

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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Kadota & Liebrand et al., Figure S1

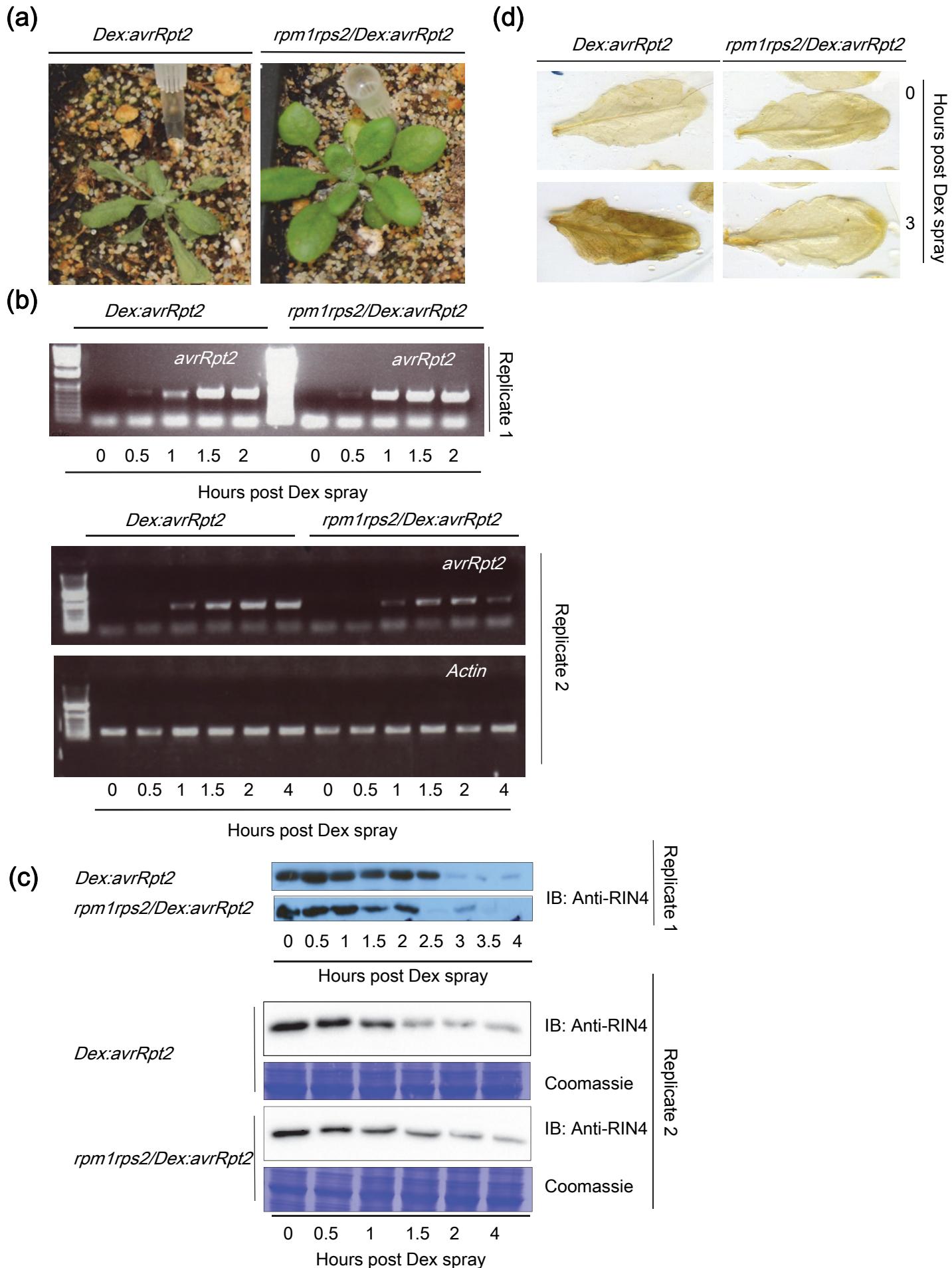


Fig. S1 Dexamethasone (Dex)-inducible *avrRpt2* expression in *Arabidopsis*. (a) Dex application to Col-0 *Dex:avrRpt2* plants results in visible cell-death and tissue collapse after 6-7 hours, while *rpm1rps2/Dex:avrRpt2* does not show cell death. Pictures were taken 7 hours post Dex application. (b) Semi-quantitative PCR analysis reveals Dex-induced *avrRpt2* expression as early as 0.5 hours after Dex application. Two replicate experiments representing a time series are shown, amplification of endogenous *Actin* was used as loading control in replicate 2 (c) Immunoblotting reveals AvrRpt2-mediated RIN4 degradation at 2-3 hours post Dex application. Two replicate experiments are shown, Coomassie stain of blots shows the rubisco band to indicate equal protein loading. (d) AvrRpt2 induces H₂O₂ production in *Dex:avrRpt2* but not in *rpm1rps2/Dex:avrRpt2*. H₂O₂ accumulation is analyzed by 3,3'-diaminobenzidine (DAB) staining.

Kadota & Liebrand et al., Figure S2

(a)

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AHA1(AT2G18960.1)859- AFTTKKDYGIGEREAQWAQAQRTLHGLQPKEVDNIFPEKGSYRELSEIAEQ
AHA2(AT4G30190.1)859- AFTMKKDYGKEEREAQWALAQRTLHGLQPKEAVNIFPEKGSYRELSEIAEQ
AHA3(AT5G57350.2)860- AFTTKQNYGIEEREAQWAHQRTLHGLQNTETANVPERGGYRELSEIANQ
AHA4(AT3G47950.1)871- AFTRQKDFGKEQRELQWAHQRTLHGLQAP-DTKMFTDRTHVSELNQMAEE
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AHA1(AT2G18960.1)910- AKRRAEIARLRELHTLKGHVESVAKLKGLDIDTAGHHYTV
AHA2(AT4G30190.1)910- AKRRAEIARLRELHTLKGHVESVVKLGLDIETP-SHYTV
AHA3(AT5G57350.2)911- AKRRAEIARLRELHTLKGHVESVVKLGLDIETA-GHYTV
AHA4(AT3G47950.1)921- AKRRAEIARLRELHTLKGHVESVVRLKGLDIETIQQAYTV
***** .:*****: ***
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(b)

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PIP2D(AT3G54820.1)251- WVGPFAGAAIAAFYHQFVLRAGAIKALGSFRSQPHV---
PIP2E(AT2G39010.1)251- WVGPFVGAAIAAFYHQFVLRAGAMKAYGSVRSQLHELHA
PIP3A(AT4G35100.2)245- WVGPFGLGALAAAAYHQYILRASAIKALGSFRSNATN---
***** * ** *** .:***.*:*** ** .**:
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(c)

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PIN3(AT1G70940.1)248- SDFYNMMGFPGGRLSNFGPADMYSVQSSRGPTPRPSNFEEENCAMASSPRF
PIN4(AT2G01420.1)245- SDFYSVMGFPGGRLSNFGPADLYSVQSSRGPTPRPSNFEEENNAVKYGFYN
PIN7(AT1G23080.2)251- SDFYSMMGFPGGRLSNFGPADMYSVQSSRGPTPRPSNFEEESCAMASSPRF
*** .:***** .:***** .:***** . *: .
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Fig. S2 Alignments of differentially phosphorylated sites in PIP, AHA and PIN proteins. Regions of differentially phosphosites of AHAs(a), PIPs(b) and PINs(c) were aligned using ClustalW. The residues in red are differentially phosphorylated residues after Dex treatment, whose total protein levels do not change significantly in abundance (see also Table S1). The residues in orange are differentially phosphorylated residues after dexamethasone treatment, for which total protein levels were not detected (see also Table S1). Identified phosphopeptides are underlined within the alignment (see also Table S6).

Kadota & Liebrand et al., Figure S3

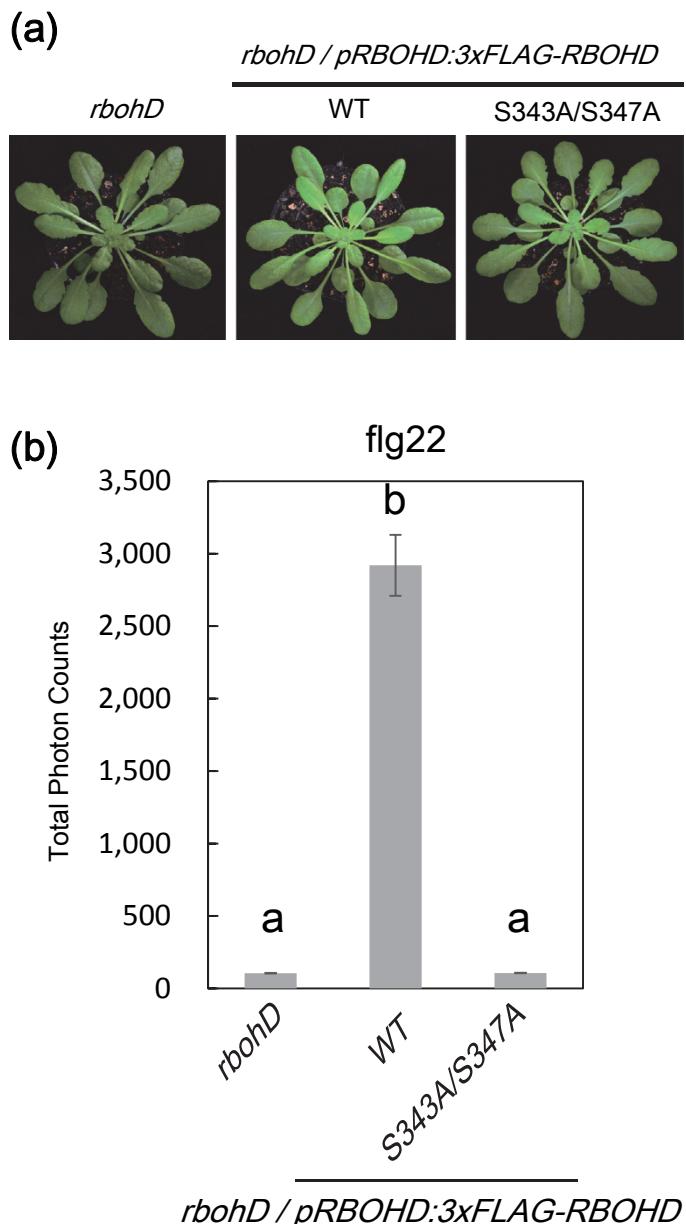


Fig. S3 RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD) phosphorylation sites S343 and S347 are required for flagellin 22 (flg22)-inducible reactive oxygen species (ROS) burst. (a) There is no growth phenotype in *rbohD* and *rbohD/pRBOHD:3xFLAG-RBOHD* (WT) and *rbohD pRBOHD:3xFLAG-RBOHD* (S343A/S347A) lines. (b) Phosphorylation of S343 and S347 is required for flg22-inducible ROS burst in *Arabidopsis*. Leaf discs were treated with 200 nM flg22 and the ROS production was measured over 40 min. Values are mean \pm SE ($n = 8$). Different letters indicate significantly different values at $p \leq 0.001$ (one way ANOVA, Tukey post hoc test). The experiments were performed three times with similar results.

Kadota & Liebrand et al., Figure S4

AtRbohD1-921	1 MKMRRGNNSSND ELG I LRGANS DTS DTES ASD RGA F -- SGPL GRP -- KRASK K NARF ADDL PKRSN --- SVA DGRGD DDE YVE TLD I RD SV A VH S V QQA AGGGGH 102
NtRbohD1-938	1 M --- QNSE NH P --- HHQHHHHS DTB I GND RASY -- SGPL SGPL NKR GCK K S ARF -- NI ESTD I G TS V GT GKS NDDA YVE TLD V RED SV A VH S V KTA -- BGDD 94
SIRbohD1-938	1 M --- QNSE NH P --- HHH HHS DTB I GND RASY -- SGPL SGPL NKR GCK K S ARF -- NI ESTD I G TS V AGA LAKS N DD A YVE TLD V RED SV A VH S V KTA -- GGAD 94
OsRbohB1-905	1 M --- A DLE AGV --- AAAT DCG NS TRS QD AATL I P SGNL GSS -- NRST I K T ARF --- KDD DEL VE TLD V O RDS V A IC E V RG DECCS-78
AtRbohD1-921	103 LEDPEL ALL T K T L E S S L N N T S L S F F R S T S S R I K N A S R E L R R V -- F S R R P S AV --- RRF DRT T S S A A I H A L K G L K F I A T K T -- A A W P A V D Q R F D K L S A D S N G L L 201
NtRbohD1-938	95 V E D P E L A L L A K G L E K K S -- T L G S A L V R N A S S R I R Q V S Q E U R R L A S L N K R P I T G --- RFD R N S A A A H A L K G L K F I S K T D G G A G W A A V E K R F D E I T A S T T G L P 193
SIRbohD1-938	95 V E D P E L A L L A K G L E K K S -- T L G S A L V R N A S S R I R Q V S Q E U R R L A S L N K R P I T G --- RFD R N S A A A H A L K G L K F I S K T D G G A G W A A V E K R F D E I T A S T T G L P 193
OsRbohB1-905	79 --- G H G T G F D S L P L S P S K S Q K L T S K L R Q V T N G L K M K S S R K A P S Q A Q Q S A K V R V K R L D R T S A A V A L K G L Q F V T A K V N D G W A A V E K R F N Q L Q D - C V L L 178
AtRbohD1-921	202 S A K F W E C L G M N K E S K D F A D Q F R A L A R R N V S C D A I T K E Q L R I F W E Q I S D E S F D A K L Q V F F D M V D K D E D G R V T E E E V A E I S L S A S A N K L S N I Q K Q A K E Y A A L I M E E L D P D 312
NtRbohD1-938	194 R A K F G E C I G M N K E S K E F A V E Y D A L A R R N I T T D S I N A Q A L K E F W D C V A D Q S F D S R L Q T F F D M V D K D A D G R I T E E E V R E I G L S A S A N R L S T I Q K Q A D E Y A A M I M E E L D P N 304
SIRbohD1-938	194 R A K F G E C I G M N K E S K E F A V E Y D A L A R R N I T T D S I N A Q A L K E F W D C V A D Q S F D S R L Q T F F D M V D K D A D G R I T E E E V R E I G L S A S A N R L S T I Q K Q S D E Y A A M I M E E L D P N 304
OsRbohB1-905	179 R S R F G K C I G M D - G S D E F A V Q F D S L A R K R G I V K O V L T D E L K D F E E Q L T Q G F D N P R L R T F D M V D K N A G R L T A E V K E L L A S A S A N K L S K I K E R A D E Y T A L I M E E L D P T 288
AtRbohD1-921	313 N A G F I M I E N L E M L L Q A P Q N S V - R M G D I - L S Q M L S D Q K U R P A K E S N P L V R V S E K I K Y F I L D N W O R L W M M L W L G C G G L F T Y R F I Q Y N K A A Y G V M Q C V G V A K G G A E 420
NtRbohD1-938	305 N L G Y I M I E N L E M L L Q A P Q N S V Q M C G E S S E N - L S Q M L S D Q K U H T O E R N P I V R V Y K S M F Y F L L D N W O R V W L L L W I G M A L G F T W K Y I Q Y E K A A Y K V M P C V G C K G A A E 413
SIRbohD1-938	305 N L G Y I M I E N L E M L L Q A P Q N S V Q M C G E S S E N - L S Q M L S D Q K U H T O E P N P L V R V Y K S F K Y F L L D N W O R V W L L W I G M A L G F T W K Y I Q Y K Q A A Y G V M Q C V G C L A K G A A E 413
OsRbohB1-905	289 N L G Y I M E D L E A L L L Q S P S E A A M S T T U N S S K S K A L S M K U A S N K E M S V R H W Q Q M Y F L E E N W R S W M L W S C I A L F I W F I O Y E N A V F G I M G Y C T T A K G A E 399
AtRbohD1-921	421 L K F I M A L I L L P V C R N T I T W L R N K I K L G T V V P P D D S L N F H K V I A S G I V V D V L L A G A H L T C D F P R L I A A D E D T Y E P M E X F G D - P T S Y W M F V K G V E G W T G I V M V V L M A F 530
NtRbohD1-938	414 L K L I M A L I L L P V C R N T I T W L R N K I R L G A A P P F D D N L N F H K V I A V A L G V G I G L S H L T C D F P R L L N A S E E Y E P M Y F G D - P T S Y W M F V K G V E G W T G I V M V L M A F 523
SIRbohD1-938	414 L K L I M A L I L L P V C R N T I T W L R N K I R L G A A P P F D D N L N F H K V I A V A L G V G I G L A H L T C D F P R L L N A S E E Y E P M Y F G D - P T S Y W M F V K G V E G W T G I V M V L M A F 523
OsRbohB1-905	400 L K F I M A L I L L P V C R N T I T W I R S K T Q G A V V P F N D N I N F H K V I A G V V G A L A G A H L T C D F P R L L H A S D A Q Y E L M K P F G E I R P P Y W W F V K G T G W T G V V M V L M A F 510
AtRbohD1-921	531 T L A T P W F F R R N K L K N L P N L K L T G F N A F W Y T H H L F I I V Y A L I V H G I K L Y L T K I V Y G K I T W M Y L A F P I I L Y A S E R L L R A F R S S I K P V K M I K V A V Y P G N V L S L H M T K P Q G F K N 641
NtRbohD1-938	524 T L A T P W F F R R N R V S L P K F H K L T G F N A F W Y T H H L F I I V Y T L F I V H G E K Y I T K Y K R I T W M Y L T P I I L Y A S E R L I R A F R S S I K V A V Y P G N V L A L H M S K P Q G F K N 634
SIRbohD1-938	524 T L A T P W F F R R G R V S F P F K F H K L T G F N A F W Y T H H L F I I V Y T L F I V H G E K Y I T K Y K R I T W M Y L T P I I L Y A S E R L I R A F R S S I K V A V Y P G N V L A L H M S K P Q G F K N 634
OsRbohB1-905	511 T L A Q P W F F R R N K L K D S N I L K K M T G F N A F W Y T H H L F I I V Y T L F I V H G E K Y I T K Y K R I T W M Y L T P I I L Y A S E R L I R R F S H I D A V G I Q V A V Y P G N V L A L Y M S K P Q G F K N 620
AtRbohD1-921	642 K S G Q F M L V N C R A V S P F E W H P F S I T S A P G D D Y L S V H I R T L G D W T R K L R T V F S E V C K P I T A G K S G L L R A D - G G - D G N L P F P K V L I D G P Y G A P Q D Y K K N D V V L L V G L G A 748
NtRbohD1-938	635 K S G Q Y M F V N C A A V S P F E W H P F S I T S A P G D D Y L S V H I R T L G D W T R Q L K T V F S E V C Q P I P N G K S G L L R A D Y L Q G - E N N P N F P R Y L I D G P Y G A P Q D Y K K N E V V L L V G L G A 743
SIRbohD1-938	635 K S G Q Y M F V N C A A V S P F E W H P F S I T S A P G D D Y L S V H I R T L G D W T R Q L K T V F S E V C Q P I P N G K S G L L R A D Y L Q G - E N N P N F P R Y L I D G P Y G A P Q D Y K K N E V V L L V G L G A 743
OsRbohB1-905	621 R S G Q Y I F I K C T A V S P Y E W H P F S I T S A P G D D Y L S V H I P T R G D W T S P I T V F S E A C R P I T E G E S G L L R A D L S K C I T D E L A R F B K L V D G P Y G A P Q D Y R E I D V L L I G L G I 731
AtRbohD1-921	749 T P M I S I L K D I I I N N M K G P D R D - - - - S D I E N N S N N - - N S K G I I F K T R K A Y F Y W V T R E Q G S F E W F K G I M D E I S I L D E E C I I B L H N Y C T S V Y E E G D A R Y 836
NtRbohD1-938	744 T P M I S I V K D I V N N M K A M D E E E N S L E D G H N - - N N M A P N S S P I A K N K G N K G S A S G G N N I N T R R A Y F Y W V T R E Q G S F D W F K G I M N E A E S M D H K G V I E B M H N Y C T S V Y E E G D A R S 853
SIRbohD1-938	744 T P M I S I V K D I V N N M K A M D E E E N S L E D G H N - - N N M A P N S S P I A K N K G N K G S A S G R N S I N T R R A Y F Y W V T R E Q G S F D W F K G I M N E A E S M D H K G V I E B M H N Y C T S V Y E E G D A R S 853
OsRbohB1-905	732 T P L I S I V K D V L N H I O G E C S V - - - - G T T E P E S S K A K K P E - - E M T K R A Y F Y W T R E G S F E W F R G V M N E V S E I S D K D V I E B L H N H C S S Y Q E G D A R S 820
AtRbohD1-921	837 A L I A M L Q S L Q H A K N G V D V V S G T R V K S H F A K P N W R Q V Y K K I I V Q H I G K R I G F V Y C G M G M I K E K K I L A L D F S R K T I T T K F D H K E N 921
NtRbohD1-938	854 A L I A M L Q S L Q H A K N G V D V V S G T R V K S H F A K P N W R Q V Y K K I I V A L N H F E A K V G V F Y C G A F A L T K E L K R O H A L D F S R K T I T T K F D H K E N 938
SIRbohD1-938	854 A L I A M L Q S L Q H A K N G V D V V S G T R V K S H F A K P N W R Q V Y K K I I V A L N H F E A K V G V F Y C G A F A L T K E L K R O H A L D F S R K T I T T K F D H K E N 938
OsRbohB1-905	821 A L I A M L Q S L Q H A K N G V D V V S G T R V K S H F A K P N W R Q V Y K K I I V A S H E N Q R V G V F Y C G E G V L V P Q R Q I S A D F T H K T I T T K F D H K E N 905

Fig. S4 Conservation of identified RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD) phosphorylation sites in the phosphoproteomic screen. An alignment of *Arabidopsis* RBOHD (AtRBOHD), *Nicotiana tabacum* RBOHD (NtRBOHD), *Solanum lycopersicum* RBOHD (SIRBOHD) and the rice RBOHD ortholog *Oryza sativa* RBOHB (OsRBOHB) is provided. Phosphorylation sites whose phosphorylation were significantly upregulated after dexamethasone treatment (one-way ANOVA+T-test p≤0.05) are shown with asterisks. Phosphorylation sites identified as differentially phosphorylated during PTI in previous studies are indicated with a square red box.

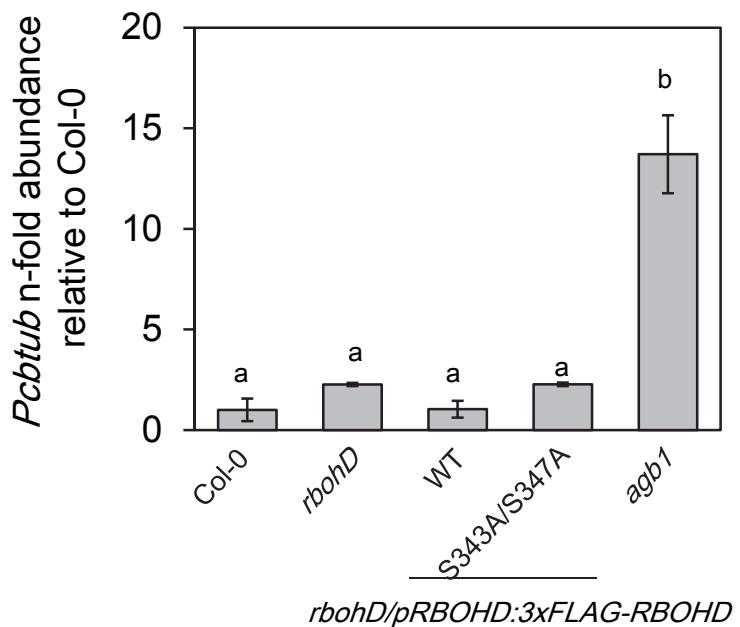


Fig. S5 The *rbohD/pRBOHD:3xFLAG-RBOHD* (S343A/SS347A) complementation line is resistant to *Plectosphaerella cucumerina* (*PcBMM* isolate). Quantification of fungal biomass 5 dpi after spray inoculation with *PcBMM* isolate (4×10^6 spores/ml) on 18 day old plants. Fungal DNA (*Pcβ*-tubulin) was quantified by qPCR using specific primers for *Pcβ-TUBULIN* and normalized to Arabidopsis *UBQUITIN10*. Bars represent averages (\pm SE) of fungal DNA levels relative to Col-0 plants, from one of two independent repetitions of the assay. Statistical analysis was performed by ANOVA , corrected with Bonferroni post hoc test. Different letters indicate significant differences ($p\leq 0.05$).

Kadota & Liebrand et al., Figure S6

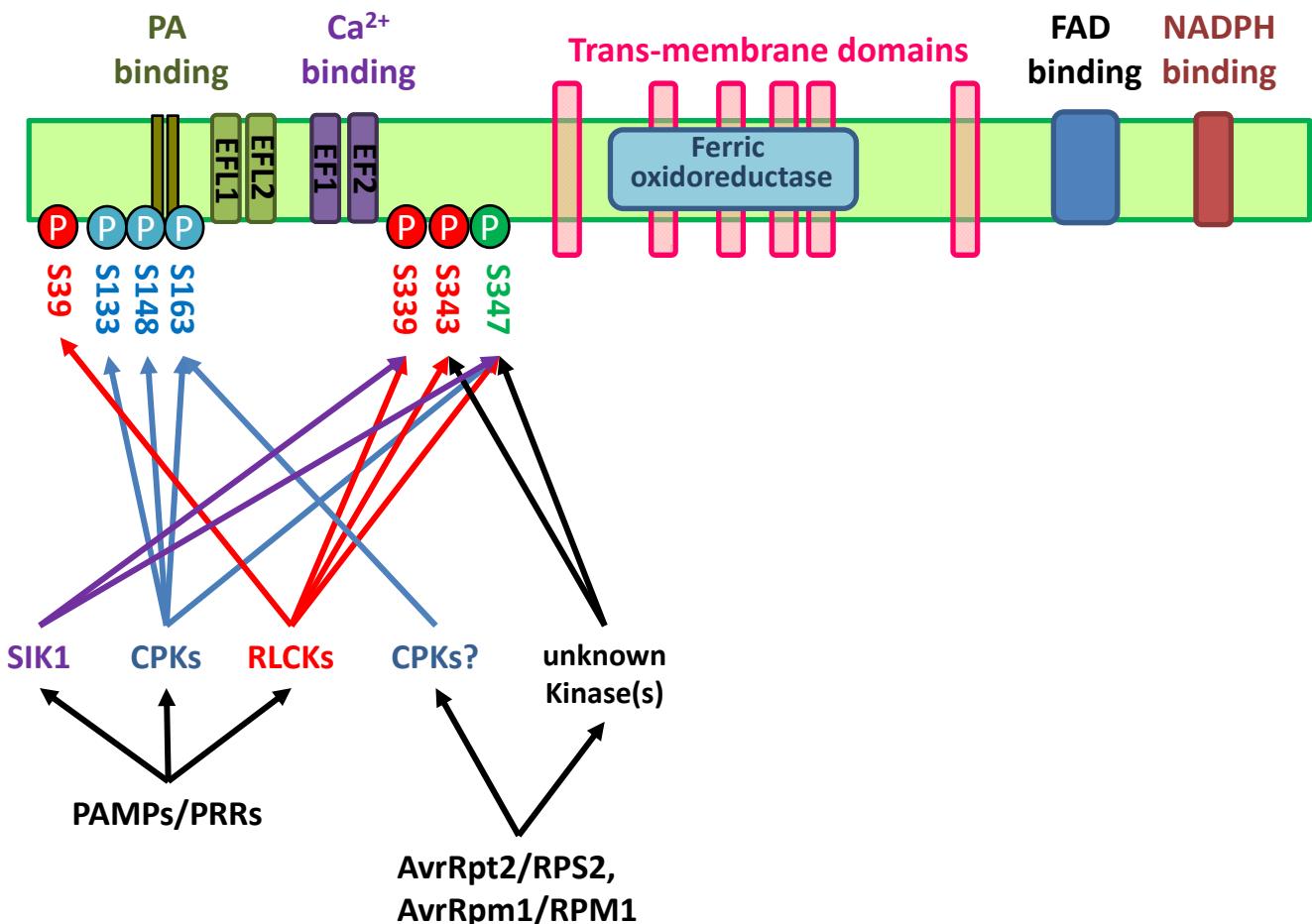


Fig. S6 Schematic representation of RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD) domains and phosphorylation sites significantly regulated during PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). RBOHD has two EF-hand motifs (EF1 and EF2), two EF-hand like motifs (EFL1 and EFL2) and possible phosphatidic acid (PA) binding sites in its N-terminal part, followed by six transmembrane domains encompassing the functional ferric oxidoreductase domain, one FAD-binding site, and one NADPH-binding site. During PTI, BIK1 phosphorylates residues S39, S339 and S343 (indicated in red), while CPKs phosphorylate residues S133, S148 and S163 (indicated in blue). MAP4K SIK1 also contributes to the phosphorylation on S339 and S347 during PTI. The residue S347 (indicated in green) is phosphorylated by both BIK1 and CPKs. We found that RESISTANT TO *P. SYRINGAE*-2 (RPS2) activation by AvrRpt2 induced phosphorylation at S163, S343, and S347. We validated that *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*) induced phosphorylation at S343 and S347, while the kinase(s) phosphorylating these sites remain to be identified.