New Phytologist Supporting Information

Full title: Quantitative phosphoproteomic analysis reveals common regulatory mechanisms between effector- and PAMP-triggered immunity in plants

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Methods S1

Plant materials and growth conditions

Arabidopsis plants were grown in a controlled growth chamber under 70 % relative humidity at 23 °C and a light intensity of 85 µE m^{-2} s^{-1}. A 10h-light and 14 h-dark photoperiod was applied. Dexamethasone (Dex)-inducible *Arabidopsis thaliana* ecotype Col-0 *GVG:avrRpt2* (*Dex:avrRpt2*) has been previously described (McNellis *et al.*, 1998). We generated *rpm1rps2/GVG:avrRpt2* (*rpm1rps2/Dex:avrRpt2*) plants by crossing *GVG:avrRpt2* plants with the *rpm1-3 rps2-101C* double mutant (Mackey *et al.*, 2003). The *Arabidopsis thaliana rbohD, rbohF, rbohDrbohF, agb1 (agb1-2), bik1 pbl1* double mutant and *pBIK1:BIK1-HA* plants were described previously (Ullah *et al.*, 2001; Torres *et al.*, 2002; Kadota *et al.*, 2014). RbohD-tagged complementation lines *rbohD/35S:FLAG-RbohD* (*rbohD/35S:FLAG-RbohD), rbohD/pRbohD:3xFLAG-RbohD* (*rbohD/pRbohD:FLAG-RbohD*) and *rbohD/pRbohD:3xFLAG-RbohD-S343A/S347A* (*rbohD/pRbohD:FLAG-RbohD-S343A/S347A*) have all been published before (Kadota *et al.*, 2014). The *rbohDrbohF/pRbohD:3xFLAG-RbohD* (*rbohDrbohF/pRbohD:FLAG-RbohD*) and *rbohD rbohF pRbohD:3xFLAG-RbohD-S343A-S347A* (*rbohDrbohF/pRbohD:FLAG-RbohD-S343A-S347A*) were generated by crossing *rbohD/pRbohD:3xFLAG-RbohD* and *rbohD/pRbohD:3xFLAG-RbohD-S343A-S347A* with *rbohDrbohF*.

Protein extraction and phosphopeptide enrichment

Frozen plant tissue was ground and solubilized in homogenization buffer (50 mM MOPS, 5 mM EDTA, 0.33 M sucrose, 0.2% (w/v) insoluble polyvinylpolypyrrolidone, 2 mM DTT, 1.5 mM ascorbate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.7 µM bestatin, 0.7 µM pepstatin A, 10 µM leupeptin, 1.4 µM E-64, 1.4 µM phenanthroline, 50 mM sodium pyrophosphate decahydrate, 25 mM sodium fluoride, 1 mM sodium molybdate, 1 mM sodium

orthovanadate, 25 mM β-glycerophosphate, pH= 5.6) at 4°C. Solubilized samples were filtered through two layers of cheesecloth and centrifuged for 15 min at 10.000 x g at 4°C to pellet cell debris. The supernatant was collected and centrifuged for 1 h at 100.000 x g at 4°C, and membrane pellets were frozen until further analysis.

In-solution trypsin digest and peptide concentration were performed as previously described (Minkoff *et al.*, 2014) with small modifications. In brief, membrane pellets were solubilized in 500 µL 8M Urea containing 1x PhosSTOP cocktail (Roche). The protein concentration in each sample was determined using the Pierce 660 nm Protein Assay (ThermoFisher Scientific). A total of 400 µg protein was diluted to 1 M Urea using 50 mM ammonium bicarbonate. DTT was added to the solution to a final concentration of 5 mM, and samples were incubated at 50°C for 45 min. Next, iodoacetamide was added to a final concentration of 15 mM and samples were incubated at room temperature in the dark for 45 min. Hereafter, trypsin was added in a ratio of 1:50 and proteins were digested by overnight incubation at 37°C while shaking. The digests were arrested by the addition of 0.3 % formic acid until samples reached a pH= 3. Peptides were concentrated using a Sep-Pak Vac 3cc 500 mg C18 column (Waters). After equilibration with 4 mL acetonitrile, the column was washed with 4 mL of 80% acetonitrile / 0.5% formic acid and thereafter with 6 mL 0.1% formic acid. Samples were passed through the column, flow-through was collected and once more passed through the column. The column was then washed with 6 mL 0.1% formic acid. Peptides were eluted slowly with 1.5 mL 80% acetonitrile / 0.5% formic acid and thereafter with 500 µL acetonitrile. Peptides were dried down by centrifugation in a speed vacuum and stored at -80°C .

Phosphopeptides were enriched using the Pierce Magnetic TiO_2 phosphopeptide enrichment kit (ThermoFisher Scientific). The manual approach protocol was followed with some alterations. In short, frozen peptides were solubilized in 300 µL 80% acetonitrile / 2% formic acid, 290 µL (~387 µg peptides) was then and added to 30 µL of prepared TiO_2 beads. The other 10 µL (~13 µg peptides) was freeze-dried and stored for total unenriched protein analysis. Samples were incubated with the beads for 20 min while shaking at room temperature. After washing three times with binding buffer and twice with washing buffer, captured phosphopeptides were eluted by incubating the beads in a shaker with 70 µL elution buffer at room temperature. Samples were centrifuged, the supernatant containing the phosphopeptides was transferred to a new tube and dried down by centrifugation in a speed vacuum. Samples were stored at -80°C until LC-MS/MS analysis.

LC-MS/MS on the QExactive

A Proxeon Easy-nLC II HPLC (ThermoFisher Scientific) with a Proxeon Nanospray Source was used in combination with a QExactive Plus Orbitrap mass spectrometer (ThermoFisher Scientific). Peptides were reconstituted in 2% acetonitrile and 0.1% formic acid and were trapped and washed on a

75 µm x 30 mm Michrom C18 trap. Subsequently, peptides were eluted and separated on a Michrom Magic C18AQ (200 µm x 150 mm) capillary reverse-phase column at a flow rate of 300 nL min^{-1}. A 120 min gradient was applied with a 2% to 35% B for 100 min, a 35% B to 80% B for 7 min and 80% B for 2 min. Subsequently a decrease of 80% to 5% B in 1 min followed by 98% A for 10 min. Solution A = 0.1% formic acid, solution B = 100% acetonitrile. The QExactive was operated in Data-Dependent Acquisition (DDA) mode using a top 15 method. Spray voltage was set to 2.2 kV, and the scan range was set to 350-1600 m/z. The maximum injection time was 30 ms. Automatic gain control was set to $1x10^6$. The MS1 resolution was set to 70,000. For MS/MS, the maximum injection time was 50 ms, the isolation window was 1.6 m/z, the scan range 200-2000 m/z, automatic gain control was set to $5x10^4$, and normalized collision energy was 27. The dynamic exclusion window was set to 15 sec, fragment MS2 resolution was 17,500. An intensity threshold of $1x10^4$ was applied, and the under-fill ratio was 1%. For unenriched total proteins, a 180 min gradient was applied, and the flow rates and volumes were adjusted accordingly.

Statistical analyses of differentially expressed phosphosites

Obtained phosphosite intensities in Maxquant were log2 transformed, and the sites matching contaminants or reverse database hits were removed. Next, phosphosites were filtered, and sites with a peptide Andromeda search engine score of >60, a phosphosite probability of >0.5 and a delta score of >5 were maintained. The phosphosite table was expanded using the "expand site table" function of Perseus. We kept and analyzed phosphosite identifications based on singly, doubly or triply phosphorylated phosphopeptides separately, indicated in the 'multiplicity' columns in Table S1. Hence some phosphosites occur more than once in our analysis (Fig. 2 and Table 1, S1-S3). The sequence window, encompassing the 15 N- and C-terminal amino acids surrounding the phosphorylated site as provided by Maxquant, was modified to highlight the phosphorylated residue in brackets and is shown in Table S1. Subsequently, we filtered for phosphosites for which at least two valid intensities were reported in the biological replicates for at least one-time point and treatment. Intensities were normalized by using the 'subtraction of the most frequent value (position of global maximum) function' per individual column. Missing intensity values were then imputed from a normal distribution per column using a width of 0.3 and 1.8 downshift to enable statistical analysis. ANOVA analysis was performed to determine statistically differentially regulated phosphosites. Phosphosites having an ANOVA p-value of ≤ 0.05 were kept and used for subsequent analysis. For generating an expression heat map, we performed Euclidean clustering per row on z-scored data using the complete linkage method in the heatmap.2 function in the statistical software R (https://www.r-project.org/). To obtain the ratios in Table S1 and highest intensities in Table S6, we followed the procedure described above except that

intensities were not normalized by subtracting the most frequent value. Log2 intensities were transformed to raw intensity values, and ratios were calculated.

Statistical analysis of differentially expressed total proteins

Raw files of the unenriched total protein samples were searched against a fasta database containing the *Arabidopsis thaliana* proteome. Detailed search parameters for Maxquant can be found in Table S5. The Maxquant Label-free quantitation (LFQ) algorithm and matching between runs were enabled (Cox *et al.*, 2014). Reverse hits, contaminants, and proteins only identified by modified peptides were removed. Protein LFQ intensities were log2 transformed, and we filtered for proteins with at least two valid intensities reported in the biological replicates for at least one time point and treatment. Missing intensity values were then imputed from a normal distribution per column using a width of 0.3 and 1.8 downshift to enable statistical analysis. ANOVA analysis ($p \leq 0.05$) was performed to determine differentially regulated proteins (Table S1). To determine protein ratios shown in Table S1, log2 intensities were transformed to raw intensity values.

Selected reaction monitoring (SRM) analysis

FLAG-RBOHD proteins were immunoprecipitated with anti-FLAG antibody from rbohD/35S:FLAG-RbohD, separated by SDS-PAGE (NuPAGe®, Invitrogen) and after staining with Coomassie Brilliant Blue G-250 CBB (SimplyBlue™ stain, Invitrogen), the proteins were cut out and digested by trypsin as described previously (Kadota *et al.*, 2014). Digested peptides were treated with H_2O_2 to oxidize the methionine residues as described previously (Kadota *et al.*, 2014). SRM of RBOHD phosphopeptides was performed by triple quadrupole MS. Transitions for phosphopeptides and control peptides, specified in Table S7, were selected based on fragmentation data acquired on an Orbitrap Fusion MS (Thermo Fisher) and imported and refined in the program Skyline (MacLean *et al.*, 2010). The peptides were analyzed using nano-spray ESI and a TQ-S MS (Waters Corp., MA, USA). The LC system consisted of a nano Acquity with a Symmetry trap (Waters, C18, 180 μm × 20 mm) to concentrate and desalt the peptides before elution to the analytical column (Waters, CSH 250 mm C18 columns, 75 μm inner diameter, 1.7 μm beads) at a flow rate of 250 nL min^{-1} with a gradient from 1% acetonitrile to 65% acetonitrile over 30 min. At least two replicate injections were performed. The resultant TQ-S files were imported into Skyline, and the peak definitions checked manually. The peak areas were then exported into Excel (Microsoft) for further analysis. The peak intensities of phosphopeptides were normalized against the intensity of control peptides (ADGGDGNLPFPK, DFADQLFR, and FADDLPK).

Semi-quantitative PCR analysis

Arabidopsis RNA was isolated using TRizol reagent according to the manufacturer's protocol (Invitrogen). RNA was quantified using nanodrop analysis, and equal amounts of RNA were used for cDNA synthesis using an oligo T primer and M-MLV Reverse Transcriptase using the companies protocol (Promega). As a control, we also performed the same reactions in the absence of M-MLV. Semi-quantitative PCR to determine *avrRpt2* expression in Arabidopsis was performed on 50 ng of cDNA. To confirm equal loading and quality of the DNA we also amplified endogenous *Actin*. PCR products were visualized under UV after separation on a 1% agarose gel containing ethidium bromide. The sequences of the primers used are listed in Table S8.

DAB-based ROS staining and luminol-based ROS burst assay

The ROS staining protocol was adapted from a described 3,3'-Diaminobenzidine (DAB, Sigma) staining protocol (Thordal-Christensen *et al.*, 1997). Arabidopsis leaves of 4-week-old plants were syringe infiltrated with *Pto DC3000* EV, *Pto DC3000* (*avrRpt2*) or *Pto DC3000* (*avrRpm1*) at a concentration of $2.5x10^7$ CFU mL^{-1}. As a control, leaves were infiltrated with 10mM $MgCl_2$ solution. Leaves were collected 2 h post bacterial infiltration and vacuum infiltrated with a 3,3′-Diaminobenzidine (DAB, Sigma) solution consisting of 1 mg mL^{-1} DAB at pH=3.8. Leaves were incubated under high humidity in a box in the dark for 6 h. Next, leaves were distained using a solution of ethanol, lactic acid and glycerol (ratio 3:1:1). For detection of PAMP-inducible ROS burst, eight leaf discs (4 mm diameter) from four 4-week-old Arabidopsis plants were sampled using a cork borer and floated overnight on sterile water. The following day the water was replaced with a solution of 17 mg mL^{-1} (w/v) luminol (Sigma) and 10 mg mL^{-1} horseradish peroxidase (Sigma) containing 200 nM flg22. Luminescence was captured over 40 min using a TriStar2 LB942 multiplate reader (Berthold) (Kadota et al., 2014).

Quantitative PCR analysis of Feruloyl CoA ortho-hydroxylase 1 (F6'H1) transcript levels

Five-week-old *Arabidopsis* plants were syringe infiltrated in the leaves with *Pto DC3000* (*avrRpt2*) (2.5×10^7 cfu mL^{-1}) or 10 mM $MgCl_2$ solution for 24 h. Gene expression of *F6'H1* in the infiltrated leaves was measured by qPCR analysis. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). The first-strand cDNA was synthesized with a ReverTra Ace-α- kit (Toyobo), and qPCR was performed using a Stratagene mx3000p real-time thermal cycler with a Thunderbird SYBR qPCR Mix kit (Toyobo; code A4251K). The relative transcript level of *F6'H1* was calculated with the standard curve method, followed by normalization to the *U-box* housekeeping gene transcript (*At5g15400*). The sequences of the primers used are listed in Table S8.

Western blotting

Unless indicated otherwise two leaf discs of Arabidopsis were ground in 150 µL 2x SDS-Laemmli buffer and homogenized. Samples were boiled for 5min in a heat block before a 5 min centrifugation step to remove any cell debris. Twenty microliters of each sample were separated by 10% SDS-PAGE. After gel electrophoresis, samples were transferred to a PVDF membrane (Immobilon-P) using semi-dry Western blotting. Membranes were blocked for 2 h in TBS (0.1 % Tween) containing 5% non-fat milk. The primary antibody was incubated overnight, and secondary antibodies were incubated for 2 h in TBS (0.1% Tween) containing 5% non-fat milk. In between incubations and before developing, blots were washed 5 times using TBS (0.1 % Tween). RIN4 was detected using anti-RIN4 (1:1000) as primary and goat-anti-Rabbit-HRP (1:2000) (BioRad) as secondary antibody. FLAG-tagged proteins were detected using anti-FLAG-HRP (1:3000) (Sigma). BIK1-HA was detected using anti-HA-HRP 3F10 (1:1000) (Roche). Detection of proteins was performed using the SuperSignal West Pico or Femto Chemiluminescent Substrate (ThermoFisher Scientific).

Pathogen strains and disease assays

The following bacterial strains were used in this study *Pseudomonas syringae pv. tomato* DC3000 transformed with pVSP61 (*Pto* DC3000 EV) , *pVSP61-AvrRpt2* (*Pto* DC3000 (*avrRpt2*)) and *pVSP61-AvrRpm1* (*Pto* DC3000 (*avrRpm1*)) (Kunkel *et al.*, 1993). Bacteria were propagated on LB media containing selection antibiotics. Five-week-old leaves of Arabidopsis were syringe-infiltrated with a 10 mM $MgCl_2$ solution containing bacteria at a concentration of $1x10^5$ CFU mL^{-1}. Three days after bacterial infiltration leaves were harvested for growth curves. Quantification of bacterial growth was performed as described previously (Kim *et al.*, 2005).

Plectosphaerella cucumerina (*Pc*) virulent isolate *PcBMM* was used for disease assays in Arabidopsis, which were performed as described previously (Torres *et al.*, 2013). After spray inoculation with *PcBMM* (4×10^6 spores mL^{-1}), Fungal DNA was quantified by qPCR at 6 dpi using specific primers for *Pcβ-tubulin* and normalized to Arabidopsis DNA using specific primers for *Ubiquitin10*. The sequences of the primers used are listed in Table S8.

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