB3 for 30 min, washed three times with PBS, and boiled in Laemmli sample buffer. All purifications were analyzed using SDS-PAGE and Coomassie stain or immunoblot, as appropriate.

Agrobacterium Infiltration, Co-IP, and BiFC-Binary vectors were transformed into Agrobacterium tumefaciens strain GV3101 by electroporation. Cells from an overnight culture were washed with sterile 20 mM MgSO4, and 300 µM MES, 10 mM MgSO4, 100 µM
acetosyringone, pH 5.2). Bacterial infiltration solutions were incubated 3 hr at 25°C with shaking before mixing the cultures, as appropriate, and infiltrating into young, nearly expanded Nicotiana benthamiana leaves. Three to five week-old plants, grown in 16 hr days under fluorescent light, were kept in the dark for 2 days following infiltration before returning to the light for 1 day. Cells were imaged by confocal microscopy with a Zeiss LSM 5 using an argon laser. The YFP signal was obtained with 514nm excitation with HFT 458/514, NFT545, and BP530-600 filters. For negative controls, cells within infiltrated regions of tissue were imaged. Autofluorescence was excited at 514nm and collected with HFT 458/514, NFT545, and LP650 filters, and mCerulean was excited at 458nm and collected with HFT 458/633, NFT490, and BP475-525 filters. Image brightness and contrast adjustments and overlays were done with ImageJ version 1.44b (http://rsb.info.nih.gov/ij).

For co-IP studies, Agrobacterium cultures were normalized to 0.6 OD600 in infiltration buffer, mixed, and co-infiltrated. N. benthamiana tissue was ground with three volumes [weight/volume (w/v)] of pre-chilled extraction buffer (50 mM Tris adjusted to pH 8.0 with MES, 0.5 M sucrose, 1 mM MgCl2, 10 mM EDTA, 5mM DTT) adapted from Liu et al. (43) with 1 mM PMSF and 1:100 plant protease inhibitor cocktail (Sigma-Aldrich) in a 4°C cold room. Homogenate was centrifuged at 4000 g for 15 min and clarified again at 2000 g for 10 min. Total protein was quantified by Coomassie Plus Assay (Pierce) and samples normalized to 2 mg/mL. Supernatant was incubated with anti-FLAG M2 Affinity Gel (Sigma-Aldrich) for 1 hr at 4°C, washed three times with PBS with 0.05% Tween-20, boiled with Laemmlı sample buffer, and analyzed by SDS-PAGE and immunoblot with anti-HA-HRP and anti-FLAG (Sigma-Aldrich).

RESULTS

A yeast two-hybrid (Y2H) screen reveals MUB-interacting E2s. AtMUB1 was used as bait in a GAL4 Y2H Arabidopsis cDNA screen of more than 18 million clones representing mRNA from tissues, cultured cells, and diverse physiological conditions (Molecular Interaction Facility; Madison, WI), and identified multiple occurrences of three E2s: AtUBC8, 9, and 10 with 20, 2, and 5 hits, respectively. To facilitate nuclear interaction of the GAL4 transcriptional subunits, a prenylation deficient mutant, AtMUB1 SAAX (C114S), was used as bait. In directed Y2H experiments, both WT CAAX and prenylation deficient SAAX forms of AtMUBs interacted with AtUBC8, 9, and 10, but results using the SAAX versions were most consistent (data not shown). Therefore, SAAX MUB bait constructs were used in subsequent experiments.

Arabidopsis MUBs specifically interact with Subgroup VI E2s. Directed Y2H experiments were performed to determine whether MUBs interact with additional E2s that were undetected due to gene expression bias in the cDNA library and to establish an interaction pattern between MUBs and E2s. Thirty-seven AtUBCs were organized into 14 subgroups by Kraft et al. (27) based on the phylogenetic relationship of the enzymatic core domain. This diverse Arabidopsis E2 family is represented in Fig. 1A with additional gene structure characteristics that validate the well-supported sequence relationships (bootstrap support in Fig. 1A). Haploid yeast strains for all six AtMUB SAAX and Ub baits were crossed with all 14 E2 subgroups as prey. In total, 35 of 37 Arabidopsis E2s and two UEVs, AtCOP10 and AtUEV1B (MMZ2), were examined. AtUEV1B was tested because it is an Arabidopsis homologue of HsMms2 (17), and it contains a C-terminal CAAX (CCVM) signal which might localize it with MUBs at a membrane. AtCOP10 was tested because, amongst the UEV family, it is most closely related to AtUBCs 8, 9, and 10 (Fig. 1A).

In this comprehensive, directed survey of MUB-E2 interactions, all six AtMUBs interacted with E2s confirming the AtMUB1 library screen results (Fig. 1B). AtMUBs interacted exclusively with Subgroup VI E2s including AtUBC8, 9, and 10, as detected in the initial Y2H screen, and also with AtUBC11, 28, 29, and 30 (Fig. 1B). AtUBC12 and 19 failed to interact, but were excluded from further analysis because growth was auto-activated with various empty bait vector controls. Within the positive Subgroup VI interactions, MUBs showed a combinatorial pattern of interaction with E2s. For instance, AtUBC9 interacted with all six AtMUBs, while AtUBCs 11 and 30 interacted only with the AtMUB3/4 subgroup (Fig. 1B).
Ironically, the original bait, AtMUB1, shows the weakest E2 interaction profile, only positive for AtUBCs 8 and 9 under the assay conditions shown in Fig. 1B. No interaction was detected between AtMUBs and a SUMO E2, AtSCE1 (data not shown), AtMUBs and AtUEVs, or between Ub and any of the Subgroup VI E2s by Y2H.

AtMUB3 and AtMUB4 yeast colonies showed robust growth with the broadest number of E2s and were used for subsequent experiments. AtMUBs 3 and 4 are representative of the two prenylation modifications: geranylgeranylation and farnesylation, respectively (26). The AtMUB3/4 subgroup also represents the residue variation seen in the Ub Ile44-homologous surface where a number of UBDs are known to interact (Fig. 2C and D) (24). The mutation of yeast non-essential residue Ub T66E disrupts binding of UBDs to the Ile44 surface while preserving Ub conjugation activity (12,44). Thr66 in Ub is substituted with a Val in AtRUB and all known animal and fungal MUBs (26). In Arabidopsis, Val and Thr are alternately found at the same position in MUB3 and 4, respectively (Fig. 2C).

Subgroup VI E2s and MUBs contain structure-predicted non-covalent interaction sites. We constructed an alignment of MUB-interacting and non-interacting E2s to identify any common sequence motif responsible for the Y2H interaction pattern. Fig. 2A illustrates sequence identity in a subregion of the E2 core encompassing β-sheets 1 through 3. As expected, there was high sequence conservation throughout the core, even when comparing representative E2s of distantly related subgroups (Supplemental Fig. 1). This relationship is evident in the number of residues with 80% identity, highlighted in black in the alignment (Fig. 2A). However, weak overall E2 conservation spanning β-sheets 1 and 2 contrasts the strong conservation within MUB-interacting E2s including an absolutely conserved S22, indicated in Fig. 2A.

The MUB-interacting Subgroup VI E2s are closely related to human Ubch5a-c and yeast Ubc4/5 E2 families. Non-covalent Ub contact residues identified on HsUbcH5b-c span β-sheets 1-3 and are also conserved in MUB-interacting E2s (Fig. 2A and B). We found that HsMUB also interacts with HsUbcH5c by Y2H (Fig. 2E). In fact, HsMUB interacts with AtUBCs 8 and 9, and AtMUBs 3 and 4 interact with HsUbcH5c, demonstrating an evolutionarily conserved interface.

Conserved sequence between Arabidopsis MUBs and Ub is depicted in Fig. 2C. Ub-E2 contact residues conserved in MUBs form a discrete Ub Ile44-proximal surface including Thr66 (Fig. 2D), and, in conjunction with E2 conservation, led to the hypothesis that a similar non-covalent interaction occurs between MUBs and Subgroup VI E2s.

MUBs interact non-covalently with E2s on a surface distinct from the E2 active site. To determine whether MUBs and E2s interact in a fashion analogous to Ub and HsUbcH5b-c, mutagenesis was performed in the predicted interaction sites based on the mutations HsUbcH5c S22R and Ub T66E (12). Homologous V86E and T86E mutations were made in AtMUB3 and AtMUB4, respectively. Likewise, AtUBC8 S22R and AtUBC9 S52R non-covalent interaction site mutations and active site C85S and C115S mutations were constructed. As shown in Fig. 3, the S22R and S52R mutations in AtUBC8 and AtUBC9 completely abolished MUB-E2 interaction by Y2H. The homologous HsUbcH5c S22R mutation also interrupted the interaction with HsMUB (Supplemental Fig. 3). Similarly, the non-covalent interaction mutants AtMUB3 V86E and AtMUB4 T86E were unable to interact with either WT or mutant E2s. Alternatively, E2 active site mutations did not interfere with MUB interaction by Y2H. Taken together with the strong sequence and structural conservation, these data support the conclusion that MUBs and Ub interact with this versatile E2 interface.

MUBs and E2s bind directly in vitro. To establish whether MUB-E2 binding is dependent upon additional eukaryotic proteins, the interaction was reconstituted in vitro. Recombinant, purified AtMUB3 was pulled-down by GST-AtUBC8 but not GST-AtUBC8 S22R or GST alone (Fig. 4A) demonstrating that Ser22 is required for direct binding to AtMUB3. We were unable to perform the reciprocal experiment because purified AtMUB3 was unstable when reapplied to metal affinity resin. We circumvented this detail by using lysates from E. coli co-expressing the proteins. Here, AtUBC8 pulled-down AtMUB3 and vice versa, but AtUBC8 S22R only weakly bound AtMUB3 (Fig. 4B). These in vitro assays confirm the Y2H interaction data and also
establish that MUB-E2 binding can occur in the absence of additional eukaryotic proteins.

MUB and E2 non-covalent interaction mutations prevent co-IP in planta. To examine the MUB-E2 interface in planta, anti-FLAG co-IP was performed on N. benthamiana leaf tissue co-infiltrated with combinations of WT or mutant FLAG-AtMUB3 CAAX and HA-AtUBC8 constructs using FLAG-GUS as a negative control. To compensate for lower AtUBC8 S22R expression, three times more total extract was loaded (Fig. 5A 4th and 5th lanes) and applied to resin relative to WT AtUBC8. WT AtUBC8 co-precipitated only with WT MUB3, but not with AtMUB3 V86E or GUS. AtUBC8 S22R failed to co-precipitate with either WT or mutant AtMUB3 (Fig. 5A).

MUBs localize E2s to the plasma membrane in planta. We additionally sought to identify the localization of the MUB-E2 interaction through BiFC of split-YFP. A full-length YFP fusion of AtMUB3 CAAX localizes to the plasma membrane in N. benthamiana leaf cells (Fig. 5B). Alternately, YFP-tagged AtMUB3 SAAX and AtUBCs 8 and 9 all localize in nuclei and cytoplasm. These MUB localization results in N. benthamiana are similar to previous observations in Arabidopsis protoplasts and stably-transformed plants (26).

When nYFP-MUB3 SAAX is co-infiltrated with cYFP-AtUBC8 or 9, fluorescence is observed in nuclei and cytoplasmic strands indicating the proteins do indeed interact (Fig. 5C). Importantly, when nYFP-MUB3 CAAX is co-infiltrated with cYFP-AtUBC8, not only is fluorescence observed, but it is localized to the plasma membrane (Fig. 5C and D). The same result was seen with AtMUB3 CAAX and AtUBC9. Likewise, AtMUB4 CAAX co-infiltration with AtUBC8 or 9 also localized fluorescence to the plasma membrane (Supplemental Fig. 4). Split-YFP fusions of MUBs or E2s did not fluoresce when co-expressed with the complementing split-YFP empty vector (Fig. 5C left and top panels). The BiFC experiments demonstrate that transgenic MUB fusions are properly recognized by prenylation machinery in plants and, as predicted, localized to the plasma membrane. These experiments also show that MUBs and E2s interact in vivo and that MUB prenylation and membrane localization is sufficient to, at a minimum, recruit E2s to the plasma membrane.

DISCUSSION

Prior to this work, the family of eukaryotic MUB proteins were determined to be associated with the plasma membrane in vivo, but remained functionally uncharacterized. Here, we provide the first evidence that MUBs are a direct physical link between the plasma membrane and the ubiquitylation system. Specifically, MUBs recruit a subset of E2s that are defined by a structurally conserved interface, which also interacts non-covalently with ubiquitin (12-14,45) and possibly other ubiquitin-like proteins.

Y2H, pull-down, and in planta co-IP assays with non-covalent surface mutations identified the interface for the MUB-E2 interaction. Co-IPs performed using MUB CAAX proteins parallel the native localization of MUBs with plasma membrane peripheral proteins. Furthermore, BiFC experiments confirm the interaction and demonstrate that prenylated MUB proteins can simultaneously bind to the plasma membrane and to E2s. Based on this, we hypothesize that MUBs recruit E2s, perhaps to regulate the homeostasis of active Ub in the plasma membrane proximal space.

The seven Arabidopsis Subgroup VI E2s that interact with MUBs are essentially composed of the core domain, are active with a wide array of E3s (27,46), and have been characterized extensively in vitro (47,48). Related E2s in animals and fungi, including the human UbcH5 and yeast Ubc4/5 families, have also served as robust model E2s. It is possible that the small size of the Subgroup VI E2s permits access to a diverse array of targets while restricted to the two-dimensional cytoplasmic surface of the plasma membrane. Here, by identifying the capacity to be recruited to the plasma membrane by MUB proteins, we add a distinguishing biological trait for this E2 family.

We were unable to detect MUB Y2H interactions with E2s outside of this subgroup, including the UEVs AtCOP10 and AtUEV1B (Fig. 1A, Supplemental Fig. 2). Published NMR studies corroborate that AtCOP10, though sister to the MUB-interacting subgroup, does not interact non-covalently with Ub (13,15).
The Mms2-related AtUEV1B and the HsUbchH5-related Arabidopsis Subgroup VI E2s also did not interact with Ub by Y2H. This result is not contradictory since the initial evaluations of Mms2 and HsUbchH5 with Ub was through sensitive NMR-based methods (12,14,16). However, this does raise the possibility that MUBs bind Subgroup VI E2s with greater affinity than the Ub-E2 interactions previously described.

Individual subgroup VI E2s likely serve specialized functions in planta as suggested by the combinatorial pattern of Y2H interactions with MUBs. For instance, the AtUBC29 and 30 proteins are 95% similar with comparable expression in planta (27), but only AtUBC29 interacts with AtMUB6. This pattern echoes previously described combinatorial UPS interactions among SCF E3 subunits (49) or between an E3 and multiple E2s (46). The modular AtMUB and E2 interactions are consistent with an expanded plant UPS compared to animals and fungi (46,48,49). Regardless of the species, MUBs may help refine ubiquitylation target selection, reaction dynamics, or localization.

MUB prenylation and E2 binding ability is reminiscent of Pex22p and Cue1p localizing E2s to endoplasmic reticulum (ER) and peroxisome membranes, respectively. In yeast, the transmembrane protein Pex22p recruits the E2 Pex4p to the outer peroxisomal membrane causing monoubiquitylation and export of Pex5p to the cytoplasm (50,51). This process appears to be conserved in plants as a functionally equivalent AtPEX4/AtPEX22 complex is needed to sustain peroxisome biogenesis (52,53). Also in yeast, transmembrane protein Cue1p recruits the E2 Ubc7p to the cytoplasmic surface of the ER for ER associated degradation (ERAD) of errant proteins (54-57). Now, with the addition of MUBs, a theme emerges where small adapter proteins anchor E2s to the cytoplasmic surface of specific membranes. Future studies will determine whether MUBs also mediate ubiquitin-directed movement of membrane proteins into the cytoplasm like Pex22p and Cue1p.

In the simplest model, MUBs could recruit an activated E2 to the plasma membrane to donate its Ub to a target or an elongating Ub-chain (Fig. 5E). Alternatively, the recruited E2 could donate its Ub to Ub-equivalent Lys29, 48, or 63 (26) on a MUB protein. Ubiquitylation of these lysines would present mono-Ub for UBD docking or establish membrane-localized reservoirs of various Ub-chains.

MUBs may also influence the dynamics of polyubiquitylating complexes. Recent studies have identified that non-covalent Ub interactions with E2s are important for regulating Ub chain formation (12-14,19,45). HsUbchH5c non-covalent interaction with Ub and the subsequent formation of high molecular weight (Ub-E2)n polymers is required for auto-polyubiquitylation of the E3 BRCA1 (12). Here, the S22R mutant of HsUbchH5c is unable support (Ub-E2)n polymer formation but can still sustain auto-monoubiquitylation of BRCA1 in vitro (12-14,45). The shared use of the E2 non-covalent interaction surface by MUB and Ub poses several possibilities where MUBs influence Ub chain formation (Fig. 5E). MUB competition with Ub for non-covalent E2 binding could promote monoubiquitylation of membrane substrates by causing a localized disruption in (Ub-E2)n polymer formation. In contrast, MUBs could take the place of a terminal Ub in a (Ub-E2)n polymer to facilitate the rapid assembly of poly-Ub chains on a membrane target. Furthermore, the binding of MUB to E2s could affect E2 activity regarding chain linkage specificity (58), enzymatic rate (15,27), and E3 selection (27,46). While the impact of MUB-E2 binding at the plasma membrane is uncertain, these models highlight the potential for key advances in understanding ubiquitylation at the plasma membrane and warrant further investigation.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: Ub: Ubiquitin; UPS: Ub/26S Proteasome System; E2 or UBC: Ubiquitin conjugating enzyme; UEV: Ubiquitin Enzyme Variant; MUB: Membrane-anchored Ubiquitin-fold.
CAAX (SAAx): Cysteine (Serine), Aliphatic, Aliphatic, other amino acid; co-IP: co-Immunoprecipitation; NLS: Nuclear Localization Signal; BiFC: Bimolecular Fluorescence Complementation; Y2H: Yeast Two-hybrid; 3-AT: 3-amino-1,2,4-triazole; IPTG: Isopropyl β-D-1-thiogalactopyranoside.

1 PDB # 1SE9
2 PDB # 2GOW
3 PDB # 2FUH

FIGURE LEGENDS

Fig 1. Arabidopsis MUBs specifically interact with Subgroup VI E2s. A. A strict consensus phylogenetic tree summarizes the sequence relationship within the core domain of Arabidopsis E2s. Subgroups are indicated with roman numerals, as described (23). The tree is rooted to outgroup AtUFC1, the E2 for UFM conjugation. Asterisks (*) indicate branches with 100% bootstrap support from 1000 pseudoreplicates. Additional E2 gene characters including the length of the N-terminal extension (N), core domain (Cr), C-terminal extension (C), and the average number of introns (I) are shaded according to the Key (inset). B. A directed Y2H assay of positive interactions identified in a screen between the baits AtMUB1-6 and ubiquitin (Ub), and preys AtUBC1-6, 8-22, 24-37 and the UEVs AtCOP10 and AtUEV1B. The entire screen and full-size tree are available as Supplemental Fig. 2. DNA binding domain bait constructs and corresponding vector controls (V) are on horizontal axis, while activation domain prey constructs and vector controls are on the vertical axis. Y2H selection is summarized for each panel (bottom) where amino acid drop outs (-) are indicated: L, leucine; Y, tyrosine; H, histidine; and 2 mM 3-Amino-1,2,4-triazole (3-AT).

Fig. 2. Arabidopsis Subgroup VI E2s and MUBs contain structure-predicted non-covalent interaction sites. A. Protein sequence alignment of α-helix 1 through β-sheet 4 of MUB-interacting and representative non-MUB-interacting E2s (identified in Fig. 1) are indicated on the vertical axis. Sequence identity of 80% (black) and 50% (grey) are highlighted. Also included are human E2s previously determined to bind Ub in this region including HsUbcH5c (12) and HsUbcH5b (14). Residues NMR-shifted by Ub non-covalent binding with the HsUbcH5 family are indicated: HsUbcH5b and c, filled shapes; UbcH5b or c, open circles. The interaction-critical HsUbcH5 Ser22 is indicated by a triangle. The homologous yeast ScUbc4 is included for reference. B. A surface rendered HsUbcH5c structure (2FUH) shows the non-covalent Ub binding interface with the C-terminus indicated (asterisk). Residues indicated with circles or a triangle in Panel A are colored onto the structure if conserved in MUB-interacting E2s: orange, closed circles (HsUbcH5 P16, V26, Q34, M38, V49); yellow, open circles (HsUbcH5 P17, A23, T36, G47); red, triangle (HsUbcH5 S22). C. Protein sequence alignment of AtMUBs and Ub. Sequence identity of 80% (black) and conservation index of 50% (grey) are highlighted. Residues indicated in Panel C with circles or a triangle are colored onto the structures if conserved between AtMUBs and Ub: teal, closed circles (Ub K6, R42, I44, K48, T66, V70); white, open circles (Ub G10, A46, G47, H68); blue, triangle (Ub T66). E. A directed Y2H assay demonstrating the interaction of HsUbcH5c with HsMUB, as well as the cross-kingdom interactions of AtMUBs with HsUbcH5c and HsMUB with AtUBCs. Assay conditions are as described in Fig. 1 except using 1mM 3-AT.

Fig. 3. Arabidopsis MUBs interact with E2s on a surface distinct from the E2 active site. A Y2H mutation analysis of the Ub-HsUbcH5 equivalent non-covalent interaction surfaces of AtMUBs (MUB3 V86E and MUB4 T86E) and AtUBCs (UBC8 S22R and UBC9 S52R), or AtUBC active sites (UBC8 C85S and UBC9 C115S) as described in Fig. 1 except with 4 mM 3-AT.

Fig. 4. Arabidopsis MUBs interact directly with E2s in vitro. A GST pull-down assay using purified proteins reconstitutes MUB E2-binding in vitro. Inputs of recombinant GST-AtUBC8, GST-AtUBC8
S22R, or GST alone were incubated with His-AtMUB3. GSH resin eluates were examined by SDS-PAGE and immunoblot, as indicated. Notably, the interaction was sensitive to the S22R mutation. *B. E. coli* co-expression lysates were purified with either GSH resin or TALON His-affinity (metal) resin, as indicated. Co-expression cultures were achieved with double antibiotic selection for His-AtMUB3 (*Amp*<sub>r</sub>) and GST-AtUBC8 or GST-AtUBC8 S22R (*Kan*<sub>r</sub>). GST-AtUBC8 and His-AtMUB3 proteins co-purify efficiently. GST-AtUBC8 S22R and AtMUB3 fail to co-purify effectively, as detected by Coomassie, despite similar input protein levels in total *E. coli* lysates detected by immunoblot, as indicated. AtUBC8 and AtMUB3 Coomassie-stained bands are identified with arrows. Sub-stoichiometric Coomassie-stained proteins migrating between 21.5 and 31 kDa were recovered with either purification as resin-binding background.

**Fig 5. Arabidopsis MUBs localize E2s to the plasma membrane in planta.** *A.* A co-IP assay with anti-FLAG resin on *N. benthamiana* leaf tissue co-transformed with WT or mutant combinations of HA-AtUBC8 and FLAG-AtMUB3 CAAX or FLAG-GUS control, as indicated. AtUBC8 co-precipitates with AtMUB3 but not with AtMUB3 V86E or GUS. AtUBC8 S22R does not co-precipitate with either AtMUB3 or AtMUB3 V86E. For AtUBC8-containing extracts, 16 µg total protein was loaded on SDS-PAGE representing input, and 450 µg total protein was applied to 40 µL anti-FLAG resin. To analyze similar amounts of UBC, three times more AtUBC8 S22R-containing extract was loaded on SDS-PAGE and applied to 40 µLs anti-FLAG resin. Equal volumes of FLAG co-IP eluates were analyzed by SDS-PAGE and immunoblot, as indicated. Asterisk (*), mouse IgG cross-reacting bands. *B.* *N. benthamiana* epidermal cells transiently expressing full-length YFP fusions of plasma membrane localizing AtMUB3 CAAX or cytoplasmic/nuclear localizing AtMUB3 SAAX, AtUBC8, and AtUBC9. YFP signal localized to cytoplasmic strands (filled arrows) and nuclei (open arrows) are indicated. *C.* BiFC of epidermal cells co-expressing split-YFP fusions of AtMUB3 and AtUBCs or empty vector controls. Combinations of N- and C-terminal YFP fragments (nY or cY, respectively) were infiltrated as vector controls or fused to the N-terminus of AtMUB and E2, as indicated. AtMUB-E2 interaction and co-localization is observed at the plasma membrane or in the cytoplasm (filled arrows) and nucleus (open arrows). *D.* An enlarged view of BiFC with nY-AtMUB3 CAAX or nY-AtMUB3 SAAX and cY-AtUBC8 highlighting fluorescence in cytoplasmic strands (filled arrows) and the nucleus (open arrows). Also shown is chloroplast autofluorescence (red) and nuclear localized AtZFP11(NLS)-Cerulean (blue). Overlap between YFP and Cerulean is indicated as white in the merge. Scale bars in all panels represent 10 µm. *E.* A model depicting prenylation-dependent membrane-localized MUB (prenyl-cysteine C) tethering an E2 to the inner surface of the plasma membrane (PM) leaving the E2 active site (cysteine C) available to bind the diglycine (GG) of Ub. Interaction-critical residues between MUB (T/V) and E2 (S) are highlighted. Various proteins that could join the complex are indicated in grey: Ub, E3, target proteins (T), and available lysines (K).
Figure 1.
Figure 3.
Figure 4.

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<tr>
<td>GST-AtUBC8 S22R</td>
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Purification:

- Metal: +
- GSH: -

**MW**

- **AtUBC8**
- **AtMUB3**

**KDa**

- 45.0
- 35.0
- 21.5
- 14.4
Figure 5.

### Table A

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![Image of Western Blot](attachment:image.jpg)

### Table B

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### Table D

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### Table E

![Diagram](attachment:diagram.jpg)
Arabidopsis membrane-anchored ubiquitin-fold (MUB) proteins localize a specific subset of ubiquitin-conjugating (E2) enzymes to the plasma membrane
Rebecca T. Dowil, Xiaolong Lu, Scott A. Saracco, Richard D. Vierstra and Brian P. Downes

J. Biol. Chem. published online February 23, 2011

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