DOLICHOL PHOSPHATE MANNOSE SYNTHASE1 Mediates the Biogenesis of Isoprenyl-Linked Glycans and Influences Development, Stress Response, and Ammonium Hypersensitivity in Arabidopsis

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The most abundant posttranslational modification in nature is the attachment of preassembled high-mannose-type glycans, which determines the fate and localization of the modified protein and modulates the biological functions of glycosyl-phosphatidylinositol-anchored and N-glycosylated proteins. In eukaryotes, all mannose residues attached to glycoproteins from the luminal side of the endoplasmic reticulum (ER) derive from the polyisoprenyl monosaccharide carrier, dolichol P-mannose (Dol-P-Man), which is flipped across the ER membrane to the lumen. We show that in plants, Dol-P-Man is synthesized when Dol-P-Man synthase1 (DPMS1), the catalytic core, interacts with two binding proteins, DPMS2 and DPMS3, that may serve as membrane anchors for DPMS1 or provide catalytic assistance. This configuration is reminiscent of that observed in mammals but is distinct from the single DPMS protein catalyzing Dol-P-Man biosynthesis in bakers’ yeast and protozoan parasites. Overexpression of DPMS1 in Arabidopsis thaliana results in disorganized stem morphology and vascular bundle arrangements, wrinkled seed coat, and constitutive ER stress response. Loss-of-function mutations and RNA interference–mediated reduction of DPMS1 expression in Arabidopsis also caused a wrinkled seed coat phenotype and most remarkably enhanced hypersensitivity to ammonium that was manifested by extensive chlorosis and a strong reduction of root growth. Collectively, these data reveal a previously unsuspected role of the prenyl-linked carrier pathway for plant development and physiology that may help integrate several aspects of candidate susceptibility genes to ammonium stress.

INTRODUCTION

The biological functions of more than 50% of proteins are determined by the covalent attachment of carbohydrates referred to as glycans, which constitute the most diverse and abundant posttranslational modifications found in nature (Apweiler et al., 1999). Data from human studies reveal that most virus and pathogenic microbes bind to cells via glycoproteins (Hart and Copeland, 2010). Furthermore, several clinical markers and therapeutic targets of cancer cells are glycoproteins (Zhao et al., 2009), plants apparently do not perform protein C-mannosylation and protein O-mannosylation (Furmanek and Hofsteenge, 2000; Lommel and Strahl, 2009). In eukaryotic organisms, the most abundant posttranslational modifications are found in nature (Apweiler et al., 1999). Although N-glycosylations occur in different taxa, including Eukarya, Bacteria, and Archaea (Weerapana and Imperiali, 2006; Jones et al., 2009), they seem to perform protein C-mannosylation and protein O-mannosylation (Furmanek and Hofsteenge, 2000; Lommel and Strahl, 2009).

In eukaryotic organisms, N-glycosylation is achieved by co- or posttranslational attachment of the preassembled core glycan, Glc3Man9GlcNAc2-Asn (Glc, Man, and GlcNAc refer to Glc, Man, and N-acetylgalactosamine) to the γ-amido group of a specific Asn in the glycosylation consensus sequence Asn-X-Ser/Thr. The reaction is catalyzed by an oligosaccharyltransferase multisubunit complex in the endoplasmic reticulum (ER) membranes (Yan et al., 2005). This modification favors the folding of N-glycoproteins due to increased solubility (Molinari, 2007; Hanson et al., 2009) and represents a signal key used by the ER-associated protein degradation machinery to determine the fate of misfolded proteins (Helenius and Aebi, 2001). Indeed, just after the transfer, the glycan is subject to trimming processes by glucosidases I and II to allow
interactions between the resulting high-Man glycan and ER chaperones for quality control (Figure 1). Following removal of the terminal Man by ER \(a_1,2\)-mannosidases, the \(N\)-glycoprotein is exported by the vesicular transport system to the Golgi complex and post-Golgi compartments where the glycan core is further modified (Helenius and Aebi, 2001; Lehrman, 2001; Lehle et al., 2006; Banerjee et al., 2007; Molinari, 2007). Mutant analyses have shown that several steps of the \(N\)-glycan synthesis pathways are vital and contribute to plant development and defense (van der Hoorn et al., 2005; Pattison and Amtmann, 2009; Saijo et al., 2009; Häweker et al., 2010).

Like \(N\)-glycoproteins, the preformed GPI anchor, \(\text{EtNP-Man}_3\text{GlcNAc}_2\)PI (referring to ethanolamine phosphate, Man, glucosamine, and phosphatidyl inositol) is attached to proteins by a multisubunit transamidase complex (Orlean and Menon, 2007; Ferguson et al., 2009) following their translocation across the ER membrane. GPI anchors permit more efficient membrane anchoring of proteins compared with acylated or prenylated proteins because they deeply penetrate into the half hydrophobic membrane leaflet (Orlean and Menon, 2007). GPI-anchored proteins are crucial components of plant cell surfaces and are involved in the control of multiple aspects of plant development, such as cellulose deposition, wall integrity, membrane raft structure, and root growth, and are implicated in plant pathogen interactions (Borner et al., 2003; Gillmor et al., 2005; Roudier et al., 2005; Debono et al., 2009). In addition, 40% of the \(\text{Arabidopsis thaliana}\) proteins predicted to possess GPI anchors are potentially modified by \(O\)-linked arabinogalactans (Ellis et al., 2010), which in

**Figure 1.** Dol-P-Man Biogenesis in Plants and Its Implication in Posttranslational Modifications.

Dolichol formed from plastid- and mevalonate-derived IPP is used for the synthesis of Dol-P-Man. The reaction is catalyzed by the DPMS complex (DPMS1, DPMS2, and DPMS3). Dolichol is also used for the synthesis of the glycan intermediate (\(\text{Man}_5\text{GlcNAc}_2\))PI-Dol. Dol-P-Man and \(\text{Man}_3\text{GlcNAc}_2\)-PP-Dol are subsequently translocated to the ER lumen by unknown flippases. Dol-P-Man is subsequently used for the biosynthesis of GPI-anchored proteins and the preassembled glycan Glc\(_3\)Man\(_6\)GlcNAc\(_2\), which is further transferred to the amido group of Asn in the \(N\)-glycosylation site, Asn-X-Ser/Thr. Following the trimming process, the decision is made to retain, export, or degrade the polypeptide. Man residues (in red) are specifically donated by DPMS for GPI anchor biosynthesis or incorporated into the glycan Glc\(_3\)Man\(_3\)GlcNAc\(_2\)-PPP- by ALG enzymes: ALG3 (Man 6th), ALG9 (Man 7th and 9th), and ALG12 (Man 8th) are indicated. Asterisks refer to subpathways not yet elucidated in plants.
addition to their biological functions are key constituents of plant gums (Pettolino et al., 2006; Seifert and Roberts, 2007).

The presence of several Man residues represents a conserved feature of the four types of protein-linked glycans (Figure 1). In the Golgi complex, only guanosine 5’-diphosphate-Man (GDP-Man) is used as the Man donor, whereas in the ER, GDP-Man and prenyl-linked mannoses are used (Helenius and Aebi, 2001). The prenyl monosaccharide dolichol P-Man (Dol-P-Man) acts as the main carrier of transferred Man residues. The synthesis of Dol-P-Man proceeds via two main steps: formation of dolichyl phosphate (Dol-P) and addition of Man from GDP-Man. In yeast and mammalian cells, Dol-P is synthesized exclusively from the mevalonic pathway between the cytosol and the ER compartments (Schenk et al., 2001). In plants, the initial steps of Dol-P synthesis operate in the plastids from deoxy xylulose phosphate-derived isopentenyl diphosphate (IPP) (40 to 50% of total IPP incorporated) and IPP derived from the cytosolic mevalonic pathway (Skorupinska-Tudek et al., 2008). Subsequently, dolichol P-Man synthase (DPMS) located on the cytoplasmic face of the ER catalyzes the transfer of Man from GDP-Man to Dol-P, and the resulting Dol-P-Man is translocated to the luminal side of the ER membrane by an unknown flippase (Sanyal and Menon, 2010). Based on available data from mammalian cells, the translocolated Dol-P-Man donates all of the Man residues used on the luminal side of the ER for the synthesis of the GPI anchor and the four mannoses of N-glycans (the 6th to the 9th Man residues) (Burda et al., 1999; Frank and Aebi, 2005) (Figure 1).

Accumulating evidence supports the existence of two classes of DPMS. In Saccharomyces cerevisiae (Orlean et al., 1988), Trypanosoma brucei (Mazhari-Tabrizi et al., 1996), Leishmania mexicana (Ilgouz et al., 1999), and Thermoplasma acidophilum (Zhu and Laine, 1996), DPMS functions as a homomeric enzyme termed DPMS1. By contrast, in mammals (Maeda et al., 2000; Maeda and Kinoshita, 2008), Schizosaccharomyces pombe (Colussi et al., 1997), and Trichoderma reessi (Kruszewska et al., 2000), the synthesis of Dol-P-Man requires the assembly of a heteromeric complex, including a catalytic DPMS1 and noncatalytic DPMS2 and DPMS3 proteins. The identification, organization, and biological role of DPMS in plants are not yet known. In this study, we analyzed how Dol-P-Man is synthesized in plants and evaluated its role using overexpressing and knock-out lines. We provide evidence that in plants, Dol-P-Man is synthesized as in mammals by three protein components referred to as DPMS1, DPMS2, and DPMS3. DPMS1 is the catalytic module and is tethered or assisted by DPMS2 and DPMS3. Our study based on the analysis of the loss of function and the overexpression of DPMS1 demonstrates the central role played by the Dol-P-Man pathway during Arabidopsis growth and development and highlights the unexpected hypersensitivity of dpms1 mutant plants to ammonium.

RESULTS

Characterization and Functional Reconstitution of DPMS

Because Dol-P-Man synthesis is catalyzed by homomeric or heteromeric (Maeda and Kinoshita, 2008) enzymes, we made use of comparative genomic analysis to identify putative plant homologs of the minimum core enzyme DPMS1 and its potential protein partners, DPMS2 and DPMS3. We performed a BLAST search against the Arabidopsis database using yeast DPMS1 and human DPMS1, DPMS2, and DPMS3 as query sequences. We noted a significant match (BLASTP, E value = 9e-47) to the peptide sequence encoded by At1g20575 that was subsequently considered to be the putative Arabidopsis DPMS1 (At-DPMS1). At-DPMS1 displayed identity 61% to human, S. pombe (61%), S. cerevisiae (28%), and rice (Oryza sativa; 85%) homologs (see Supplemental Figure 1 online). Using the same procedure, we retrieved putative Arabidopsis DPMS2 (At1g74340) and DPMS3 (At1g48140) proteins that have E values of 4e-14 and 0.001 and sequence identity 57 and 31% to human DPMS2 and DPMS3, respectively (see Supplemental Figures 2 and 3 online). Using the prediction program HHM TOP (http://www.enzim.huehnten/submit.html), we noted the presence of transmembrane domains in DPMS2 (two transmembrane helices, 7-30 and 49-73) and DPMS3 (two transmembrane helices, 7-27 and 36-55). Previous studies based on the relatedness to the bacterial spore coat forming protein SpSA have shown that human DPMS1 and S. cerevisiae DPMS1 have similar tertiary structure, in spite of their divergence (Maeda and Kinoshita, 2008). These studies indicate that in human and S. cerevisiae, Tyr-12, Asp-44, Asp-97, and Arg-212 are key active residues (Lamani et al., 2006; Maeda and Kinoshita, 2008). These residues are conserved in Arabidopsis DPMS1 (see Supplemental Figure 1 online).

To analyze precisely the requirements for Arabidopsis Dol-P-Man synthesis, we used purified recombinant DPMS1, DPMS2, and DPMS3 proteins (see Supplemental Figure 4 online). Recombinant DPMS1 alone did not catalyze Dol-P-Man synthesis (Figure 2, lane 1). Similarly, recombinant DPMS2 and DMPS3 did not display DPMS activity either individually or when both proteins were combined (Figure 2, lanes 2, 3, and 7). Dol-P-Man was synthesized when DPMS1 + DPMS2 + DPMS3 were associated (Figure 2, lane 4), and the reaction was linear with respect to time for 45 min. When DPMS1 and DPMS3 alone were coincubated (i.e., in the absence of DPMS2), up to 20% of the activity could be recovered (Figure 2, lane 6), whereas only 5% of the activity was obtained in the presence DPMS1 + DPMS2 alone (Figure 2, lane 5).

Because Arabidopsis accumulates dolichols from Dol14 to Dol32 (Jozwiak et al., 2009), and due to the paucity of data characterizing Dol-P-Man, scale-up reactions were performed using a reaction mixture containing nonlabeled Dol14 to analyze further the reaction product of Arabidopsis DPMS. The DEAE-cellulose fraction eluted with dichloromethane/methanol (7/3v/v) containing ammonia (0.28 N) and 50 mM ammonium acetate was subjected to liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) analysis to characterize the putative Dol14-P-Man product with an expected molecular mass of 1215. LC-ESI-MS analysis revealed under basic conditions (pH 8) the presence of an ion with a mass-to-charge ratio (m/z) of 1196 that had the highest intensity, corresponding to the dehydrated form [M-H]- (Figure 3A). A [M-H]- form with m/z 1214 was also identified, but with a lower intensity, and was nevertheless the best candidate for the identification of the Man residue and the phosphate group that gave a loss of m/z 241 corresponding to [Man-PO3] (see Supplemental Figure 5 online). These data agree with the fact...
Assays were performed using purified recombinant DPMS1, DPMS2, and DPMS3 either alone (lanes 1 to 3) or in different combinations: DPMS1 + DPMS2 + DPMS3 (lane 4), DPMS1 + DPMS2 (lane 5), DPMS1 + DPMS3 (lane 6), and DPMS2 + DPMS3 (lane 7). The TLC plate was developed using the solvent mixture (dichloromethane/methanol/water; 10:10:3, v/v/v), and reaction products were analyzed by autoradiography. The origin (Or) and the position of Dol-14-P-Man (arrow) are indicated.

All considered, these data demonstrate that DPMS1 is the catalytic module of DPMS, but the enzyme activity requires the coinucbation of DPMS1 with DPMS2 and DPMS3. Thus, DPMS2 and DPMS3 are probably orthologs of mammal DPMS2 and DPMS3 (Maeda and Kinoshita, 2008). These data suggest that the catalytic activity of Arabidopsis DPMS1 is more stringently modulated by DPMS3 than by DPMS2. These observations are in good agreement with previous data observed in the case of mammalian cells and in contrast with those reported for S. cerevisiae, where DPMS1 alone catalyzes the synthesis of Dol-P-Man without the assistance of additional proteins (Orlean et al., 1988).

To characterize further the role of DPMS1, DPMS2, and DMSP3, we searched independent insertion mutants corresponding to each locus among publicly available Arabidopsis T-DNA mutant databases. T-DNA insertion lines of DPMS1 (dpms1-1 and dpms1-2), DPMS2 (dpms2-1 and dpms2-2), and DPMS3 (dpms3-1 and dpms3-2) were obtained. The resulting homozygous insertion mutants were identified by PCR, RT-PCR, and immunoblot analyses. DPMS transcripts did not accumulate in the homozygous mutant lines, compared with wild-type plants (see Supplemental Figures 6A to 6C online). Further analysis using anti-DPMS1 antibodies revealed the absence of DPMS1 in dpms1-1 and dpms1-2 mutants (see Supplemental Figure 6A online). The capacity of each dpms mutant to catalyze the synthesis of Dol-P-Man was tested in vitro using crude microsomal membranes. The synthesis of Dol-P-Man could be demonstrated from microsomal membranes from dpms2 and dpms3 but was reduced to ~20 to 30% of the wild-type level (see Supplemental Figure 7A online). By contrast, in microsomal membranes isolated from dpms1, the synthesis of Dol-P-Man could not be demonstrated (see Supplemental Figure 7A online). The DPMS deficiency of dpms1 plants was restored following the expression of DPMS1 in a dpms1 mutant background (see Supplemental Figure 7A online). Thus, as shown above, in the absence of DPMS1, Dol-P-Man was not synthesized, regardless of the presence of functional DPMS2 and DPMS3.

Our data suggest that Arabidopsis DPMS1, DPMS2, and DPMS3 may interact or form a complex in vivo. The binding of DPMS1, DPMS2, and DPMS3 to each other has been previously demonstrated using mammalian cells transfected with epitope-tagged proteins (Maeda et al., 2000). To determine whether Arabidopsis DPMS proteins function as parts of a protein complex, we transiently coexpressed green fluorescent protein (GFP)-tagged Arabidopsis DPMS1, DPMS2, and DPMS3 in Nicotiana benthamiana leaves. As a control, leaves were transfected with Agrobacterium tumefaciens harboring an empty GFP vector. Total proteins of transfected leaves were solubilized with digitonin before immunoprecipitation using antibodies directed against Arabidopsis DPMS1 (anti-DPMS1) covalently linked to protein A-Sepharose. Following immunoprecipitation, bound proteins were eluted with an acidic buffer and subjected to immunoblot analysis using anti-GFP (Figure 4A). Immunoblot analysis of the immunoprecipitates obtained using anti-DPMS1 indicates that no immunoprecipitate was recovered from leaves transfected with the empty GFP vector (Figure 4A, lane 6). On the other hand, the anti-GFP antibodies revealed interactions between DPMS1, DPMS2, and DPMS3 (Figure 4A, lane 7). Collectively, these data indicate that DPMS1, DPMS2, and DPMS3 coimmunoprecipitate and probably interact in vivo. Using the same protocol, one could note interactions between DPMS1 + DPMS2 (Figure 4A, lane 8), DPMS1 + DPMS3 (Figure 4A, lane 9), and DPMS2 + DPMS3 (Figure 4A, lane 10). To assess the role of DPMS3 further, we analyzed its interaction with DPMS1 and DPMS2 using an in vitro pull-down assay. We used biotin-labeled DPMS3 and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) as a control to pull down interacting proteins from an Escherichia coli extract expressing recombinant DPMS1 and DPMS2 as thioredoxin fusion proteins. After extensive washing to remove nonspecifically bound proteins, the acidic pH buffer eluate was subjected to SDS-PAGE analysis (Figure 4B). DPMS3 pulled down two prominent polypeptides having molecular masses of 43.8 kD (P1) and 25.2 kD (P2) (Figure 4B, lane 3) that were excised and used for in-gel tryptic digestion and sequence analysis by nanoliquid chromatography-MS/MS. The MS analysis of interacting proteins from the pull-down assay revealed that DPMS2 and DPMS1 are both DPMS3 binding proteins (see Supplemental Table 1 online). Further analysis using individually
recombinant extracts containing DPMS1 (Figure 4B, lanes 4 and 5) or DPMS2 (Figure 4B, lanes 6 and 7) revealed that both interact with DPMS3. The specificity was ascertained by the fact that these polypeptides were not pulled down by Rubisco (Figure 4B, lane 8). These data are reminiscent of the behavior of mammalian DPMS proteins in which DPMS3 could represent the binding module for DPMS1 and DPMS2 (Ashida et al., 2006).

Finally, the subcellular localization of DPMS protein components was analyzed by confocal microscopy using GFP-tagged DPMS1, DPMS2, and DPMS3 that were transiently expressed in Arabidopsis leaves. The results indicate that DPMS1 as well as DPMS2 and DPMS3 were localized in the ER membranes like the GFP-tagged marker At-RTNLB2 (Nziengui et al., 2007) (Figure 5).

Hypersensitivity of dpms1 Plants to Ammonium

During the first step in characterizing dpms mutants, we observed that dpms2 (dpms2-1 and dpms2-2) and dpms3 (dpms3-1 and dpms3-2) plants were phenotypically indistinguishable from wild-type plants when grown on Murashige and Skoog (MS, M0221) medium or soil. On the other hand, dpms1 (dpms1-1 and dpms1-2) grown on MS (M0221) exhibited a chlorotic phenotype and developed short roots (Figures 6A and 6B). To analyze further the specificity of this phenomenon, we generated additional mutants by RNA interference (RNAi)-mediated reduction of DPMS1 expression (DPMS1-RNAi). We isolated three DPMS1-RNAi lines and confirmed the low expression of DPMS1 by RT-PCR in lines 2 and 3 (see Supplemental Figure 6D online). Chlorophyll content and root growth were drastically reduced in DPMS1-RNAi3 plants (Figure 6B). The extent to which the DPMS activity was reduced in the DPMS1-RNAi lines was evaluated using microsomal membranes, revealing background activities in DPMS1-RNAi2 and DPMS1-RNAi3 lines compared with wild-type plants (see Supplemental Figure 7B online). Thus, the phenotype analysis of DPMS1-RNAi plants recapitulated that observed for T-DNA dpms1 plants. Unexpectedly, upon transfer to soil, dpms1 and DPMS1-RNAi plants progressively lost their chlorotic phenotype and accumulated chlorophylls to near wild-type levels (Figures 6C to 6G). The root growth of soil-grown dpms1 plants was increased but remained shorter compared with that of wild-type plants. The seed coat of dpms1 seeds displayed a wrinkled architecture (shown in Figure 8P). Functional complementation of the dpms1 mutant phenotype was observed following transformation of dpms1 plants with the DPMS1 gene (Figure 6G). Taken together, these observations suggested that endogenous factors or the composition of the MS culture medium may play an important role in dpms1 mutant phenotypes. After several modifications of the mineral composition of the medium, we established a correlation between the presence of 20.6 mM NH₄⁺ in MS (M0221) and the appearance of

![Figure 3](image_url). UPLC-MS/MS Analysis of Dol₁₄-P-Man Produced by the Arabidopsis DPMS Complex.

The reaction product obtained from the nonradioactive DPMS assay was subjected to DEAE-cellulose column chromatography and the fraction eluted with dichloromethane/methanol (7:3, v/v) containing ammonia (0.28 N), and 50 mM ammonium acetate was analyzed using two MRM modes with negative electrospray ionization (ESI⁻).

(A) Dol₁₄-P-Man with the transition of m/z 1196 > 1094 corresponding to [M-H₂O-H-] = 1196 and the characteristic fragment of m/z = 1094 representing partial loss of Man [Dol₁₄-P-(C₂H₃O)-H-].

(B) MRM transition of m/z 1196 > 1052 corresponding to [M-H₂O-H-] = 1196 and to another characteristic fragment of m/z = 1052, representing the loss of Man [Dol₁₄-P-Man-H-].
Agrobacterium GFP-DPMS2, or GFP-DPMS3 constructs. Leaves were infected similarly with Agrobacterium strain GV3101 individually harboring the GFP-DPMS1, N. benthamiana

Figure 4. Interaction between DPMS Protein Components. (A) N. benthamiana leaves were infiltrated with 1:1:1 or 1:1 mixtures of Agrobacterium strain GV3101 individually harboring the GFP-DPMS1, GFP-DPMS2, or GFP-DPMS3 constructs. Leaves were infected similarly with Agrobacterium harboring an empty GFP vector control. Infiltrated leaves were collected 72 h after for immunoblot analysis. Coomassie blue–stained protein bands of control (lane 1), GFP-DPMS1 + GFP-DPMS2 + GFP-DPMS3 transfected leaves (lane 2), GFP-DPMS1 + GFP-DPMS2 transfected leaves (lane 3), GFP-DPMS1 + GFP-DPMS3 transfected leaves (lane 4), and GFP-DPMS2 + GFP-DPMS3 transfected leaves (lane 5) were used for immunoprecipitation with anti-DPMS1. The anti-DPMS1 immunoprecipitates were used for immunoblot analysis with anti-GFP as shown: lane 6, immunoprecipitates from control leaves; lane 7, immunoprecipitates from leaves expressing GFP-DPMS1 + GFP-DPMS2 + GFP-DPMS3, and lane 8, immunoprecipitates from leaves expressing GFP-DPMS1 + GFP-DPMS3 and GFP-DPMS2 + GFP-DPMS3 (lane 10).

(B) Recombinant DPMS3 was biotinylated and used as a bait to pull down recombinant DPMS1 and DPMS2. For a negative control, Rubisco protein was used as bait. The soluble extracts obtained from E. coli overexpressing recombinant DPMS1 and DPMS2 as thioredoxin fusion proteins were incubated with purified DPMS3 or Rubisco immobilized on agarose beads for 2 h at 6°C before extensive washing and elution with acidic buffer, pH 2.8. SDS-PAGE and Coomassie blue–stained proteins from total proteins from induced bacteria harboring an empty vector (lane 1; pBAD/TOPO ThioFusion), 1:1 mixture of total proteins from induced bacteria expressing DPMS1 and DPMS2 (lane 2), DPMS1-interacting proteins (lane 3; P1, 43.8 kD and P2, 25.2 kD) that were subsequently subjected to nanoLC-MS/MS analysis; total proteins from induced bacteria expressing DPMS1 (lane 4), DPMS3-interacting protein (lane 5; P1, 43.8 kD), total proteins from induced bacteria expressing DPMS2 (lane 6), DPMS3-interacting protein (lane 7; P2, 25.2 kD), and Rubisco interacting proteins (lane 8).

chlorotic leaves. We compared the effects of MS culture media differing predominantly in having variable NO₃⁻/NH₄⁺ ratios. When dpms1 mutants were cultivated on MS (M 0238) medium (modification number 4, NH₄⁺ free) containing exclusively NO₃⁻ (39.39 mM) as mineral nitrogen, the chlorophyll content was retained, and the visible phenotype was indistinguishable from that observed for the wild-type plants, except that root growth was reduced to ~30 to 50% (see Supplemental Figure 8 online). To test further the sensitivity and the specificity of the response of dpms1 and DPM51-RNAi plants to NH₄⁺, we analyzed the effect of increasing concentrations of NH₄⁺ in the culture medium. Cultivation on medium (MS, M 0238) containing increased concentrations of NH₄⁺ decreased both the chlorophyll pigments and root growth of dpms1 and DPM51-RNAi plants, compared with plants grown on the same medium devoid of NH₄⁺ (Figures 6A and 6B). The severity of the ammonium-induced effects on dpms1 plants increased and became nonreversible and lethal with longer exposure time. These analyses revealed that the presence of NH₄⁺ in MS medium was responsible for the chlorotic phenotype of dpms1 plants. The chlorophyll content of wild-type plants was not changed by any of the NH₄⁺ concentrations used; only root growth was slightly reduced at higher NH₄⁺ concentrations (Figure 6I). Thus, our data demonstrate that functional DPM51 is vital in the adaptation of Arabidopsis to increased external NH₄⁺ concentration. It has been previously shown that NH₄⁺ induces a dramatic decrease of root growth in Arabidopsis in the absence of K⁺ (Cao et al., 1993). As the concentration of K⁺ (20.04 mM) in the MS medium usually supports optimal Arabidopsis growth, we can conclude that K⁺ could not alleviate the toxic effect of NH₄⁺. Therefore, our data suggest that dpms1 mutants and DPM51-RNAi plants are NH₄⁺ hypersensitive, regardless of the presence of K⁺. Our data unveil a previously unsuspected connection between the synthesis of Dol-P-Man and the sensitivity of plant to ammonium. To explore further the connection between the Dol-P-Man pathway and NH₄⁺ in Arabidopsis, we analyzed the behavior of Arabidopsis srd5a3-like mutants. SRD5A3 encodes the reductase catalyzing the later steps of dolichol synthesis from polyenol in mammals and yeast and probably in plants (Cantagrel et al., 2010) (see Supplemental Figure 9A online). The Arabidopsis genome contains two Arabidopsis SRD5A3-LIKE genes, At2g16530 and At1g72590 (see Supplemental Figure 10 online). We analyzed two independent homozygous knockout lines, srd5a3-like1-1 and srd5a3-like1-2, corresponding to At2g16530 (see Supplemental Figures 9B and 9C online). We found that srd5a3-like1 and srd5a-like2 display chlorotic phenotypes remarkably similar to those observed for dpms1 (Figures 6A and 6B) when grown in the presence of ammonium (see Supplemental Figures 9D and 9E online). Although additional work is needed to evaluate fully the role of Arabidopsis SRD5A3-LIKE genes, these results extend the fact that knockout of DPM51 potentiates ammonium sensitivity in Arabidopsis. Previous studies established a link between GDP-Man pyrophosphorylase (GMPase) and ammonium sensitivity in Arabidopsis using vtc1 and hsn, two allelic GMPase-deficient mutants (Qin et al., 2008; Barth et al., 2010). Therefore, we determined the GMPase activity of wild-type plants (940 ± 50 nmol/h/mg protein) and dpms1 plants (915 ± 30...
nmol/h/mg protein) was nearly identical. Because ascorbic acid (AsA) is produced via GMPase activity, we determined the AsA content of wild-type and dpms1 plants. The AsA content of dpms1 plants (2700 ± 200 nmol/g fresh weight) was lower than that of wild-type plants (3500 ± 300 nmol/g fresh weight). However, when dpms1 plants were supplemented with 5 mM galactono-1,4-lactone, the proximal AsA precursor, the wild-type phenotype was not restored. Therefore, our data indicate that AsA deficiency could not account for the dpms1 mutant phenotype. In addition, the data suggest that when Dol-P-Man is not synthesized in Arabidopsis, the resulting GDP-Man precursor is not redirected toward the synthesis of AsA. Thus, downstream steps involving the use of Dol-P-Man are probably key determinants of Arabidopsis NH4+ sensitivity. To analyze further the consequence of DPMS1 deficiency, total proteins from wild-type and dpms1 were subjected to affinodetection using con-canavalin A, a high Man-specific lectin (Faye and Chrispeels, 1985). Several reactive bands corresponding to glycoproteins bearing N-linked glycans could be observed in the wild-type and in the dpms1 mutant. The different electrophoretic mobility of reactive polypeptides suggests a modified glycosylation status between wild-type plants and dpms1 mutants (see Supplemental Figure 11 online). Decreased Dol-P-Man availability makes it likely that N-glycosylation and GPI anchor attachment are perturbed or impaired. We therefore tested both types of post-translational modifications using anti-Protein Disulfide Isomerase (PDI) (Lukowitz et al., 2001) and anti-Plasmodesmata Callose Binding (PDCB1) protein (Simpson et al., 2009) antibodies as genuine markers of N-glycosylated and GPI-anchored proteins, respectively. Immunoblot analysis revealed that PDI migrated slightly faster in dpms1-1 and dpms1-2 mutants than in the wild-type plants, consistent with a modified N-glycosylation pattern (Figures 7A and 7B). On the other hand, for PDCB1 we detected in the wild type several polypeptide bands with a molecular mass of 19 to 22 kD (Figures 7C and 7D) due to the fact that PDCB is encoded by a multigene family in Arabidopsis (Simpson et al., 2009). In dpms1-1 and dpms1-2, the lower molecular mass PDCBs was not detected, whereas the content of the higher molecular mass species was reduced, thus suggesting a GPI anchor deficiency (Figures 7C and 7D).
Effect of Modulated Expression of DPMS1

Loss-of-function mutants and functional rescue of a mutant phenotype have been widely used to assess the role of plant gene products. The limitations of this strategy using only insertional mutants have been discussed previously (Hirschi, 2003). Previous data have shown that the availability of Dol-P-Man modulates the structure and the composition of *S. cerevisiae* cell walls (van Berkel et al., 1999; Orłowski et al., 2007). Therefore, we decided to generate Arabidopsis lines overexpressing DPMS1 using a Rubisco promoter that possesses enhancer elements activated by several plant transcription factors. In two selected lines, the overexpression of DPMS1 occasionally resulted in altered stem branch diameter and morphology (Figures 8A to 8D). This phenomenon was paralleled by the appearance of wrinkled seed coat architecture and by perturbation of the vascular bundle arrangements very similar to that observed in the clavata1 mutant that is altered in a receptor kinase gene (Deyoung and Clark, 2008) (Figures 8E to 8P). Notably, the seed coat architecture of overexpressing lines nearly matched that of dpms1 mutants and is indicative of the formation of aberrant cell walls.

Because Dol-P-Man is at the convergence of several pathways, we examined how overexpression of DPMS1 might influence the expression of several genes known to encode enzymes implicated in or catalyzing upstream and downstream steps of glycan synthesis. These included the biosynthetic enzyme GDP-Man pyrophosphorylase (also known as VTC1, for vitamin C1) (Conklin et al., 1997), dolichol prenyltransferase (DolS) or Leaf Wilting 1 (LEW1) (Zhang et al., 2008), UDP-N-acetylgalactosamine:dolichol...
phosphate N-acetylglucosamine-1-P transferase (GPT) (Koizumi et al., 1999), two mannosyltransferases called Asn-linked glycosylation (ALG) genes, ALG11 (Zhang et al., 2009) and ALG3 (Henquet et al., 2008), and the N-linked glycan trimming enzymes, α-glucosidase I (GCS1) and an uncharacterized α-glucosidase-like enzyme (GCSL) (Boisson et al., 2001). Real-time RT-PCR using soil-grown plants revealed that VTC1, DolS, DPMS1, DPMS2, DPMS3, ALG11, ALG3, GPT, and GCS1, which sequentially mediate the synthesis of Dol-P-Man and its downstream processing, were induced in the DPMS1-overexpressing lines compared with dmps1 mutants as shown by the expression profile observed for DPMS1-Ov2 and dmps1-1 (Figure 9A). Except for ALG11 and ALG3, a similar trend was observed between the DPMS1-overexpressing lines and wild-type plants (Figure 9A).

DISCUSSION
Multimodular Organization of Plant DPMS
Man residues transported by Dol-P play a key role in the biosynthesis of N-glycoproteins, O- and C-mannosylated proteins, and GPI-anchored proteins. In addition, the removal of the terminal Man residue donated by Dol-P-Man is the last enzymatic step before glycoproteins can exit the ER. If folding is not complete at this step, the attached glycan dictates the

DPMS and ER Stress Status
It has been shown in yeast that the alteration in the protein glycosylation pathways alone induces a buildup of misfolded proteins in the ER lumen and leads to ER stress. During ER stress, the most prominent change is represented by the transcriptional induction of genes controlling the pathway known as the unfolded protein response (UPR) (Ng et al., 2000). For instance, the UPR is constitutively triggered in the dolichol phosphate Man-deficient Chinese hamster ovary (Foulquier et al., 2002). It has also been reported that in fibroblasts from patients suffering from congenital disorders of glycosylation type-I, the UPR is moderately but constitutively induced (Lecca et al., 2005). Finally, Arabidopsis lef mutants affected in the biogenesis of dolichols are more sensitive to tunicamycin (Zhang et al., 2008), which is an inhibitor of N-acetylglucosamine transferase that acts as a potent inducer of the UPR (Martinez and Chrispeels, 2003; Iwata and Koizumi, 2005). Thus, due to the central role of Dol-P-Man in protein glycosylation, we tested whether DPMS1-deficient or DPMS1-overexpressing Arabidopsis plants have an altered UPR in the presence or absence of tunicamycin. We analyzed the expression of several ER stress marker genes, including UPR marker genes using soil-grown plants or plants treated with tunicamycin. Marker genes included three bZIP membrane-bound transcription factors (bZIP17, bZIP28, and bZIP60) that globally regulate the induction of ER stress response genes (Iwata and Koizumi, 2005; Che et al., 2010; Liu and Howell, 2010), including the UPR markers Bip2, Bip3, calnexin, and calreticulin. Gene expression monitored by real-time RT-PCR revealed that UPR marker genes were constitutively expressed in DPMS1-overexpressing plants compared with dmps1 and wild-type plants (Figure 9B). The expression profile of DPMS1-overexpressing plants was paralleled by the induced expression of two transcriptional regulators of oxidative stress responses, RCD1 (Katiyar-Agarwal et al., 2006) and RD29A (Liu et al., 1998), suggesting that the ER stress in DPMS1-overexpressing plants is balanced by concomitant upregulation of antioxidant pathways. When plants were treated with tunicamycin to induce the UPR, the magnitude of the response of the genes encoding bZIP17, bZIP28, and bZIP60 transcription factors, which globally regulate the stress, was lower in DPMS1-overexpressing plants compared with dmps1 (Figure 9C). These data are consistent with ER stress being already present in plants overexpressing DPMS1 and suggest that dmps1 and wild-type plants are more responsive to tunicamycin treatment than are DPMS1-overexpressing plants.
degradation of the polypeptide chain (Tokunaga et al., 2000; Lehrman, 2001; Molinari, 2007) (Figure 1). Although the synthesis of Dol-P-Man has been studied in detail in *S. cerevisiae* and in human cells, the protein components catalyzing its synthesis in plants were unknown. Two prototypic organizations of DPMS have been proposed. The first is represented by the yeast enzyme, which comprises a homomeric protein called DPMS1 (Maeda and Kinoshita, 2008). The second type is represented by the mammalian enzyme, which is composed of three proteins, including DPMS1, DPMS2, and DPMS3 (Maeda and Kinoshita, 2008). We determined the component enzymes involved in the synthesis of Dol-P-Man from *Arabidopsis*. We provide evidence that *Arabidopsis* DPMS1, DPMS2, and DPMS3 are functional orthologs of mammalian genes involved in Dol-P-Man synthesis. Like human DPMS1, we show that *Arabidopsis* DPMS1 has no activity when expressed in *E. coli*, thereby pointing to a functional difference with *S. cerevisiae* (Colussi et al., 1997; Maeda and Kinoshita, 2008). As observed in mammalian cells (Tomita et al., 1998), the synthesis of Dol-P-Man could be demonstrated in vitro using a reconstituted enzyme system containing DPMS1, DPMS2, and DPMS3 (Figure 2). To examine the contribution of each protein, we tested the activity of DPMS1+DPMS2, DPMS1+DPMS3, and DPMS2+DPMS3 (Figure 2). We observed that whereas the combinations of DPMS1+DPMS2 and DPMS1+DPMS3 displayed 5 and 20% reduced synthesis of Dol-P-Man, respectively, compared with DPMS1+DPMS2+DPMS3, the DPMS2+DPMS3 combination was inactive (Figure 2). Taken together, these data suggest that *Arabidopsis* DPMS1 represents the catalytic core of

Figure 8. Phenotypic Analysis of DPMS1 Overexpression in Arabidopsis.

(A) and (B) Close-up view of the wild type (A) and a DPMS1-overexpressing plant DPMS1-Ov1 (B).
(C) Cylindrical stem of wild-type (Col-0) plants and flattened stem of DPMS1-Ov1 (Ov1) and DPMS1-Ov3 (Ov3).
(D) Higher magnification of (C).
(E) to (G) Sections of the flattened stem of DPMS1-Ov1 stained with phloroglucinol (pink color) to detect lignin in the secondary cell walls of vascular tissues.
(H) to (J) Stem sections of dpms1-1 plants stained as in (E) to (G).
(K) to (M) Stem sections of wild-type plants stained as in (E) to (G).
(N) to (P) Scanning electron micrographs of seed coat from wild-type plants (N) compared with wrinkled seed coats of DPMS1-Ov1 (O) and dpms1-1 (P) plants.
Bars = 1.5 cm in (A) and (B), 0.5 cm in (C) and (D), 250 μm for stem sections in (E) to (M), and 100 μm for seeds in (N) to (P).
Multiple Roles for DPMS in Plants

The biogenesis of Dol-P-Man involves the cooperation of several plant cell compartments. Previous studies led to the isolation of the enzyme complex including DPMS2 and DPMS3, as shown in mammalian cells (Maeda and Kinoshita, 2008). Consistent with these data, we show that endogenous DPMS1, DPMS2, and DPMS3 are compartmentalized in the ER membranes (Figure 5) and probably form a complex in vivo as shown by pull-down assay and affinity interaction analyses (Figure 4). Although DPMS2 is associated with catalytically active DPMS complex, its precise function is unknown. It has been suggested that it may stabilize the DPMS complex (Maeda and Kinoshita, 2008). Our data suggest that the trimerization of DPMS could be facilitated by the fact that DPMS3 interacts with both DPMS1 and DPMS2 (Figure 4). Because DPMS2 enhances up to twofold the activity of GPI-N-acetylgalcosaminyltransferase, which is involved in the biosynthesis of the GPI anchor, DPMS2 has also been considered as a regulator of the GPI pathway (Watanabe et al., 2000; Maeda and Kinoshita, 2008). This led to the suggestion that the biosynthesis of GPI and Dol-P-Man are probably coregulated (Orlean and Menon, 2007). It has also been shown that the binding of Dol-P is increased in the presence of DPMS2 (Maeda et al., 1998). In mammalian cells, deficiency of DPMS1 (Sugiyama et al., 1991; Tomita et al., 1998), DPMS2 (Maeda et al., 1998), and DPMS3 (Ashida et al., 2006) drastically blocks or reduces the biosynthesis of Dol-P-Man and leads to N-glycosylation defects. The requirement for DPMS3 is supported by the fact that mammalian cell DPMS3 acts as a coiled-coil protein that tethers DPMS1 to the ER membranes (Ashida et al., 2006; Maeda and Kinoshita, 2008; Lefebre et al., 2009). Furthermore, a single missense mutation in mammalian DPMS3 drastically reduces the catalytic activity of DPMS1 and leads to muscular dystrophy (Lefebre et al., 2009). In mammalian cells, it has also been shown that stable expression of DPMS1 requires DPMS3, whereas DPMS2 is required for the stable expression of DPMS3 (Maeda et al., 1998; Maeda et al., 2000; Ashida et al., 2006). Notably, mammalian DPMS1 is degraded by the proteasome in the absence of DPMS3 (Ashida et al., 2006). Interestingly, database (http://atted.jp/) searches revealed that in Arabidopsis, the expression of DPMS1 is correlated with that of DPMS3. DPMS3 ranks third on the list with a Pearson’s correlation coefficient of 0.56. However, in Arabidopsis, dpms2 and dpms3 mutants still retain DMPS activity, thus pointing to a key difference between plants and animals (see Supplemental Figure 7A online).

**Figure 9.** Quantitative Analysis of the Expression of Dolichol and Glycan Biosynthetic Genes and Stress Marker Genes.

(A) Real-time PCR quantification of dolichol and glycan biosynthetic genes and stress marker genes from 32-d-old soil-grown wild-type, dpms1-1, and DPMS1-overexpressing (DPMS1-Ov1) plants. The expression values for dpms1-1 and DPMS1-Ov1 are shown relative to the wild type, which is set to 1 (dashed line). Results are calculated from means values ± SD of triplicates from three independent biological samples. Dol-P-Man and glycan pathway genes (zone I): GDP-Man pyrophosphorylase/Man-1-pyrophosphatase VTC1, Dolichol synthase DolS, DPMS1, DPMS2, DPMS3, mannosyltransferases ALG11 and ALG3, GPT. Glycan trimming genes (zone II): GCS1, α-glucosidase GCSL.

(B) Real-time PCR quantification of stress marker genes from 32-d-old soil-grown wild-type, dpms1-1, and DPMS1-overexpressing (DPMS1-Ov1) lines. The expression conditions are presented as in (A). UPR and ER stress markers (zone I): BZIP17, BZIP28, BZIP60, Bip2, Bip3, Calnexin, and Calreticulin. Oxidative stress markers (Zone II): RCD1 and RD29A.
Arabidopsis LEW1, which encodes a cis-prenyltransferase involved in the biosynthesis of dolichol (Zhang et al., 2008). lew1 mutants display a leaf-wilting phenotype (Zhang et al., 2008). We show that among the three genes encoding the Arabidopsis DPMS enzyme complex, only mutations in the DPMS1 gene encoding the catalytic module lead to an apparent phenotype, characterized by extensive chlorosis, reduction of root growth, and a wrinkled seed coat under normal culture conditions (Figures 6 and 8). Thus, our data point to nonlethal phenotypes when DPMSs are knocked out. Interestingly, Arabidopsis ALG3, which mediates the incorporation of the 6th Man of N-glycans from Dol-P-Man, has been identified (Henquet et al., 2008; Kajiuara et al., 2010). However, alg3 mutants are undistinguishable from the wild-type plants when grown under normal or stress conditions, including salt stress and low and high temperatures. Arabidopsis ALG12/EBS4 encodes the enzyme sequentially catalyzing the transfer of the (8th) Man residue from peratures. Arabidopsis ALG12/EBS4 mutants, incomplete glycans are transferred to polypeptides, and the degradation of defective brassinosteroid receptors is inhibited (Hong et al., 2009). Thus, in plants, mutations affecting the synthesis and the attachment of protein glycans are silent or associated with diverse developmental and physiological deficiencies that could lead to lethal phenotypes (Pattison and Amtmann, 2009; Zhang et al., 2009). Interestingly, short or premature glycans could be attached to glycoproteins in plants (Henquet et al., 2008). By contrast, DPMS1 is essential for cell viability in S. cerevisiae (Orlean, 1992), whereas knockout of mice DPMS2 blocks the synthesis of the GPI anchor and is embryo-lethal (Nozaki et al., 1999). Several human congenital disorders associated with Dol-P-Man biosynthesis and metabolism have been described (Haeuptle and Hennet, 2009). Detailed analysis revealed that in humans, Dol-P-Man deficiency gradually affects two subpathways of protein glycosylation. Mild Dol-P-Man deficiency due to DPMS3 mutations affects mainly O-mannosylation pathways and leads to α-dystroglycanopathies. On the other hand, severe Dol-P-Man deficiencies perturb N-glycosylation and GPI pathways and provoke abnormal clotting and epilepsy (Lefebre et al., 2009). DPMS1 deficiency caused a reduction of PDCB1 accumulation, consistent with an impairment of GPI anchor biosynthesis (Figures 7C and 7D). This feature is consistent with previous reports showing that the impairment of GPI attachment to phospholipase C in T. brucei (Garg et al., 1997) or yeast Gas1p (Meyer et al., 2000) leads to lumenal ER retention, premature secretion, or degradation of proteins. Dol-P-Man is the key donor for the synthesis of the core glycan of GPI anchors (Orlean and Menon, 2007). Arabidopsis mutants affected in the early steps of GPI anchor synthesis have been characterized. These include peanut, which encodes GPI-mannosyl transferase I that catalyzes the transfer of the first mannosyl residues to the GPI anchor (Gillmor et al., 2005), and seth1 and seth2, which encode protein components of GPI-N-acetylglucosaminyltransferase (Lalanne et al., 2004). In contrast with dpms1, Arabidopsis peanut is lethal (Gillmor et al., 2005), and seth1 and seth2 are male sterile (Lalanne et al., 2004). GPI anchor synthesis is complex and requires at least 18 genes (Orlean and Menon, 2007). Due to the complexity of the pathway, few studies have been made using purified enzymes. For instance, GPI-mannosyltransferase I, which catalyzes the first incorporation of a Man residue into the GPI anchor, is a multicomponent protein complex (Orlean and Menon, 2007). Data from human, yeast, Plasmodium falciparum, and T. brucei reveal that the protein components are species specific and are not interchangeable (Kim et al., 2007), thus making cross-study comparisons difficult. A potentially attractive possibility is that plants could synthesize dolichol or lipid-linked phosphate Man through an alternative pathway. In this view, it is worth noting that the stringent requirement for the presence of a reduced α-isoprene unit in Dol-P-Man can be fulfilled by a monoterpenoid derivative, such as citronellol phosphate (Russ et al., 1993), synthetic lipid phosphates (Wilson et al., 1993), and various derivatives (Sprung et al., 2003). Finally, the Arabidopsis genome contains several genes encoding Golgi-localized nucleotide sugar transporters (GONSTs) predicted to import GDP-Man in the Golgi (Handford et al., 2004). Thus, one cannot exclude the transport of GDP-Man in the ER lumen via GONST-like transporters and its use in the biosynthesis of N-glycoproteins and GPI-anchored proteins in dpms1 plants.

We show that the overexpression of DPMS1 could change the architecture of Arabidopsis stems and the vascular bundle arrangements and provoke the formation of a wrinkled seed coat (Figure 8), consistent with the important role played by GPI-anchored proteins in cell wall formation (Gillmor et al., 2005). This phenomenon is paralleled by a broad induction of the expression of several genes encoding Golgi-localized nucleotide sugar transporters (GONSTs) predicted to import GDP-Man in the Golgi (Handford et al., 2004). Thus, one cannot exclude the transport of GDP-Man in the ER lumen via GONST-like transporters and its use in the biosynthesis of N-glycoproteins and GPI-anchored proteins in dpms1 plants. The plant cell 2007). Due to the complexity of the pathway, few studies have...
**DPMS1, a Candidate Susceptibility Gene for Ammonium Toxicity**

Although primary nitrogen assimilation in plants proceeds via its reduced form (i.e., ammonium), Arabidopsis, like the other members of the Brassicaceae, is sensitive to the presence of ammonium in the culture medium (Britto and Kronzucker, 2002). Interestingly, an allosteric regulation of ammonium transporter has been demonstrated that involves the phosphorylation of the Arabidopsis ammonium transporter AMT1 to reduce ammonium absorption (Lanquart et al., 2009). We show that the Dol-P-Man pathway mediated by DPMS1, DPMS2, and DPMS3 is linked to ammonium sensitivity in Arabidopsis. Whereas the loss-of-function mutants dpms2 and dpms3 retained a wild-type phenotype, we found that the loss-of-function mutant dpms1 and DPMS1-RNAi exacerbate the hypersensitivity of Arabidopsis to ammonium and, as a result, the leaves become strongly chlorotic and the root growth is drastically reduced. The strong depletion of DPMS1 transcript and protein coincided with background DPMS activity in DPMS1-RNAi2 and DPMS1-RNAi3 lines (see Supplemental Figures 6D and 7B online).

On the other hand, dpms2 and dpms3 have ~20 to 30% of DPMS activity and do not display a bleached phenotype. Extrapolation from in vitro data to the in vivo situation is difficult because the integration of DPMS1 in the ER membranes is probably not optimal in the absence of DPMS2 or DPMS3. Thus, the resulting topological changes are likely amplified in vitro and could lower the DPMS activities measured from dpms2 and dpms3 microsomes. Alternatively, it could be possible that the reduction of DPMS activity to ~20 to 30% of wild-type activity did not confer sensitivity to ammonium. The chlorotic phenotype disappears when dpms1 mutant plants were grown on soil, consistent with the usually low ammonium concentration, which has been estimated to be 10 to 1000 times lower than that of nitrate (Marshner, 1995). The mechanism underlying the hypersensitivity to NH₄⁺ is not well understood. Multiple factors have been proposed to account for ammonium toxicity in plants. The toxicity has been attributed to increased proton efflux and acidification of the external medium and to the reduction of dicarboxylic acid and cation concentrations (Barker et al., 1966; Kirkby, 1968; Raven and Smith, 1976). It has also been proposed that the high demand for carbohydrate skeletons, such as oxaloacetate and ketoglutarate, for nitrogen incorporation may lead carbon shortage (Schjoerring et al., 2002). Perturbation of hormonal homeostasis has also been proposed (Barker, 1999).

Finally, it is well known that the toxicity of NH₄⁺ is exacerbated by K⁺ deprivation (Wall, 1939) and that K⁺ can alleviate the toxic effect of NH₄⁺ (Cao et al., 1993). In addition to their physicochemical properties (i.e., charge, size, and hydration energy) (Wang et al., 1996; White, 1996), a reciprocal influence between K⁺ and NH₄⁺ has been described during their absorption (ten Hoopen et al., 2010). Indeed, the Arabidopsis potassium transporter KAT1 has affinity for NH₄⁺ (Schachtman et al., 1992). This could contribute to the chlorotic phenotype and the severe reduction of root growth of dpms1 plants in the basal MS medium (M0221), which contains 20.04 mM K⁺ and 20.61 mM NH₄⁺ (Figure 6).

Arabidopsis GDP-GMPase, also known as VTC1, catalyzes the conversion of d-Man-1-P into GDP-Man. It has been shown recently that mutation in the Arabidopsis GDP-GMPase gene causes NH₄⁺ hypersensitivity associated with the inhibition of root growth (Qin et al., 2008; Barth et al., 2010). It has been suggested that the strong inhibitory effect of NH₄⁺ on GMPase activity could account for NH₄⁺ hypersensitivity (Qin et al., 2008). Because, DPMS1 acts downstream of GMPase and due to the fact that DPMS1 and dpms1 plants have the same GMPase activity, our data indicate the sensitivity of GMPase to NH₄⁺ alone is not sufficient to explain the NH₄⁺ sensitivity of Arabidopsis.

Additionally, our data from dpms1 and those obtained from vtc1 plants (Barth et al., 2010) suggest that AsA is not directly implicated in the NH₄⁺ sensitivity. Thus, the NH₄⁺ sensitivity results primarily from the perturbation of Dol-P-Man biogenesis and its downstream utilization, rather than at the level of the branch leading to AsA. Because Dol-P-man is required for the synthesis of N-glycoproteins, GPI-anchored proteins and arabinogalactan proteins, one may reasonably expect that these different pathways are affected. GPI-anchored proteins are overrepresented in lipid rafts that are relatively enriched in phytosterols (Kierszniowska et al., 2009) that could modulate the function of mineral transporters. With respect to the chlorotic phenotype, it is worth noting that some Arabidopsis and rice proteins destined for the plastids are postranslationally modified by N-glycosylation (Villarejo et al., 2005; Nanjo et al., 2006; Radhamony and Theg, 2006). Rice α-amylase, for instance, is a glycoprotein transported from the ER-Golgi compartment to plastids via the secretory pathway (Kitajima et al., 2009). In addition, it has been shown recently that the blockage of the secretory pathway by brefeldin A induces starch accumulation in Arabidopsis plastids (Hummel et al., 2010). The above data, coupled with the fact that blocking starch catabolism triggers plastid senescence (Stettler et al., 2009), suggest that further exploration of the link between starch hydrolysis and ammonium susceptibility is needed.

The biological activity of several proteins is dependent on postranslational modifications, among which the covalent attachment of glycans is highly prevalent. Dol-P-Man is implicated as a Man donor in the formation of N-glycoproteins, GPI-anchored proteins and arabinogalactan proteins, which are collectively predicted to represent more than 50% of proteins in Arabidopsis. Our data reveal that the synthesis of Dol-P-Man is mediated by three genes that encode DPMS1, DPMS2, and DPMS3 proteins, which assemble into a functional DPMS complex where DPMS1 represents the catalytic module. We show through loss of function and overexpression studies that DPMS1, in contrast with DPMS2 or DPMS3, exerts a broad influence on various aspects of Arabidopsis development, including root growth, seed architecture, vascular bundle organization, activation of UPR, induction of chlorosis, and sensitivity to ammonium. Given the growing implications of glycan-linked proteins in a wide range of biological functions, including receptor–ligand interaction, signal transduction, pathogenesis, endocytosis, and cell wall organization, further understanding of how Dol-P-Man impacts the glycome network and influences plant development remain important challenges for future work.
Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana plants in the Columbia background were usually grown on MS basal salt medium (M0238; Ducha Biochimie) containing Suc (3%) and agar (8 g/L) as described previously (Bouvier et al., 2006). To test the effect of ammonium on plant growth, the seeds were germinated on MS medium (M 0238, modification number 4, NH₄Cl free; Ducha Biochimie) supplemented with increasing NH₄⁺ concentrations as indicated in the text. Alternatively, the seeds were grown on damp soil (Bouvier et al., 2006). For tunicamycin treatment, 21-d-old MS-grown (M 0238) Arabidopsis plants were transferred to the same liquid MS medium containing 5 μg/mL of tunicamycin for 5 h. Leaves from 2-month-old Nicotiana benthamiana plants grown under greenhouse conditions were used.

Screening of T-DNA Insertion Mutants

The T-DNA insertion mutants of Arabidopsis (Columbia ecotypes) corresponding to the different genes were obtained. The following lines were used: GKV_767A10 (dpms1-1), SALK_030487C (dpms1-2), SALK_111777 (dpms2-1), SALK_111779 (dpms2-2), SALK_037120 (dpms3-1), SALK_051037 (dpms3-2), SALK_006421 (srd5a3-like1-1), and SALK_113221 (srd5a3-like1-2). Segregation analysis (resistant:susceptible, 3:1) based on the antibiotic resistance, genomic PCR, and RT-PCR analyses using appropriate primers (see Supplemental Table 2 online) were performed to characterize the homozygous lines as described previously (Bouvier et al., 2006). Antibodies raised against recombinant Arabidopsis DPMS1, as described previously (Bouvier et al., 2006), were also used for immunological analysis of dpms1 mutants.

Histological and Microscopy Analysis

Hand-cut section of Arabidopsis stems from wild and mutant plants were treated with phloroglucinol-HCl (0.1% phloroglucinol in 20% HCl from WVR International) for 2 min to stain lignin. Sections were washed with water and examined under a light microscope. The seed surface morphology was analyzed using scanning electron microscopy without any special sample preparation. Mature seeds were collected and directly placed into a Hitachi Tabletop microscope TM-1000 and examined by scanning electron microscopy at an operating voltage of 15 kV. Images were taken using the software supplied by manufacturer.

Molecular Cloning and Plant Transformation

Primers used to generate the different constructs are listed in Supplemental Table 2 online. For lines overexpressing DPMS1, we cloned DPMS1 cDNA into the ImpactVector 1.1 (Plant Research International) previously digested by NcoI and BglII. The resulting expression cassette was subcloned (Ascl-PacI) in the binary vector pBIN Plus (Plant Research International) before transformation into Agrobacterium tumefaciens strain GV3101 and infiltration according to the floral dip procedure and kanamycin selection (Clough and Bent, 1998). For the complementation of dpms mutant plants, genomic fragments corresponding to the coding region were individually inserted into the XbaI site of the pCAMBIA-1300 vector (http://www.cambia.org/daisy/cambia/materials/vectors). T1 plants were selected on MS containing hygromycin. For the DPMS1-RNAi construct, we made use of a short inverted repeat of DPMS1 cDNA (hairpin). Two 87-bp fragments amplified from the DPMS1 cDNA containing NcoI and BglII at their 5’ termini and EcoRI at their 3’ termini were digested by EcoRI, self-ligated, and digested with NcoI and BglII. The 194-bp fragment was then ligated to the NcoI-BglII sites of the ImpactVector 1.1. Transient expression assays were performed using GFP-DPMS1, GFP-DPMS2, GFP-DPMS3, and At-RNLB2 inserted into the Nicotiana plasmid site of pCATSGFP vector before insertion into the binary vector (Bouvier et al., 2006). Transient coexpression of GFP-DPMS1, GFP-DPMS2, and GFP-DPMS3 in N. benthamiana was performed as described previously using 1:1:1 or 1:1 mixtures of Agrobacterium GV3101 harboring the GFP constructs (Mialoundama et al., 2009). In parallel, control leaves were infected with Agrobacterium harboring an empty vector. The leaves were harvested 72 h after inoculation and processed for SDS-PAGE and immunoblot analysis using anti-DPMS1 and anti-GFP antibodies (Invitrogen) (used at a 1/2500 dilution).

Gene Expression Analysis

Relative expression of the different genes was evaluated by quantitative PCR using total RNA isolated from the different plant lines using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer’s instructions. Three independent plants were used, and quantitative PCR was performed in triplicate from each biological sample. The different gene-specific primers were designed using Probe Finder software (Roche) and are listed in Supplemental Table 2 online. Two micrograms of total RNA were reverse transcribed in a total volume of 40 μL with 2.5 μM oligo(dT)₂₀; 0.5 mM deoxynucleotide triphosphate, and 400 units of SuperScript III reverse transcriptase (Invitrogen). One microliter of cDNA was quantified using gene-specific primers in a total volume of 10 μL SYBR Green I Master Mix (Roche) in a Light Cycler 480 II apparatus (Roche) according to the manufacturer’s instructions. The cycle threshold (Ct) was used to determine the relative expression level using the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001). The relative expression level of each gene was analyzed using GenEx Pro software (MultiD analyses; www.gene-quantification.de/datan.html) after normalization by reference with the internal references TIP41-LIKE and GAPDH.

Subcellular Localization of DPMS

Transient Expression of GFP-tagged DPMSs in Arabidopsis leaves were performed using the above-described GFP-DPMS1, GFP-DPMS2, and GFP-DPMS3 constructs as described previously (Eastmond et al., 2010). The reticulon-like protein At-RNLB2 fused with GFP (GFP-AfRTNLB2) was used as an ER marker protein (Nziengui et al., 2007). Leaf tissues expressing the individual GFP constructs were analyzed by confocal microscopy as described previously (Bouvier et al., 2006).

Recombinant DPMS Proteins and in Vitro Reconstitution of DPMS Complex

DPMS cDNAs were subcloned into a pBAD-TOPO vector using the pBAD-TOPO Thi fusion vector (Invitrogen) using the primers listed in Supplemental Table 2 online, as described previously (Bouvier et al., 2006). After induction with 0.001% Ara, the bacterial cells containing the recombinant His-tagged DPMS proteins were disrupted using a French press in the presence of lysis buffer A (50 mM Tris-HCl, pH 7.6, 1% digitonin, 0.1% dodecylmaltoside, 50 mM KCl, and 10 mM imidazole). The resulting mixture was centrifuged at 10,000g for 15 min. The supernatant was mixed with nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography (Qiagen). Ni-NTA agarose beads were rinsed three times batchwise with buffer B (50 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.1% digitonin, and 10 mM imidazole) to remove nonspecifically bound proteins. The washed beads (200 mL) were subsequently mixed with 1 mL buffer C, which contained 50 mM Tris-HCl, pH 7.6, 50 mM KCl, and 5 mg/mL of liposomes prepared from identical amounts of phosphatidylcholine and phosphatidylethanolamine using a Vibra Cell sonicator instrument (Sonics and Materials) as described previously (Maeda et al., 2000). The His tag–coupled proteins were eluted from the Ni-NTA agarose beads with buffer C containing 150 mM imidazole. In the reconstitution assay, identical amounts of recombinant DPMS1, DPMS2, or DPMS3 in buffer C were combined. The purified proteins were used for enzyme assay and SDS-PAGE analysis.
In-Gel Digestion and NanoLC-MS/MS Analysis of DMPS Component Proteins

The gel bands were predigested using a MassPREP automated system (Waters). The gel slices were washed twice in 25 mM ammonium hydrogen carbonate and acetonitrile. The disulfide bonds were subsequently reduced in 10 mM dithiothreitol (Sigma-Aldrich) at 57°C, and then the Cys residues were alkylated in 55 mM iodoacetamide (Sigma-Aldrich). Following dehydation with acetonitrile, the proteins were cleaved in the gel using 125 ng of modified porcine trypsin (Promega), 125 ng bovine pancreas chymotrypsin (Roche), or 130 ng of Pseudomonas fragi AspN (Promega). The peptides were extracted sequentially with 1% trifluoroacetic acid in acetonitrile/water (60:40 v/v) and 100% acetonitrile before concentration using a Speed Vac system. The extracts were analyzed using an ultra-high-pressure LC system (NanoAcquity; Waters) coupled to a high-resolution nanoelectrospray-quadrupole-time of flight type mass spectrometer (Maxis; Bruker Daltonics). The samples were trapped on a 20 × 0.18-mm, 5-μm Symmetry C18 precolumn (Waters), and the peptides were separated on a nanoACQUITY UPLC BEH130 C18 column (Waters; 75 μm × 200 mm, 1.7 μm particle size). The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Trapping was performed during 3 min at 5 μL/min with 99% of solvent A and 1% of solvent B. Elution was performed at a flow rate of 450 nL/min using 6 to 35% gradient (solvent A to B) over 9 min at 45°C followed by 90% (solvent B) over 1 min before the reconditioning the column using 99% of solvent A over 6 min. Maxis equipped with a nanosprayer was operated in the positive ion mode. The MS scan was performed over an m/z range of [50;2200] using Tunemix (Agilent Technologies). Online correction of this calibration was performed using two Tunemix ions (m/z 299.2945 and 922.0098) as recalibration masses. The capillary voltage was set to 4500 V and the end plate offset to −500 V. For MS/MS experiments, the system was operated with automatic switching between MS and MS/MS modes. The MS scan was performed over an m/z range of [50;2200] at 0.2 s/scan. The MS/MS scan time was automatically set depending on the intensity of the selected precursor peak on the MS spectrum (from 0.1 to 1.4 s/scan over the same m/z range). The three most abundant peptides, preferably doubly and triply charged ions, were selected on each MS spectrum for further isolation and collision-induced dissociation using an optimized collision energy depending on the charge state and the m/z of the ion. The selected peptides are then excluded for 0.3 min. The complete system was fully controlled by Compass Hystar (Bruker). The MS data collected during the analysis were processed and converted to mascot generic files (mgf) using Compass Data Analysis 4 software (Bruker). The mgf peak list files were then submitted to the Mascot search engine (Matrix Sciences; version 2.3.01) in a local server. Searches were performed against a composite target decoy database generated using internally developed tools (http://msuda.strasbg.fr) and containing the National Center for Biotechnology Information protein sequences of Arabidopsis and common contaminants (trypsin and human keratin) downloaded on the 23rd of September 2010. Searches were performed with a tolerance of 5 ppm for precursor ions and 0.02 D for fragment ions, allowing a maximum of one missed cleavage site for trypsin and chymotrypsin and three missed cleavages sites for AspN. Carbamidomethylation of Cys residues, oxidation of Met residues, and acetylation of protein N-terminal residues were searched as variable modifications. The spectra that yielded identifications were manually inspected.

Purification of Dolichols and Assay of DPMPS Activity

Ginkgo biloba seeds obtained from the botanical garden (Strasbourg, France) were used to extract and purify dolichols with chain length of 14 to 18 isoprene residues, according to published procedures (Tateyama et al., 1989). The resulting dolichol fraction was further purified by preparative HPLC using a Zorbax C18 column eluted using a linear gradient of solvent A (methanol/propan-2-ol/water, 12:8:1 v/v/v) to solvent B (hexane/ propan-2-ol, 7:3 v/v) (Skorupinska-Tadeu et al., 2003).

Dol-P-Man synthase was synthesized by coupling the synthesis of Dol-P to that of Dol-P-Man. First, purified Dol-14 (10 μg) was incubated at 25°C for 10 min with (15 μg) purified recombinant polypropenyl-dolichol kinase cloned from Streptococcus mutans DGK (Hartley et al., 2008) in a reaction mixture (100 μL) containing 50 mM Tris-HCl, pH 7.6, 2 mM [γ-32P]ATP (10 mCi/mmol; Hartmann Analytic), 1 mM DTT, 5 mM MgCl₂, and 0.1% Tween 80. Upon completion of this step, 50 μL of liposomes containing purified DPMPS protein components (equivalent to 20 μg) as specified in the text were added to the reaction mixture supplemented with 2 mM GDP-Man, and the reaction was allowed to proceed for 30 min. Unlabeled Dol-P-Man was used as the reaction condition except nonradioactive ATP (2 mM) was used in the incubation medium. In the case of Arabidopsis microsomal membranes, the leaves were homogenized in the medium containing 50 mM Tris-HCl, pH 7.6, 0.25 M Suc, 1 mM EDTA, 0.5% polyvinylpyrrolidone, and 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 14,000g for 15 min, and the resulting supernatant was centrifuged at 100,000g for 1 h to pellet the microsomal membrane fraction. The microsomal membrane fraction was suspended in 50 mM Tris-HCl, pH 7.6, containing 0.25 M Suc and 1 mM DTT. Microsomal preparations equivalent to 250 μg of protein were incubated at 25°C for 30 min in the reaction mixture containing 50 mM Tris-HCl, pH 7.6, 2 mM [γ-32P]ATP (10 mCi/mmol; Hartmann Analytic), 1 mM DTT, 5 mM MgCl₂, Dol-14 (10 μg), 0.1% Tween 80, and 2 mM GDP-Man for 30 min. The reaction products were extracted using dichloromethane/methanol (2:1 v/v). Following centrifugation, the dichloromethane phase was adsorbed onto a mini DEAE-cellulose column prepared as described previously (Camara and Monéger, 1977). The column was sequentially eluted with dichloromethane and dichloromethane/methanol (7:3 v/v), and the fraction containing the Dol-P-Man was eluted with dichloromethane/methanol (7:3 v/v) containing ammonia (0.28%) and 50 mM ammonium acetate. The eluate was concentrated and subjected to thin layer chromatography (TLC) on silica gel plate developed with dichloromethane/methanol/water (10:10:3 v/v/v), and the position of Dol14-P-Man (retention factor = 0.7) was detected by autoradiography. Radioactivity incorporated in the products was quantified by a liquid scintillation counter. For incubation with nonradioactive ATP, the Dol-P-Man band was scraped and eluted with dichloromethane/methanol (2:1 v/v) before ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) analysis.

UPLC and MS Analysis of Dol-P-Man

Characterization of Dol-14-P-Man recovered from the DEAE-cellulose column was performed using UPLC-MS/MS. All analyses were performed using a Waters Quattro Premier XE equipped with an ESI source and coupled to an Acquity UPLC system (Waters) with diode array detector. UV spectra were recorded from 200 to 500 nm. Chromatographic separation was achieved using an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters), coupled to an Acquity UPLC BEH C18 precolumn (2.1 × 5 mm, 1.7 μm; Waters). The temperature of the column oven was 40°C, and the injection volume was 3 μL. Eluents were methanol/acetonitrile/water (60/30/9.99, v/v/v), basified with 1 mM ammonium acetate and 0.01% piperidine (solvent A) and ethanol 99.99%; basified with 1 mM ammonium acetate and 0.03% piperidine (solvent B). The flow rate was 0.31 mL/min. Chromatographic conditions were as follows: 100% (A) for 1 min; gradient from 100% (A) to 100% (B) for 5 min, 100% (B) and an isocratic run of 5 min at 100% of B, followed by a gradient from 100% (B) to 100% (A) for 1 min to the initial conditions that was maintained for 3 min. The total run time was 15 min. Nitrogen generated from pressurized air in a N2G nitrogen generator (Mistral) was used as the drying and nebulizing gas. The nebulizer gas flow was set to ~50 L/h, and the desolvation gas flow to 700 L/h. The interface
temperature was set at 300°C and the source temperature at 120°C. The capillary voltage was set at 3 kV, and the cone voltage was optimized for each Dol-P-Man depending on the length of the isoprenoid skeleton. Full-scan and selected ion recording analyses were used to determine the m/z of Dol-P-Man. Fragmentation was performed by collision-induced dissociation with argon at 1.0 × 10⁻⁴ mbar. The collision energy was optimized using daughter scan monitoring and MRM. MS conditions for Dol₄₊⁻⁻P-Man were set after optimization as follows: polarity ES⁺, capillary 3 kV, cone 40 V. Low mass and high mass resolution were 13 for both mass analyzers, ion energies 1 and 2 were 0.5 V, entrance and exit potential were 2 and 1 V, and detector (multiplier) gain was 650 V. Collision-induced dissociation of deprotonated parent ions was accomplished with collision energy of 10 and 15 V. Daughter scan monitoring, neutral loss, and MRM permitted the identification of Dol₄⁺⁻⁻P-Man. The prominent compound was identified as a deprotonated parent ion [M-H₂O-H⁻], and the predominant daughter fragment ions 1094 and 1052 corresponding to partial and total Man loss were obtained by daughter scan and subsequently used for MRM transitions. The combination of chromatographic retention time, parent mass, and the two more intense daughter ions analysis was used to selectively monitor Dol₄⁺⁻⁻P-Man (1196 > 1094) and (1196 > 1052). Data acquisition and analysis were performed with the MassLynx software (ver.4.1) running under Windows XP professional on a Pentium PC.

Affinity Interactions ofDPMS Protein Components

Total leaf proteins were prepared from N. benthamiana leaves expressing various combinations of GFP-DPMS1, GFP-DPMS2, and GFP-DPMS3 constructs. Leaf tissues were ground into a fine powder in liquid nitrogen before extraction with the buffer (50 mM Tris–HCl, pH 7.6, 2 mM PMSF, 0.2% Triton X-100, and 0.3% digitonin). Anti-DPMS1-Sepharose beads were prepared using dimethylpimelimidate (Schneider et al., 1982). Proteins derived from leaf extracts were centrifuged, and the supernatant was incubated for 6 h at 4°C to 100 μL of anti-DPMS1-Sepharose beads in the presence of the incubation buffer (50 mM Tris–HCl, pH 7.6, 0.1 M KCl, 2 mM PMSF, and 0.3% digitonin). Immunoadsorbed complexes were washed by centrifugation using the same buffer containing 0.25 M KCl before elution of bound proteins with 0.1 M Gly, pH 2.5. The eluate was neutralized to pH 7.6 and subjected to SDS-PAGE and immunoblot analyses using anti-GFP (Invitrogen) as described previously (Bouvier et al., 2006).

The pull-down assay was performed using DPMS3 as bait to pull down putative DPMS1 and DPMS2 preys. Streptavidin agarose beads (Thermo Scientific) (200 μL) were incubated for 30 min at 25°C with biotin-labeled DPMS3 (250 μg) prepared using the Pierce pull-down biotinylated protein-protein interaction kit (Thermo Scientific). Purified Rubisco protein (Sigma-Aldrich) was used as negative control bait. Next, total Escherichia coli lysates (500 μg) obtained after French press disruption and centrifugation of bacterial cells expressing recombinant DPMS1 and DPMS2 were incubated for 2 h at 4°C with biotinylated protein-protein interaction kit streptavidin agarose in a medium containing 50 mM Tris–HCl, pH 7.6, 3% digitonin, 2 mM PMSF, 0.2% Triton X-100, and 0.1 M KCl. The beads were washed by centrifugation with 50 mM Tris–HCl, pH 7.6, buffer containing 2 mM PMSF, 0.3% digitonin, and 0.25 M KCl to remove nonspecifically bound proteins. DPMS3 interacting proteins were eluted with the acidic pH 2.8 buffer.

Affindetection of Glycoproteins

Total proteins were extracted using 25 mM Tris, pH 8.3, containing 250 mM Gly and 0.1% SDS and subjected to SDS-PAGE analysis before electrophoretic transfer onto nitrocellulose membranes. Concanavalin A binding proteins were detected using jack bean (Canavalia ensiformis) Concanavalin A-peroxidase (Sigma-Aldrich) and Amersham enhanced chemiluminescence reagent. PDI and PDCB1 were used as markers for N-glycoproteins and GPI-anchored proteins and probed using anti-PDI (used at a 1/2500 dilution) (Łukowicz et al., 2001) and anti-PDCB1 (used at a 1/2500 dilution) (Simpson et al., 2009) antibodies.

Ascorbic Acid Analysis

Arabidopsis leaves were pulverized in liquid nitrogen, and the resulting powder was homogenized in 1 h HClO₄. Following centrifugation and neutralization with K₂CO₃, the supernatant was saved for ascorbate determination as described previously (Foyer et al., 1983).

GMPase Activity

Plant leaves were pulverized in liquid nitrogen, and the resulting powder was homogenized in the extraction buffer containing 50 mM Tris–HCl, pH 7.6, and 2 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000g for 10 min, and the supernatant (100 μg protein) was used for enzyme assay in a reaction mixture (200 μL final volume) containing 50 mM Tris–HCl, pH 7.6, 2 mM 2-mercaptoethanol, 5 mM MgCl₂, 2 mM GTP, and 2 mM d-Man. The reaction was performed at 30°C for 1 h. The reaction was quenched by boiling, and GDP-Man was analyzed by HPLC as described previously (Albermann et al., 2000). Protein concentration was determined using the Pierce bicinchoninic protein assay kit (Pierce).

Chlorophyll Determination

Total chlorophyll content was determined in 80% acetone extracts (Lichtenhaller and Welbourn, 1983).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative databases under the following accession numbers: At-DPMS1 (Arabidopsis; At1g20575), Os-DPMS1 (Oryza sativa; NP_001051751), Sp-DPMS1 (Schizosaccharomyces pombe; CAB11700), Hs-DPMS1 (Homo sapiens; EAW75608), Rn-DPMS1 (Rattus norvegicus; NP_001100014), Tb-DPMS1 (Trypanosoma brucei; AJ866775), Lm-DPMS1 (Leishmania mexicana; AJ131960), Sc-DPMS1 (Saccharomyces cerevisiae; YAB68116), At-DPMS2 (At1g74340), Os-DPMS2 (NP_001172795), Hs-DPMS2 (AAH15233), Rn-DPMS2 (NP_062125) Sp-DPMS2 (NP_595676), At-DPMS3 (At1g48140), Os-DPMS3 (EAY87536), Hs-DPMS3 (BAA96291), Rn-DPMS3 (NP_001102801), Cd-DPMS3 (Candida dubliniensis; XP_002419846), At-SRD5A3-like1 (At2g16530), At-SRD5A3-like2 (At1g27590), Hs-SRD5A3 (NM_024592), At-RTNBL2 (At4g11220), TIP41-LIKE (At4g34270), GAPDH (At1g3440), and α-Tubulin (At1g04820).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Multiple Sequence Alignment of DPMS1 with Homologous Proteins.

Supplemental Figure 2. Multiple Sequence Alignment of DPMS2 with Homologous Proteins.

Supplemental Figure 3. Multiple Sequence Alignment of DPMS3 with Homologous Proteins.

Supplemental Figure 4. Analysis of Recombinant DPMS Proteins Expressed in E. coli.

Supplemental Figure 5. Neutral Loss Analysis of Dol₄⁺⁻⁻P-Man.

Supplemental Figure 6. Characterization of Arabidopsis dpms Mutants and Transgenic Lines.

Supplemental Figure 7. Chromatographic Analysis of Dol₄⁺⁻⁻P-Man Synthesized by Microsomal Membranes Isolated from Wild-Type
Arabidopsis, dpms Mutants, Complemented Mutants, and DPMS1-RNAi Lines.

Supplemental Figure 8. Ammonium Sensitivity of dpms1 Compared with Wild-Type Plants.

Supplemental Figure 9. Characterization and Ammonium Sensitivity of srd5a3-like1 Mutants.

Supplemental Figure 10. Multiple Sequence Alignment of SRD5A3 with Homologous Proteins.

Supplemental Figure 11. Affinodetection Analysis of Total Leaf Proteins Using Concanavalin A.

Supplemental Table 1. In-Gel Digestion and NanoLC-MS/MS of DPMS3-Interacting Proteins.

Supplemental Table 2. Primer Sequences Used for Cloning, Genotyping, and Expression Studies.

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DOLICHOL PHOSPHATE MANNOSE SYNTHASE1 Mediates the Biogenesis of Isoprenyl-Linked Glycans and Influences Development, Stress Response, and Ammonium Hypersensitivity in Arabidopsis

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