SHORT COMMUNICATION

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AtPAP2 is a tail-anchored protein in the outer membrane of chloroplasts and mitochondria

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To date, Arabidopsis purple acid phosphatase 2 (AtPAP2) is the only known plant protein that is dual-targeted to chloroplasts and mitochondria by a C-terminal targeting signal. Using in vitro organelle import and green fluorescence protein (GFP) localization assays, we showed that AtPAP2 is located on, but not imported across the outer membrane (OM) of chloroplasts and mitochondria and exposed its N-terminal enzymatic domain to the cytosol. It was also found that a short stretch of 30 amino acids (a.a.) at the C-terminal region (a.a. 615–644) that contains a stretch of 18 hydrophobic residues, a WYAK motif and 8 hydrophilic residues is sufficient for dual-targeting. Mutation of WYAK to WYAE had no effect on dual-targeting ability suggesting that the charge within this flanking region alone is not an important determinant for dual-targeting.

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Tail-anchored (TA) proteins possess an N-terminal functional domain and a single transmembrane domain (TMD) at the C-terminus followed by a hydrophilic tail.[1] Newly synthesized TA proteins are released from free ribosomes with the C-terminal hydrophobic region inserted into various membranes, such as the endoplasmic reticulum, chloroplast outer envelope, mitochondrial outer membrane and the peroxisomal membrane.[2] The functional domain of TA proteins orients to the cytosol and the TMD of TA proteins is inserted into membranes post-translationally. Sorting of proteins by the C-terminal tail (CT) to their specific intracellular destinations is essential for their functions.[3] For instance, overexpression of a C-terminal TMD-truncated AtPAP2 in Arabidopsis abolishes its faster plant growth phenotype.[4]

Over 500 proteins in Arabidopsis have been predicted to have TA structures, of which, 130 have had their subcellular localization experimentally confirmed based on either GFP targeting or mass spectrometry.[2] Most TA proteins were assigned to the ER and secretory membranes, 27 proteins to mitochondria and 32 proteins to plastids.[2,5] These include several isoforms of Tom20 and Tom22 (also known as Tom9) of the mitochondrial outer membrane translocon[6,7] and the GTPase receptors of the outer membrane translocon of plastids, including AtToc33, AtToc34.[5,8]

Arabidopsis purple acid phosphatase 2 (AtPAP2) is the only plant TA protein shown to be dual-targeted to chloroplasts and mitochondria.[4] It was predicted to carry a putative N-terminal signal peptide, a phosphatase domain and a transmembrane domain (TMD) followed by a short hydrophilic C-terminal tail (CT) (a.a. 614–636) by the TMHMM analysis.[4] AtPAP2 was detected in the membrane fraction using immunoblotting.[4] An in vivo targeting assay using chimeric GFP vectors showed that the C-terminal TMD motif of AtPAP2, but not the predicted N-terminal signal peptide, can direct GFP to both plastids and mitochondria in Arabidopsis PSB-D protoplasts.[4] In transgenic Arabidopsis, deletion of the N-terminal signal peptide did not affect the AtPAP2 overpression phenotype such as earlier flowering and enhanced seed yield, whereas deletion of the C-terminal TMD domain abolished the fast-growing phenotype.[4] The goal of this study was to verify the precise localization of AtPAP2 and to examine its dual-targeting capabilities by in vitro import and GFP localization assays.

AtPAP2 is Sensitive to Externally Added Proteases

To verify if AtPAP2 is imported into mitochondria and plastids, in vitro import assays using isolated organelles were performed.[11] The [^{35}S] Met-labeled precursor of AtPAP2 was synthesized using rabbit reticulocyte TNT in vitro transcription/translation lysate (Promega, Melbourne, Australia). The precursor was incubated with isolated Arabidopsis mitochondria under conditions that support import, followed by proteinase K treatment or rupture of the outer membrane following import and subsequent proteinase K treatment (**Fig. 1**).[12] Mitochondria was re-isolated and proteins resolved on sodium dodecyl sulfate-PAGE (SDS-PAGE) and gels were stained, dried, exposed to a BAS TR2040S plate for 24 h and visualized using a BAS 2500 (Fuji, Tokyo). Results showed that the 74 kDa precursor protein of AtPAP2 was located on the outer membrane of

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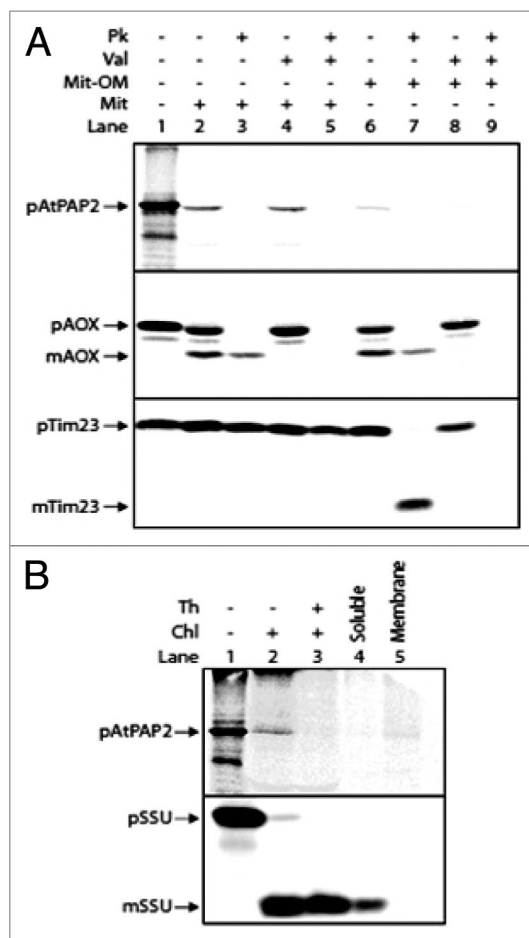

Figure 1. AtPAP2 protein import experiments. (A) In vitro import of radiolabeled AtPAP2 into isolated mitochondria. Lane 1, precursor protein alone. Lane 2, precursor protein incubated with Arabidopsis mitochondria. Lane 3, as in lane 2 except that proteinase K was added after mitochondrial incubation to degrade unimported proteins; Lanes 4 and 5 as in lanes 2 and 3, except that valinomycin was added prior to import to dissipate the membrane potential. Lanes 6–9, as lanes 2–5, except that the mitochondrial outer membrane was ruptured following import and prior to proteinase K digestion. Import control of Tim23 and AOX verified that the import of Tim23 and AOX precursors were inhibited in the presence of valinomycin due to the lack of a membrane potential (lanes 1–5*)* and the inner membrane was intact, as indicated by the proteases-insensitive Tim23 and AOX facing to the ruptured mitochondria outer membrane (lanes 6–9). (B) Import of AtPAP2 into isolated chloroplasts. Lane 1, ^{35}S-labeled AtPAP2 precursor protein; lane 2, AtPAP2 precursor protein incubated with Arabidopsis chloroplasts; lane 3, as lane 2, except that thermolysin was added after import to degrade un-imported proteins. Lanes 4 and 5, soluble and membrane fractions following alkaline extractions of chloroplasts. Abbreviations: Chl, chloroplast; Mit, mitochondria; PK, proteinase K; Th, thermolysin; Val, valinomycin; p, precursor protein band; m, mature protein band. (+), presence; (-), absence.

the mitochondria: AtPAP2 was sensitive to protease digestion before and after rupture of the outer membrane of mitochondria (**Fig. 1A, lanes 3, 5, 7 and 9**) in a valinomycin-independent manner (**Fig. 1A, lanes 4 and 5**). Valinomycin depolarizes the membrane potential across the inner membrane of mitochondria. Precursor proteins such as alternative oxidase (AOX) and component 23 of the inner mitochondrial membrane translocase (TIM23), located in the mitochondrial inner membrane are insensitive to added protease when the outer membrane was ruptured (**Fig. 1A**). The addition of valinomycin blocks the import of proteins into the inner mitochondrial membrane and rupture of the mitochondria OM results in the complete digestion of the precursor proteins (**Fig. 1A**).

In isolated chloroplasts, membrane-integrated AtPAP2 was digested by treatment with thermolysin, while the precursor of the small subunit of Rubisco (SSU) was imported into chloroplasts and processed into the mature thermolysin-insensitive form (**Fig. 1B**). These results clearly indicate that AtPAP2 is located on the outer membranes of mitochondria and chloroplasts and its N-terminal functional phosphatase domain is orientated toward the cytosol consistent with being typical TA proteins. If the large N-terminal portion was located inside the outer membrane of mitochondria and plastids, a large protease protected fragment would be evident upon import following protease treatment of intact organelles. Furthermore, transient expression in rice and tobacco BY-2 protoplasts showed that all the GFP constructs fused with the TMD/CT region of AtPAP2 were co-localized with mitochondrial (F1-RFP) and plastidic (plastid-mCherry) markers and generated "ring-like" signals surrounding these organelles (**Fig. 2**). These indicated that AtPAP2 proteins were localized to the outer membranes of these two organelles but not imported across the outer membrane.

AtPAP2 Dual-localization is Dependent on a C-terminal Targeting Signal

To characterize the targeting sequence at the C-terminus of AtPAP2, a comprehensive series of GFP C-terminal chimeric constructs were generated. The GFP fusion constructs were transiently introduced into onion epidermal cells and GFP localization was examined by fluorescence microscopy (**Fig. 3**). When the full length TMD/CT of AtPAP2 (residues 615–656, P2C1) and TMD/CT lacking the conserved EE doublets in the C-tail (residues 615–653, P2C2) were appended to the C-terminus of GFP, respectively, the fusion proteins were targeted to both plastids and mitochondria, as evidenced by the co-localization with plastid marker (SSU-RFP) and mitochondrial marker (mito-mCherry) (data not shown). The dual targeting characteristics could be maintained even by a shorter sequence (residues 615–644, P2C3). However, the AtPAP2 C-tail alone (residues 634–656, P2C4), without the hydrophobic TMD motif, was unable to target GFP to plastids and mitochondria, and instead this fusion protein diffused in the cytosol. This indicated that the TMD is essential for dual-targeting to mitochondria and plastids.

While 500 proteins in Arabidopsis are predicted to have a TA structure, how they are sorted to the various membranes and maintain their localization specificity remains unknown. Using proteomic methods, 42 and 25 Arabidopsis proteins were identified on the outer membranes of mitochondria[13] and chloroplasts,[14] respectively. Of these, 13 mitochondrial and 5 chloroplast proteins are putative TA proteins (data not shown), of

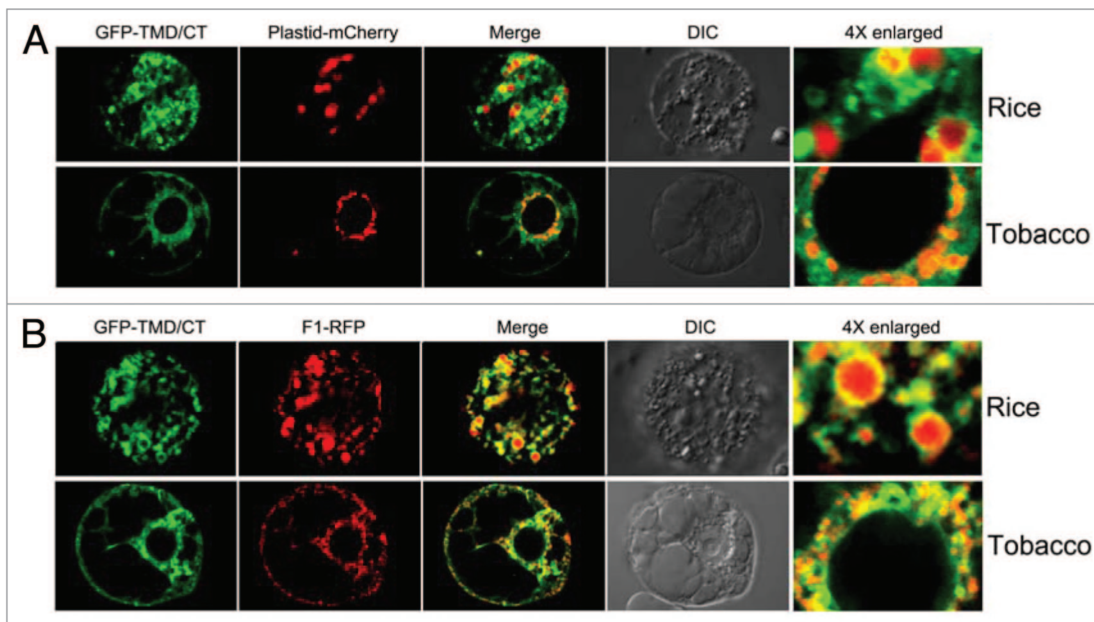

Figure 2. Targeting of the GFP fused with C-terminal tail of AtPAP2 in rice and tobacco BY-2 protoplasts. The GFP fusion construct (P2C1) were co-transfected with the plastidial (plastid-mCherry) marker (A) and mitochondrial (F1-RFP) marker (B). Scale bar, 50 μm.

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which AtToc33,[5] AtToc34 [5] and TOM20 [15] have been well characterized previously (**Table 1**). Different sorting pathways for plastid OM proteins (Toc34, Toc159 and Toc75) have been proposed.[16] Soluble sorting factors, such as the ankryin repeat containing protein AKR2A, which interacts with AtToc33, AtToc34 and OEP9 at their C-termini, assists their transport to the plastid membrane.[5]

To date AtPAP2 is the only TA protein dual-targeted to the outer membranes of both chloroplasts and mitochondria. The hydrophobicity of the TMD[17] and the positively charged amino acids Lys (K) and Arg (R) flanking the TMD sequence were proposed to be determinants for the sorting of TA proteins.[18] Our results showed that the alteration of a positively charged residue (K) to a negatively charged residue (E) adjacent to the first a.a. of TMD sequence did not affect the dual targeting ability. This suggests the TMD motif and the hydrophilic residues at the C-terminus of the TMD motif are the major determinants of the dual-targeting. Helical wheel projections of the TMDs of TA proteins targeted to OM of chloroplasts and mitochondria is showed in **Figure 4**. Generally, the TMDs of TA proteins (AtToc 33/34) targeted to plastid OM contain K, Q and P, which are not favored by hydrophobic transmembrane helix. In contrast, the TMDs of TA proteins targeted to mitochondria OM (AtTom20 and AtTom22) are very hydrophobic. For AtPAP2, the 18 aa of the TMD are extremely hydrophobic and unexpectedly, contain 5 glycine residues. Due to its high conformational flexibility, it is entropically unfavorable to include glycine residues in the relatively constrained α-helical structure. The inclusion of glycine residues may increase the flexibility of the TMD of AtPAP2, and whether this facilitates binding to sorting proteins specific to both chloroplasts and mitochondria OM is a subject for further studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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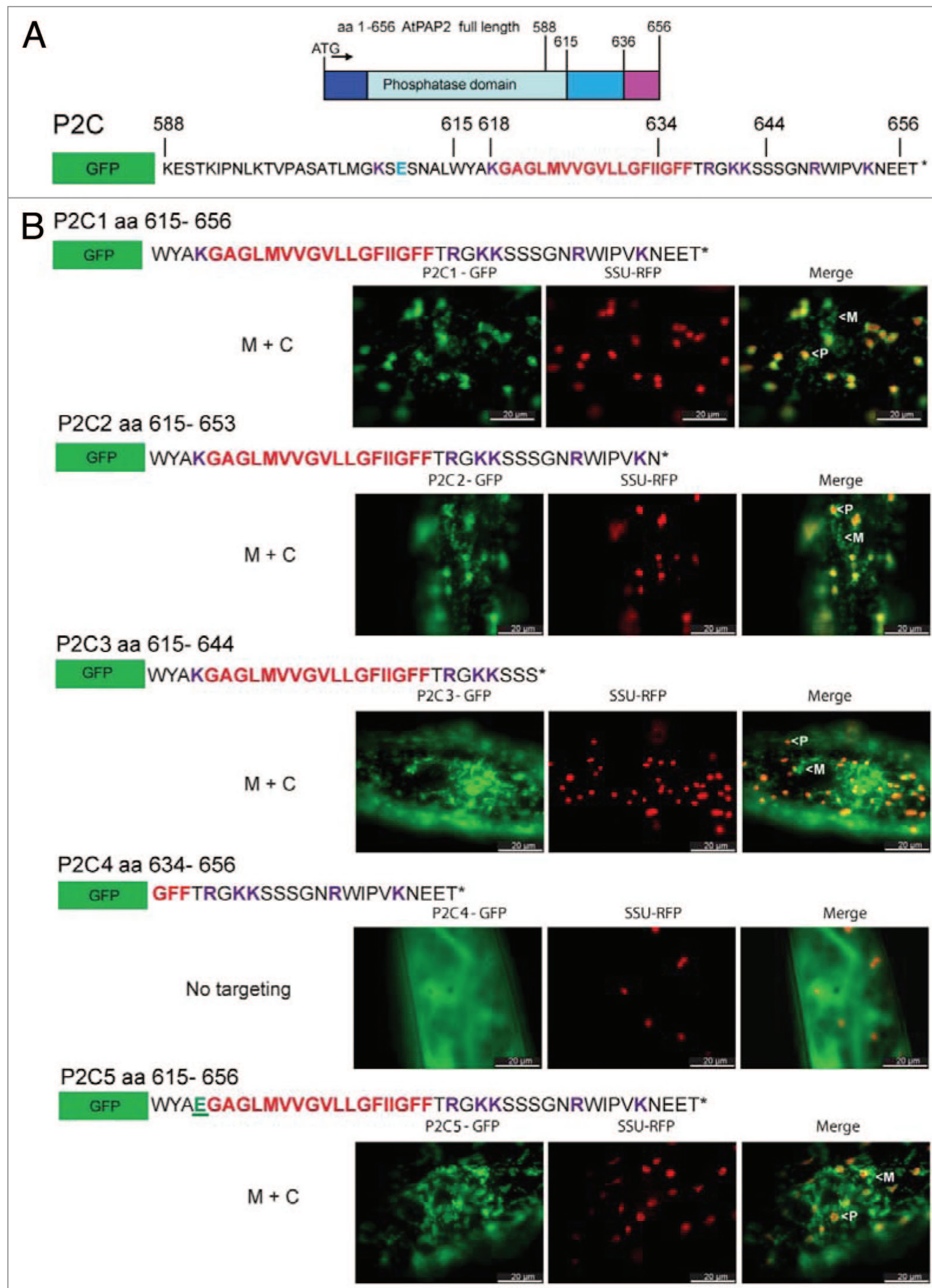

Figure 3. Targeting of GFP fused with various C-terminal extensions of AtPAP2. Hydrophobic motifs are in red; positively and negatively charged residues are in purple and blue, respectively; *denotes stop codon. (A) Full length AtPAP2 has been previously shown to be dual-targeted to the outer-membranes of plastids and mitochondria, by its C-terminal sequence.[4] (B) A series of GFP constructs containing AtPAP2 with a number of modified C-terminal extensions were biolistically transformed into onion epidermal cells, alongside a plastidial RFP marker. Plastids and mitochondria have been identified in the merged micrograph by a (P or M), respectively. The K to E residue substitution in P2C5 has been shown in green.

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Table 1. C-terminal hydrophobic motifs of AtPAP2 and preprotein receptors of TOC and TOM

Name	AGI	Sequence (N-terminus to C-terminus)	Hydrophobicity (TMD) K&D		Positive charges	Negative charges
			H	μH		
Plastids						
AtPAP2	At1g13900	LMG KSE SNA LWY AKG AGL MVV GVL LGF IIG FFT RGK KSS SGN RWI PVK P*	2.39	0.66	7	1
AtToc33	At1g02280	KKM VDG SYS DDK GKK LIP LII GAQ YLI VKM IQG AIR NDI KTS GKP L*	1.51	1.32	8	4
AtToc34	At5g05000	KLV EGP NPN ERG KKL IPL MFA FQY LLV MKP LVR AIK SDV SRE SKP AWE LR*	1.73	0.17	8	4
Mitochondria						
AtTom20–1	At3g27070	KNK KSS DEK YIV MGW VIL AIG VVA CIS FRK LR*	2.37	0.56	7	2
AtTom20–2	At1g27390	KKR NTE FTY DVC GWI ILA CGI VAW VGM AKS LGP PPP AR*	2.17	0.56	5	2
AtTom20–3	At3g27080	NKK SSD AKY DAM GWV ILA IGV VAW ISF AKA NVP VSP PR*	2.13	0.43	5	2
AtTom20–4	At5g40930	QKK TSE FKY DVF GWV ILA SYV VAW ISF ANS QTP VSR Q*	1.97	0.49	4	2
AtTom22-I	At1g04070	KKL LKS TGK AAW IAG TTF LIL AVP LIL ELE QDH RLG EID FEQ ASL LGT PPV GAM L*	2.14	0.07	5	6
AtTom22-V	At5g43970	SKK LLR STG KAA WIA GTT FLI LVV PLI IEM DRE AQI NEI ELQ QAS LLG APP SPM QRG L*	2.31	0.18	6	5

The mean hydrophobicities were calculated by HydroMCalc (http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html#Hiscale) using Kyte and Doolittle (K&D) hydrophobicity scale. H and μH are the total sum of all residue hydrophobicity indices and the vectorial sum of all the hydrophobicity indices divided by the number of residues, respectively. Hydrophobic motifs are underlined; * denotes stop codon.

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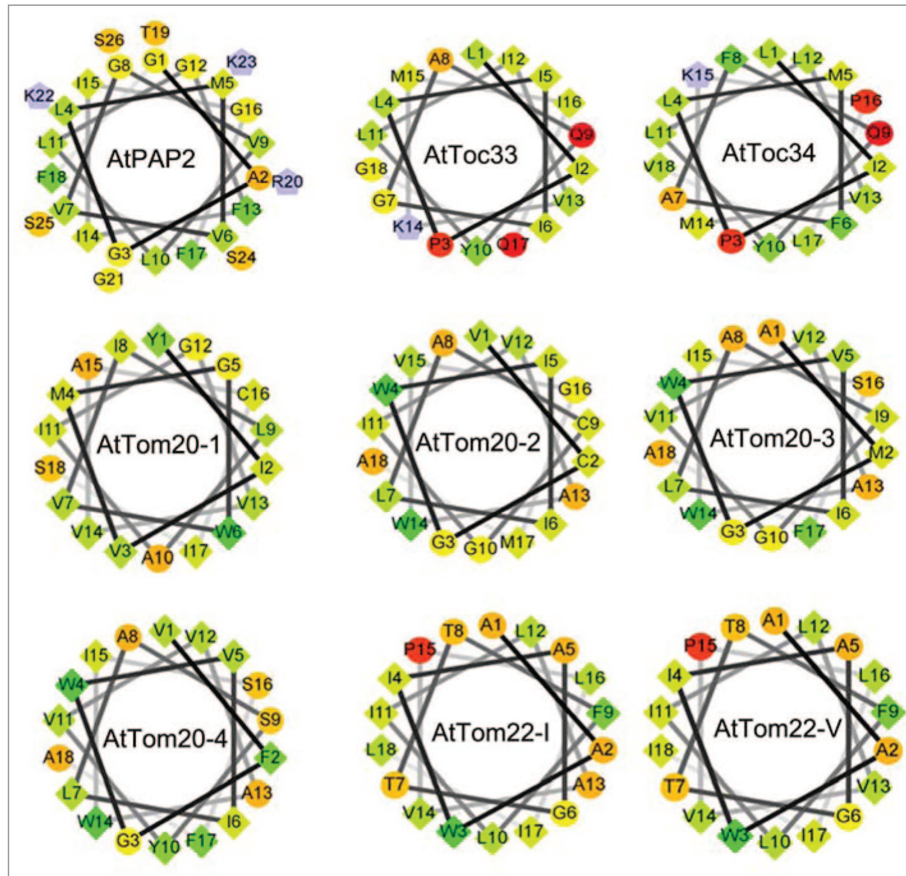

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Figure 4. Helical wheel projections of the predicted C-terminal helices of AtPAP2 and TOC and TOM complex members. The C-terminal hydrophobic sequences were analyzed by the program (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Numbers indicate the order of the sequence of amino acids. Hydrophobicity is highlighted based on Kyte-Doolittle scale[19] (Diamond green very hydrophobic and yellow lowest hydrophobic; circle red very hydrophilic). Residues that are potentially positively charged are presented as pentagons.