Abstract

Proteases are essential regulators of plant stress responses, yet the mechanisms of their functions remain poorly understood. Cysteine proteases, including metacaspases, vacuolar processing enzymes, and papain-like cysteine proteases, have been implicated in regulating plant cell death triggered by biotic and abiotic stress. The unicellular green alga *Chlamydomonas reinhardtii* (Chlamydomonas) with its simpler genome compared to higher plants serves as a good model for the study of these enzymes. However, the lack of molecular characterisation of these proteases limits further functional studies.

In this work, we investigated the molecular mechanisms of selected stress-associated cysteine proteases of Chlamydomonas. We performed a biochemical characterisation of both Chlamydomonas metacaspases, CrMCA-I and CrMCA-II, which revealed information about their cleavage preferences, selectivity, and activation, providing knowledge for further investigation of their cellular functions. As part of this work, the first successful recombinant production and characterisation of a plant type I metacaspase in soluble form was achieved in *E. coli* by the removal of a short, hydrophobic sequence, unique to plant type I metacaspases. Additionally, we evaluated novel metacaspase-targeting activity-based probes, which selectively detect active metacaspase forms, enabling us to study their maturation mechanisms *in vitro*.

Furthermore, we investigated the presence and activity of cysteine proteases during cell death induced by 5 mM hydrogen peroxide (H_2O_2) in Chlamydomonas using fluorogenic peptides, activity-based probes and proteomics. Experiments aimed at detecting active metacaspases using activity-based probes in Chlamydomonas extracts revealed an unexpected activity of a previously uncharacterised papain-like cysteine protease, CrCEP1. The protease was found to ligate peptides to the N-termini of the photosystem-II proteins PsbO, PsbP and PsbQ, revealing its dual protease and transpeptidase activity, with potential physiological implications.

The results presented in this work enhance our understanding of the two Chlamydomonas metacaspases and the papain-like cysteine protease CrCEP1 and pave the way for further research into the roles of proteases regulating stress response in Chlamydomonas.

Keywords:
Chlamydomonas reinhardtii, stress-induced cell death, metacaspases, papain-like cysteine proteases, activity-based probes, transpeptidation.