New protein fluorescent labeling methods for carbonylation and S-acylation studies in plants

MSc Andrea Román[1,2], MSc Salvador Priego[1,2], Ph.D. Antonio J. Castro[1], **Dr Juan De Dios Alche**[1,3]
[1]Plant Reproductive Biology and Advanced Imaging Laboratory (BReMAP), Estación Experimental del Zaidín (CSIC), Granada, Spain, [2]These authors contributed equally to this work,, Spain, [3]University Institute of Research on Olive Grove and Olive Oils (INUO), Jaén, Spain

Background
Pollen, the male gametophyte, plays a pivotal role in plant reproduction, being essential for successful fertilization and seed production in Angiosperms. Thus, understanding its biology is crucial for ensuring optimal crop yields. Carbonylation is an irreversible post-translational modification (PTM) characterized by the non-enzymatic addition of carbonyl groups to amino acid residues, leading to protein malfunction and cell death. Carbonylation leads to disruption of protein structure and function. On the other hand, S-acylation or palmitoylation is another PTM that involves the reversible attachment of palmitic acid to specific Cys residues of proteins by the action of protein S-acyltransferases (PATs). This PTM plays crucial roles response to biotic and abiotic stresses, hormonal signaling, cell polarization and expansion, and cytoskeleton organization [1].

Protein labeling techniques for in vivo studies have garnered considerable attention, with cycloaddition reactions emerging as promising methods due to their compatibility with diverse conditions. However, the cytotoxic effects of copper catalysts have limited their widespread application [2]. This study aimed to develop innovative protein labeling methods for assessing protein carbonylation and S-acylation in plant tissues.

Methods
For S-acylation studies, we developed a new method for incorporating fluorescence-labeled palmitic acid into proteins using an azide-alkyne cycloaddition reaction. This method was experimentally validated using an in vitro culture system for pollen. In a first approach, the synthetic lipid containing the azide functional group was added to the culture medium, either in the presence or not of a chemical PAT inhibitor [3], being incorporated to proteins by the enzymatic machinery of germinating pollen grains. Then, pollen was fixed and the fluorescent labeling reaction was performed and visualized by fluorescence microscopy. Alternatively, the fluorescent labeling was carried out exogenously. Then, copper was removed using size exclusion techniques, thus eliminating its cytotoxic effect. Finally, pollen was germinated in the presence of the palmitol-fluorochrome conjugate and the dynamics of the S-acylome was monitored by using time-lapse fluorescence microscopy.

For detecting carbonylated proteins, we developed two different methods. First, we carried out derivatization of carbonyl groups by DNPH (2,4-dinitrophenylhydrazine) [4] to hydrazone groups, which were further detected using an Alexa Fluor 488-conjugated antibody. Alternatively, we used the fluorescent probe BzCH (7-hydrazinyl-4-methyl-2H-1-benzopyran-2-one,), which is capable of binding specifically to carbonyl groups [5]. These two methods were further experimentally validated as above in the presence or absence of the ROS inducer Paraquat in the culture medium. Moreover, this method also allowed to quantify protein carbonylation by conducting derivatization assays followed by image processing and fluorescence quantification.

Results
Both fluorescent labeling approaches revealed the dynamics of S-acylated proteins in growing pollen tubes, showing a similar distribution pattern of S-acylated proteins. Thus, a high

fluorescent signal was observed in the cytoplasm of the vegetative cell and, to a lesser extent, in the cytoplasm of the pollen tube. Fluorescence significantly decreased in the presence of the PAT inhibitor, suggesting that was mostly due to the activity of PAT enzymes. Moreover, the exogenous labeling of the synthetic lipid allowed the real-time monitoring of the S-acylome in a single growing pollen tube by using time-lapse microscopy.

On the other hand, carbonylated proteins were found to accumulate in the cytoplasm of the vegetative cell of non-germinated pollen grains, suggesting that this PTM may be a marker of loss of pollen viability. In addition, we observed a consistent accumulation pattern of carbonylated proteins at the apical region of the pollen tube. Interestingly, protein carbonylation levels significantly increased in the presence of Paraquat in the germination medium, suggesting that ROS-mediated oxidative stress may triggers carbonylation, leading to pollen tube growth arrest.

Conclusions
In this work we have developed a novel and reliable fluorescent labeling method to study S-acylation of plant proteins, overcoming limitations associated with cytotoxic catalysts during cycloaddition reactions. Moreover, we have set up two methods to localize carbonylated proteins in plant tissues by using fluorescent probes.

This research was funded by projects PID2020-113324GB-100 and TED2021-130015B-C22 from MICIIN/AEI, both of them cofinanced by the ERDF program of the European Union. Salvador Priego and Andrea Román were granted with a PhD fellowship from Junta de Andalucía and MICINN, respectively.

Keywords:

carbonylation, cycloaddition, pollen, S-acylation, fluorescent-probes

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