Profiling Thimet Oligopeptidase-Mediated Proteolysis in *Arabidopsis thaliana*

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NO – PHOTO / RECORD

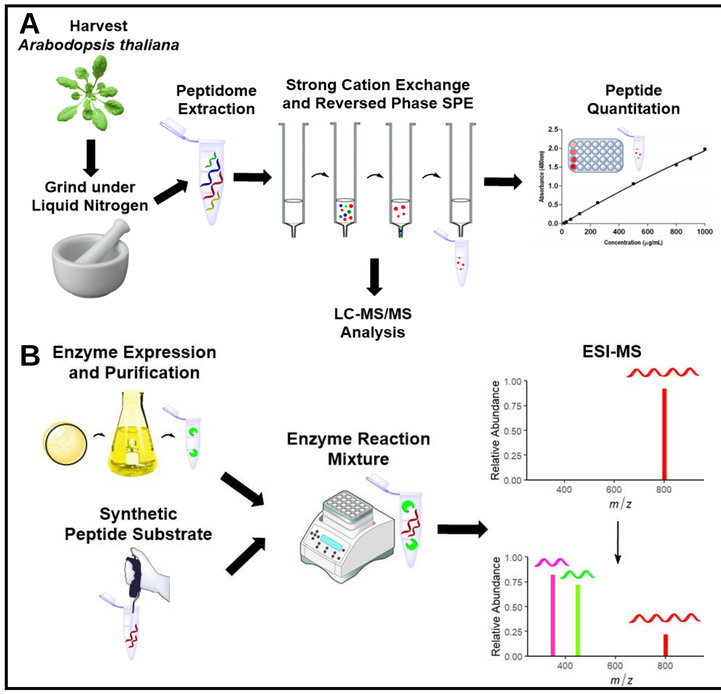
Overview

- Plant stress continues to reduce agricultural yields causing substantial economic losses, making it crucial to understand how plants perceive stress signals to elicit responses for survival.
- Uncovering the role of peptidases in plant stress response is limited by the ability to identify substrates.
- Here we present a comparison between *Arabidopsis thaliana* wildtype and *top1top2* null mutants to reveal putative TOP substrates *in vivo*; substrate candidates were then validated via *in vitro* enzyme assays.

Introduction

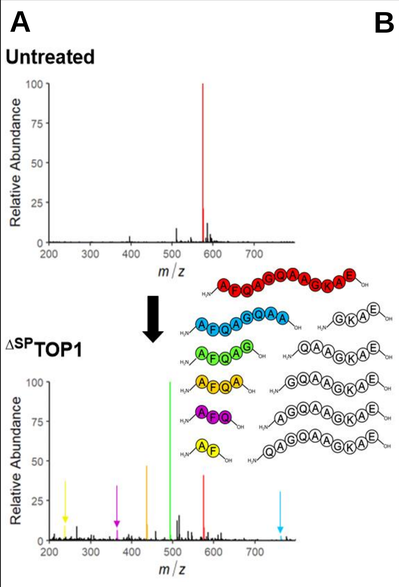
- Plants have evolved various strategies to maintain protein homeostasis (proteostasis). Proteolysis is recognized as critical for the plant's defense against pathogens and adaptive responses to environmental stress [1-2].
- Thimet oligopeptidases (TOPs) are zinc-dependent peptide hydrolases that are critical components in plant response to oxidative stress triggered by pathogens or abiotic factors. TOPs are required for a fully functioning immune response to pathogens that activate effector-triggered immunity [3-4]. The Arabidopsis proteome contains two TOP enzymes.
- TOP1 contains an N-terminal signal peptide that mediates its localization to chloroplasts and mitochondria [4,5]. It was shown to cleave presequences containing eight to 23 amino acids *in vitro* and is hypothesized to act downstream of organellar proteases for peptide degradation and organelle import processing [5]. When the N-terminal signal peptide is cleaved, the enzyme's mature form, denoted $^{\Delta SP}$TOP1, has been shown to have increased activity due to stronger interactions with the substrate [4].
- TOP2 is located in the cytosol, where it is predicted to act downstream of the 20S proteasome, degrading proteasome-generated peptides during stress [4,5].
- Further differential characterization of the TOP1/TOP2 null mutant with the wild type (WT) will allow for the delineation of TOP peptide substrates which will generate insights into the relevance of TOP-mediated proteolytic pathways in proteostasis.

Methods



A. General workflow for label-free quantitative peptidomics. Six-week-old plant rosette leaves were ground under liquid N_2 before extracting peptides with 10% TCA in acetone. Peptides were isolated from small molecules with SCX SPE and desalted with RP SPE before peptide quantitation. Peptide concentrations across replicates were normalized before LC-MS/MS analysis. **B.** To initiate the enzyme assay, TOP1, $^{\Delta SP}$TOP1, or TOP2 was added at a peptide:TOP ratio of 10:1 and incubated at 23°C for 30 min. Samples were resuspended in 50% methanol/0.1% formic acid and directly infused via ESI on a Q Exactive HF-X hybrid MS for intact mass analysis. The mass spectrometer was operated at a resolving power of 120k, positive polarity, spray voltage of 3 kV, with 150–2000 m/z range, and collecting 100 scans/sample for averaging.

Results



B

Originating Protein	Peptide Sequence	TOP1 Cleavage Sites	ΔSP TOP1 Cleavage Sites	TOP2 Cleavage Sites
Chaperonin 60 subunit β 1	AGVNKLADLVGVTLGPK	**AGVNKLADLVGVTL**↓GPK	**AGVNKLADLVGVTL**↓GPK **AGVNKLA**↓**DLVGVTL**↓GPK **AGVNKL**↓**ADLVGVTL**↓GPK AGVNKLADLVG↓**VTL**↓GPK	ND
30S ribosomal protein S5	SDDEHHFEASESGAS	ND	**SDDEHHFE**↓ASESGAS	ND
Oxygen-evolving enhancer protein 3-2	FDATRKEAEAM	**FDATRKE**↓AEAM	**FDATRKE**↓**AEAM** **FD**↓**ATRKE**↓**AEAM**	**FDATRKE**↓AEAM
Eukaryotic translation initiation factor 5A-2	VMLRPASPGTGVIAGGA	**VMLRPASPGTG**↓VIAGGA **VMLRPASPG**↓**TGVIAGGA**	**VMLRPASPGTG**↓**VIAGGA** **VMLRPASPG**↓**TGVIAGGA** **VMLRPA**↓SPGTGVIAGGA **VML**↓RPASPGTGVIAGGA	**VMLRPASPG**↓TGVIAGGA
60S ribosomal protein L28-1	MDPYKYRPA	**MDPYKY**↓RPA	**MDPYKY**↓RPA	**MDPYKY**↓RPA
Catalase-2	AADKDQAVVLATT	ND	**AADKDQAVVL**↓ATT AADKD↓**QAVVL**↓ATT	ND
Cysteine synthase 1	FQTIDNLDYAARSKSSPD	ND	**FQTIDNLD**↓YAARSKSSPD	ND
Stress-induced protein KIN2	AFQAGQAAGKAE	**AFQAGQAA**↓GKAE **AFQAG**↓QAAGKAE	**AFQAGQAA**↓GKAE **AFQAG**↓QAAGKAE **AFQA**↓GQAAGKAE **AFQ**↓AGQAAGKAE **AF**↓QAGQAAGKAE	**AFQAGQAA**↓GKAE **AFQAG**↓QAAGKAE
Phosphoglycerate kinase 1	GKVLPGVIALDEAIPVT	ND	GKVLPGVIALD↓**EAIPVT** **GKVLPG**↓**VIAL**↓**DEAIPVT** **GKVLP**↓GVIALDEAIPVT	ND
Bifunctional protein FolD 2	MLLRNTVDGAKRVFGE	MLLRNTVD↓**GAKRVFGE** **MLLR**↓NTVDGAKRVFGE	MLLRNTVDGAK↓**RVFGE** MLLRNTVDG↓**AKRVFGE** MLLRNTVD↓**GAKRVFGE** **MLLR**↓NTVDGAKRVFGE **MLL**↓RNTVDGAKRVFGE	MLLRNTVD↓**GAKRVFGE** **MLLR**↓NTVDGAKRVFGE

A. The untreated AFQAGQAAGKAE peptide was detected in one charge state (red, m/z 574.79, +2 charge state). The N-terminal products AFQAGQA (blue, m/z 763.37, +1 charge state), AFQAG (green, m/z 493.24, +1 charge state), AFQA (orange, m/z 436.22, +2 charge state), AFQ (purple, m/z 365.18, +1 charge state), and AF (yellow, m/z 237.12, +1 charge state) indicate the five cleavage sites detected for $^{\Delta SP}$TOP1. All observed masses match with the theoretical peptide masses within 3 ppm mass error. **B.** Synthetic AtTOP peptide substrates found to be cleaved by TOPs after analysis with ESI-MS. The arrows represent identified sites of cleavage (ND: none detected). Cleaved peptide products that are bolded and underlined were uniquely detected in the enzyme-treated samples compared to the bare synthetic peptide samples.

Conclusions

- Putative TOP substrates were identified from differential label-free quantitative peptidomics between wildtype and *top1top2* knock-out mutant.
- 10 substrates were validated using *in vitro* enzyme assays with heterologously expressed TOPs and synthetic candidate peptides.
- The enzymatic assays allowed for the identification of TOP cleavage sites, revealing a slight preference for hydrophobic amino acids surrounding the site of hydrolysis.
- The validated TOP substrates are derived from proteins involved in various cellular processes, including stress response, photosynthesis, biogenesis, protein folding, glycolysis and antioxidant defense.

References

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