Investigating the Roles of Protein P0 from *Cucurbit aphid-borne yellows virus* in ATG8 Processing in the Autophagy Degradation Pathway

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Background

Plants exhibit a common immune defense mechanism called RNA silencing that eliminates virus RNAs. During RNA silencing, small double stranded RNAs derived from virus replication intermediates are bound by ARGONAUTE (AGO) proteins and integrated into the RNA-induced silencing complex (RISC), which guides the degradation of viral RNAs. Cucurbit aphid-borne yellows virus (CABYV) is a member of the *Polerovirus* genus of RNA viruses with a broad host range including cucurbit and non-cucurbit species. CABYV is spread by aphids that feed on phloem, and its impact can result in up to 50% reductions in crop yields. The CABYV P0 protein ($P0^{CA}$) is a key virulence protein that allows virus infection to overcome RNA silencing by causing degradation of AGO proteins. $P0^{CA}$ has been found to behave as an F-box protein that targets AGO protein for degradation.

Plants posses two key pathways for protein degradation that rely on ubiquitination to target proteins for destruction via the 26S proteasome or autophagy. P0 proteins appear to target AGO proteins to membranes with the autophagy-related protein ATG8, which is a marker for the autophagy compartments in the cell (Fig 1) and AGO degradation is blocked using chemical inhibitors of the pathway.

Our lab has previously observed that P0 from the Turnip yellows virus (TuYV, $P0^{Tu}$) interferes with the processing of ATG8 by the protease ATG4B, one of the first steps in the autophagy pathway (Fig 1). TuYV is a closely-related species of poleroviruses and we predict that $P0^{CA}$ would show a similar effect on ATG8 processing when co-expressed *in planta*.

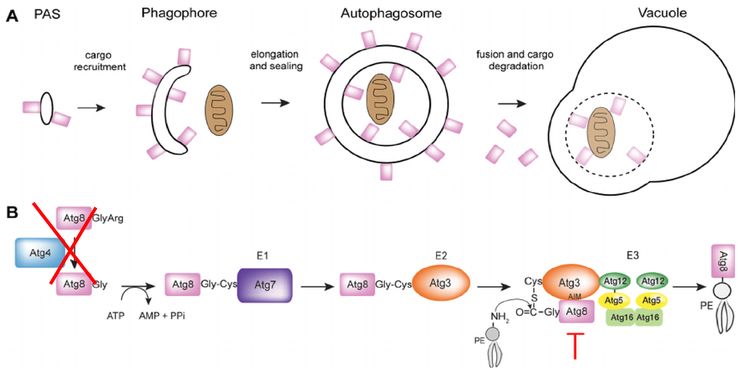

Figure 1: Diagram representing the ATG8 processing by ATG4 cleavage and conjunction to the lipid phosphatidylethanolamine (PE) to mark membranes of the autophagy degradation pathway. P0 protein may target AGO proteins for degradation by interacting with ATG8, thereby also interfering with processing of a subset of the ATG8 protein pool.

Hypothesis

- This project aims to investigate P0 from CaBYV for interference with ATG8 processing by coexpressing wild-type and mutant versions of $P0^{CA}$ with a recombinant fusion protein of isoform ATG8c.
- We hypothesize that the wild-type $P0^{CA}$ and a mutant retaining VSR activity will result in accumulation of unprocessed ATG8c:EGFP:HA recombinant protein, while a mutant $P0^{CA}$ protein defective in VSR activity will lead to full processing for the ATG8c:EGFP:HA protein, as exhibited by detection of only the EGFP:HA fragment.

Methods

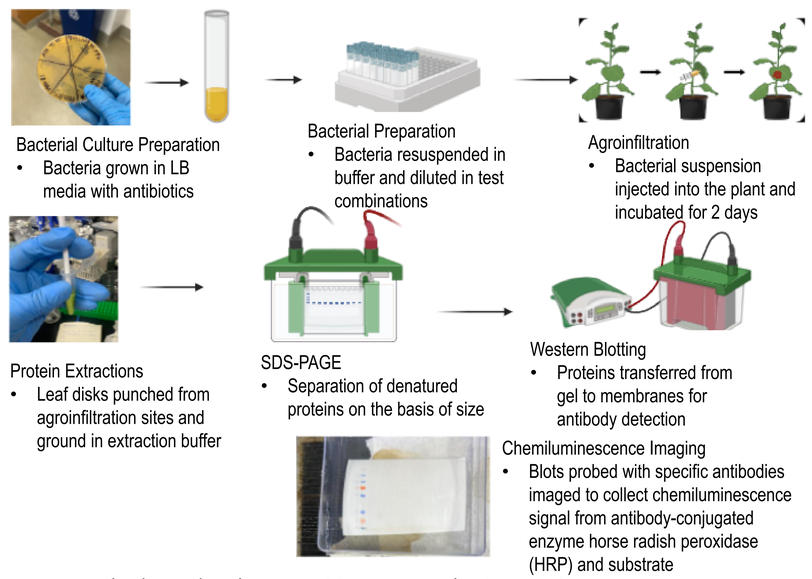

Figure 2: Methods used in this project to grow agrobacteria cultures from previously generated clones stored as glycerol stocks, including: $P0^{CA}$, its mutants, ATG8c, GFP6, and pBIN61 empty vector. Cultures were combined in combinations at OD = 0.1 for agroinfiltration and proteins were extracted 2 days later. After separation by SDS-PAGE and transfer to PVDF membranes, blots were probed with anti-GFP antibodies for one hour, washed, then imaged for chemiluminescence to detect HRP activity on substrate.

Results

$P0^{CA}$ activity was tested for interference with processing of ATG8 using the ~45 kDa recombinant ATG8c:EGFP:HA protein, as previously observed for $P0^{Tu}$. Only the ~25 kDa free EGFP:HA fragment was detected, showing full processing of the ATG8c:EGFP:HA fusion protein.

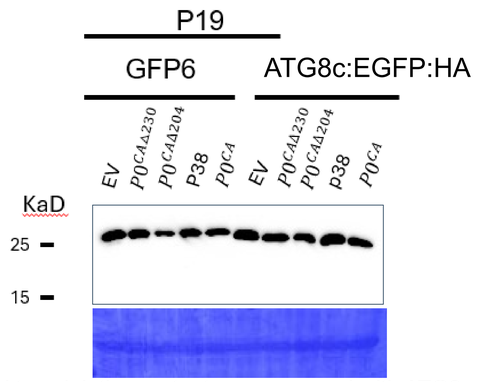

Figure 3: $P0^{CA}$ and two deletion mutants coexpressed with ATG8c:EGFP:HA or a control green fluorescent protein (GFP6) to test for interference of processing of ATG8, as previously observed for $P0^{Tu}$. The VSR protein from Turnip crinkle virus (P38) was examined as a VSR protein that blocks RNA silencing by binding to AGO proteins without targeting them for degradation. pBIN61 empty vector (EV) was included as a negative control. Combinations of constructs lacking VSR activity had the VSR protein P19 added to equalize protein expression (P19 VSR functions by binding to dsRNAs, not by interacting with AGO proteins). Equal loading of proteins was ensured by staining the blot with Coomassie blue after antibody detection to visualize the prominent RuBisCO band.

Conclusion

The P0 proteins from TuYV and CAYBV were previously shown to have conserved amino acid sequences, similar VSR functions, a conserved interaction with the Arabidopsis protein SKP1, and both elicit hypersensitive response in the *Nicotiana glutinosa* accession TW59, with $P0^{Tu}$ eliciting a stronger response.

We hypothesized the $P0^{CA}$ would share an activity we previously observed for $P0^{Tu}$, which causes accumulation of the unprocessed ATG8c:EGFP:HA fusion protein, which suggests a physical interaction between P0 proteins and the ATG protein on autophagic membranes. Our results showed that $P0^{CA}$ did not interfere with ATG8 processing as anti-GFP antibodies detected only the ~25 kDa GFP fragment that is released during processing of the full-length fusion protein, and two deletion constructs with C-terminal residues removed, did not have any change in phenotype versus the wild-type $P0^{CA}$ protein. Our inclusion of an unrelated VSR protein P19 ensured all proteins were expressed at a similarly high level so our results were not related to protein expression levels.

Our observations suggest that the difference we have seen in $P0^{CA}$ versus $P0^{Tu}$ elicitation of HR in *Nicotiana glutinosa* may result from a functional difference that is also reflected in the differences we observed here in the effects of these P0 proteins on processing of the ATG8 protein by ATG4B. The P0 protein is the most rapidly evolving protein of the poleroviruses due to its interaction with the plant immune system and immune pressure likely selecting advantageous mutations. It is likely that interference with ATG8 processing does not increase virus fitness if the it decreases the ability of TuYV to target AGO proteins for degradation and this activity may not be an important and ubiquitous function of all P0 proteins.

Future Work

Future investigation of the $P0^{Tu}$ interaction with ATG8 is necessary using approaches like coimmunoprecipitation. These studies should also address whether ATG8 interacts with the P0 proteins from other polerovirus species like CABYV to understand how the VSR activity of P0 proteins has evolved.

References

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Methods:

Bacterial Culture Preparation

• Bacteria grown in LB media with antibiotics

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• Bacteria resuspended in buffer and diluted in test combinations

Agroinfiltration

• Bacterial suspension injected into the plant and incubated for 2 day

Protein Extractions

• Leaf disks punched from agroinfiltration sites and ground in extraction buffer

SDS-PAGE

• Separation of denatured proteins on the basis of size

Western Blotting

• Proteins transferred from gel to membranes for antibody detection

Chemiluminescence Imaging

• Blots probed with specific antibodies imaged to collect chemiluminescence signal from antibody-conjugated enzyme horse radish peroxidase (HRP) and substrate

Figure 2:

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