# Deciphering the S-Nitrosylome of *Arabidopsis thaliana* during the defense response

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The biological effects of nitric oxide (NO) are mainly mediated through S-nitrosylation of cysteine thiols. We have used the biotin switch method combined with mass spectrometry analysis to identify targets of S-nitrosylation in *Arabidopsis thaliana* cell suspension cultures and leaves challenged with the pathogenic bacteria *Pseudomonas syringae* pv. *tomato*. The NO targets belong to different functional categories, and include proteins previously identified in plants and mammals as well as novel targets. Our data support the idea that NO orchestrates the whole plant physiology through covalent modification of proteins.

One of the earliest events occurring in plants following pathogen infection is a strong oxidative burst and increases in the NO level being the correct intracellular balance between these molecules crucial for the establishment of resistance [1, 2]. The reversible covalent binding of NO to a specific cysteine residue (S-nitrosylation) is the main mechanism by which NO exerts its function and several candidates have been identified in plants [3, 4]. In order to uncover the role of this type of modification during basal resistance (the first line of active defense in plants) and R-mediated resistance (a strong form of race-specific resistance), we aim to identify S-nitrosylated proteins in Arabidopsis plants and cell extracts challenged with virulent Pst and avirulent *Pst avrRpt2*.

Protein extracts were subjected to the biotin switch method converting S-nitrosylated Cys to biotinylated Cys [3]. Extracts (10 mg for protein purification and 300 μg for detection of biotinylated proteins) were first incubated with 30 mM methyl methanethionsulfonate (MMTS) and 2.5% SDS for blocking free cys residues. Treatment with GSNO (500 μM) was used to generate S-nitrosothiols in vitro and the treatment with the reducing agent DTT (20mM) was performed as a control that the labelling observed corresponds to originally S-nitrosylated proteins. Biotinylated proteins were visualized by immunoblotting using anti-biotin antibody and were purified by affinity chromatography on a neutravidin matrix, and bound proteins were eluated with 100 mM β-mercaptoethanol. For protein identification the purified proteins were trypsin digested and subjected to LC/MS/MS analysis using a Surveyor/MicroAS HPLC system in tandem with an LTQ mass spectrometer (ThermoFisher Scientific, USA). The MS/MS spectra obtained were analysed using the Bioworks 3.2 software (ThermoFisher Scientific, USA), and the SEQUEST search engine (Thermo, MA), against a non-redundant NCBI and MSDB databases and their reverse, allowing for variable cysteine modification by β-mercaptoethanol and organism restriction to Arabidopsis. The FDR value was adjusted to 1% taking as a reference the Xcorr values obtained from the search using the reverse database (DCn >0.1).

The results obtained indicate more intense labeling following *P. syringae* infection, especially those from *PstavrRpt2*- treated samples. In addition more S-nitrosylated proteins were purified from leaves or cells challenged with *P.syringae* compared to control and non-treated samples. This indicates that leaves and cells undergoing a defense reaction contain higher levels of S-nitrosothiols, which is consistent with the increased NO production during the incompatible interaction [2].

The 127 NO-target proteins identified cover basically any process of cellular physiology and include cytoskeleton proteins (11%), enzymes involved

in carbon and nitrogen mobilization (41%), pathogen- and stress-related proteins (10%), enzymes of the antioxidant defense system (6%) and signaling and regulating proteins (14%). The majority of the NO targets were not exclusive to a given treatment, which is in agreement with the dynamic nature of S-nitrosylation, and suggests that the right balance between both redox states are needed for the correct function of the protein.

Comparison of S-nitrosylation targets in animal systems [5, 6] with the proteins identified in this work and in previous studies in plants [4] reveals common targets between animals and plants. About 75% of the proteins identified here represent novel potential targets for S-nitrosylation, while others have been identified in previous studies in animals and plant systems.

These results provide clues on the possible NO targets during basal and R-mediated resistance, and suggest that NO-specific modification of these enzymes allows the coordinated modulation of redox signalling and orchestrates the outcome of compatible and incompatible interactions. While we are planning to identify the cys residues involved and perform mutational studies with purified proteins and transgenic of selected candidates, we have conducted infection experiments with Arabidopsis loss-of-function insertion mutants for redox- and defense-related genes. The differential behaviour of these mutants from wild-type control plants in both, compatible and incompatible interactions, and the monitoring of the progress of disease symptoms provide evidence that these mutants are compromised in the activation of basal and R-mediated defense responses.

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