Research paper

Biochemical characterization of Arabidopsis APYRASE family reveals their roles in regulating endomembrane NDP/NMP homeostasis

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Summary Statement
The seven apyrase enzymes from Arabidopsis localize to the plant endomembrane. Our analyses indicates that five members (AtAPY1, 2, 4, 5, 6) exhibit lumenal NDPase activities while AtAPY3 has NTPase activity. AtAPY7 displayed no NTDPase activity.

Short (page heading title): Arabidopsis apyrase family

Abstract

Plant apyrases are nucleoside triphosphate diphosphohydolases and have been implicated in an array of functions within the plant including the regulation of extracellular ATP. Arabidopsis encodes a family of seven membrane bound apyrases (AtAPY1 to 7) comprised of three distinct clades all of which contain the five conserved apyrase domains. With the exception of AtAPY1 and AtAPY2, the biochemical and the subcellular characterization of the other members are currently unavailable. In this research, we have shown all seven Arabidopsis apyrases localize to internal membranes comprising the *cis*-Golgi, ER and endosome, indicating an endo-apyrase classification for the entire family. In addition all members, with the exception of AtAPY7, can function as endo-apyrases by complementing a yeast double mutant (Δ*ynd1*Δ*gda1*) which lacks apyrase activity. Interestingly to note that complementation of the mutant yeast using well characterized human apyrases could only be accomplished by using a functional ER endo-apyrase (NTPDase6), but not the ecto-apyrase (NTPDase1). Furthermore, the substrate specificity analysis for the Arabidopsis apyrases AtAPY1 to 6 indicated that each member has a distinct set of preferred substrates covering various NDPs and NTPs. Combining the biochemical analysis and subcellular localization of the Arabidopsis apyrases family, the data suggests that their possible roles in regulating endomembrane NDP/NMP homeostasis.

Keywords

Arabidopsis apyrase, human, yeast, endomembrane

Abbreviations: NTPDase**,** nucleoside triphosphate diphosphohydolases; ACR, apyrase conserved regions; ATPases, adenosine triphosphatases; ER, endoplasmic reticulum; dKO, double knock-out

Introduction

The apyrase class of enzymes (EC 3.6.1.5) are nucleoside triphosphate diphosphohydolases (NTPDases) that belong to the GDA1_CD39 nucleoside phosphatase superfamily and contain five apyrase conserved regions (ACRs). They are active against both nucleotide tri- and diphosphates (NTP, NDP), converting them to nucleotide monophosphates (NMP). Apyrases have been identified in an array of species, including plants, mammals, insects, fungi and bacteria [1]. The NTPDase activity requires divalent cations (Mg^{2+}, Ca^{2+}) and are distinct from the adenosine triphosphatases (ATPases) due to their broader substrate activities and insensitivities to F-type, P-type, and V-type ATPase inhibitors [2].

In mammals, apyrases were initially characterized as having cell surface ATPase activity (ecto-apyrase). The human apyrases are the most extensively characterized family and comprise cell surface localized ecto-apyrases (NTPDases 1, 2, 3 and 8) and endo-apyrases which are associated with the endoplasmic reticulum (ER), Golgi and intracellular vesicles (NTPDase 4, 5, 6, 7) [1]. The plasma membrane localized apyrases are mainly involved in the regulation of extracellular ATP to prevent desensitization of purine receptors [3]. In contrast, the intracellular localized ER/Golgi human endo-apyrases are involved in the conversion of NDP to NMP to both drive lumenal glycosylation reactions and produce co-substrates (NMP) for the membrane localized nucleotide sugar antiporters [4]. *Saccharomyces cerevisiae* (yeast) encodes two apyrases, namely GDA1 and YND1 [5, 6]. The yeast GDA1 protein is an NDPase with preferential activity against GDP [7]. YND1 has a broader substrate specificity and can readily hydrolyze both NDPs and NTPs, although with a preference for GDP [6]. The functions of these two yeast apyrases are somewhat redundant as *YND1* can partially complement glycosylation defect phenotypes when expressed in the Δ*gda1* background. Interestingly, yeast cells (Δ*ynd1*Δ*gda1*) lacking both apyrases are still viable [6].

In plants, the involvement of extracellular ATP as a potential signaling molecule has been proposed for a number of years [8]. A number of studies have demonstrated that plant cells release significant quantities of ATP into their extracellular matrix when they are mechanically stimulated [9], wounded [10], during growth [11] and during stomatal opening [12]. Recently, with the characterization of a plasma membrane localized ATP receptor kinase [13] a role for plant apyrases in the regulation of extracellular ATP has been strengthened. In the reference plant *Arabidopsis thaliana*, a total of seven NTPDases have been identified based on the presence of the ACRs [14]. Among the seven members, APYRASE 1 (*AtAPY1* At3g04080) and APYRASE 2 (*AtAPY2*: At5g18280) have been the most extensively investigated. Both AtAPY1 and AtAPY2 have been shown to play numerous physiological roles in pollen development, vegetative growth and stomata opening/closure [12, 15, 16]. Collectively, these responses were attributed to defects in ecto-nucleotide signaling responses. However, recently both AtAPY1 and AtAPY2 have been identified in plant Golgi proteomes [17] and their localizations confirmed by fluorescent protein tagging [18, 19]. In addition, knocking out either *AtAPY1* or *AtAPY2* affects latent lumenal UDPase/GDPase activity in microsomal preparations from Arabidopsis which resulted in a minor change to the galactose content of their cell walls [18]. Furthermore, the conditional suppression of *AtAPY1* in the *atapy2* background resulted in structural changes to the cell wall [20]. These data provide strong evidence to support of the hypothesis that AtAPY1 and AtAPY2 functions as plant endo-apyrases and are necessary for lumenal glycosylation. However, this functionally defined role as an endo-apyrase would not necessarily preclude a role

as regulators of ecto-ATP/ADP concentration via secretary mechanism, as has been recently argued [14] based on data showing that immunochemical [16] and genetic [20] suppression of AtAPY1 and AtAPY2 results in an increase in extracellular ATP.

Aside from AtAPY1 and AtAPY2, a further five apyrase members are encoded by Arabidopsis (AtAPY3 to 7), although their biochemical and physiological functions remain elusive. Some initial characterization of AtAPY6 and AtAPY7 has been undertaken, with double knock-out plants (*atapy6atapy7*) resulting in late anther dehiscence, exine deformation and low male fertility [21]. These structural changes to the pollen cell wall in combination with an internal localization for AtAPY6 tagged lines further support roles as endo-apyrases involved in polysaccharide biosynthesis [21]. Consequently, in an effort to resolve the functional roles of the Arabidopsis apyrase family, we sought to systematically investigate their subcellular localizations and determine their substrate specificities and relate these findings to functional roles in the context of the well characterized apyrase family members from humans.

Experimental

Cloning procedures for heterologous protein expression

The Arabidopsis apyrase family members *AtAPY3* (At1g14240), *AtAPY4* (At1g14230), *AtAPY5* (At1g14250), *AtAPY6* (At2g02970) and *AtAPY7* (At4g19180) were cloned from a mixed organ Arabidopsis cDNA library using primers designed based on sequences in The Arabidopsis Information Resource (TAIR) [22] (Table S1). PCR products were recombined into pDONR™/Zeo by BP reaction (Life Technologies) and verified by sequencing. The genes *AtAPY1* (At3g04080) and *AtAPY2* (At5g18280) were previously cloned using a similar approach [17]. For transient subcellular localizations, the *AtAPY1* to *7* pDONR™/Zeo constructs were recombined into the N-terminal YFP and C-terminal YFP Gateway® compatible pBullet vectors [23] by LR reactions (Life Technologies). The human apyrase cDNA sequences were obtained from the Mammalian Gene Collection [24] and comprised *ENTPD1* (BC047664.1), *ENTPD6* (BC025980.2) and *ENTPD7* (BC122857.1). These sequences were codon optimized for yeast expression (Figure S1), synthesized (GenScript), recombined into the pDONR™/Zeo vector by BP reaction (Life Technologies) and verified by sequencing. The *AtAPY7* sequence was codon optimized (Figure S1) and synthesized (GenScript) for yeast expression. For yeast complementation assays, the pDONR™/Zeo constructs were recombined into a pDR-Leu Gateway® yeast expression vector [17].

Chromosomal deletion the GDA1 locus from Saccharomyces cerevisiae

The chromosomal *GDA1* locus (YEL042W) was replaced with URA3 (orotidine-5'-phosphate) by homologous recombination as previously described [25]. Genomic DNA was extracted from the *Saccharomyces cerevisiae* wild type strain BY4741 (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) using YeaStar™ Genomic DNA Kit (Zymoresearch) and used as template. The yeast *GDA1* gene was cloned by PCR using the pGDA1 primers and inserted into pENTR™/D-TOPO® (Life Technologies) to be used as the templates to create the knockout cassette border sequence. A further round of PCR was undertaken using the pGDA1-R primers to create a BamHI site used to replace GDA with URA3. The resultant product was cloned into pENTR™/D-TOPO®. The URA3 gene was amplified by PCR from the vector pRS416-GPD [26] and restriction sites BamHI and MscI were added by PCR using the URA3 primers. The URA3 PCR product was digested with BamHI and MscI and ligated into the pENTR/D-TOPO-GDA backbone to create the knockout cassette pGDA-URA3-tGDA. To knockout the chromosomal *GDA1* gene, the *Saccharomyces cerevisiae* strain Δ*ynd1* (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *ynd1Δ0*) obtained from the Yeast Knockout Collection (Thermo Scientific) was transformed with the linearized vector pGDA-URA3-tGDA using Frozen-EZ Yeast Transformation II Kit™ (Zymoresearch). The transformants were selected on solid medium containing Yeast Nitrogen Base (YNB) without amino acids (Becton, Dickinson and Company) supplemented with 2 % (w/v) glucose and 1X CSM-Ura (Sunrise Science Products). Genomic DNA was isolated from candidate transformants using YeaStar™ Genomic DNA Kit (Zymoresearch). The integrity of the *GDA1* locus was examined by PCR using the following primer sets: left border using primers GDA-L; middle using primers GDA-M and right border using primers GDA3-R. The presence of the inserted URA3 sequence was examined by PCR using the primers URA3-ORF. Primers are detailed in Table S1.

Yeast transformation and complementation assay

The Δ*gda1*Δ*ynd1* dKO yeast strain was transformed with the various plasmids using the EZ-YEAST™ transformation kit (MP Biomedicals) and selected on solid media containing Yeast Nitrogen Base (YNB) without amino acids (Becton, Dickinson and Company) supplemented with 2 % (w/v) glucose and 1X CSM-Leu-Ura (Sunrise Science Products). Complementation was assessed by growing single colonies overnight at 30 °C in liquid media as described above. Liquid cultures were serially diluted and spotted on solid selection media as outlined above.

Monosaccharide analysis of the yeast cell wall

Sample extraction and preparation procedures were undertaken according to previously described methods employing TFA hydrolysis [27]. Cultures (50 mL) were grown until OD = 1.0 to 1.2 and cells harvested by centrifugation at 2000 x g for 5 min. Cells were disrupted in 0.5 mL of 10 mM Tris-HCl (pH 8) using glass beads and a vortex. Cell walls were collected by centrifugation (3800 x g for 5 min) and washed in cold distilled water and dried in a vacuum concentrator. Cell wall pellets were hydrolyzed with 1 mL of 2 N trifluoroacetic acid (TFA) at 100 °C for 4 hours. Samples were lyophilized and re-suspended in 1 mL water prior to analysis. Monosaccharide composition was performed using High Performance Anion Exchange Chromatography on a Dionex ICS 3000 equipped with a pulse amperometric detector as previously described [28]. The monosaccharide composition of yeast samples was calculated by linear regression from a five point standard curve comprising glucose, mannose and glucosamine loaded before, during and after the sample set.

RNA extraction and RT-PCR

Total RNA was isolated from yeast strains using YeaStar™ RNA Kit (Zymo Research). Approximately 300 ng of total RNA was treated with DNase (Invitrogen) and used as template for cDNA synthesis by SuperScript III Reverse Transcriptase (Invitrogen). PCR was undertaken using Taq 2X Master Mix (New England Biolabs Inc.) using conditions as provided by the manufacturer. The RT-PCR of apyrase transcripts was undertaken with *att*B1 and *att*B2 primers (Table S1). The yeast *UBC6* gene (ubiquitin-conjugating enzyme) was used as a control.

Immunoblotting

Total protein was isolated from overnight yeast cultures as previously described [29]. The protein was quantified by Bradford (Thermo Scientific) [30]. A total of 5 µg total protein was re-suspended in 0.2 M Tris–HCl, pH 6.5, 8 % (w/v) SDS, 8 % (v/v) 2-mercaptoethanol, 40 % (v/v) glycerol, and 0.04 % (w/v) bromophenol blue and boiled for 5 min. Samples were subjected to 10 % (w/v) SDS-PAGE and blotted onto PVDF membrane. Heterologous expressed proteins were detected using the Universal antibody (UNI) against the Gateway® *att*B2 site [29], followed by incubation with a secondary antibody and detection by chemiluminescence using the Protein Detector™ LumiGLO® Western Blotting Kit (KPL Inc).

Yeast microsomal preparations

The yeast membranes were isolated from the complemented Δ*gda1*Δ*ynd1* dKO yeast strains by initial disruption with glass beads in 400 µL of chilled extraction buffer (20 mM Tris-HCl, 10 mM MgCl2, 1 mM EDTA, 5 % (v/v) Glycerol, 1 mM DTT, 1 mM PMSF and 1 x Roche cOmplete Protease Inhibitor Cocktail). The cells were centrifuged at 5000 x *g* for 10 min at 4 °C and supernatants collected. The supernatant was centrifuged at 50000 x *g* for 1 hour at 4 °C and

the resultant membrane pellet was re-suspended in 10 mM Tris buffer (pH 7.5) for the NTPDase assay.

Measurement of NTPDase activity

A total of 50 µg microsomal protein was incubated in 500 µl reaction buffer (3 mM NDP or NTP or NMP [Sigma-Aldrich], 3 mM MnSO4, 30 mM Tris-MES, pH 6.5, and 0.03 % (v/v) Triton X-100) for 1 h at room temperature. The released phosphate was measured using the Malachite Green Phosphate Assay (ScienCell Research Laboratories) with slight modifications, namely that 100 µL of reagent A and 100 µL of reagent B were each added to the 50 µL solution. The incubation times were undertaken according to the protocol.

Particle Bombardment

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen). Particle bombardments for transient localizations were conducted according to previous methods [23]. Essentially, 0.6 µg plasmid DNA was added to a 25 µL microcarrier/glycerol solution containing 400 µg of microcarriers (1 µm gold, Bio-Rad). Followed by 25 µL of 2.5 M $CaCl_2$ and 10 µL of 0.1 M spermidine. The solution was mixed for 10 min at 3000 rpm and supernatant removed. The pellet was washed with 100 % (v/v) ethanol and re-suspended in 20 µL of 100 % (v/v) ethanol, loaded onto a macrocarrier and air dried. The macrocarrier was placed onto the hepta adapter macrocarrier holder (leaving the other 6 empty). Fresh epidermal peels from yellow onions or whole Arabidopsis rosettes harvested from 6 to 8-week old plants were bombarded under vacuum (28 inHg) at a target distance of 6 cm and a helium pressure of 1100 psi. Arabidopsis rosettes were bombarded on 1 % (w/v) agar plates containing half strength Murashige and Skoog basal salt mixture. After bombardment, plant material were kept on plates overnight in the dark until imaging by confocal microscopy [23].

Phylogenetic Analysis and Informatics

The Arabidopsis apyrase protein sequences were obtained from The Arabidopsis Information Resource [22]. The human and plant apyrase protein sequences were obtained from GenBank [31] while yeast sequences were from the Saccharomyces Genome Database [32]. Phylogenetic trees were created using MEGA6 [33], with sequences aligned using MUSCLE (using UPGMB and default parameters), phylogenetic reconstruction was undertaken using Maximum Likelihood with 1000 Bootstrap Replications. Protein domains were obtained from InterProScan [34] and predicted transmembrane helices from TMHMM [35]. Protein features were visualized using DOG (Domain Graph, version 1.0) [36].

GenBank Accessions

The following sequences have been deposited at GenBank: AtAPY1/At3g04080 (JQ937231); AtAPY2/At5g18280 (JQ937238); AtAPY3/At1g14240 (JF830008); AtAPY4/At1g14230 (JF830009); AtAPY5/At1g14250 (JF830010); AtAPY6/At2g02970 (JF830011); AtAPY7/At4g19180 (JQ965809).

Results

The Apyrase family of Arabidopsis thaliana

A total of seven loci have been identified in the Arabidopsis genome that contain the apyrase domain, namely AtAPY1 (At3g04080), AtAPY2 (At5g18280), AtAPY3 (At1g14240), AtAPY4 (At1g14230), AtAPY5 (At1g14250), AtAPY6 (At2g02970) and AtAPY7 (At4g19180). A recent phylogenetic analysis of several hundred plant apyrases indicated that they fall into three major clades [14]. The seven member Arabidopsis apyrase family contain representatives in each clade and are clustered into the AtAPY1-2 clade I (GDA1-like), the AtAPY3 to 6 (clade II) and AtAPY7 in clade III (Figure 1A).

Since the eight *Homo sapiens* (human) apyrase genes (NTPDase1 to 8) have been characterized both biochemically and genetically [1] and the two apyrase enzymes from yeast, (GDA1 and YND1) have been extensively characterized [6], we undertook a phylogenetic analysis with several plant apyrases, the human apyrase family and the two yeast enzymes (Figure 1B). The clade I (GDA-like) Arabidopsis members (AtAPY1 and AtAPY2) form a distinct clade with the other characterized plant apyrases, human apyrases and the yeast GDA1 enzyme (Figure 1B). Although NTPDase6, GDA1, AtAPY1 and AtAPY2 appear to have a substrate preference for NDPs [5, 18, 37], a number of the plant apyrases in this clade have been associated with exhibiting NTPase activity, namely StAPY3 [38] and PsAPY2 [39]. In humans, NTPase activity is associated with the secreted ecto-apyrase clade members (NTPDase1 to 3 and NTPDase8) and all display Type IV-A membrane protein topology (Figure S2). In contrast, plant members of the GDA-like clade are typical Type II membrane proteins (Figure 1A). The Arabidopsis AtAPY7 is only weakly associated with the human ecto-apyrase clade, it has a similar membrane topology and can also be classed as a Type IV-A membrane protein (Figure 1A).

The final group of Arabidopsis apyrase members forms an independent cluster (clade II) comprising AtAPY3 to 6. The apyrase members AtAPY3, AtAPY4 and AtAPY5 are recurrent tandem gene duplications on chromosome 1. All three contain a single putative N-terminal transmembrane domain typical of Type II membrane proteins. In contrast, AtAPY6 (clade II) would appear to be Type IV-A membrane protein (Figure 1A).

Disruption of the yeast apyrases GDA1 and YND1 affects the yeast cell wall composition

The the Δ*ynd1*Δ*gda1* strain was previously created by crossing the *Δynd1::URA3* haploid (*XGY4*) with the *Δgda1::LEU2* haploid (*G2-11*). The Δ*ynd1*Δ*gda1* dKO cells (KAI1) showed slow growth compared to the single mutants and the wild-type strain on YPAD plates at 30°C [6]. However, the laboratory that created this Δ*ynd1*Δ*gda1* line has lost the original strain. We sought to recreate the Δ*ynd1*Δ*gda1* strain by replacing the *GDA1* open reading frame (ORF) with the *URA* ORF by a heterologous exchange in the Δ*ynd1* single mutant background [25, 40]. The Δ*ynd1*Δ*gda1* double knock-out (dKO) was verified using primer sets designed to assess the presence of the *GDA* ORF (Figure 2A). Only the *URA* ORF was detected by PCR in the Δ*ynd1*Δ*gda1* dKO strain indicating that the *URA* ORF had successfully replaced the *GDA1* ORF in the Δ*ynd1* single mutant background (Figure 2A).

The yeast cell wall contains β(1→3)-D-glucan, β(1→6)-D-glucan, chitin, and mannoproteins which are mostly synthesized at the plasma membrane [41]. Cell wall mannoproteins are

synthesized in the ER/Golgi lumen and are dependent on the delivery of GDP-mannose from the cytosol, a process that is driven by the co-transport of GMP generated by apyrases (GND1/YND1) in the ER/Golgi lumen [42]. We analyzed the monosaccharide composition of a TFA hydrolyzed insoluble fraction extracted from the Δ*ynd1*Δ*gda1* dKO strain after growth in modified YNB media after 2 days at 30ºC. The composition of mannose in this insoluble fraction was about 50% less than that found in wild-type (BY4741) cells (Figure 2B). The Δ*ynd1*Δ*gda1* dKO strain also contained less cell wall material including non-lumenal derived polymers, namely β(1→3)-D-glucan, β(1→6)-D-glucan, and N-acetylglucosamine from chitin (Figure 2C); highlighting the importance of the mannoprotein component in the construction of the yeast cell wall. Finally, similar to previously reported results [6], the newly generated Δ*ynd1*Δ*gda1* dKO also exhibits very slow growth on modified YNB media (data not shown).

Yeasts lacking endogenous apyrase activity are complemented by human endo-apyrases

The human apyrases represent a biochemically well characterized family of enzymes with varying subcellular locations and activities. The members of the human apyrase family comprise the ER lumenal GDA-like NDPases (e.g. NTDPase6), an intracellular membrane associated clade with NDPase/NTPase activity (e.g. NTPDase7) and the ecto-apyrase group with NTPase activities (e.g. NTPDase1). We were interested in assessing the ability of these defined classes of apyrases to complement the Δ*ynd1*Δ*gda1* dKO yeast strain generated above. The GDA-like human NTDPase6, with a substrate specificity for NDPs, was able to recover the growth phenotype observed in the Δ*ynd1*Δ*gda1* dKO strain (Figure 3A). Neither the NTPDase1 (ecto-apyrase) nor the NTPDase7 (intracellular with reported NDPase/NTPase activities) were able to complement the growth phenotype exhibited by the Δ*ynd1*Δ*gda1* dKO strain (Figure 3A). NTPDase1 has a mixed NTP/NDP substrate specificity [43], while NTPDase7 has a preference for NTPs [44]. The presence of the yeast codon optimized human apyrase gene transcripts was verified by RT-PCR (Figure S3). Overall, these results indicate that subcellular context as well as substrate specificity are necessary for complementation of this yeast Δ*ynd1*Δ*gda1* dKO strain.

Yeast lacking endogenous apyrases can be complemented by Arabidopsis apyrases

In order to assess the *in vivo* activities of the Arabidopsis apyrase family, we performed a complementation assay in the Δ*ynd1*Δ*gda1* dKO as described above. Previously, the Arabidopsis clade I apyrase members AtAPY1 and AtAPY2 were shown to act as lumenal NDPases through the independent complementation of the glycosylation phenotype associated with the Δ*gda1* mutant background as well as the hygromycin sensitivities of the Δ*ynd1* mutant related defects in the cell wall [18]. When these clade I Arabidopsis apyrases were expressed in the Δ*ynd1*Δ*gda1* dKO background, both AtAPY1 and AtAPY2 were able to complement the growth phenotype when compared to yeast harboring the empty vector (pDR-Leu, Figure 3A). These results support our previous findings that both AtAPY 1 and AtAPY2 are able to function as internal Golgi lumenal NDPases. The heterologous expression of the clade II Arabidopsis apyrase members (AtAPY3 to 6) in the Δ*ynd1*Δ*gda1* dKO background revealed that AtAPY4, AtAPY5 and AtAPY6 were all able to complement the growth defect phenotype of the Δ*ynd1*Δ*gda1* dKO yeast strain (Figure 3A), demonstrating these enzymes are also able to function as internal Golgi lumenal NDPases. In contrast, AtAPY3 exhibited relatively weak complementation compared to other members of this clade (Figure 3A). The clade III Arabidopsis apyrase AtAPY7 was unable to complement the growth phenotype of the Δ*ynd1*Δ*gda1* dKO strain (Figure 3A).

An analysis of the monosaccharide composition of the insoluble cell wall fractions from the complemented Δ*ynd1*Δ*gda1* dKO strains further supported a role for the Arabidopsis apyrases as lumenal NDPases (Figure 3B). The proportion of mannose in cell wall extracts significantly increased in all the complemented strains with the AtAPY5 construct resulting in near wild-type levels (Figure 2B). The AtAPY3 construct was the least able to recover cell wall mannose, reflecting the reduced growth phenotype. The AtAPY4 construct resulted in only a marginal increase in cell wall mannose compared to AtAPY3, but was very capable of complementing the growth phenotype (Figure 3A). The ability to recover mannose in cell wall extracts of the Δ*ynd1*Δ*gda1* dKO yeast likely reflects the activity of each apyrase with respect to the substrate GDP, (derived from lumenal GDP-mannose). Cell extracts were not analyzed from cells harboring the AtAPY7 construct as it exhibited no complementation of the growth phenotype.

To ensure the Arabidopsis apyrases were being adequately expressed in the Δ*ynd1*Δ*gda1* dKO background, we analyzed protein extracts by immunoblotting. Evidence for the expression of constructs containing AtAPY1 to 5 in the Δ*ynd1*Δ*gda1* dKO background was apparent (Figure S4). A faint band was detected for AtAPY6 when 25 µg microsomal protein was analyzed by immunoblotting and indicated some full-length product, however no evidence for the AtAPY7 protein could be obtained. Previously we had observed processing of the AtAPY1 construct when expressed of in the Δ*gda1* background [18]. In this instance it is possible that the C-terminal is processed from AtAPY6 and AtAPY7. As a consequence we undertook RT-PCR analysis to verify the presence of all apyrase transcripts in the Δ*ynd1*Δ*gda1* dKO. Evidence for the presence of all transcripts was apparent for all constructs (Figure S3).

The Arabidopsis AtAPY1 to 6 exhibit apyrase-like activities

Microsomal preparations from the seven Arabidopsis apyrase members expressed in the Δ*ynd1*Δ*gda1* dKO strain were used to measure latent NTPDase activity via inorganic phosphate release using malachite green [18]. The GDA-like clade I members AtAPY1 and AtAPY2 exhibited a clear preference towards the nucleotide substrates UDP (0.7 µmol Pi h^{-1} μg^{-1}) and UDP/GDP (1 to 3 µmol Pi h^{-1} μg^{-1}) respectively (Figure 4), supporting previous reports indicating they both function as UDP/GDPases [18, 19]. The clade II member AtAPY3 has a strong preference toward NTPs (8 to 12 µmol Pi h^{-1} μg^{-1}) but also has significant activities toward ADP and GDP with 4 to 6 µmol Pi h^{-1} μg^{-1} (Figure 4). In contrast, other members of the clade II apyrase family displayed an array of substrate preferences. No significant NTPase or NDPase activity could be detected for AtAPY4 except a slight affinity for CTP, while AtAPY5 demonstrated the highest level of NDP activity measured in our assay, ranging from 10 to 18 µmol Pi h^{-1} μg^{-1} (Figure 4). AtAPY6 appears to have a broad range of substrate activities toward all NTP and NDP substrates analyzed, with values from 0.5 to 2.5 µmol Pi h^{-1} μg^{-1} (Figure 4). Finally, the single clade III representative AtAPY7 displayed no detectable NTPase or NDPase activity under our experimental conditions (data not shown), although the presence of the protein could not be confirmed. In summary, the AtAPY1 to 6 Arabidopsis enzymes all exhibit classic apyrase-like NTPase and/or NDPases activities, with an absence of nucleotide monophosphate activity.

Subcellular localization of the Arabidopsis apyrase family

Previously, two members of the Arabidopsis apyrase family (AtAPY1 and AtAPY2) implicated as ecto-apyrases were shown to localize to Golgi membranes [17-19]. In an effort to resolve the

subcellular distribution of Arabidopsis apyrases and subsequently their potential roles within the cell, we sought transiently co-localize all seven members using N- and C-terminal YFP fusions. As previously observed, both AtAPY1 and AtAPY2 localize to the *cis*-Golgi when either the N- or C-terminal YFP construct was used (Figure 5). Similarly *cis*-Golgi localization results were identified for AtAPY4, AtAPY5 and AtAPY7 using either the N- or C-terminal YFP constructs (Figure 5). These data indicate that AtAPY1, 2, 4, 5, 7 are likely *cis*-Golgi resident proteins. Neither the AtAPY3 nor the AtAPY6 constructs significantly overlapped with the *cis*-Golgi marker (Figure 5). The AtAPY3 C-terminal YFP construct resulted in an internal punctate signal with minimal *cis*-Golgi marker overlap. Further analysis with a *trans*-Golgi marker (CFP-VTI12) and an endosomal marker (CFP-RabF2a) indicted that AtAPY3 likely localizes to the endosome (Figure 6). Since the C-terminal AtAPY6 construct produced a diffuse web-like structure, we co-localized this construct using an ER marker (Figure 6). This resulted in a significant signal overlap indicating co-localization with the ER marker. Identical results were obtained by transient localizing in Arabidopsis rosette leaves by particle bombardments (Figure S5). The outcome of these localization experiments is summarized in Table 1.

Discussion

The apyrase family of Arabidopsis would appear to be representative of plant species with members present in each phylogenetic clade [14]. Based on their subcellular distributions, the seven members of the Arabidopsis apyrase family are endo-apyrases. Their subcellular distributions are remarkably similar to the human endo-apyrase members with the majority localized to the Golgi apparatus, a single ER localized candidate and a single member localized to an intracellular vesicle (Figure 7). Our biochemical analysis indicated a wide range of substrate preference for most members of the family, providing evidence for functional diversity.

The yeast and human apyrase families

The recreation of the Δ*ynd1*Δ*gda1* dKO strain enabled an investigation of the cell wall of yeast lacking any substantive lumenal apyrase activity. The results indicated that only the mannose content derived from mannoproteins was being substantially affected, with some compensation by D-glucan and chitin occurring. Similar observations have been made with *gda1* single mutant in *Candida albicans* where chitin levels were found to increase in their cell walls [45]. The reduced mannose content of the wall also appeared to affect the total amount of cell wall material, supporting an integrated process for the biosynthesis and construction of the yeast cell wall [41]. The elimination of apyrase activity in yeast did not completely prevent the production of cell wall derived mannose, indicating that the transport of GDP-Man was still able to occur, likely at a reduced rate, without the counter substrate GMP. This is in contrast to deletion of *VRG4*, the Golgi resident GDP-Man transporter from yeast, which is lethal [46], as is the *VIG9* null mutant, the GDP-mannose pyrophosphorylase essential for the biosynthesis of GDP-Man [47]. Thus, it is possible that lumenal GDP (or some other molecule) is able to be utilized as a counter substrate to enable the delivery of some GDP-Man into the Golgi lumen.

The regeneration of the Δ*ynd1*Δ*gda1* dKO strain enabled us to examine the function of human apyrase family members. We selected the well-characterized ecto-apyrase NTPDase1 to assess the complementation of the mutant yeast strain with a secreted apyrase. Although complementation of the yeast dKO mutant strain with human apyrases was not as strong as our results with Arabidopsis apyrases, there was clear complementation by Golgi localized human apyrase NTPDase6. Minimal complementation was observed for the vesicle localized human apyrase NTPDase7 or the ecto-apyrase NTPDase1 (Figure 3). These results indicate that subcellular localization and biochemical function are important components of endo-apyrase yeast complementation assays.

Clade I Arabidopsis apyrases: AtAPY1 and AtAPY2

The Arabidopsis apyrases AtAPY1 and AtAPY2 are related to yeast GDA1-like (Clade I) and are the most extensively characterized plant apyrases. While they have been implicated to function at the plasma membrane as ATPases and ADPases regulating ecto-ATP/ADP concentrations [16, 49] recent evidence provides a distinct functional role in Arabidopsis for both AtAPY1 and AtAPY2, namely as endo-apyrases residing in the Golgi lumen with UDPase and GDPase activities [18, 19]. We have now demonstrated that both enzymes exhibit a clear substrate preference for UDP, as would be expected for apyrases responsible for the turnover of UDP after glycosylation reactions within the Golgi lumen.

Several plant apyrases associated with the GDA1-like clade have been implicated as ecto-apyrases through their association with NTPase activity and apoplastic localizations [38, 39]. However, since the human ecto-apyrase NTPDase1 appears to require glycosylation for NTDPase activity [48] progression through the secretory pathway to provide glycan maturity and NTDPase function may be required. Whether this is also a feature of plant apyrases is currently unknown, but the fact that AtAPY1 and AtrAPY2 are both able to complement endo-apyrase activity in yeast, localize to the *cis*-Golgi and possess NDPase activities *in vitro*, it would indicate a central role for these enzymes as Arabidopsis endo-apyrases involved in the conversion of NDPs to NMPs as an important component of endomembrane glycosylation as previously discussed [18, 19].

Clade II Arabidopsis apyrases: AtAPY3, AtAPY4 and AtAPY5

The Arabidopsis clade II apyrase members have a diverse topology, with AtAPY3 to 5 exhibiting a single putative N-terminal transmembrane domain, while AtAPY6 appears to possess both an N- and C- terminal transmembrane domain. The mixed topology for this clade is not unique to Arabidopsis, with examples in both *Glycine max* and *Vitis vinifera* to name a few [14]. AtAPY3, AtAPY4 and AtAPY5 occur as recurrent tandem duplications and share 68% identity, all three are expressed during Arabidopsis development with AtAPY3 predominately in the roots and both AtAPY4/AtAPY5 in the vegetative rosette [50]. Based on this information, it may be possible to speculate that these enzymes undertake similar functions at different developmental stages. However, the biochemical and localization analyses would support a more varied functional role in Arabidopsis.

The *AtAPY3*-YFP construct localized to small punctate structures with minimal overlap to the *cis*-Golgi or *trans*-Golgi markers, however there was considerable overlap with the late endosomal marker, RabF2a [51, 52]. This non-Golgi localization was recently confirmed in *Nicotiana benthamiana* [53]. The enzyme exhibited a clear substrate preference for NTPs and was unable to successfully complement the yeast Δ*gda1*Δ*ynd1* dKO strain, which could have been due to poor expression or high protein turnover (Figure S4). Among the reported intracellular human NTPDases (NTPDase4, 5, 6 and 7), only NTPDase7 displays a strong preference for NTPs and is also reported to localize to internal vesicles [44]. However, no specific functional role for NTPDase 7 has been reported [1]. Yeast complementation involving NTPDase7 also resulted in poor growth of the Δ*gda1*Δ*ynd1* dKO yeast strain. While similar results were observed for AtAPY3, it is possible that subcellular localization played an important role in these experiments. Given its NTP preference and subcellular localization, a possible role in intra-cellular signaling through an involvement with the GTP-binding/GTPase regulatory networks [54] or NTP secretion [55] would be conceivable.

In contrast, both AtAPY4-cYFP and nYFP-AtAPY5 localized to the *cis*-Golgi and their coding regions were able to complement the yeast Δ*gda1*Δ*ynd1* strain. AtAPY5 exhibited the highest specific activities for NDPs of all the Arabidopsis apyrases, which resulted in the high mannose yield from cell wall extracts of complemented yeast strain. The biochemical analysis of AtAPY4 resulted in the lowest NDPase activates measured, exhibiting a substrate preference for CTP. However, even with this reduced NDPase activity, its localization to the Golgi lumen likely assisted in the positive complementation phenotype in yeast Δ*gda1*Δ*ynd1* cells. Overall, these results suggest *in planta* endo-apyrase roles for these enzymes with functional roles related to

their NTPDase activities which could constitute lumenal NDPase activity during specific aspects of vegetative growth. Whether AtAPY4 also functions as a lumenal NTPase requires further investigations.

The clade II Arabidopsis apyrase: AtAPY6

Similar to AtAPY6, all four human ecto-apyrases are reported to contain both N- and C-terminal transmembrane domains. Based on these structural characteristics, we initially considered AtAPY6 a potential ecto-apyrase. However, *AtAPY6*-YFP constructs were localized to the ER, biochemical assays indicate broad NDP/NTP substrate preferences and its heterologous expression in yeast complemented the Δ*gda1*Δ*ynd1* strain, resulting in a high amount of mannose recovered from cell wall extracts. In contrast, the human ecto-apyrase NTPDase1 was unable to successfully complement the yeast mutant strain when compared to results from the human GDA1-like apyrase, NTPDase6.

Similar to other members of clade II, AtAPY6 exhibits a defined expression pattern during Arabidopsis development, namely mature pollen [50]. A recent analysis of AtAPY6 confirmed its high expression in mature pollen and an analysis of *atapy6* mutants indicated a minor role in pollen development associated with abnormal exine patterning [21]. The localization of AtAPY6 to the ER and its broad substrate specify is unique amongst the Arabidopsis apyrase family. The only other ER localized apyrase is the human NTPDase5, which is thought to remove the inhibiting effects of UDP and support the efficient re-glucosylation of proteins enabling correct folding of glycoproteins [56]. These observations in combination with the biochemical and molecular data would support an endo-apyrase role for AtAPY6. Finally with its ER localization, it may have a role in supporting glucosylation of nascent *N*-glycans [57] through the turnover of UDP; inhibition of which is most evident in maturing pollen [21].

Clade III Arabidopsis apyrase: AtAPY7

The final member of the seven apyrase-like proteins encoded by Arabidopsis and the only member of clade III is AtAPY7. While localization of the *AtAPY7*-YFP construct seems to indicate *cis*-Golgi localization, the construct was unable to complement the Δ*gda1*Δ*ynd1* yeast strain. Furthermore, biochemical analysis of microsomal fractions showed no NTPDase activity. Although it is possible that the heterologous expression in yeast was unsuccessful, we were able to detect *AtAPY7* transcripts in the transformed yeast cells. The AtAPY7 protein sequence contains the well characterized five apyrase conserve regions (ACRs) indicating it is a member of the apyrase family [21].

A recent molecular analysis of AtAPY7 determined that it was ubiquitously expressed in a range of Arabidopsis tissues and developmental stages. An analysis of *atapy7* mutants also indicated minor aberrations to the pollen exine as observed in *atapy6* mutants. Interestingly, dKO mutants lacking both *AtAPY6* and *AtAPY7* produced relatively normal plants but with low male fertility from collapsed pollen which further resulted in reduced seed set [21]. Given the likelihood that AtAPY7 does not appear to function as a typical apyrase, it is difficult to explain the synergistic effects observed in *atapy6atapy7* lines. The expression pattern for *AtAPY7* would not indicate a specific role in pollen development, indicating its function could be associated with an important lumenal process which is in demand during pollen maturation. A more detailed analysis of AtAPY7 function needs to be undertaken to determine its role in pollen development.

554
555 Overall our results would indicate that at least for the reference plant Arabidopsis, all members
556 of the apyrase family are localized internally within the endomembrane system. Although there
557 is evidence that individual apyrase members from other plant species can be secreted, we saw no
558 evidence for this with Arabidopsis apyrase family members. Indeed, biochemical evidence and
559 yeast complementation experiments would suggest an overall preference for NDPs over NTPs.
560 These findings along with a number of recent studies [18, 19, 58] do not necessarily preclude the
561 existence of an ecto-apyrase in Arabidopsis, however they do indicate that further investigations
562 are required. Interestingly, many of the characterized plant ecto-apyrases are encoded by legume
563 species and appear to have roles in host-pathogen interactions such as nodulation [59]. Since the
564 extracellular space is where symbiotic interactions initially occur, it is possible that apyrases
565 from legume species have evolved to undertake ecto-apyrase functions associated with these
566 interactions. Given Arabidopsis is incapable of forming such nitrogen fixing associations, it is
567 conceivable that this function never evolved in certain plant lineages. However, further is
568 necessary to determine whether this is the case.
569

Author Contribution

Tsan-Yu Chiu, Dominique Loqué and Joshua L. Heazlewood designed the experiment. Tsan-Yu Chiu, Jeemeng Lao and Bianca Manalansan performed the experiments. Tsan-Yu Chiu, Joshua L. Heazlewood, Dominique Loqué and Stanley J. Roux analyzed the data. Tsan-Yu Chiu, and Joshua L. Heazlewood wrote the manuscript.

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Figure Legends

Figure 1. The apyrase family of *Arabidopsis thaliana*.
(A) Schematic protein structure of the seven Arabidopsis apyrase proteins outlining the apyrase conserved domain GDA1_CD39 and predicted transmembrane helices (TMD). The clade designations are based on plant sequences.
(B) Phylogenetic tree of Arabidopsis apyrase family with yeast, human and previously reported plant apyrases, including pea (PsAPY1 and PsAPY2), potato (StAPY3), soybean (GS52 and GS50) and *Dolichos biflorus* (DbLNP). The phylogenetic tree was created using MEGA6 using MUSCLE (1000 replicas). The percentage of replicate trees is shown on the branches. The bar indicates branch length.

Figure 2. Generation and cell wall analysis of Δ*gda1*Δ*ynd1* yeast double knockout strain.
(A) RT-PCR analysis of wild-type (BY4741), Δ*ynd1* and Δ*gda1*Δ*ynd1* yeast strains outlining the creation of the double knockout strain by replacing the *GDA1* locus with *URA3* by homologous recombination. Characterization of cell wall material (TFA hydrolyzed) by anion exchange chromatography from overnight cultures of wild-type (BY4741) and Δ*gda1*Δ*ynd1* yeast strains **(B)** expressed and mole (%) **(C)** expressed as g/g fresh weight (FW) (n=3 ± SE).

Figure 3. Complementation of the Δ*gda1*Δ*ynd1* yeast double knockout strain.
(A) Assessment of functional complementation of Δ*gda1*Δ*ynd1* by growth complementation on YNB-Leu-Ura media using a serial dilution. The pDR-Leu is an empty vector control.
(B) Characterization of cell wall material in the Δ*gda1*Δ*ynd1* double knockout (dKO) line complemented by expressing Arabidopsis apyrases AtAPY1 to 6. Cell wall material from overnight cultures was hydrolyzed by TFA and analyzed by anion exchange chromatography. The (*) indicates a significant difference in the mannose content (p<0.01) between the complemented lines and the Δ*gda1*Δ*ynd1* double knockout line (n=3 ± SE).

Figure 4. Specific activity of the Arabidopsis apyrase enzymes AtAPY1 to 6.
Latent NTPDase activity was assessed using isolated microsomes from overnight cultures of Δ*gda1*Δ*ynd1* double knockout strain expressing the Arabidopsis apyrase genes. The NTP substrates are displayed as filled bars, while the NDP substrates are shown as empty bars. Activity is expressed in µmole Pi/hour/µg total protein (n=3 ± SE).

Figure 5. Subcellular localization of the Arabidopsis apyrase family.
Localization of transiently expressed Arabidopsis apyrase proteins using an N- or C-terminal Yellow Fluorescent Protein (YFP) using particle bombardment in onion epidermal cells. For each construct (three panels), the first contains the protein of interest (apyrase), the second contains the organelle marker and the third shows the overlay image. Subcellular localization of apyrase proteins was undertaken using either an N-terminal YFP (nYFP) or a C-terminal YFP (cYFP). The *cis*-Golgi marker was α-mannosidase I fused to Cyan Fluorescent Protein (CFP). Scale = 10µm.

Figure 6. Subcellular localization of AtAPY3 and AtAPY6.
The punctate structures identified using the *AtAPY3*-CFP construct was assessed using a *trans*-Golgi marker (CFP-VTI12) and an endosomal marker (CFP-RabF2a). Overlap in signal was

observed with the endosomal marker (arrows). The ER localization of AtAPY6 was confirmed using the ER localization marker, WAK2-CFP-HDEL in combination with the *AtAPY6*-cYFP construct. Scale = 10μm.

Figure 7. Schematic diagram summarizing the subcellular localization, putative topology and major specific activity of the Arabidopsis apyrase family.

824 **Table 1. Summary of subcellular localizations for the Arabidopsis apyrase family.**

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AGI	Name	Onion (C-YFP)	Onion (N-YFP)	Arabidopsis (C-YFP)	SUBA[1] (MS)	SUBA[2] (FP)	Inferred Location
AT3G04080.1	AtAPY1	*cis*-Golgi	*cis*-Golgi	*cis*-Golgi	Golgi	Golgi	*cis*-Golgi
AT5G18280.1	AtAPY2	*cis*-Golgi	*cis*-Golgi	*cis*-Golgi	Golgi	Golgi	*cis*-Golgi
AT1G14240.1	AtAPY3	endosome	-	endosome	-	-	endosome
AT1G14230.1	AtAPY4	*cis*-Golgi	-	*cis*-Golgi	-	-	*cis*-Golgi
AT1G14250.1	AtAPY5	-	*cis*-Golgi	*cis*-Golgi	-	-	*cis*-Golgi
AT2G02970.1	AtAPY6	ER	-	ER	-	-	ER
AT4G19180.1	AtAPY7	*cis*-Golgi	*cis*-Golgi	*cis*-Golgi	-	-	*cis*-Golgi

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827 [1]Subcellular location by proteomic analyses as outlined in the SUBcellular Arabidopsis database [60].
828 [2]Subcellular location by fluorescently tagged protein as outlined in the SUBcellular Arabidopsis database [60]

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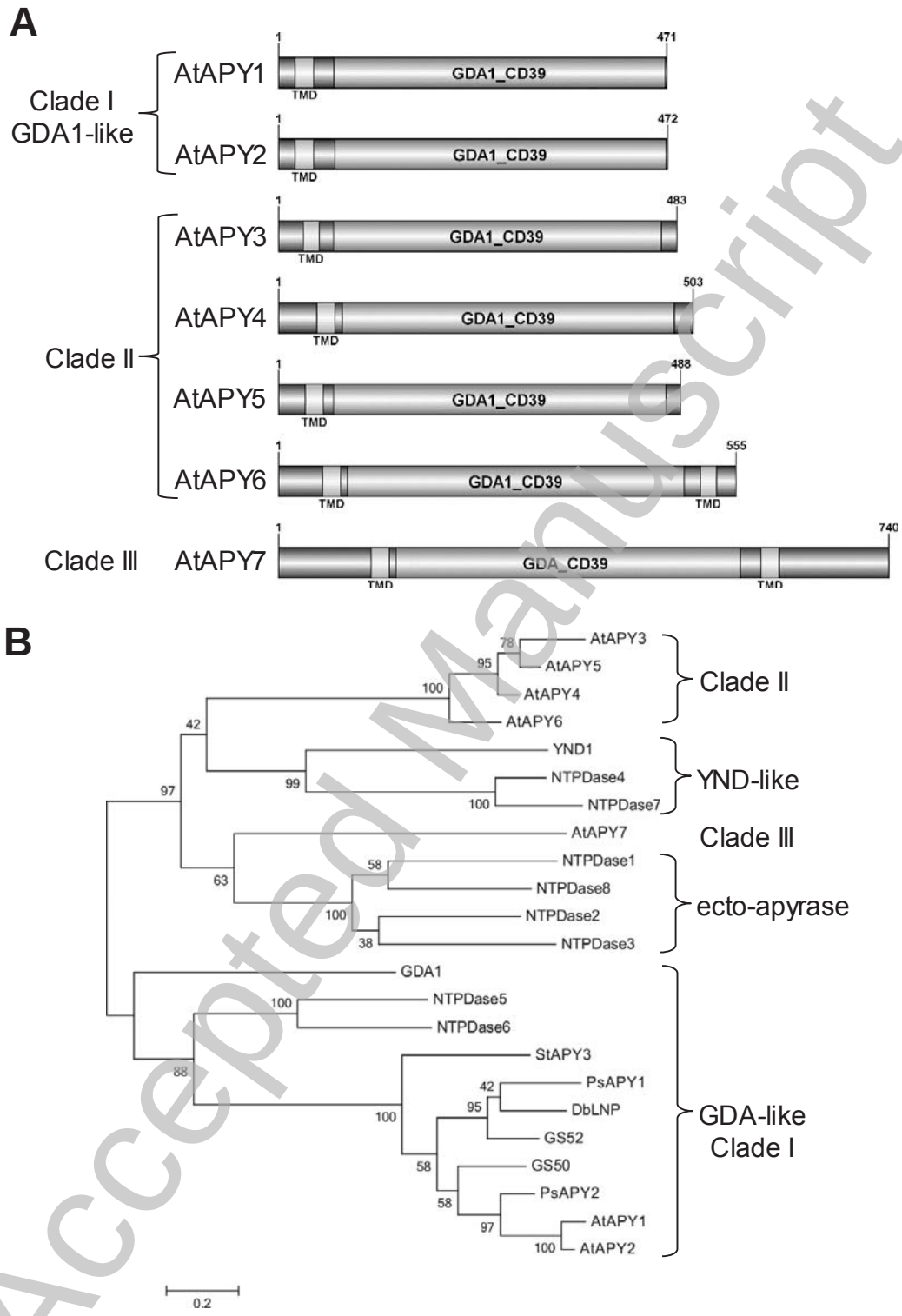
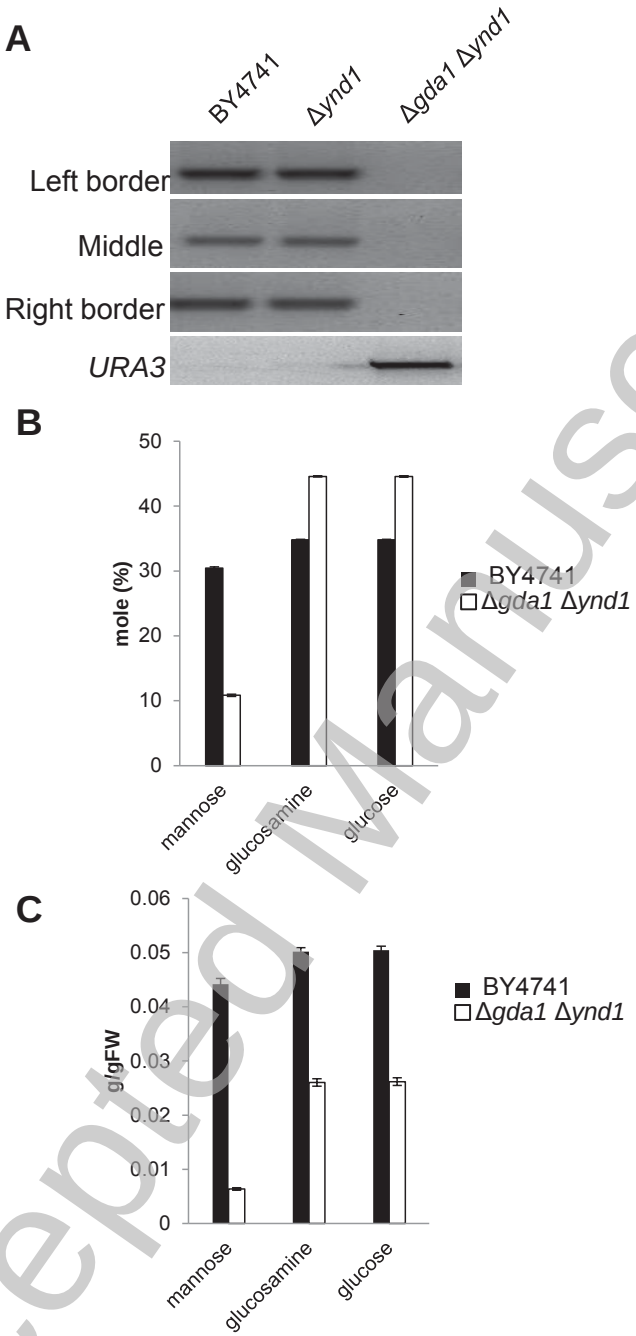


Figure 1.



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834 Figure 2.

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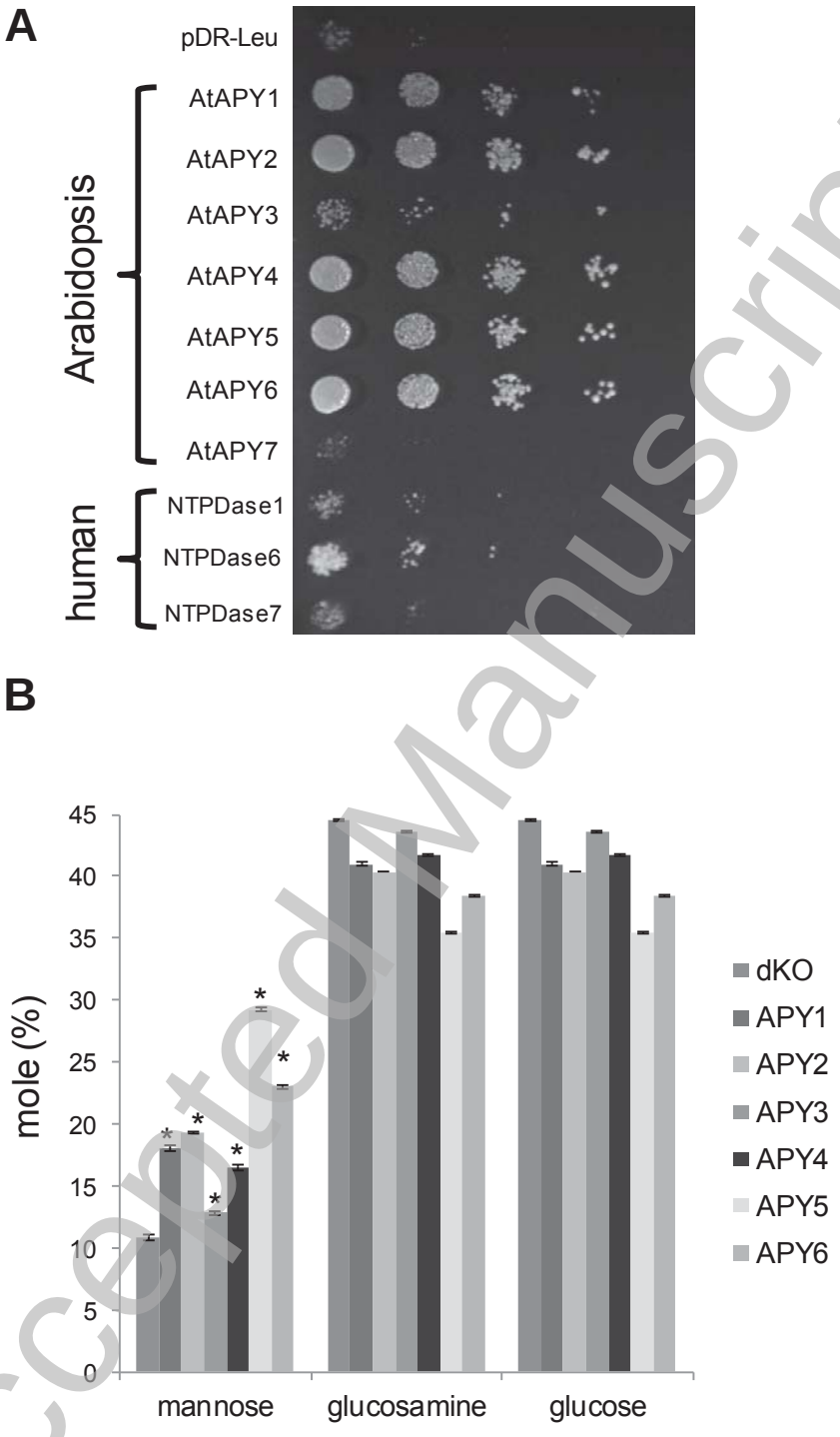

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837 Figure 3.

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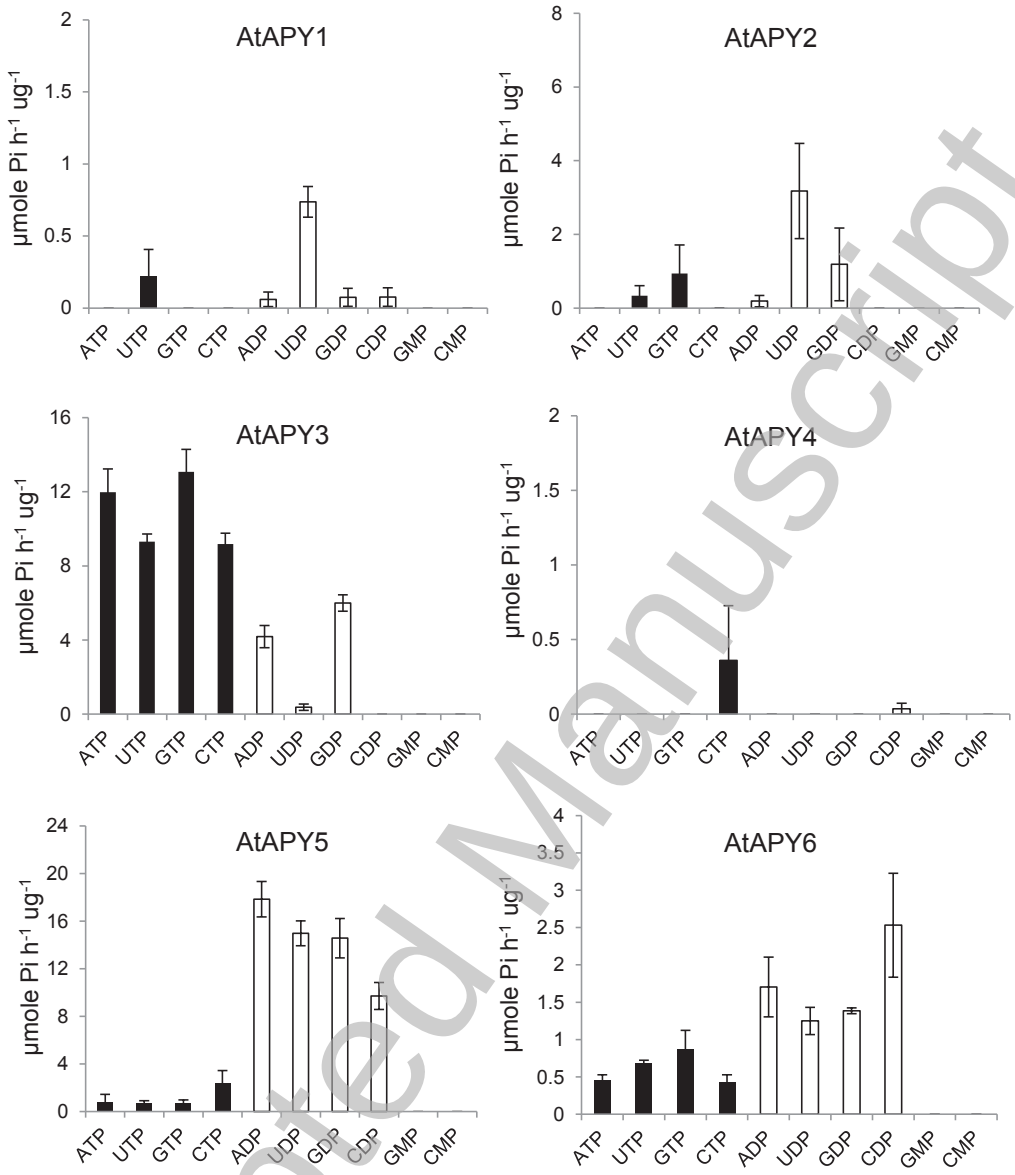

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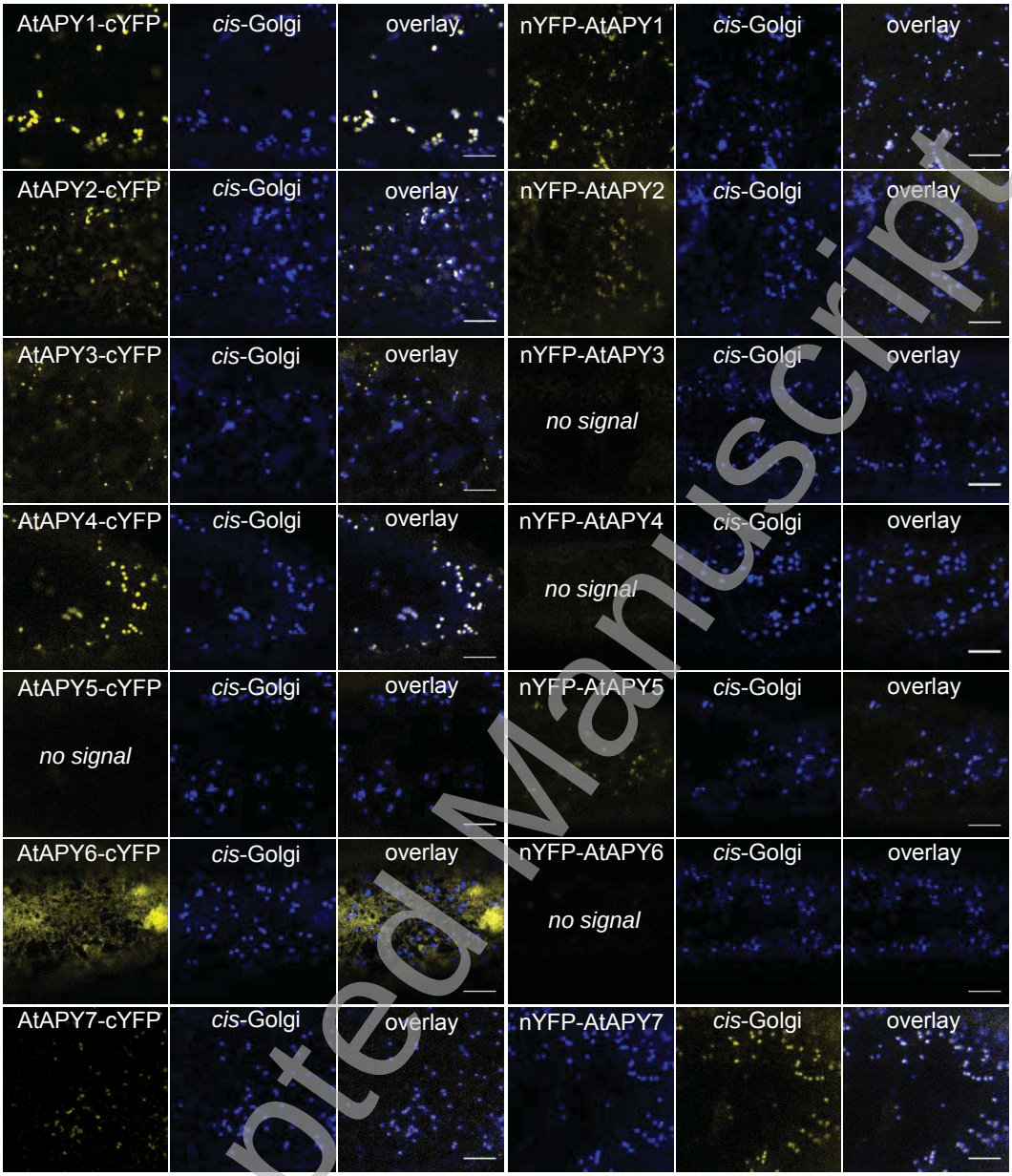

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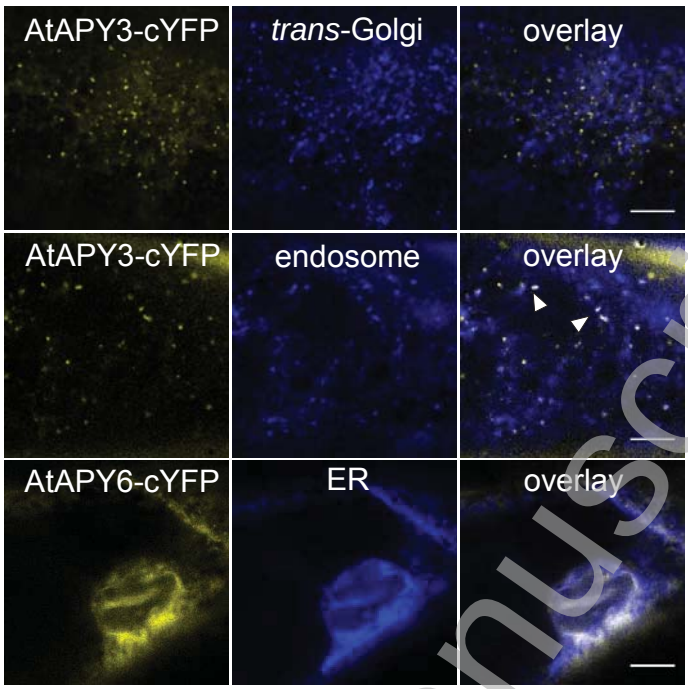
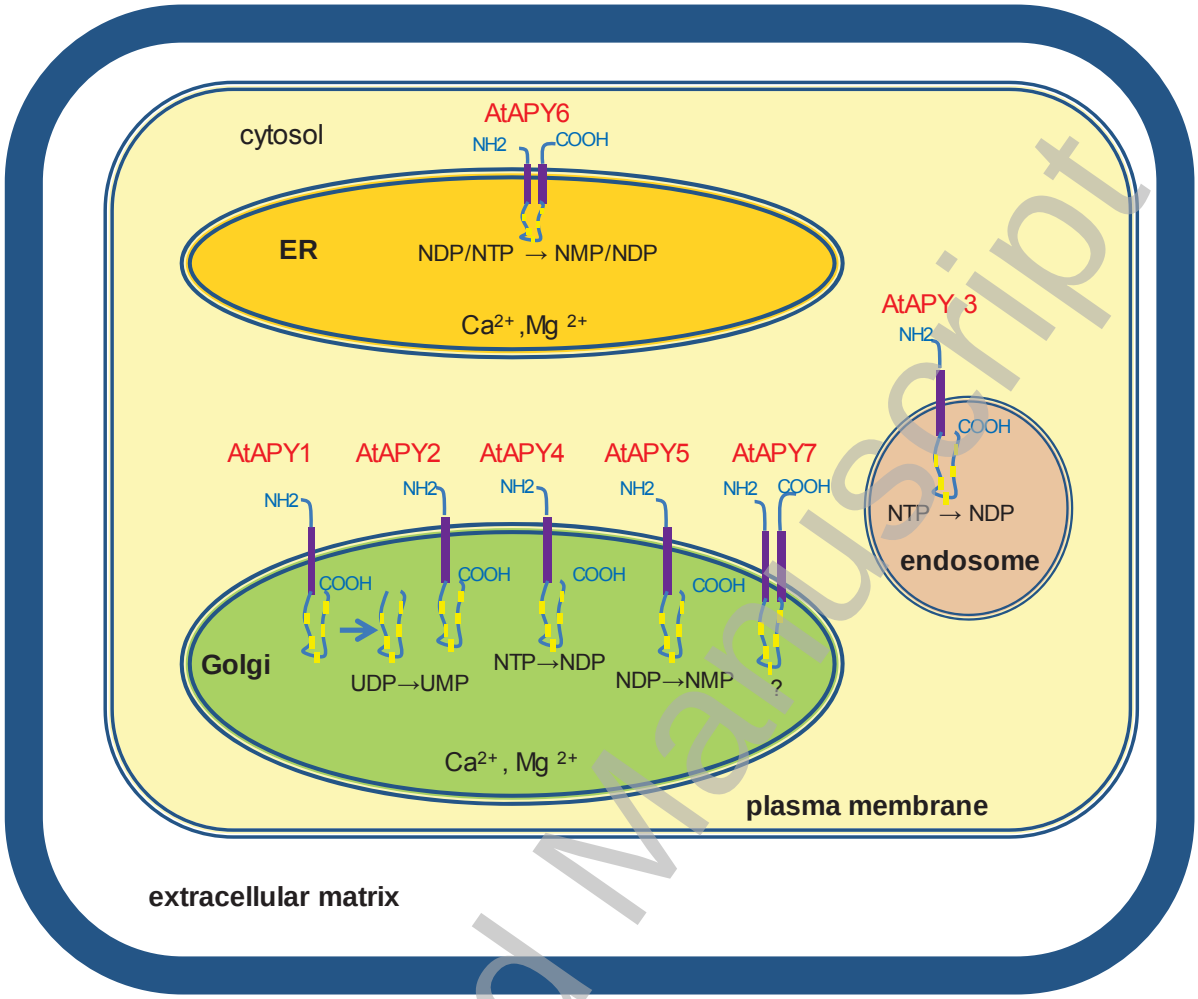


Figure 6.



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857 Figure 7.

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