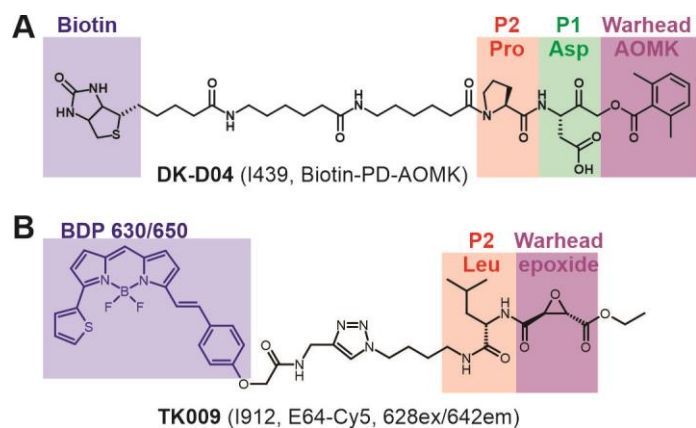


## New Phytologist Supporting Information

*Title: Activity-based proteomics uncovers suppressed hydrolases and a neo-functionalised antibacterial enzyme at the plant-pathogen interface.*

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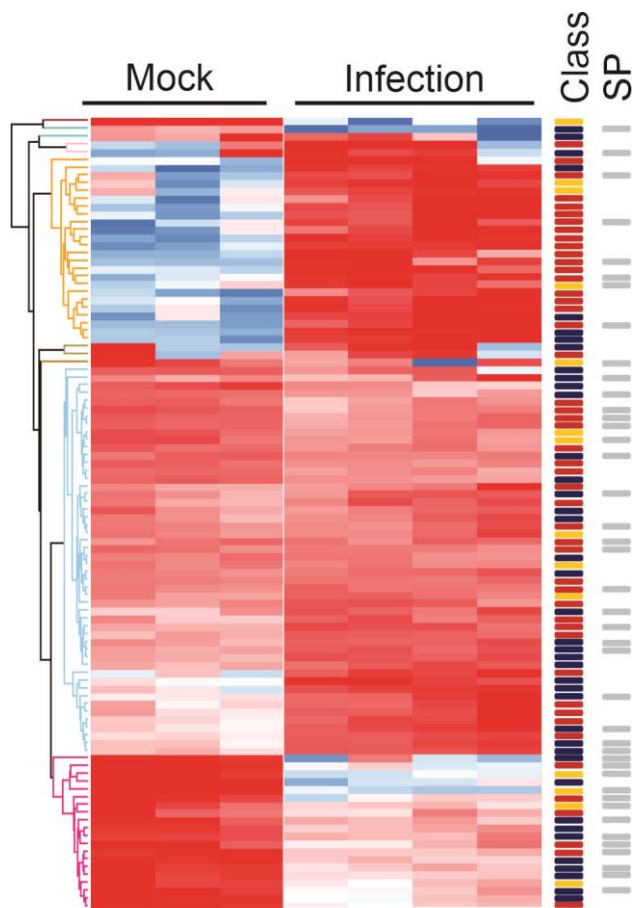
Article acceptance date: 16.2.2023.



**Figure S1** Structure of additional activity-based probes.

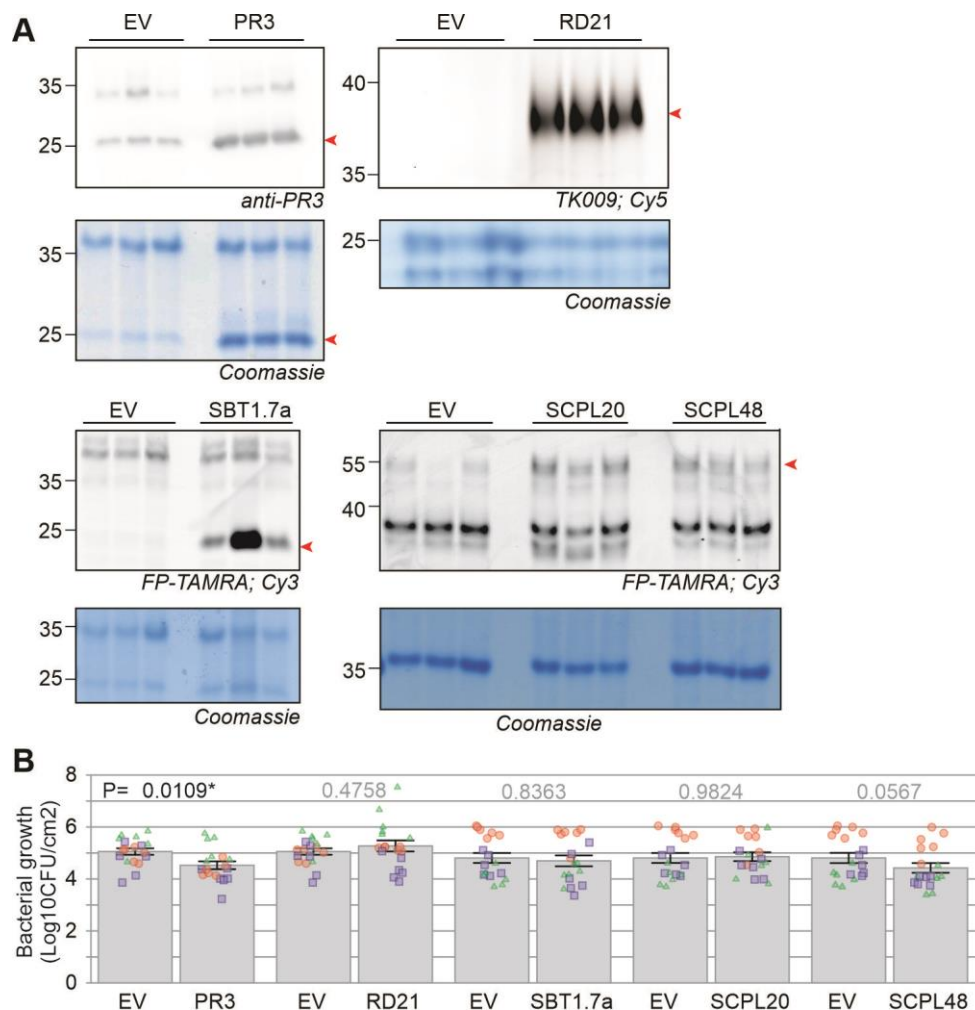
(A) Structure of DK-D04 (Biotin-PD-AOMK), a probe for biotinylation of VPEs.

(B) Structure of TK009 (Cy5-E64), a probe for fluorescent labelling of PLCPs.



**Figure S2.** Replicate activity-based proteomics experiment.

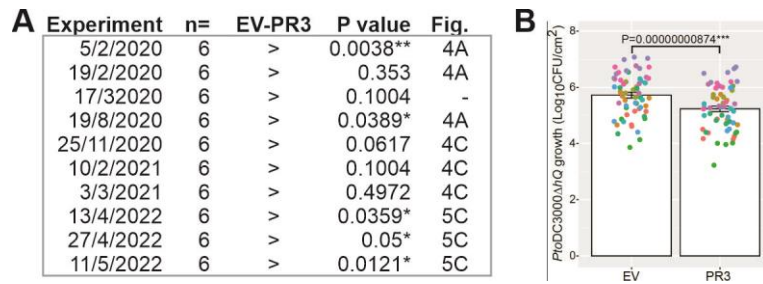
Heatmap of 105 active hydrolases detected by activity-based proteomics (experiment ACE136), grouped by category (left) and are annotated as SH(red) / GH(blue) / CP(yellow); hydrolases with a SignalP-predicted signal peptide are marked in grey (right). *N. benthamiana* plants were infected with *Pto*DC3000( $\Delta hQ$ ) or water (mock); apoplastic fluid was collected at 2 days post infection (2dpi). Apoplastic fluid was labelled for active serine hydrolases (SHs), glycosyl hydrolases (GHs) and cysteine proteases (CPs) followed by affinity purification of the labelled proteins and MS analysis.



**Figure S3** Transient hydrolase expression and agromonas assays.

(A) Confirmation of hydrolase expression. Hydrolase genes were transiently overexpressed by agroinfiltration of *N. benthamiana*. AF was isolated in triplicate at day-4 and labelled with fluorescent probes selective for papain-like proteases (TK009) or Ser hydrolases (FP-TAMRA). Labelled samples were separated on protein gels, which were scanned for fluorescence and stained with Coomassie Blue. *NbPR3* accumulation was detected with an anti-PR3 antibody and detected by Coomassie Blue staining.

(B) *NbPR3* reduces bacterial growth. Hydrolases were transiently overexpressed by agroinfiltrations. At day 2, agroinfiltrated leaves were infiltrated with  $10^6$  CFU/ml *PtoDC3000*( $\Delta hQ$ ). At day 5, leaf extracts were generated, diluted and spotted on plates containing CFC antibiotics to select for *P. syringae*. Bacterial growth is shown in Log<sub>10</sub>CFU/cm<sup>2</sup>. Bars show the mean value of 18 biological replicates performed over three separate experiments, and error intervals represent the standard error. P-values were calculated by two-way ANOVA followed by post-hoc comparison using the Dunnett test to examine the effect of hydrolase overexpression on bacterial growth.



**Figure S4** Ten replicate infection assays show increased resistance upon *NbPR3* expression.

**(A)** Ten experimental replicates, each having n=6 biological replicates. Bacterial growth on *NbPR3* expressing leaves was always lower (>) than the EV control, and statistically significant for 5 of the 10 experiments. **(B)** All (6x10=) 60 datapoints of the ten experiments summarised in one figure. The p-value was calculated by a 2-way ANOVA, using the experimental data as a block.

*NbPR3* and the empty vector (EV) control were transiently expressed by agroinfiltration. Two days later the same leaf was infiltrated with  $10^6$  CFU/ml bacteria and bacterial population densities were determined three days later, expressed  $\text{Log}_{10}\text{CFU}/\text{cm}^2$ .

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NbPR3 27 IGSIVTSDLFNEMLKNRNDVRCPANGFYTYDAFIAAANSFPGFGTTGDDTARRKEIAAFF 86
CHN-A 80 LGSILSSSMFDQMLKHRNDNACQKGFYSYNAFINAARSFPGFGTSGDITARKREIAAFF 139

NbPR3 87 GQTSHE TTGGSLSAE--PFTGGYCFVRQNDQ-----SERYYGRGPIQLTN 129
CHN-A 140 AQTSHET TGGWATAPDCPYAWGYCWLREQSGPGDYCTPSGQWPCAPGRKYFGRGPIQISH 199

NbPR3 130 RNNYEKAGTAIQDDLNNPDLVATDATISFKTAIWFWMTAQDNKPSSHDVIIGSWTPSAA 189
CHN-A 200 NYNYGPCGRAIGVDLLNNPDLVATDPVISFKSALWFWMTFQSPKPSCHDVIIGRWQPSAC 259

NbPR3 190 DQAANRVPGYGVITNIIINGGIECGMGRNDAVEDRIGYRRCGMLNVAPGENLDCYNQRN 249
CHN-A 260 DRAANRIPGFGVITNIIINGGLECGRGTDSRVQDRIGFYRRCSTLGVSPGDNLDCGNQRS 319

NbPR3 250 FAQG 253
CHN-A 320 FNGG 323

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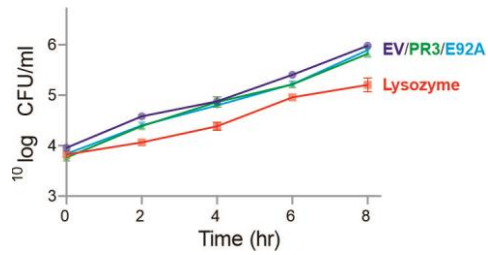
**Figure S5** Protein sequence alignment of *NbPR3* and endochitinase CHN-A.

The mature *NbPR3* protein sequence was aligned with the class I endochitinase CHN-A from *N. tabacum*. Residues that differ from *NbPR3* (grey), key catalytic residues (red) and other residues relevant for chitinase activity (green) are highlighted.

Nbenthamiana	1	-----MEFSGSPLVLFFCC	13
Ntabacum	1	-----MEFSGSPMALFFCC	13
Nattenuata	1	-----MEFSGSPLALFFCC	13
Nsylvestris	1	-----MEFSGSPMALFFCC	13
Ntomentosiformis	1	-----MEFSGSPLTLFFCC	13
Pepper	1	-----MKFLSSPLAL-TC	12
Potato	1	-----MEFLGSQMVLC	12
Tomato	1	-----MVLCC	5
Arabidopsis	1	MKTNLFLFLIFSLLLLSLSSAEQCGRQAGGALCPNGLCCSEFGWCGNTEPYCKQPGCQSQC	60
Rice	1	-----MTTTTTRFVQLAACAAAS	18
Nbenthamiana	14	VFFLFLTGSLAQGIGSIVTSDLFNEMLKNRNDVRCPANGFYTYDAFIAAANSFPGFGTTG	73
Ntabacum	14	VFFLFLTGSLAQGIGSIVTSDLFNEMLKNRNDGRCPANGFYTYDAFIAAANSFPGFGTTG	73
Nattenuata	14	VFFLFLTGSLAQGIGSIVTSDLFNEMLKNRNDGRCPANGFYTYDAFIAAANSFPGFGTTG	73
Nsylvestris	14	VFFLFLTGSLAQGIGSIVTSDLFNEMLKNRNDGRCPANGFYTYDAFIAAANSFPGFGTTG	73
Ntomentosiformis	14	VFFLFLTGSLAQGIGSIVTNDLFNEMLKNRNDGRCPANGFYTYDAFIAAANSFPGFGTSG	73
Pepper	12	VFFLFLTGSLAQDVGSIIVTNDLFNMLKNRDDRCPAKGFYTYDAFIAAANSFPGFGTTG	71
Potato	12	VFWLFLTGSLAQDVGSIIVTSDLFNEMLKNRNDRCPAKGFYTYDAFIGAANSFSGFGTTG	71
Tomato	6	VFLFLTGSLAQDVGSIIVTSDLFNEMLKNRNDRCPAKGFYTYDAFIAAANSFPGFGTTG	65
Arabidopsis	61	TPGGTP-PGPTGDLSGIISSSQFDMLKHRNDACPARGFYTYNAFIITAAKSFPGFGTTG	119
Rice	19	LLAVAASGAAQGVGSVITQAVFNSMLPNRDNSSQCPARGFYTYDAFIAAANSFPAFGTSG	78
Nbenthamiana	74	DDT-ARRKEIAAFFGQTSHEETTGGSL-SA-EPFTGGYCFVRQNDQ-----	115
Ntabacum	74	DDT-ARRKEIAAFFGQTSHEETTGGSL-SA-EPFTGGYCFVRQNDQ-----	115
Nattenuata	74	DDT-ARRKEIAAFFGQTSHEETTGGSL-SA-EPFTGGYCFVRQNDQ-----	115
Nsylvestris	74	DDT-ARRKEIAAFFGQTSHEETTGGSL-SA-EPFTGGYCFVRQNDQ-----	115
Ntomentosiformis	74	DDT-ARRKEIAAFFGQTSHEETTGGSL-SA-EPFTGGYCFVRQNDQ-----	115
Pepper	72	DDT-ARRKEIAAFFGQTSHEETTGGSL-SADGPFAGGYCFVREGNQ-----M	115
Potato	72	DDT-ARRKEIAAFFGQTSHEETTGGSL-SADGPFAGGYCFVREGNQ-----V	115
Tomato	66	DDT-ARRKEIAAFFGQTSHEETTGGSL-SADGPFAGGYCFVREGNQ-----M	109
Arabidopsis	120	DTA-TRKKEVAAFFGQTSHEETTGGWATAPDGPYSWGYCFKQEQNPASDYCEPSATWPCAS	178
Rice	79	GSAELIRRELAAFFGQTSHEETTGGTR-GSSDQFQWGYCFKEINKA-----T	124
Nbenthamiana	116	SERYYGRGPIQLTNRNNEYKAGTAIQDVLVNNPDLVATDATISFKTAIWFWMTAQDNKPS	175
Ntabacum	116	SERYYGRGPIQLTNRNNEYKAGTAIQDVLVNNPDLVATDATISFKTAIWFWMTPQDNKPS	175
Nattenuata	116	SERYYGRGPIQLTNRNNEYKAGTAIQDVLVNNPDLVATDATISFKTAIWFWMTPQDNKPS	175
Nsylvestris	116	SERYYGRGPIQLTNRNNEYKAGTAIQDVLVNNPDLVATDATISFKTAIWFWMTPQDNKPS	175
Ntomentosiformis	116	SERYYGRGPIQLTNRNNEYKAGNAIRQDVLVNNPDLVATDATISFKTAIWFWMTPQDNKPS	175
Pepper	116	GSGFYGRGPIQLTGQSNYDLAQQAIGQDLVNNPDLVATDATVDFKTAIWFWMTPQGNKPS	175
Potato	116	GSGFYGRGPIQLTGQSNYDLAQQAIGQDLVNNPDLVATDATVDFKTAIWFWMTPQGNKPS	175
Tomato	110	GSGFYGRGPIQLTGQSNYDLAQQAIGQDLVNNPDLVATDATVDFKTAIWFWMTPQGNKPS	169
Arabidopsis	179	GKRYYGRGPIQLSWNNYNYGLCGRAIGVDLLNPNPDLVANDAVIAFKAAIWFWMTPQPPKPS	238
Rice	125	SPPYYGRGPIQLTGQSNYQAAGNALGLDLVGNPDLVSTDAVVSFKTAIWFWMTPQGNKPS	184
Nbenthamiana	176	SHDVIIGRWTPSAADQAANRVPGYGVITNIINGGIECGMGRNDAVEDRIGYYRRCGMLN	235
Ntabacum	176	SHDVIIGRWTPSAADQAANRVPGYGVITNIINGGIECGIGRNDAVEDRIGYYRRCGMLN	235
Nattenuata	176	SHDVIIGRWTPSAADQSANRAPGYGVITNIINGGIECGIGRNDAVEDRIGYYRRCGMLN	235
Nsylvestris	176	SHDVIIGRWTPSAADQAANRVPGYGVITNIINGGIECGIGRNDAVEDRIGYYRRCGMLN	235
Ntomentosiformis	176	SHDVIIGRWTPSAADQSANRAPGYGVITNIINGGIECGVGNDAVEDRIGYYRRCGMLN	235
Pepper	176	CHDVIIGRWTPSAADTSANRQPGYGVITNIINGGIECGKQGNPQVEDRIGFYRYCYTILN	235
Potato	176	CHDVIIGRWTPSAADTSANRQPGYGVITNIINGGIECGKQGNPQVEDRIGFYRYCYTILN	235
Tomato	170	CHDVIIGRWTPSAADASANRQPGYGVITNIINGGIECGKQGNPQVEDRIGFYRYCYTILN	229
Arabidopsis	239	CHAVIAGQWQPSDADRAAGRLPGYGVITNIINGGLECGRGQDGRVADRIGFYRYCYNIFG	298
Rice	185	CHDVIIGRWTPSAADTAAGRVPYGVITNIINGGIECGVGNDAVDRIGYKRYCDMLG	244
Nbenthamiana	236	VAPGENLDCYNQRNFAQG-----	253
Ntabacum	236	VAPGENLDCYNQRNFGQG-----	253
Nattenuata	236	VAPGENLDCYNQRNFGQG-----	253
Nsylvestris	236	VAPGENLDCYNQRNFGQG-----	253
Ntomentosiformis	236	VAPGDNLDCYNQRNFAQG-----	253
Pepper	236	VAPGDNLDCYNQRNFAEA-----	253
Potato	236	VAPGDNLDCYTQRNFAEA-----	253
Tomato	230	VAPGDNLDCYDQRNFAEA-----	247
Arabidopsis	299	VNPGGNLDCYNQRSFVNGLLEAAI	322
Rice	245	AGYGSNLDCYNQRNFAS-----	261

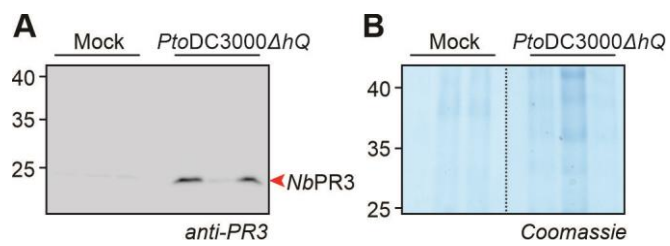
**Figure S6** Protein alignment of putative *NbPR3* orthologs from diverse plant species.

ClustalO alignment of putative *NbPR3* orthologs from several *Nicotiana* species, pepper, potato, tomato, *Arabidopsis thaliana* and rice. Differences with *NbPR3* (grey), key catalytic residues (red) and other residues relevant for chitinase activity (green) are highlighted.



**Figure S7** *NbPR3* does not impact bacterial growth *in vitro*.

AF from agroinfiltrated plants expressing *NbPR3* or its E92A mutant or the empty vector (EV) control, with or without added lysozyme (1 mg/ml), was incubated with OD = 0.001 *PtoDC3000*. Samples were taken at various time points and colony forming units (CFU) was determined by plating out dilution series. Error bars represent standard error of n=3 replicates.



**Figure S8** *NbPR3* accumulates in the apoplast upon infection.

*N. benthamiana* leaves were infiltrated with water (mock) or *PtoDC3000(ΔhQ)* and AF was isolated from three biological replicates at 2dpi and equal volumes were analysed by western blot with the anti-PR3 antibody (**A**) and on gel, stained with Coomassie Blue (**B**).