Identification of the pokeweed antiviral protein interactome by co-immunoprecipitation-mass spectrometry (coIP-MS)

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INTRODUCTION

- Pokeweed antiviral protein (PAP) is a ribosome-inactivating protein (RIP) expressed in the American pokeweed plant, *Phytolacca americana*.
- RIPs function enzymatically to remove an adenine base from the large ribosomal subunit, halting protein translation (1).
- However, PAP has been shown to localize predominantly to the extracellular space (2), sequestered away from its ribosomal target, where its function remains unknown.
- When expressed transgenically in plants, PAP confers a broad-spectrum antiviral activity (3) and is therefore suspected to play a role in plant defense.

OBJECTIVE

- The purpose of this work is to identify PAP-protein *in vivo* interactions using co-immunoprecipitation coupled with mass spectrometry (coIP-MS).
- Identification of PAP-protein interactions will elucidate PAP function in the cell through guilt by association.

RESULTS

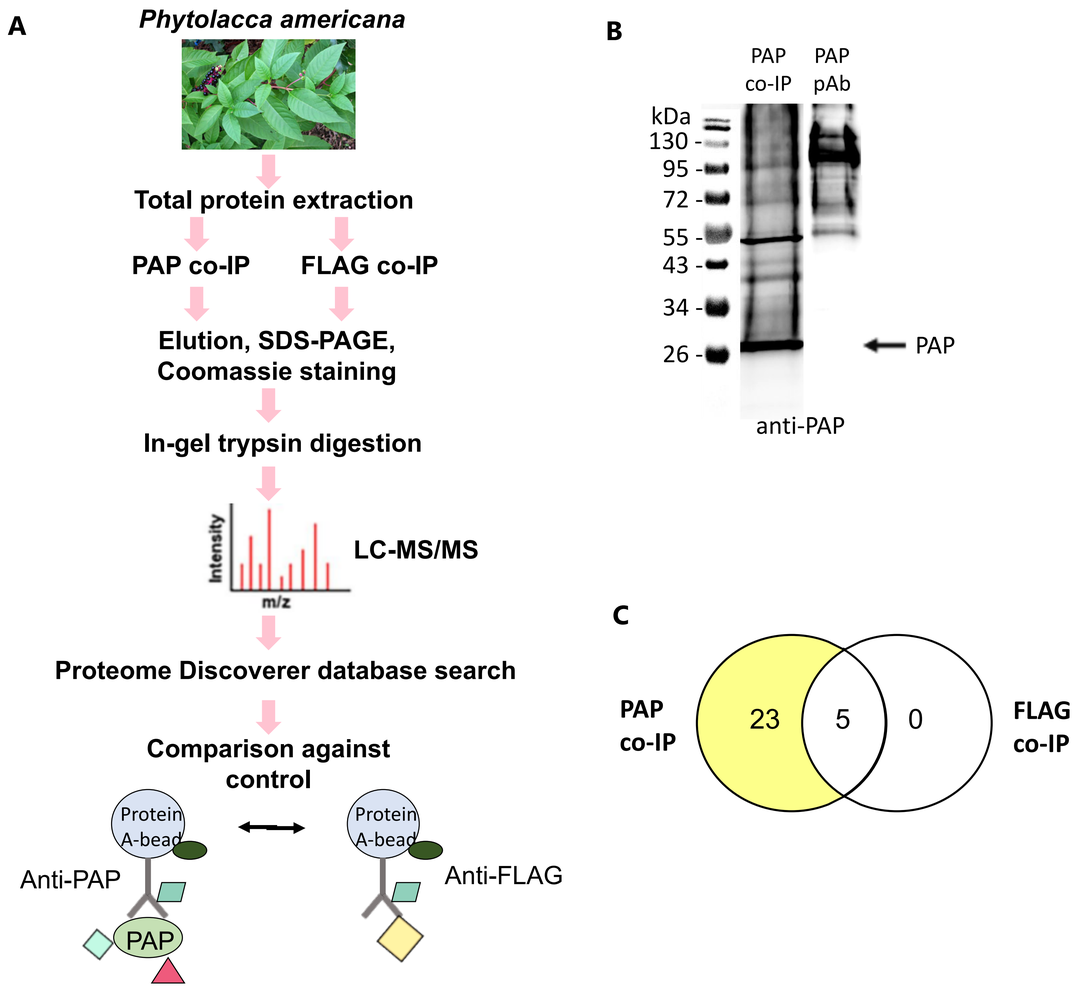

Figure 1. Co-immunoprecipitation-mass spectrometry for mapping of the PAP-protein interactome. (A) CoIP-MS workflow. Total protein extraction was performed on 6-leaf *Phytolacca americana* plants and lysate was incubated with either PAP or FLAG rabbit IgG antibody crosslinked to protein A magnetic beads for co-immunoprecipitation. Antibody-bound protein complexes were eluted in 2x Laemmli buffer and separated via SDS-PAGE, followed by Coomassie staining and in-gel trypsin digestion. Extracted peptides were analyzed by LC-MS/MS and obtained mass spectra were compared to a pokeweed protein database using Proteome discoverer (ThermoFisher). Peptide identification was done by setting peptide hits > 2 and the false discovery rate below 1% based on decoy database. Proteins identified in both control and experimental samples were removed from the list of putative PAP interactors. **(B)** Total protein from pokeweed lysate was co-immunoprecipitated with a PAP antibody crosslinked to protein A-magnetic beads with dimethyl pimelimidate. Samples that were not boiled were analyzed by Western blot and probed with PAP pAb. **(C)** Venn diagram showing number of proteins pulled-down by PAP co-IP compared to the FLAG control IP. Specific PAP interactors are shown shaded in yellow.

Table 1. PAP specific interactors ordered by gene ID, with homologous protein description, subcellular localization and function as obtained from Uniprot database. Proteins chosen for further interaction validation are highlighted in blue.

Pokeweed gene ID	Homologous protein description	Homologous protein species	Subcellular location	Biological function
PHYAM_013847	Ribulose bisphosphate carboxylase small chain 1 (RBCS-1)	*Mesembryanthemum crystallinum*	chloroplast	carbon fixation
PHYAM_003256	30S ribosomal protein S5 (RPS5)	*Spinacia oleracea*	chloroplast	translation
PHYAM_025849	30S ribosomal protein S17 (RPS17)	*Spinacia oleracea*	chloroplast	translation
PHYAM_025507	40S ribosomal protein S14 (RPS14)	*Zea mays*	cytoplasm	translation, small ribosomal subunit assembly
PHYAM_013566	60S ribosomal protein L23 (RPL23A)	*Arabidopsis thaliana*	cytoplasm	translation
PHYAM_009893	Tubulin beta-6 chain (TUBB6)	*Oryza sativa subsp. japonica*	cytoskeleton	microtubule cytoskeleton organization, mitotic cell cycle
PHYAM_026164	Cysteine protease (XCP1)	*Arabidopsis thaliana*	extracellular space, lysosome, vacuole	programmed cell death, cellular development, proteolysis in protein catabolism
PHYAM_007276	Non-specific lipid-transfer protein (IWF1)	*Beta vulgaris*	extracellular	defense response, lipid transport
PHYAM_003326	Elongation factor 1-alpha (REFA1)	*Oryza sativa subsp. japonica*	cytoplasm	translational elongation
PHYAM_010495	ATP synthase subunit beta (atpB)	*Agapanthus africanus*	chloroplast	ATP synthesis
PHYAM_026431	Chlorophyll a-b binding protein CP26 (LHCB5)	*Arabidopsis thaliana*	chloroplast	photosynthesis
PHYAM_025787	Ribulose bisphosphate carboxylase large chain (RBCL)	*Basella alba*	chloroplast	carbon fixation
PHYAM_000542	Peroxisomal (S)-2-hydroxy-acid oxidase	*Spinacia oleracea*	peroxisome	oxidative photosynthetic carbon pathway
PHYAM_006846	Tubulin alpha-3 chain (TUBA3)	*Hordeum vulgare*	cytoskeleton	microtubule-based process
PHYAM_028184	Antiviral protein 2 (PAP2)	*Phytolacca americana*	unknown	viral defense, negative regulation of translation
PHYAM_027772	40S ribosomal protein S26-1 (RPS26A)	*Arabidopsis thaliana*	cytoplasm	translation
PHYAM_002561	Carbonic anhydrase	*Spinacia oleracea*	chloroplast	carbon utilization
PHYAM_026698	ATP synthase gamma chain (ATPC)	*Nicotiana tabacum*	chloroplast	ATP synthesis
PHYAM_012933	Chlorophyll a-b binding protein 36 (CAB36)	*Nicotiana tabacum*	chloroplast	photosynthesis
PHYAM_012451	Antiviral protein alpha	*Phytolacca americana*	extracellular	Antiviral defense, negative regulation of translation
PHYAM_024627	30S ribosomal protein S21 (RPS21)	*Spinacia oleracea*	chloroplast	translation
PHYAM_023348	Polygalacturonase inhibitor (PGIP)	*Pyrus communis*	extracellular	fungus defense
PHYAM_006163	Magnesium-chelatase subunit ChlD (CHLD)	*Nicotiana tabacum*	chloroplast	photosynthesis, chlorophyll biosynthetic process

SUMMARY

- CoIP-MS was used to pull-down PAP and its interactors in pokeweed leaves and 23 PAP-specific interactors were identified by mass spectrometry (FDR < 0.01).
- Interaction of PAP with PHYAM_026164 and PHYAM_007276 implicates PAP in plant defense processes when localized to the extracellular space.
- Future work includes validation of selected PAP interactors by co-expressing PAP and either PHYAM_026164 or PHYAM_007276 in tobacco leaves and performing a reverse co-IP: the protein interactor will be immunoprecipitated and presence of PAP in the co-immunoprecipitated population will be shown by Western blot
- Effect of PAP-protein interactions on plant viral resistance will be tested by overexpression of PAP and protein interactors in tobacco leaves followed by infection with tobacco mosaic virus (TMV); qRT-PCR will be used to quantify viral load
- Previous RIP studies have focused on RIP-nucleic acid interactions; this work will represent the first investigation of a RIP-protein interactome.

1. Brigotti, M., Rambelli, F., Zamboni, M., Montanaro, L., Sperti, S. (1989). *Biochem J*, 257(3): 723–727.
2. Ready, M. P., Brown, D. T., Robertus, J. D. (1986). *Proc. Natl. Acad. Sci. U.S.A,* 83: 5053–5056.
3. Di, R., Tumer, N.E. (2015). *Toxins*, 7(3): 755–772.

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