Cysteine reactivity profiling to unveil redox regulation in phytopathogens

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Running head: Cys reactivity profiling

Reactivity-based chemical proteomics is a powerful technology based on the use of tagged chemicals that covalently react with surface-exposed residues on proteins in native proteomes. Reactivity profiling involves the purification, identification and quantification of labelled peptides by LC-MS/MS. Here, we have detailed a protocol for reactivity profiling of Cys residues using iodoacetamide probes, displaying >1000 reactive Cys residues in the proteome of phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*DC3000). Comparative reactivity profiling of *Pto*DC3000 treated with or without hydrogen peroxide (H_2O_2) identified ~200 H_2O_2-sensitive Cys residues in antioxidant enzymes, metabolic enzymes and transcription regulators. Interestingly, half of these H_2O_2-sensitive Cys residues are more reactive in response to H_2O_2 and several proteins have multiple Cys residues with opposite reactivities in response to H_2O_2 exposure.

Keywords: Reactivity profiling; biotinylated iodoacetamide; BIAM; LC-MS/MS; redox sensitive Cys residues; hydrogen peroxide*; Pseudomonas syringae* pv. *tomato* DC3000.

1. Introduction

Reactivity profiling is an emerging chemical proteomics method, primarily developed in pharmaceutical research [1-2]. Reactivity profiling is used in two ways: competitive profiling and comparative profiling [3-6]. Competitive profiling is used to screen chemical libraries to discover new drug targets, whereas comparative profiling identifies differentially reactive residues in biological samples generated from two different conditions.

Reactivity profiling is based on the use of reactivity probes that consist of a reactive group and an affinity tag or chemical tag. Reactivity probes have a simple structure to minimise steric hindrance when compared to the more selective activity-based probes that contain an additional binding group. The reactivity probes used here carry an iodoacetamide (IAM) reactive group to alkylate cysteine (Cys) residues that are exposed on proteins in native proteomes.

Cys reactivity profiling with IAM-based probes has been established with different probes and approaches [2,4,7-9]. The method typically includes 4-5 steps: 1) labelling with reactivity probes (*in vitro* or *in vivo*); 2) coupling to biotin by click-chemistry; 3) digesting the labelled proteome with e.g. trypsin; 4) enrichment of labelled peptides on various avidin beads; 5) identification and quantification of the labelled peptides by LC-MS/MS.

In this chapter, we describe the method that we developed using the commercially available biotinylated IAM (BIAM; **Figure 1A**). By using BIAM, we omit the click-chemistry reaction (step-2) (**Figure 1B**). We also employ label-free quantification (LFQ) instead of isotopic labelling, which is often used for Cys reactivity profiling [10]. LFQ simplifies the procedure and mitigates the risks of sample loss. This method allows us to detect ~1000 labelled peptides from only 200 µg proteins.

To provide a step-by-step protocol, we show comparative profiling of the bacterial plant pathogen *Pseudomonas syringae* exposed to hydrogen peroxide (H_2O_2) as an example. *P. syringae* bacteria are exposed to H_2O_2 during early stages of infection, generated by plant respiratory burst

oxidase homologs (RBOHs [11-12]). This experiment aims to identify redox-sensitive Cys residues in pathogen proteins by mimicking the host-delivered oxidative burst through exposure to 10 mM H_2O_2.

Following this procedure, we identified ~1000 labelled peptides assigned to ~760 unique Cys residues and ~200 redox-sensitive Cys residues in antioxidant enzymes, metabolic enzymes and transcription regulators (**Figure 2A** and **Supplemental Table S1**). Interestingly, half of the 192 Cys residues that show differential reactivity upon H_2O_2 treatment, are more reactive in H_2O_2-treated samples (**Figure 2A**). These results indicate that this method uncovers not only Cys residues that become oxidised, but also Cys residues becoming more reactive during oxidative stress. Moreover, several proteins have multiple sites with opposite reactivities in response to H_2O_2 treatment. For instance, two glutaraldehyde 3-phosphate dehydrogenases (GAPDHs), Gap1 and Gap2, show a complete oxidation of the active site residue, consistent with the literature [13], whilst the other Cys residues have unaltered reactivity or even an increased reactivity upon H_2O_2-treatment (**Figure 2B**). Taken together, this experiment provides a good example of intriguing observations to be made using Cys reactivity profiling following the protocol detailed below.

2. Materials

2.1 H_2O_2 treatment of Pseudomonas syringe *cultures*

1. *Pseudomonas syringae* pv. *tomato* DC3000 frozen stock, stored at –80 ºC.
2. Rifampicin (25 mg/mL): dissolve 125 mg of rifampicin in 5 ml DMSO, vortex well and store at −20º C in 500 µL aliquots.
3. Liquid NYG medium: 5 g/L of Bacto™ peptone, 3 g/L of Bacto™ yeast extract, 20 g/L of glycerol; autoclave at 121ºC and 100 kPa for 15 min.
4. Solid NYG medium: NYG medium supplemented with 1.5 % Agar.
5. Minimal (mannitol-glutamate (MG)) medium: 10 g/L of mannitol, 2 g/L of L-glutamic acid, 0.5 g/L of KH_2PO_4, 0.2 g/L of NaCl; adjust pH to 7.0 with 1N KOH and adjust pH to 7.4 and the final volume to 900 mL with distilled water, autoclave at 121 °C and 100 kPa for 15 min and add sterile–filtered (0.22 µm membrane filter) $MgSO_4$ to a final concentration of 0.8 mM (0.2 g/L).

6. 30 % (w/w) hydrogen peroxide solution in H_2O (Sigma Aldrich). Please beware that the stock can expire when not properly stored.

7. 0.22 µM syringe filter units (Millipore).

8. Incubator with shaker for incubating bacterial cultures at 28 °C, 220 rpm.

9. Spectrophotometer and UV cuvettes for measuring optical density (OD) at 600 nM.

2.2 Protein isolation and BIAM labeling

1. Protein extraction buffer: 1×PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4); filter sterilized using 0.22 µm membrane filter.

2. Protein LoBind tubes, 1.5 mL (Eppendorf).

3. Ultrasonic disintegrator/Probe sonicator (Sanyo MSE Soniprep 150).

4. BOECO Tube Rotator RS-24 (BOECO, BOE 8024000) or equivalent.

5. DC Protein Assay (Bio-Rad, 5000116).

2.3 BIAM labeling

1. BIAM (N-(Biotinoyl)-N'-(Iodoacetyl)Ethylenediamine (Invitrogen™).

2. Dimethyl sulfoxide (DMSO).

2.4 Peptide preparation by FASP

Please make all solutions for FASP fresh on the day of use.

1. Acetonitrile (ACN) for UHPLC and LC-MS (Sigma-Aldrich).

2. Trifluoroacetic acid (TFA), LC-MS Grade.

3. 1.0 M TEAB (triethylammonium bicarbonate), pH 8.5±0.1(Sigma-Aldrich).

4. Urea Reagent Plus®, ≥99.5 %, pellets (Sigma-Aldrich).

5. Tris-(2-carboxyethyl)-phosphine-hydrochloride (TCEP, Sigma-Aldrich).

6. 2-chloroacetamide (Sigma-Aldrich).

7. Lysyl Endopeptidase® (Lys-C), Mass Spectrometry Grade (Wako).

8. 50 mM Tris-HCl, pH 8.5; filter sterilized through 0.22 µm membrane filter.

9. Trypsin Gold, Mass Spectrometry Grade (Promega).

10. 100% acetic acid (HAc).

11. Vivacon 500 centrifugal concentrators (10,000 MWCO) (Sartorius).

12. Refrigerated centrifuge (Eppendorf, 5427 R).

13. Digital Heating Shaking Drybath (Thermo Scientific) or equivalent.

14. Vacuum centrifuge with cold trap for volatile organics (Thermo Scientific).

2.4 Purification of biotinylated peptides

Please make all solutions for purification fresh on the day of use.

1. Acetonitrile (ACN) for UHPLC and LC-MS (Sigma-Aldrich).

2. Formic acid (FA, Merck).

3. Monomeric Avidin (Invitrogen).

4. Ammonium bicarbonate (NH_5CO_3)

5. Gilson Diamond Filter Tips Sterile DFL10ST, 0.1-10 µL (Gilson).

6. Visiprep™ SPE Vacuum Manifold DL (Supelco).

7. Vacuum pump (Welch Vacuum Piston Pump 2522C).

2.5 Sequencing labeled peptides

1. Trap column: 5 mm, PepMap RSLC, C18, 300 µm ID particle size 2 µm (Thermo Fisher).

2. C18 reversed-phase capillary column: 50 cm, PepMap RSLC, EASY-Spray column, C18, 75 µm ID particle size 2 µm (Thermo Fisher).

3. Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher) with a Dionex UltiMate™ 3000 HPLC (Thermo Fisher).

4. V-bottom 96-well plate.

5. Loading buffer: 5 % formic acid, 5 % DMSO. Make fresh before use.

6. Mobile phase A: 0.1 % formic acid, 5 % DMSO. Make fresh before use.

7. Mobile phase B: 100 % acetonitrile, 0.1 % formic acid, 5 % DMSO. Make fresh before use.

2.6 Data acquisition and interpretation

1. Computer with operating system: Windows (64-bit) with .NET Framework, 4.7.2 or higher.
2. MaxQuant1.5.3.30 (https://maxquant.org/ [14-15]).
3. MSFileReader 16.0 (Thermo Fisher).
4. Perseus 1.5.5.3 data analysis software (https://maxquant.org/perseus/).
5. Microsoft Excel (www.microsoft.com).

3. Methods

3.1 H_2O_2 treatment of Pseudomonas syringae *cultures*

How you prepare the proteome defines the potential coverage of labelling (*see* **Note 1**).

1. Revive *Pseudomonas syringae* p.v. *tomato* DC3000 from the glycerol stock by streaking on LB agar medium containing 25 µg/mL rifampicin and incubate the plate for 48 h at 28ºC.
2. Pick a single bacterial colony from the plate and inoculate 10 mL liquid NYG medium containing 25 µg/mL rifampicin and grow the bacteria overnight, shaking at 28ºC.
3. Centrifugate the overnight grown bacterial culture at 2000 x g for 10 min at room temperature (RT).
4. Remove the supernatant and suspend the pellet in 10 mL minimal medium.
5. Measure the optical density at 600 nm (OD_{600}) and dilute the bacterial suspensions with minimal medium to OD_{600} = 0.5.
6. Dilute the bacterial cultures 1:100 in 50 mL minimal medium to OD_{600} = 0.005 and incubate them for ~8 hours until they reach OD_{600} = 0.3-0.5.
7. Centrifugate the bacterial cultures at 2000 x g for 10 min at RT; remove the supernatant and suspend the pellet in 40 mL minimal medium.
8. Split the bacterial suspensions into two to make two identical 20 mL bacterial suspensions in 50 mL falcon tubes.

9. Treat one of the 20 mL bacterial suspensions with 20.4 µL of 9.8 M H_2O_2 (10 mM final concentration) and another with the same amount of water as a mock treatment for 20 min at 28ºC.

10. Centrifugate the bacterial cultures at 2000 x g for 10 min at RT, remove the supernatant and suspend the pellet in 1 mL 1 x PBS.

11. Transfer the bacterial suspensions to 1.5 mL tubes and centrifugate them at 2000 x g for 2 min at RT.

12. Remove the supernatant and snap-freeze the pellet in liquid nitrogen.

3.2 Protein isolation

Important: you need to minimise protein oxidation during protein isolation (*see* **Note 2**).

1. Add 100 µL 1 x PBS per one 1 mL bacterial cultures with OD_{600} = 1.0.
2. Lyse the bacterial cells by sonication on ice, 3 times for 10 seconds each.
3. Centrifugate the lysate at 15,000 x g for 10 min at 4ºC.
4. Transfer the supernatant into a 1.5 mL tube.
5. Determine the protein concentration using a suitable assay.
6. Dilute extract to 1 mg protein/mL with 1 x PBS.

3.3 Labelling with BIAM

Important: labelling conditions are dictated by used probes (*see* **Note 3**).

1. Dispense 200 µL of the lysate into 1.5 mL tubes
2. Add 2 µL of 1 mM BIAM (10 µM final concentration) or DMSO as a no-probe control.
3. Incubate the labelling mixtures for 1 hour at RT while tumbling on a tube rotator.

3.4 Peptide generation by FASP

In this step, proteins will be digested with trypsin/Lys-C using Filter Aided Sample Preparation (FASP), originally described in [16] (*see* **Note 4**).

1. Assemble the Vivacon 500 filter units and wash them with 200 µL of 0.1 % TFA in 50 % ACN by centrifugation at 15,000 *x g* for 15 min at 20ºC. There should be thin layer of liquid. If not, the filters might be leaking.
2. Prewash the filter with 200 µL of 8 M urea in 100 mM TEAB by centrifugation at 15,000 x g for 15 min at 20ºC.
3. Load the labelling mixtures on the filter and centrifugate at 15,000 x g for 20 min at 20ºC. This step removes excess probe.
4. Wash the filter with 200 µL of 8 M urea in 0.1 M TEAB by centrifugation at 15,000 x g for 20 min at 20ºC.
5. Repeat step 4 a further 4 times. Detergent-containing samples need to be washed intensively until the flow-through no longer forms bubbles.
6. Reduce the proteins by adding 4 µL of 500 mM TCEP in 200 µL of 8 M urea in 0.1 M TEAB (final concentration 10 mM) on the filter and incubate them for 30 min at room temperature.
7. Alkylate the proteins by adding 20 µL of 500 mM 2-chloroacetamide in water (final concentration 50 mM) on the filter and incubate them for 30 min at room temperature in the dark.
8. Centrifugate the samples at 15,000 x g for 20 min at 20ºC.
9. Wash the filter with 200 µL of 6 M urea in 50 mM TEAB and centrifugate them at 15,000 x g for 20 min at 20ºC.
10. Repeat step 9 once.
11. Change to a fresh collection tube for peptide collection.
12. Prepare the 1 µg/µL Lys-C stock solution by dissolving 20 µg Lys-C in 20 µL 50 mM Tris-HCL (pH 8.5).
13. Dilute 5 µL of 1 µg/µL Lys-C stock solution with 95 µL of 1 M urea in 50 mM TEAB. Load 1 µg Lyc-C per 40 µg protein on the filter with a total volume of 100 µL and incubate for 4 hours at 37ºC.
14. Prepare the 1 µg/µL trypsin stock solution by dissolving 100 µg of Lys-C in in 100 µL 50 mM acetic acid (2.875 mL acetic acid solution in 1 µL MS water).

15. Dilute 5 µL of 1 µg/µL trypsin stock solution with 95 µL of 50 mM TEAB. Load 1 µg trypsin per 40 µg protein on the filter with a total volume of 100 µL and incubate for 16-20 hours at 37ºC.

16. Seal the lid with Parafilm to prevent evaporation.

17. Collect the digested peptides by centrifugation at 15,000 x g for 20 min at 20ºC.

18. Transfer the peptide-containing flow-through in 1.5 mL tubes.

19. Wash the remaining peptides off the filter with 150 µL 0.1 % TFA by centrifugation at 15,000 x g for 15 min at 20ºC.

20. Wash the remaining peptides off the filter with 150 µL 0.1 % TFA in 50 % ACN by centrifugation at 15,000 x g for 15 min at 20ºC.

21. Pool the flow-through collected from steps 16–19 in 1.5 mL tubes.

22. Dry the peptides by vacuum centrifugation.

3.5 Purification of biotinylated peptides

In this step, biotinylated peptides are purified using SPE (solid-phase extraction) operated through a vacuum manifold to increase the sample processing capacity (*see* **Note 5**).

1. Dissolve the 200 µg peptides in 200 µL of 25 mM ammonium bicarbonate (1 µg/µL final concentration) using sonication in a water bath, if necessary.

2. Assemble the Visiprep™ SPE Vacuum Manifold DL, place the Gilson Diamond Filter Tips on the disposable liner and fasten the valves.

3. Wash the filter tips with 400 µL of 0.1 % formic acid (FA) 50 % acetonitrile (ACN) by applying the vacuum.

4. Repeat Step-3 once.

5. Wash the filter tips with 400 µL of 25 mM ammonium bicarbonate by applying the vacuum.

6. Repeat Step-5 twice.

7. Load 150 µl monomeric avidin beads on the filter tips.

8. Wash the filter tips with 200 µL of 25 mM ammonium bicarbonate by applying the vacuum.

9. Repeat Step-8 once.

10. Place the new collection tube and slowly pass the peptides solutions through the filter tip by applying the vacuum.

11. Reload the flow-through on the filter tips.

12. Repeat Step-11 once.

13. Wash the filter tips with 200 µL of 25 mM ammonium bicarbonate by applying the vacuum.

14. Repeat Step-12 once.

15. Close the valves and place the new collection tube.

16. Load 200 µL of 0.4 % TFA in 30 % acetonitrile and incubate for 30 min.

17. Elute the peptides by applying the vacuum in the collection tube.

18. Load another 200 µL of 0.4 % TFA in 30 % acetonitrile straightaway.

19. Dry down the biotinylated peptides by vacuum centrifugation.

3.6 Sequencing labeled peptides

LFQ requires individual MS runs of all samples sequentially (*see* **Note 6**).

1. Reconstitute the peptides in 10-20 µL of 5 % DMSO, 5 % formic acid solution and add the sample to a V-bottom 96-well plate.

2. Place the plate in the Dionex UltiMate™ 3000 UHPLC system.

3. Trap the peptides on a C18 PepMap100 pre-column (300 µm i.d. x 5 mm, 100 Å, Thermo Fisher) using solvent A (0.1 % formic acid in water) at a flow rate of 10 µL/min.

4. Separate peptides on an in-house packed analytical column (75 µm i.d. packed with ReproSil-Pur 120 C18-AQ, 1.9 µm, 120 Å, Dr. Maisch GmbH).

5. Acquire data in data-dependent acquisition (DDA) mode.

6. Aquire full scan MS spectra in the Orbitrap (scan range 350-1500 m/z, resolution 70000, AGC target 3e6, maximum injection time 50 ms).

7. Select the 10 most intense peaks for HCD fragmentation at 30 % of normalised collision energy at resolution 17500, AGC target 5e4, maximum injection time 120 ms with first fixed mass at 180 m/z.

8. Select charge exclusion for unassigned and 1+ ions.

3.7 Data acquisition and interpretation

1. Download the Uniprot reference proteome for *Pseudomonas syringae* pv. *tomato* (strain ATCC BAA-871/DC3000), Proteome ID; UP000002515.

2. Submit the RAW spectra to Andromeda [17].

3. Annotate the spectra using MaxQuant (1.5.3.30) with default settings [18].

4. Select oxidation of methionine residues (16 Da) and acetylation of the protein N-terminus (42 Da), modification of cysteine by alkylation with iodoacetamide (57 Da) or with BIAM ($C_{14}H_{22}O_3N_4S$, 326.1413 Da) as the “Variable Modifications”. Set the “Enzyme specificity” was set to “Trypsin/P” with two missed cleavages allowed. Switch on “Label-free protein quantification”. Retention times are recalibrated based on the in-built nonlinear time-rescaling algorithm. Switch on the “match between runs” option with the maximal match time window set to 0.7 min and the alignment time window set to 20 min. The quantification is based on the “value at maximum” of the extracted ion current. At least two quantitation events are required for a quantifiable protein.

5. For further analysis and filtering of the results, upload the text file for modification sites (e.g. BIAMSites.txt) to Perseus v1.5.5.3. [16]. MaxQuant generates several text files. The file with modification sites appears with the name you set in the MaxQuant configuration. Use the modification sites of your interest.

6. Load the columns named “Intensity (sample name)_(1,2,3...)” as main column. The rest of the columns are default. One sample can have multiple columns depending on how many modifications per peptides are allowed in MaxQuant.

7. To have only one column per sample and the appropriate number of rows per site, process the data by using “Expand site table”.

8. Log2-transform the MS intensities.

9. Create categorical groups by selecting categorical annotation rows (e.g. Control, H_2O_2).

10. Optionally, import protein annotations (e.g. added annotations: mainAnnot.pseudomonas_syringae_pv._tomato_(strain_dc3000).txt.gz)

11. Filter the data, e.g. detected at least three times in at least one group.

12. Impute the missing values from normal distribution using default settings.

13. Normalise the values by subtracting from the median of each column.

14. Visualise the data with a volcano plot using default settings (**Figure 2A**).

4. Notes

1. It is critical to select the appropriate conditions that create the proteome containing the proteins of your interest. We chose a minimal medium because the virulence gene expression (e.g. type III secretion system (T3SS) genes) of *P. syringae* are suppressed in rich media [19]. The transient exposure of *P. syringae* to H_2O_2 is aimed to mimic the oxidative burst which *P. syringae* experiences in the plant apoplast during the infection [12].

2. The lysis method and buffer should be carefully considered to prevent alteration of the redox balance before labelling. For instance, high concentrations of reducing agents (e.g. DTT and TCEP) will change the redox status of the lysates. Conversely, too much exposure to air may cause oxidation of extracts. Also catalases, peroxidases and other redox enzymes will change the redox balance.

3. There are several probes for reactive thiols [20]. The probe concentration and incubation time depend on the potency of the used probe and the proteome and should be adjusted accordingly.

4. The FASP protocol takes advantage of MW filter microfuge tubes and includes all steps from removal of excess probes, buffer exchange, protein denaturation, disulphide-bond reduction, alkylation to digestion on one filter column, whilst minimising sample loss. Also, an appropriate molecular weight (MW) cut-off column efficiently filters out the contaminants such as intact trypsin and Lys-C that have MWs more than 20 kDa from the flow-through that is your peptide pool at the final step.

5. The vacuum manifold can handle 12 samples simultaneously but it is easier to handle less than 6 samples. When assembling, please be aware of inserting the disposable liner inside the collection

tubes and make sure that the unused ports are tightly closed and do not apply more vacuum than 20 Hg (0.6772778 bar).

6. LFQ analysis requires the MS analysis of all samples, intermitted by MS standards. If included, the no-probe controls should be analysed first. A prerun with a probe-labelled sample is useful to adjust the loading, if needed.

5. Acknowledgements

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6. References

[1] Dennehy MK, Richards KA, Wernke GR, Shyr Y, Liebler DC (2006) Cytosolic and nuclear protein targets of thiol-reactive electrophiles. Chem Res Toxicol 19:20–9

[2] Weerapana E, Wang C, Simon GM, Richter F, Khare S, Dillon MB, Bachovchin DA, Mowen K, Baker D, Cravatt BF (2010) Quantitative reactivity profiling predicts functional cysteines in proteomes. Nature 468:790–5

[3] Deng X, Weerapana E, Ulanovskaya O, Sun F, Liang H, Ji Q, Ye Y, Fu Y, Zhou L, Li J, Zhang H, Wang C, Alvarez S, Hicks LM, Lan L, Wu M, Cravatt BF, He C (2013) Proteome wide quantification and characterization of oxidation- sensitive cysteines in pathogenic bacteria. Cell Host Microbe 13:358–70

[4] Abegg D, Frei R, Cerato L, Prasad Hari D, Wang C, Waser J, Adibekian A (2015) Proteome-wide profiling of targets of cysteine reactive small molecules by using ethynyl benziodoxolone reagents. Angew Chem 54:10852-7

[5] Backus KM, Correia BE, Lum KM, Forli S, Horning BD, González-Páez GE, Chatterjee S, Lanning BR, Teijaro JR, Olson AJ, Wolan DW, Cravatt BF (2016) Proteome-wide covalent ligand discovery in native biological systems. Nature 534:570–4

[6] Maurais AJ, Weerapana E (2019) Reactive-cysteine profiling for drug discovery. Current opinion in chemical biology 50:29-36.

[7] Abo M, Li C, Weerapana E (2017) Isotopically-labeled iodoacetamide-alkyne probes for quantitative cysteine-reactivity profiling. Molecular Pharmaceutics 15:743-9

[8] Tian C, Sun R, Liu K, Fu L, Liu X, Zhou W, Yang Y, Yang J (2017) Multiplexed thiol reactivity profiling for target discovery of electrophilic natural products. Chem Biol 24:1416-27

[9] Zanon PRA, Lewald L, Hacker SM (2020) Isotopically labeled desthiobiotin azide (isoDTB) tags enable global profiling of the bacterial cysteinome. Angew Chem Int Ed 59:2829-36

[10] Metcalfe C, Cresswell P, Ciaccia L, Thomas B, Barclay AN (2011) Labile disulfide bonds are common at the leucocyte cell surface. Open Biol 1:110010

[11] Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones JD, Doke N (2003) *Nicotiana benthamiana* gp91phox homologs NbrbohA and NbrbohB participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant Cell 15:706-18

[12] Fones H, Preston GM (2012) Reactive oxygen and oxidative stress tolerance in plant pathogenic Pseudomonas. FEMS Microbiol Lett 327:1-8

[13] Peralta D, Bronowska AK, Morgan B, Dóka É, Van Laer K, Nagy P, Gräter F, Dick TP (2015) A proton relay enhances H_2O_2 sensitivity of GAPDH to facilitate metabolic adaptation. Nat Chem Biol 11:156-63.

[14] Tyanova S, Temu T, Carlson, A, Sinitcyn P, Mann M, Cox J (2015) Visualization of LC-MS/MS proteomics data in MaxQuant. Proteomics 15:1453–6

[15] Tyanova S, Temu T, Cox J (2016) The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc 11:2301–19

[16] Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, Mann M, Cox J (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 13:731-40

[17] Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M (2014) Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol Cell Proteomics 13:2513-26

[18] Cox J, Mann M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26:1367-72

[19] Stauber JL, Loginicheva E, Schechter LM (2012) Carbon source and cell density-dependent regulation of type III secretion system gene expression in *Pseudomonas syringae* pathovar tomato DC3000. Res Microbiol 163:531-9

[20] Hoch DG, Abegg D, Adibekian A (2018). Cysteine-reactive probes and their use in chemical proteomics. Chem comm, 54:4501-12

FIGURES

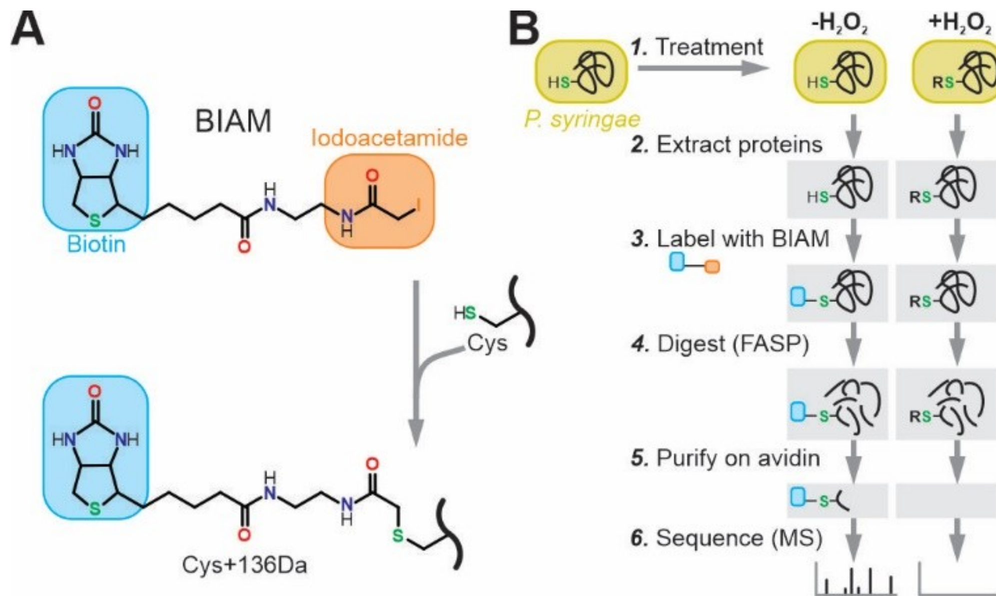

Figure 1. Concept of Cys reactivity profiling with BIAM

(A) Alkylation of Cys residues with BIAM. BIAM contains iodoacetamide as a reactive group (organe) and biotin as affinity tag (blue). Labeled peptides have 136 Da adducts on Cys residues.

(B) Comparative Cys reactivity profiling of *P.syringae* during oxidative stress consists of six steps: 1) Mock-treatment and hydrogen peroxide (H_2O_2) treatment of two identical *P.syringae* cultures; 2) Protein extraction under non-denaturing conditions; 3) Labeling with BIAM; 4) Peptide preparation using FASP; 5) Purification of BIAM labeled peptide on monomeric avidin beads; 6) Sequence labeled peptides by LC-MS/MS.

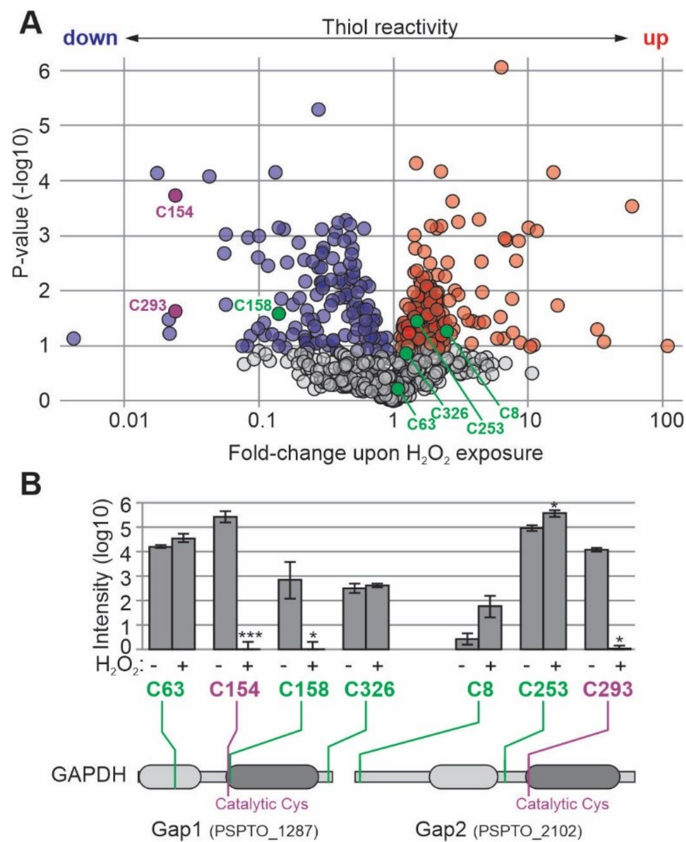

Figure 2. Comparative Cys reactivity profiling in *Pseudomonas syringae*

(A) Comparative Cys reactivity profiling on *P. syringae* exposed to H_2O_2 displays significantly altered Cys reactivities. Volcano plot showing all BIAM-labelled peptides identified at least three times, plotted against statistical significance (P-value, y-axis) and fold-change of intensities of labelled peptides in mock-treated versus H_2O_2 treated proteome (x-axis). The peptides with significantly decreased or increased mass spectra intensities upon H_2O_2 treatment were highlighted in blue and red, respectively. Gap1/Gap2-derived peptides are indicated.

(B) GAPDH proteins Gap1 and Gap2 carry multiple Cys residues with opposite reactivities upon H_2O_2 treatment. The graph shows the fold change of MS intensity of each labelled peptide of Gap1 and Gap2. Cartoons depict the PFAM domain structures with labelled Cys residues. Highlighted are catalytic Cys residues (purple) and remaining Cys (green).