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Research

Diverse NLR immune receptors activate defence via the RPW8-NLR NRG1

Baptiste Castel[1], Pok-Man Ngou[1], Volkan Cevik[1,2], Amey Redkar[1,3], Dae-Sung Kim[1,4], Ying Yang[1,5], Pingtao Ding[1] and Jonathan D. G. Jones[1]

[1]The Sainsbury Laboratory, University of East Anglia, Norwich Research Park, Colney Lane, Norwich, NR4 7UH, UK; [2]The Milner Centre for Evolution, Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, UK; [3]Department of Genetics, University of Córdoba, Córdoba 14071, Spain; [4]Department of Plant Sciences, College of Life Sciences, Wuhan University, Wuhan 430072, China; [5]Center for Plant Science Innovation, Beadle Center, University of Lincoln-Nebraska, Lincoln, NE 68588, USA

Author for correspondence:
Jonathan D. G. Jones
Tel: +44 1603 450327
Email: jonathan.jones@tsl.ac.uk

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Summary

- Most land plant genomes carry genes that encode RPW8-NLR Resistance (R) proteins. Angiosperms carry two RPW8-NLR subclasses: ADR1 and NRG1. ADR1s act as 'helper' NLRs for multiple TIR- and CC-NLR R proteins in Arabidopsis. In angiosperm families, *NRG1* co-occurs with *TIR-NLR Resistance* (*R*) genes. We tested whether NRG1 is required for signalling of multiple TIR-NLRs.
- Using CRISPR mutagenesis, we obtained an *nrg1a-nrg1b* double mutant in two Arabidopsis accessions, and an *nrg1* mutant in *Nicotiana benthamiana*.
- These mutants are compromised in signalling of all TIR-NLRs tested, including WRR4A, WRR4B, RPP1, RPP2, RPP4 and the pairs RRS1/RPS4, RRS1B/RPS4B, CHS1/SOC3 and CHS3/CSA1. In Arabidopsis, NRG1 is required for the hypersensitive cell death response (HR) and full oomycete resistance, but not for salicylic acid induction or bacterial resistance. By contrast, *nrg1* loss of function does not compromise the CC-NLR R proteins RPS5 and MLA. RPM1 and RPS2 (CC-NLRs) function is slightly compromised in an *nrg1* mutant. Thus, NRG1 is required for full TIR-NLR function and contributes to the signalling of some CC-NLRs.
- Some NRG1-dependent R proteins also signal partially via the NRG1 sister clade, ADR1. We propose that some NLRs signal via NRG1 only, some via ADR1 only and some via both or neither.

Introduction

The plant immune system involves both cell surface receptors that detect extracellular pathogen-associated molecular patterns (PAMPs) and intracellular receptors that detect pathogen 'effector' proteins which, if not detected, usually contribute to pathogen virulence (Jones & Dangl, 2006). Most *Resistance* (*R*) genes cloned encode nucleotide-binding, leucine-rich repeat (NLR) immune receptors (Kourelis & Van Der Hoorn, 2018). These receptors are widely deployed during breeding for crop disease resistance (Borhan *et al.*, 2010; Jones *et al.*, 2014; Witek *et al.*, 2016). A better understanding of NLR-mediated immunity could facilitate their deployment to safeguard crops from pathogens, reducing the need for chemical applications.

Most NLRs comprise an N-terminal domain, a central nucleotide-binding (NB) domain shared by apoptotic protease activating factor 1 (APAF-1), plant Resistance (R) proteins and a cell death protein 4 (CED-4) domain (NB-ARC), and C-terminal leucine-rich repeats (LRRs). At their N-termini, they usually carry a Toll/Interleukin-1 receptor/Resistance protein (TIR) domain or non-TIR N-terminus, often with Coiled-Coils (CC). A phylogenetically distinct NLR subset carries an N-terminal Resistance to Powdery Mildew 8 (RPW8) domain. The corresponding TIR-NLR, CC-NLR and RPW8-NLR proteins, on activation, trigger a complex network of responses, including gene induction, the production of reactive oxygen species (ROS) and salicylic acid (SA), transcriptional reprogramming and a form of cell death called the 'hypersensitive response' (HR), resulting in resistance (Jones & Dangl, 2006).

The RPW8-NLR NbNRG1 was first identified in *Nicotiana benthamiana* (*Nb*) as required for resistance to *Tobacco mosaic virus* (TMV) mediated by the *N* gene (Peart *et al.*, 2005). *N* encodes a TIR-NLR that activates resistance on recognition of the TMV replicase component p50. NRG1 is widespread in angiosperms, suggesting an important role in immunity (Collier *et al.*, 2011; Shao *et al.*, 2016).

Two additional 'helpers' have been described in plants. ADR1 is part of a conserved clade within angiosperms and contributes to the function of RPP2, RPP4, RPS2, SNC1, CHS3 and RRS1 in *Arabidopsis thaliana* (Arabidopsis) (Bonardi *et al.*, 2011; Dong *et al.*, 2016). Interestingly, ADR1s are also RPW8-NLRs, phylogenetically close to the NRG1 clade (Collier *et al.*, 2011; Shao *et al.*, 2016). The NRC helper NLRs in Solanaceae are required for the sensor NLRs Rpi-blb2, Mi2-5, Sw5b, R8, R1, Prf, Rx,

Bs2 and CNL-119900, amongst others (Wu *et al.*, 2017). In both cases, the helper clade is relatively conserved and partially redundant, whereas their associated sensors are expanded and diversified. Phylogenetic analyses reveal that the conserved NRG1 clade and TIR-NLRs co-occur in angiosperm genomes: all clades that lack NRG1 also lack TIR-NLRs (Collier *et al.*, 2011; Shao *et al.*, 2016). Conceivably, all RPW8-NLRs are helper NLRs, with ADR1 signalling downstream of CC-NLRs and TIR-NLRs, and NRG1s signalling downstream of TIR-NLRs only.

We set out to test whether NRG1 is a helper for other TIR-NLRs. We generated *nrg1a-nrg1b* loss-of-function double mutants in Arabidopsis and a mutant of the single *NRG1* copy in *N. benthamiana* using clustered and regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated9 (Cas9), and tested for loss of TIR-NLR activities (Li *et al.*, 2013). We found that NRG1 is at least partially required for the signalling of the TIR-NLRs WRR4A, WRR4B, RPP1, RPP2 and RPP4, the NLR pairs CHS1/SOC3, CHS3/CSA1 and RRS1/RPS4, and the CC-NLRs RPS2 and RPM1, but is not required for the signalling of the CC-NLRs RPS5 and MLA7. Other recent studies have also suggested a requirement of NRG1 for RPP1-mediated signalling and have identified Roq1, an additional NRG1-dependent TIR-NLR (Brendolise *et al.*, 2018; Qi *et al.*, 2018). Surprisingly, we found that NRG1 is required for RRS1/RPS4-mediated HR, but not bacterial disease resistance conferred by RRS1/RPS4. We discuss the potentially complementary roles of NRG1 and its sister clade ADR1. We propose that some NLRs signal via NRG1 only (e.g. CSA1/CHS3), some via ADR1 only (e.g. RPS2) and some via both (e.g. RRS1/RPS4).

Materials and Methods

Plant material and growth conditions

The *A. thaliana* (Arabidopsis) accessions used in this study are Wassilewskija-2 (Ws-2) and Columbia-0 (Col-0). Ws-2_*rrs1-1-rps4-21-rps4b-1* (Saucet *et al.*, 2015), Ws-2_*eds1-1* (Parker *et al.*, 1996) and Col-0_*eds1-2* (Falk *et al.*, 1999) have been published. Seeds were sown directly on compost and plants were grown at 21°C, with 10 h of light and 14 h of dark, at 75% humidity. For seed collection, 5-wk-old plants were transferred under long-day conditions: 21°C, with 16 h of light and 8 h of dark, at 75% humidity. For *N. benthamiana*, seeds were sown directly on compost and plants were grown at 21°C, with cycles of 16 h of light and 8 h of dark, at 55% humidity.

CRISPR-mediated generation of knock-out alleles

Four CRISPR constructs (*CRISPR-1*, *CRISPR-2*, *CRISPR-3* and *CRISPR-4*) were assembled using the Golden Gate cloning method and expressed via *Agrobacterium tumefaciens* strain GV3101 in Arabidopsis Ws-2, Col-0 or *N. benthamiana*. For *CRISPR-1*, three sgRNAs targeting both *AtNRG1A* and *AtNRG1B* (GCTCATTACCAAACCTGAAA[nGG], GTGGAAAGCTGGTCTGAAG[nGG] and GATGATTTGTTCTCATCGAAA[nGG]) were designed and assembled by PCR to an sgRNA backbone in a Golden Gate-compatible fashion (Supporting Information Methods S1 and Castel *et al.*, 2018). sgRNAs were assembled with the *AtU6-26* promoter in the Golden Gate-compatible level 1 vectors *pICH47751*, *pICH47761* and *pICH47772*, respectively. A human codon-optimized allele of *Cas9* was assembled with the *AtUBI10* promoter and *Nos* terminator in Golden Gate-compatible level 1 vector *pICH47742*. These four level 1 vectors, together with a FAST-Red selectable marker in level 1 vector *pICH47732*, were assembled in the binary vector *pAGM4723*, resulting in the *CRISPR-1* final vector. For *CRISPR-2*, only one sgRNA targeting both *AtNRG1A* and *AtNRG1B* (GTGGAAAGCTGGTCTGAAG[nGG]) was used. A plant codon-optimized allele of *Cas9* containing a potato *IV2* intron was assembled with the *AtRPS5a* promoter and *Pisum sativum rbcS E9* terminator in *pICH47811*. They were cloned in the binary vector *pICSL4723* in a similar fashion as *CRISPR-1*, resulting in the *CRISPR-2* final vector. For *CRISPR-3*, one sgRNA targeting *NbNRG1* (CAGTATTCGATGACATCGAG[nGG]) was used. A human codon-optimized allele of *Cas9* was assembled with the *35S* promoter and *Mas* terminator in Golden Gate-compatible level 1 vector *pICH47742*. The sgRNA and Cas9 expression vectors, together with a glufosinate resistance plant-selectable marker in level 1 vector *pICH47732*, were assembled in *pICSL4723*, resulting in the *CRISPR-3* final vector. For *CRISPR-4*, two sgRNAs targeting *WRR4B* in Ws-2 (AATCGCTTCCGTGAGAGCTG[nGG] and TACATAGTGTACTATCTAAA[nGG]) were used. A plant codon-optimized allele of *Cas9* was assembled with the *AtUBI10* promoter and *Ocs* terminator in Golden Gate-compatible level 1 vector *pICH47742*. The sgRNA and Cas9 expression vectors, together with a FAST-Red selectable marker in level 1 vector *pICH47732*, were assembled in *pAGM4723*, resulting in the *CRISPR-4* final vector. For *CRISPR-2* sgRNA specifically, 67 bp of the *AtU6-26* terminator was included by PCR at the sgRNA 3′-end. Expression of *CRISPR-1* in Arabidopsis Ws-2 resulted in c.1153delG and c.1159_1160insT mutations in *NRG1A* and *NRG1B*, respectively. Expression of *CRISPR-2* in Arabidopsis Col-0 resulted in c.1153delG and c.1159_1160insTATTTTTGGTCCTC mutations in *NRG1A* and *NRG1B*, respectively. Expression of *CRISPR-3* in *N. benthamiana* resulted in a c.638_644delAAGAGAA mutation in *NbNRG1*. Expression of *CRISPR-4* in Arabidopsis Ws-2 resulted in a c.181_182insA mutation in *WRR4B*. All of these mutations cause a codon reading frame shift and early stop codons in or before the NB-ARC domain encoding region. The progeny of the mutants were analysed and we selected lines without T-DNA and *NRG1* mutation at the homozygous state. We refer to these lines as Ws-2_*nrg1a-nrg1b*, Col-0_*nrg1a-nrg1b*, *N. benthamiana*_*nrg1* and Ws-2_*wrr4b*.

Gene cloning and expression

CHS1 was cloned in a '35S–Ocs' expression cassette with a C-terminal V5 tag via the USER method (Geu-Flores *et al.*, 2007). Briefly, *CHS1* was PCR amplified from Col-0 genomic DNA template with GGCTTAAUATGTCTACTTCTTATTCTTTTTTGTTGGC and AACCCGAUCCTCTTTGGGATGCTTC

CA primers, and V5 was amplified from pICSL50012 using ATCGGGTUCCGGAAGAGGATCGCATC and GGTTTAA UTCACTTGTCATCGTCATCCT primers, with KAPA HiFi Uracil+ enzyme, following the manufacturer's protocol. Amplicons were assembled in pICSLUS0001-OD with the USER method (Methods S1 and Geu-Flores *et al.*, 2007). Similarly, WRR4 was amplified with its 5′ and 3′ regulatory sequences from Oy-0 genomic DNA with GGCTTAAUCGACGAAGCATC AGACAAGG and GGTTTAAUTCAGTGATGCATGGTGG AGT, and assembled in LBJJ233-OD.

To clone *NRG1*, *NRG1A* and *NRG1B*, promoter, coding sequence and terminators were domesticated (i.e. *BsaI* sites were removed by synonymous transitions) and assembled in a single vector by Gibson Assembly (Gibson *et al.*, 2009). Single vectors were then assembled with corresponding tags by the Golden Gate method (Engler *et al.*, 2009). Further details are provided in Methods S1.

Bacterial growth assay

Pseudomonas syringae pv. *tomato* strain DC3000 carrying pVSP61:AvrRps4-HA, pVSP61:AvrRpt2 or pVSP61 empty vector was grown on selective king's B (KB) medium agar plates for 48 h at 28°C. Bacteria were harvested from the plates, resuspended in infiltration buffer (10 mM $MgCl_2$, pH 5.6) and the concentration was adjusted to an optical density at 600 nm (OD_{600}) = 0.001 (5×10^5 colony forming units (CFU) ml^{-1}). The abaxial surfaces of 5-wk-old Arabidopsis leaves were hand infiltrated with a 1-ml needleless syringe. For quantification, leaf samples were harvested with a 6-mm-diameter cork borer, resulting in a *c.* 0.283 cm^2 leaf disc. Two leaf discs per leaf were harvested and used as a single sample. For each condition, four samples were collected just after infiltration and eight samples were collected 72 h after infiltration. Samples were ground in 200 µl of infiltration buffer, serially diluted (5, 50, 500, 5000 and 50 000 times) and spotted (6–10 µl per spot) on selective KB medium agar plates to grow for 48 h at 28°C. The number of colonies (CFU per drop) was monitored and bacterial growth was expressed in CFU cm^{-2} of leaf tissue.

HR assay in Arabidopsis

Pseudomonas fluorescens engineered with a type III secretion system (Pf0-1 EtHAn; Thomas *et al.*, 2009) carrying pBS46:AvrRps4, pBS46:AvrRps4KRVY, pBS46:PopP2, pBS46:PopP2_C321A, pBS46:AvrRpt2, pVSP61:AvrRpm1 or pVSP61:AvrPphB, and *P. syringae* pv. *tomato* strain DC3000 carrying pVSP61:AvrRps4-HA, pVSP61:AvrRpt2 or pVSP61 empty vector, were grown on selective KB medium agar plates for 48 h at 28°C. Bacteria were harvested from the plates, resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6) and the concentration was adjusted to OD_{600} = 0.05, 0.1 or 0.2 (2.5×10^7, 5×10^7 or 10^8 CFU ml^{-1}). The abaxial surfaces of 5-wk-old Arabidopsis leaves were hand infiltrated with a 1-ml needleless syringe. Cell death was monitored 24 h after infiltration.

Electrolyte leakage assay

Pf0-1 (T3S) strains were grown and infiltrated as for HR assays. Leaf discs were taken with a cork borer from infiltrated leaves. Discs were dried, washed in deionized water for 30 min before being floated on deionized water (16 discs per sample, three samples per biological replicate for three biological replicates). Electrolyte leakage was measured on a LAQUAtwin-EC-33 (Horiba, Kyoto, Japan) conductivity meter at the indicated time points.

Oomycete pathogen propagation and inoculation

For the propagation of *Albugo candida*, zoospores were suspended in water (*c.* 10^5 spores ml^{-1}) and incubated on ice for 30 min. The spore suspension was then sprayed on plants using a Humbrol® (Hornby Hobbies Ltd, Sandwich, UK) spray gun (*c.* 700 µl per plant) and plants were incubated at 4°C in the dark overnight. Infected plants were kept under 10 h light (20°C) and 14 h dark (16°C) cycles. Plants were scored as susceptible if a pathogen was capable of accomplishing its life cycle and sporulation was macroscopically visible within 3 wk after plant inoculations.

For the propagation of *Hyalanoperonospora arabidopsidis* (*Hpa*), 1-wk-old Arabidopsis seedlings were sprayed with fresh *Hpa* spores at a concentration of 10^4 spores ml^{-1} using a Humbrol® spray gun (*c.* 700 µl per plant). Sprayed seedlings were covered with a plastic lid and were kept under 10 h light (16°C) and 14 h dark (16°C) cycles. Susceptibility was measured as the number of spores per plant. Approximately 80 plants were bulked in 2 ml of water and spores were counted using a haemocytometer. Results are expressed as the number of spores per plant.

Transient gene expression in *N. benthamiana* leaves

Agrobacterium tumefaciens strains were streaked on selective media and incubated at 28°C for 48 h. A single colony was transferred to liquid lysogeny broth medium with appropriate antibiotic and incubated at 28°C for 24 h in a shaking incubator (200 rotations per minute). The resulting culture was centrifuged at 2000 ***g*** for 5 min and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6) at OD_{600} = 0.4 (2×10^8 CFU ml^{-1}). For co-expression, each bacterial suspension was adjusted to OD_{600} = 0.4 in the final mix. The abaxial surfaces of 5-wk-old *N. benthamiana* were infiltrated with a 1-ml needleless syringe. Leaves were phenotyped for cell death at 5 d post-inoculation (dpi).

Identification of ADR1 in the *N. benthamiana* genome

The NB-ARC amino acid sequence of NbNRG1 (DLPLQEL KVKLLEEKEKVVVLSAPAGCGKTTLAAMLCQEDDIKDK YRDIFFVTVSKKANIKRIVGEIFEMKGYKGPDFASEHAAV CQLNNLLRRSTSQPVLLVLDDVWSESDFVIESFIFQIPGF KILVTSRSVFPKFDTYKLNLLSEKDAKALFYSSAFKDSIPY VQLDLVHKAVRSCCGFPLALKVVGRSLCGQPELIWFNR VMLQSKRQILFPTENDLLRTLRASIDALDEIDLYSSEATT LRDCYLDLGSFPEDHRIHAAAILDMWVERYNLDED) was

used as a tBLASTN query sequence on the 'N. benthamiana Genome v1.0.1 predicted cDNA' database from Sol Genomic Network (Fernandez-Pozo *et al.*, 2015). Three transcripts were identified: *Niben101Scf02118g00018.1* (*NbNRG1*), *Niben101Scf03844g01015.1* (*NbNRG2*, pseudogene) and *Niben101Scf02422g02015.1* (uncharacterized). The other results were not considered because of low coverage or low identity. The *Niben101Scf02422g02015.1* predicted transcript does not start with a START codon and does not finish with a STOP codon, and so could be incomplete. *Niben101Scf02422g02015.1* was used as BLASTN query on 'N. benthamiana Genome v1.0.1 Contigs' to identify the gene in its genomic context. We retrieved the *Niben101Scf02422g02015.1* genomic sequence on contig Niben101Scf02422Ctg032 and identified a START codon 23 bp upstream and a STOP codon 564 bp downstream of *Niben101Scf02422g02015.1*, framing a 2487-bp gene including exons and introns (Fig. S1a). The resulting protein was analysed using the SMART protein domain annotation resource (Letunic & Bork, 2017). It contains an N-terminal RPW8 domain, a central NB-ARC domain and C-terminal LRRs. A phylogenetic reconstruction of the Niben101Scf02422g02015.1 NB-ARC domain, together with the NB-ARC domains of AtADR1, AtADR1-L1, AtADR1-L3, AtADR1-L3, AtNRG1A, AtNRG1B, AtDAR5, NbNRG1 and AtZAR1 as outer group, places Niben101Scf02422g02015.1 in the ADR1 clade. MUSCLE was used to generate phylogenetic trees using the Maximum Likelihood method with 100 bootstraps. This analysis was carried out using MEGA7 software.

Gene expression measurement by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

For gene expression analysis, RNA was isolated from three biological replicates and used for subsequent RT-qPCR analysis. Briefly, RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and treated with RNase-Free DNase Set (Qiagen). Reverse transcription was carried out using SuperScript IV Reverse Transcriptase (ThermoFisher, Waltham, MA, USA). qPCR was performed using a CFX96 Touch™ Real-Time PCR Detection System. Primers for qPCR analysis of *ICS1* (*Isochorismate Synthase1*) are CAATTGGCAGGGAGACTTACG and GAGCTGATCTGATCCCGACTG. Primers for qPCR analysis of *PR1* (*Pathogenesis-Related1*) are ATACACTCTGGTGGG CCTTACG and TACACCTCACTTTGGCACATCC. Primers for qPCR analysis of *WRR4A* are GCAAGATAGCGAGC TCCAGA and GCAAGAAACATACAAGTCCTCCA. Primers for qPCR analysis of *EF1α* are CAGGCTGATTGTGCTG TTCTTA and GTTGTATCCGACCTTCTTCAGG. Data were analysed using the double delta Ct method (Livak & Schmittgen, 2001).

For *CHS3-2D* and *CSA1*, RT-PCR was conducted on cDNA using 3′-GCGAGGTCACTGCAATTCTC-5′ and 3′-TGTCT GACACTCCAACCACA-5′ primers for *CHS3* and 5′-TGGTG TTTGAAGGAGCTTGC-3′ and 5′-CATGAGCAGCTTGTA CGGAC-3′ primers for *CSA1*, with Taq DNA Polymerase (NEB, Ipswich, MA, USA), following the manufacturer's protocol.

Trypan blue staining

Whole plants were boiled for 1 min in stain solution (10 ml lactic acid, 10 ml glycerol, 10 g phenol, 10 mg trypan blue and water in a final volume of 10 ml, mixed in a 1 : 1 ratio with ethanol) and then decolorized in chloral hydrate (2.5 g chloral hydrate and water in a final volume of 1 ml). They were mounted in 60% glycerol and examined under a microscope.

SA measurement

Pseudomonas fluorescens engineered with a type III secretion system (Pf0-1 EtHAn) (Thomas *et al.*, 2009) carrying pBS46: AvrRps4 or pBS46:AvrRps4KRVY was grown on selective KB medium agar plates for 48 h at 28°C. Bacteria were harvested from the plates, resuspended in infiltration buffer (10 mM $MgCl_2$, pH 5.6) and the concentration was adjusted to $OD_{600} = 0.05$ (2.5×10^7 CFU ml^{-1}). The abaxial surfaces of 5-wk-old Arabidopsis leaves were hand infiltrated with a 1-ml needleless syringe. Leaves were harvested at 24 h post-inoculation and freeze dried. SA was extracted from 10 mg of ground dry tissue with 400 µl of 10% methanol and 1% acetic acid in water on ice for 30 min. The solution was centrifuged at 16 000 ***g*** for 10 min. A second extraction was carried out on the pellet in the same conditions and both supernatants were mixed. Samples were analysed on an Acquity UPLC attached to a TQS tandem mass spectrometer (both from Waters, Milford, MA, USA). Detection was by negative electrospray MS. The spray chamber conditions were a desolvation temperature of 600°C, desolvation gas at 900 l h^{-1}, cone gas at 150 l h^{-1} and nebulizer pressure of 7.0 bar. The spray voltage was 1.5 kV in negative mode.

Protein extraction and western blot

Proteins were extracted from leaf tissue using TruPAGE™ LDS Sample Buffer (Sigma-Aldrich) following the manufacturer's recommendations. They were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by immunoblotting. Tris-glycine polyacrylamide (PAA) gels were prepared with 5% polyacrylamide for the stacking gel, and 10% or 12% polyacrylamide for resolving gels, in this study. The prestained protein ladder (PageRuler, ThermoFisher) was used as molecular weight marker. Proteins were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Burlington, MA, USA) using a semi-dry transfer apparatus supplied by Trans-Blot Turbo (Bio-Rad). Membranes were blocked for 1 h at room temperature or overnight at 4°C in TBST (Tris-buffered saline with 0.1% Tween) containing 5% (w/v) nonfat dry milk. Membrane incubation with horseradish peroxidase (HRP)-conjugated antibodies (Anti-FLAG M2, 1 : 10 000 dilution, Sigma; Anti-GFP, 1 : 10 000 dilution, Santa Cruz Biotechnology, Santa Cruz, Dallas, TX, USA) was carried out in TBST supplemented with 5% milk by gentle agitation at room temperature for 1 h. The membrane was then rinsed three times in TBST (10 min) and once in TBS (Tris-buffered saline). Chemiluminescence detection for the proteins of interest was

carried out first by incubating the membrane with developing reagents (SuperSignal West Pico & West Femto, Thermo Fisher Scientific), and then using an ImageQuant LAS 4000 (GE Healthcare Life Sciences, Buckinghamshire, UK).

Results

Identification of Cas9-induced *nrg1a-nrg1b* mutants in Arabidopsis and an *nrg1* mutant in *N. benthamiana*

Of the five RPW8-NLRs from *A. thaliana* accession Col-0, three belong to the ADR1 clade (ADR1, ADR1-L1 and ADR1-L2) and two belong to the NRG1 clade (NRG1A (At5g66900) and NRG1B (At5g66910)) (Collier *et al.*, 2011). Two genes encode proteins that resemble NRG1A and NRG1B, but do not share the canonical RPW8, NB-ARC and LRR structure. At5g66590 (referred to here as NRG1C) comprises a partial NB-ARC domain and LRRs, but no RPW8 domain. At5g66630 (DAR5) has RPW8, NB-ARC and LIM (Lin11, Isl-1 & Mec-3) domains, but no LRR. The *N. benthamiana* genome carries a single copy of *NRG1* and a pseudogene called *NRG2* (Peart *et al.*, 2005). We identified *Niben101Scf02422g02015.1* as an additional RPW8-NLR encoding gene. A phylogenetic reconstruction of all the RPW8-NLRs from Arabidopsis and *N. benthamiana* positions *Niben101Scf02422g02015.1* in the ADR1 clade (Fig. S1b). Thus, *N. benthamiana* contains one copy of *ADR1* and one copy of *NRG1*. We used CRISPR-Cas9 to generate null alleles of *AtNRG1A* and *AtNRG1B* in *A. thaliana* accessions Ws-2 and Col-0, and a null allele of *NbNRG1* in *N. benthamiana*. We assembled three CRISPR constructs using Golden Gate cloning (see the Materials and Methods section; Fig. S2a; Methods S1). *CRISPR-1* and *CRISPR-2* constructs both targeted Arabidopsis *NRG1A* and *NRG1B*, but differed in the promoter used to express *Cas9*. The *CRISPR-3* construct targeted *N. benthamiana* NRG1. We identified insertion or deletion (indel) mutations causing a reading frame shift resulting in early stop codons in or before the NB-ARC domain region for all three genes (Fig. S2b). After screening of self-progenies, we identified Ws-2_*nrg1a-nrg1b*, Col-0_*nrg1a-nrg1b* and *N. benthamiana_nrg1* lines that carry homozygous *nrg1* mutations and lack T-DNA.

NRG1 mediates RRS1/RPS4-mediated HR, but not bacterial resistance, in Ws-2

In Arabidopsis Ws-2, RRS1-R and RPS4 comprise a TIR-NLR pair that recognizes the effectors PopP2 from *Ralstonia solanacearum* and AvrRps4 from *P. syringae*. Their paralogues RRS1B and RPS4B also recognize AvrRps4, but not PopP2. RRS1/RPS4 and RRS1B/RPS4B activation results in HR, defence gene induction and bacterial resistance (Narusaka *et al.*, 2009; Saucet *et al.*, 2015). We tested whether these responses require NRG1. We delivered the effector AvrRps4 using *P. syringae* pv. *tomato* strain DC3000 and assessed cell death (Fig. S3). We observed fewer leaves displaying cell death in Ws-2_*nrg1-nrg1b* than in Ws-2 wild-type (WT). Cell death was not completely abolished in Ws-2_*nrg1-nrg1b*. However, some combinations indicate that DC3000 can induce AvrRps4-independent cell death (Fig. S3), such as DC3000 with empty vector in WT, or with AvrRps4 in an *rrs1-rps4-rps4b* mutant. DC3000 carries a diverse set of effectors that influence immunity and could mask the AvrRps4-induced HR. To test for HR after delivery of a single effector, we used the *P. fluorescens* strain Pf0-1 carrying a type III secretion system (Pf0-1 EtHAn) (Thomas *et al.*, 2009). With the Pf0-1 system, AvrRps4 induces a specific HR in Ws-2 and Col-0, as a result of the activation of RRS1/RPS4 and RRS1B/RPS4B (Saucet *et al.*, 2015). The RRS1/RPS4 and RRS1B/RPS4B-mediated HR is completely lost in both Col-0_*nrg1a-nrg1b* and Ws-2_*nrg1a-nrg1b* backgrounds (Fig. 1a). This observation confirms that the cell death observed with DC3000_AvrRps4 in Ws-2_*nrg1a-nrg1b*, Ws-2_*eds1* and Ws-2 WT is independent of AvrRps4 (Fig. S3). However, AvrRpt2, AvrRpm1 and AvrPphB, which activate the CC-NLRs RPS2, RPM1 and RPS5, respectively (Debener *et al.*, 1991; Kunkel *et al.*, 1993; Simonich & Innes, 1995), induce HR after delivery from Pf0-1, even in an *nrg1a-nrg1b* mutant background. We conducted an electrolyte leakage assay to test whether the HR caused by the CC-NLRs RPS2, RPM1 and RPS5 was quantitatively reduced in Col-0_*nrg1a-nrg1b* (Fig. 1b). Indeed, RPS2- and RPM1-mediated HR, but not RPS5-mediated HR, as assayed by ion leakage assay, were also partially reduced at an early time point. This reduction was not seen at later time points for RPM1. These data indicate that NRG1 is fully required for the HR mediated by the TIR-NLRs RRS1/RPS4 and RRS1B/RPS4B, partially required for the HR mediated by the CC-NLRs RPM1 and RPS2, and not required for the HR mediated by the CC-NLR RPS5. Both *AtNRG1A* and *AtNRG1B* can complement Ws-2_*nrg1a-nrg1b* loss of TIR-NLR-mediated HR, indicating that they are redundant for this function (Fig. 1a).

We used the *P. syringae* pv. *tomato* strain DC3000 to test for bacterial resistance (Xin & He, 2013). Bacterial growth on Ws-2 is reduced for DC3000 expressing AvrRps4, as a result of recognition by RRS1/RPS4 and RRS1B/RPS4B (Saucet *et al.*, 2015). Surprisingly, this AvrRps4-dependent reduced growth is unaltered in an *nrg1a-nrg1b* double mutant (Fig. 2a). Although DC3000 can trigger AvrRps4-independent cell death, the AvrRps4-dependent HR is lost in *nrg1a-nrg1b* (Figs 1, S3). Thus, loss of NRG1 function abolishes HR from activation of RRS1/RPS4 and RRS1B/RPS4B, but does not compromise the activation of disease resistance to bacteria in Arabidopsis. The maintenance of RPS2-mediated HR in the absence of NRG1 correlates with resistance to DC3000 expressing AvrRpt2 in Col-0_*nrg1a-nrg1b* (Fig. 2b).

The activation of RRS1/RPS4 also results in the induction of SA-related genes, such as *ICS1* and *PR1* (Sohn *et al.*, 2014). SA plays a crucial role as a signalling molecule that activates plant immunity (Durner *et al.*, 1997). We measured changes in SA levels and the expression of SA-responsive *PR1* and SA biosynthetic gene *ICS1* in response to Pf0-1_AvrRps4, and, as a control, to Pf0-1_AvrRps4^{KRVY}, a Pf0-1 strain carrying a nonrecognized allele of AvrRps4 (Sohn *et al.*, 2012a,b). *PR1* and *ICS1* both show RPS4/RRS1/AvrRps4-dependent gene induction, even in

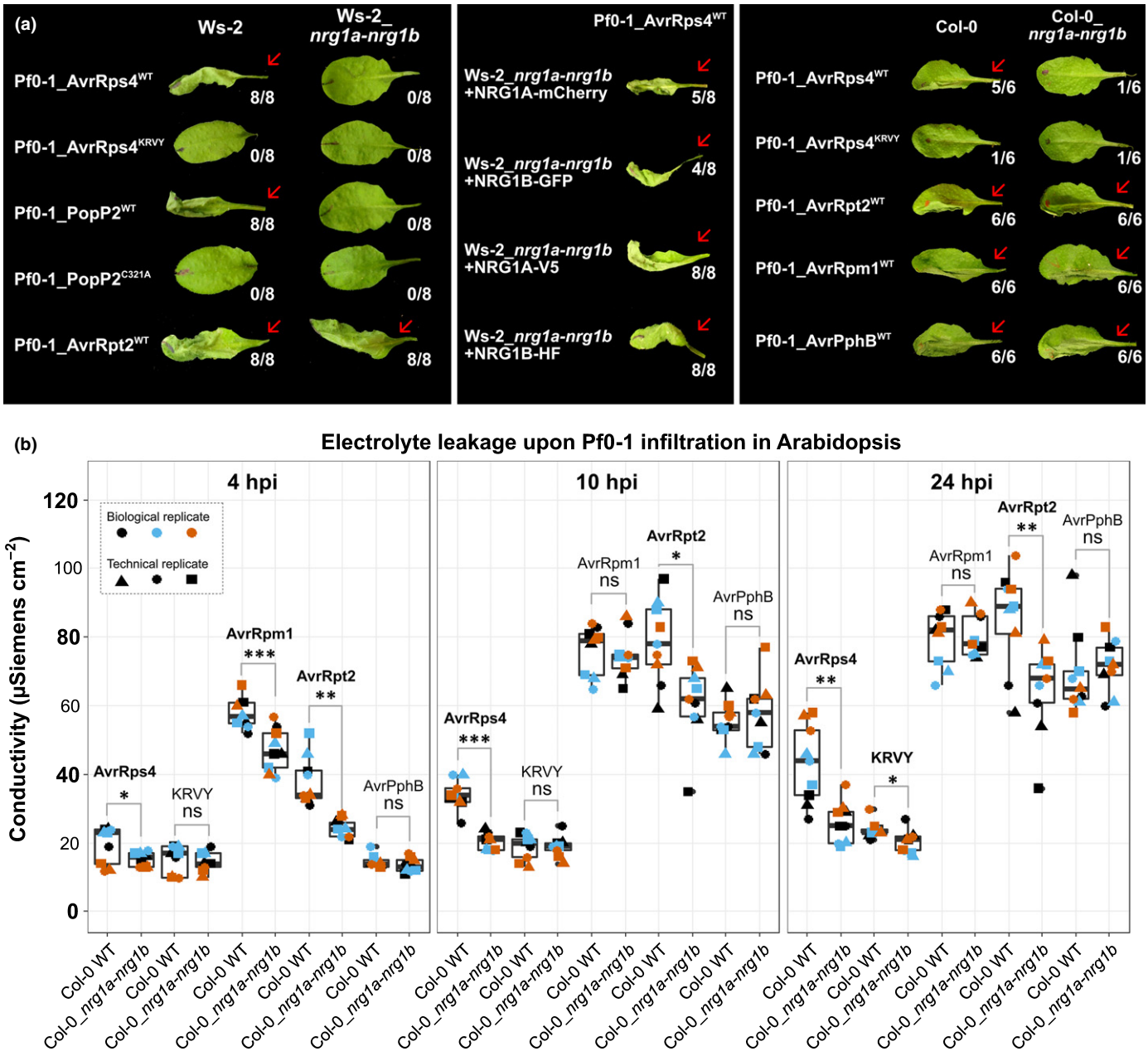

Fig. 1 NRG1 is required for the RRS1/RPS4-mediated hypersensitive response (HR). (a) Arabidopsis leaves were infiltrated with *Pseudomonas fluorescens* strain Pf0-1 expressing the indicated effectors at an optical density at 600 nm (OD_{600}) = 0.2. For Ws-2, the whole leaf was infiltrated. For Col-0, half of a leaf was infiltrated. Photographs were taken at 24 h post-inoculation (hpi). The red arrows show an HR, indicating NLR activation by the cognate effector. The numbers indicate the number of plants displaying HR of the total number of plants tested. PopP2- and AvrRps4-mediated HR is lost in Ws-2_*nrg1* (Ws-2_*nrg1a-nrg1b*) and Col-0_*nrg1* (Col-0_*nrg1a-nrg1b*). AvrRps4^{KRVY} (AvrRps4$^{KRVY-AAAA}$) and PopP2^{C321A} are mutant alleles unable to trigger HR in wild-type (WT) plants, used as a negative control for HR in WT. Indeed, they did not trigger HR. AvrRpt2, AvrRpm1 and AvrPphB are CC-NLR-activating effectors used as positive control for HR in *nrg1*. They all trigger HR in both WT and *nrg1* mutant lines. HR is recovered in lines complemented with either NRG1A or NRG1B, tagged with mCherry, green fluorescent protein (GFP), V5 or His-FLAG (HF). (b) HR was quantified by the electrolyte leakage assay. Col-0 WT or Col-0_*nrg1a-nrg1b* plants were infiltrated in the same conditions as for the photographs. Sixteen discs were collected, rinsed and immersed in 10 ml of water. The electroconductivity was measured at 4, 10 and 24 hpi. Boxplots represent nine data points (three biological replicates × three technical replicates). Colours indicate biological replicates (plants grown at different times); shapes indicate technical replicates (different plants inoculated with the same inoculum). Significance was calculated with *t*-tests and the *P* value is indicated: ns (nonsignificant), $P > 0.05$; *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$.

the absence of NRG1 (Figs 3, S4). SA is weakly induced by Pf0-1_AvrRps4^{KRVY}, probably as a result of PAMP-triggered immunity (PTI), and strongly induced by Pf0-1_AvrRps4 (Fig. 3b). SA induction is not reduced in Ws-2_*nrg1a-nrg1b*. Thus, NRG1 is not required for RRS1/RPS4-mediated elevation of SA levels and induction of defence genes.

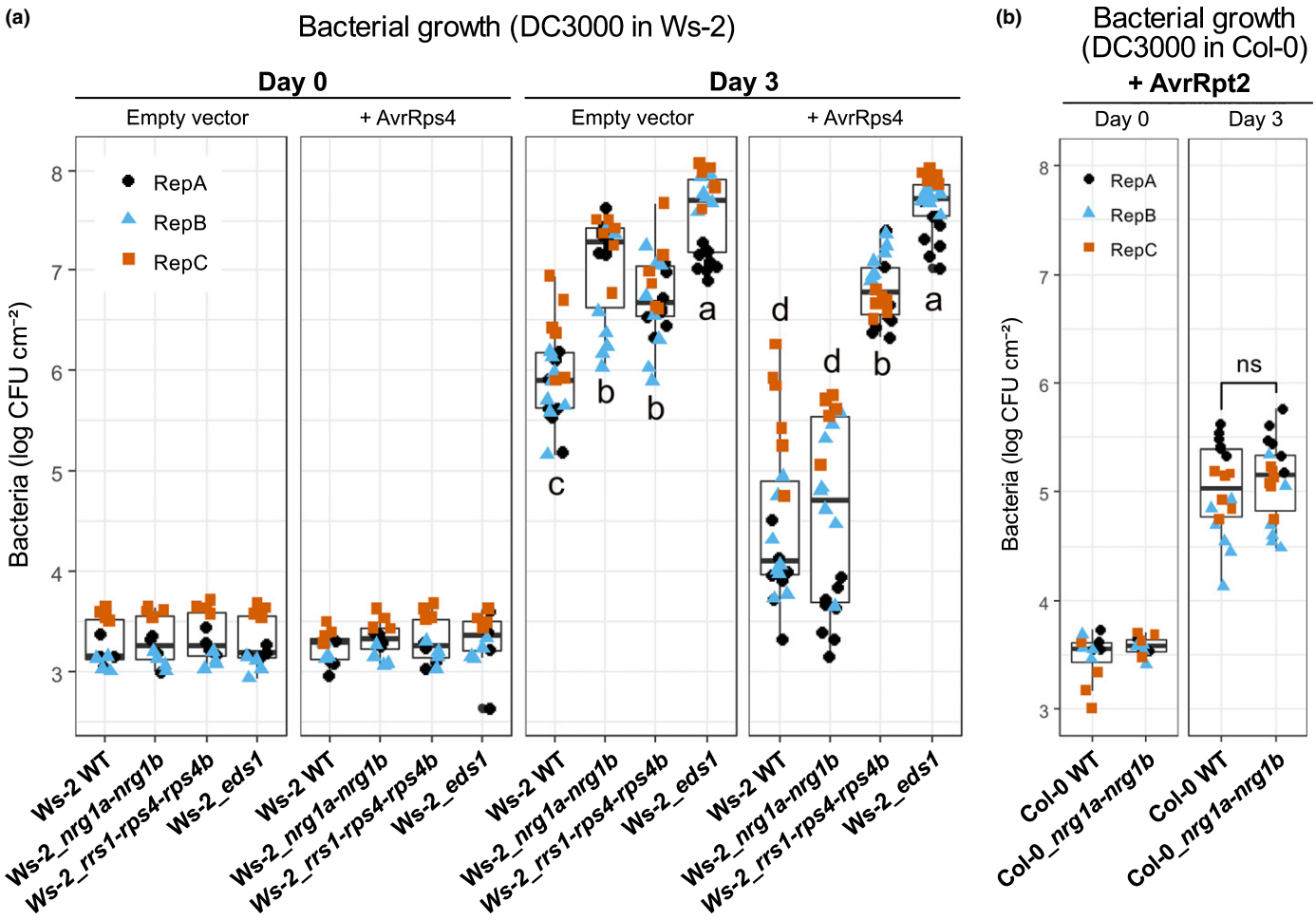

Fig. 2 NRG1 is not required for bacterial resistance. Bacterial growth assay in Arabidopsis using *Pseudomonas syringae* pv. *tomato* strain DC3000 (DC3000). One leaf per plant was infiltrated with DC3000 expressing or not (a) AvrRps4 or (b) AvrRpt2 at an optical density at 600 nm (OD_{600}) = 0.001. Bacterial quantification was performed just after infiltration (0 d post-inoculation, dpi) and at 3 dpi. Each dot represents one individual plant. Colours and shapes represent three biological replicates. (a) Letters below or above the bars indicate significant differences ($P < 0.05$) as determined by a one-way ANOVA followed by *post hoc* Tukey's honestly significant difference (HSD) analysis. (b) The growth of DC3000_AvrRpt2 was not different in Col-0 wild-type (WT) and Col-0_*nrg1a-nrg1b*, as indicated by a nonsignificant (ns) *t*-test ($P = 0.418$). Rep, biological replicate. CFU, colony-forming unit.

Arabidopsis *nrg1* mutants show impaired TIR-NLR-dependent resistance to oomycete pathogens

Arabidopsis accession Col-0 resists *Hyaloperonospora arabidopsidis* (downy mildew or *Hpa*) races Emoy2 and Cala2 via the TIR-NLRs RPP2 and RPP4, respectively (Van Der Biezen *et al.*, 2002; Sinapidou *et al.*, 2004). In Ws-2, *Hpa* race Cala2 is resisted via the TIR-NLR RPP1_WsA (Botella *et al.*, 1998). We tested whether Cala2 and Emoy2 resistance requires NRG1 (Figs 4, S5). We found that the hyphal growth in cotyledons was more extensive in Ws-2_*nrg1a-nrg1b* than in Ws-2 WT for Cala2 (Figs 4, S5). Larger numbers of spores were produced on *nrg1a-nrg1b*-infected plants, but not to the level of *eds1 (enhanced disease resistance 1*, an Arabidopsis mutant strongly affected in resistance mediated by TIR-NLRs; Wiermer *et al.*, 2005). Some spores were observed in the resistant WT plant; these could either be spores that persist from the inoculation or fresh spores from rare sporangiophores sometimes observed in cotyledons of the WT plant (Fig. S5g). We conclude that RPP1- and RPP2-mediated resistance to Cala2 and RPP4-mediated resistance to Emoy2 partially requires NRG1.

Arabidopsis Col-0 and Ws-2 also contain TIR-NLRs that confer resistance to the oomycete *Albugo candida* (*Ac*), the cause of white rust. For instance, the TIR-NLR WRR4A confers resistance to the white rust race AcEm2 (Borhan *et al.*, 2008). We expressed an allele of *WRR4A* from Arabidopsis accession Oy-0 in the AcEm2-compatible accession Ws-2 and observed full resistance (Fig. 5a). However, Ws-2_*nrg1a-nrg1b* transformed with *WRR4A* was partially susceptible, showing that WRR4A requires NRG1 to activate full resistance to white rust. All transgenic lines expressed *WRR4A* at different levels within the same range (Fig. 5b). Thus, the partial loss of resistance in Ws-2_*nrg1a-nrg1b_WRR4A* is not a result of low expression of *WRR4A*, but rather of the loss of *NRG1*.

Ws-2 lacks *WRR4A* but contains a paralogue, called *WRR4B*, which confers resistance to the white rust races Ac2V, Ac7V and AcBoT (Cevik *et al.*, 2019). We expressed a Cas9 construct targeting *WRR4B* with two sgRNAs and recovered a homozygous

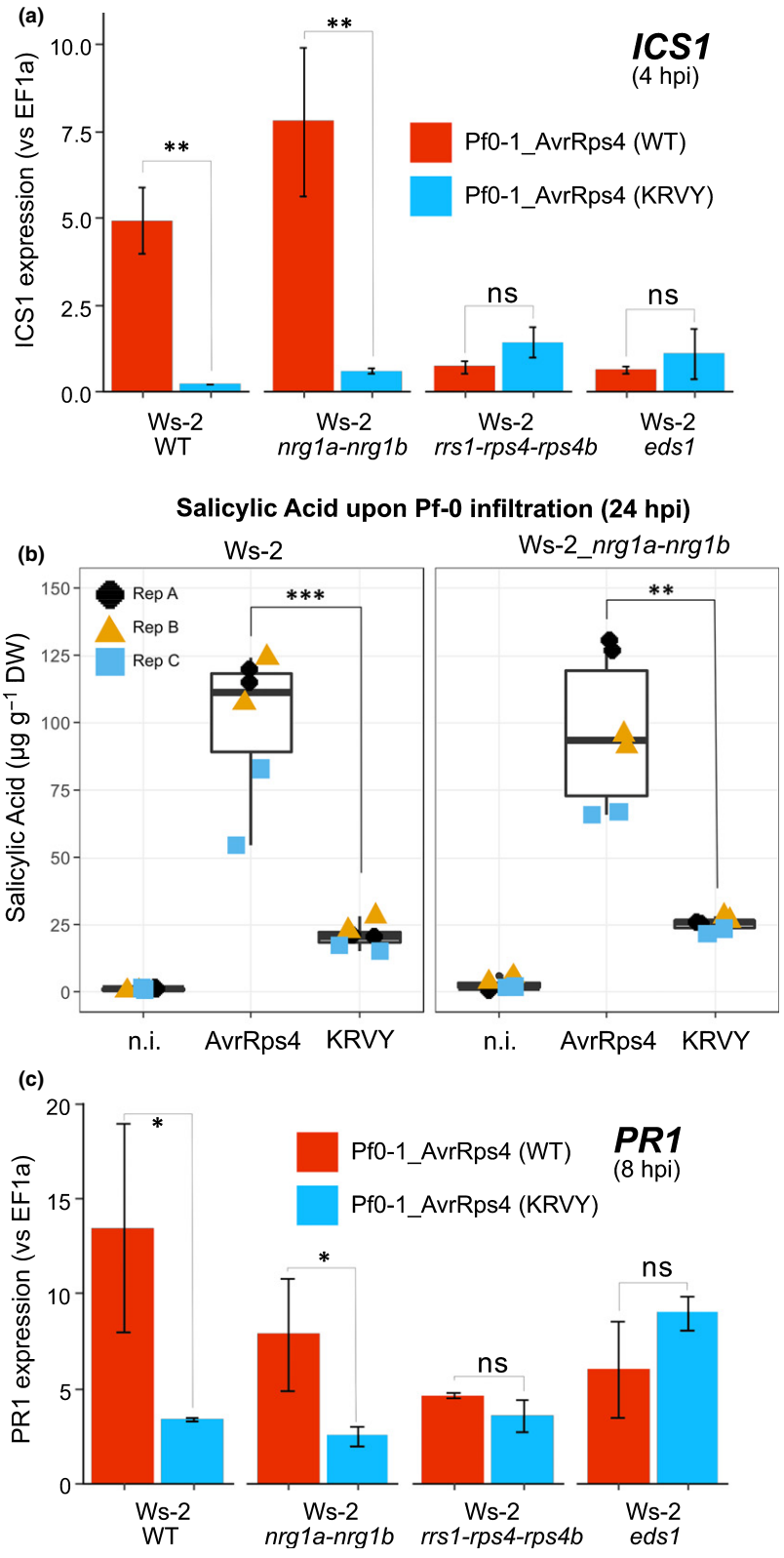

Fig. 3 NRG1 is not required for salicylic acid (SA) pathway activation. (a, c) Induction of *ICS1* and *PR1* on Pf0-1 strain infiltration. Arabidopsis leaves were infiltrated with bacteria at an optical density at 600 nm (OD_{600}) = 0.2. Samples were collected at 4 h post-inoculation (hpi) for *ICS1* expression and at 8 hpi for *PR1* expression. Values represent the expression level relative to *EF1α*, using the 'double delta Ct' method. Three lines were used and bulked for each treatment. Error bars represent SE of three technical replicates. Three biological replicates all showed induction of *ICS1* and *PR1* by AvrRps4 in both WT and Ws-2_*nrg1-nrg1b*. (b) Induction of SA on Pf0-1 strain infiltration. Arabidopsis leaves were infiltrated with bacteria at OD_{600} = 0.05. Samples were collected at 24 hpi. SA was extracted from 10 mg of dry weight and SA was quantified by ultrahigh-pressure liquid chromatography. Colours and shapes represent three biological replicates, each represented by two independent extractions from the same set of infiltrated leaves. n.i., non-infiltrated. Significance was calculated with *t*-tests and the *P* value is indicated: ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mutant line (see Materials and Methods). This line is partially susceptible to Ac2V and Ac7V, but still resists AcBoT (Fig. 5c). Similarly, Ac2V and Ac7V, but not AcBoT, can complete their life cycle on Ws-2_*nrg1a-nrg1b* (Fig. 5c). Thus, both WRR4B and NRG1 are required for full Ac2V and Ac7V resistance, and the phenotypes of both loss-of-function mutants are similar, suggesting that WRR4B also requires NRG1 to confer white rust resistance.

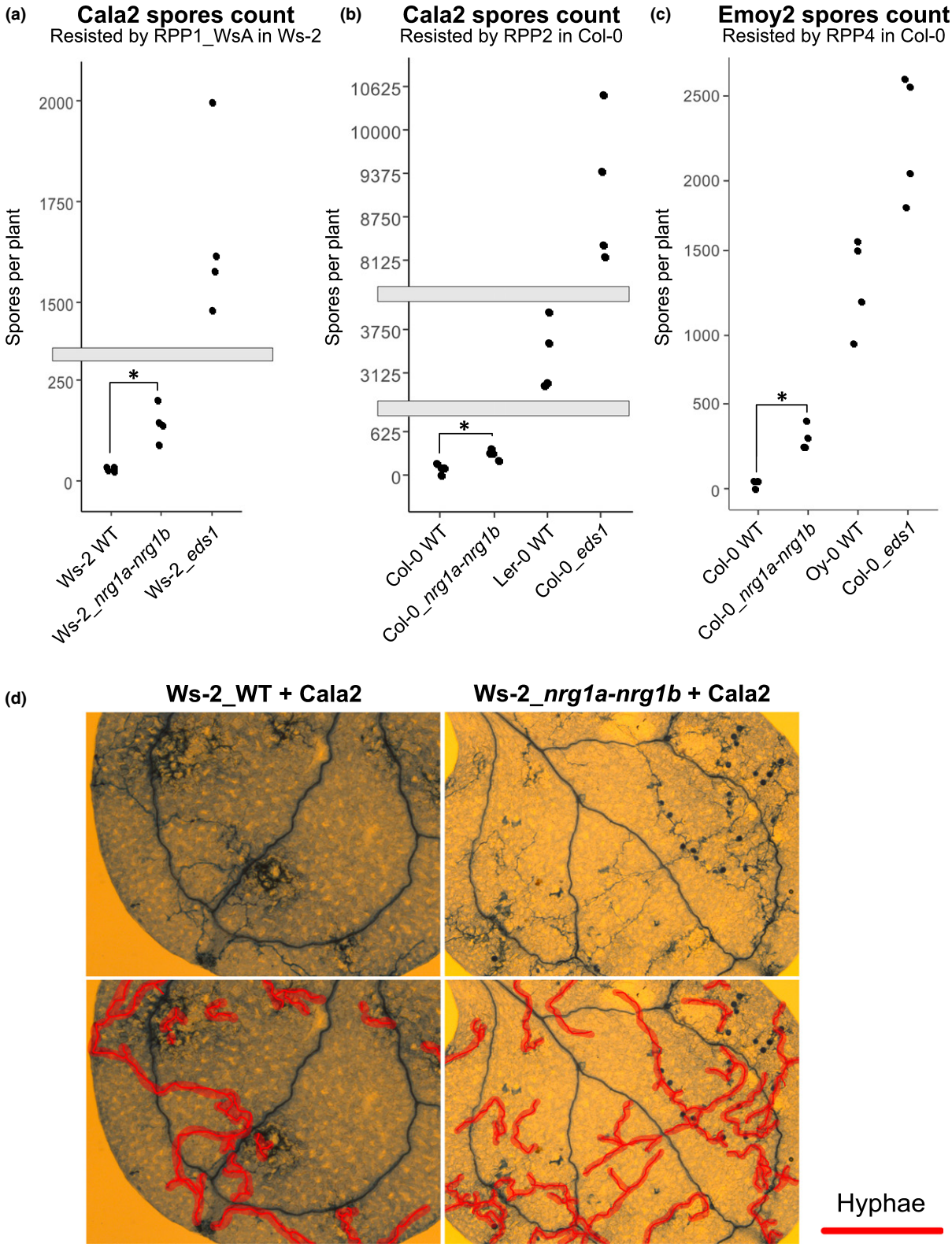

Fig. 4 *nrg1* mutants are partially compromised in resistance to downy mildew. Seven-day-old plants were spray inoculated with *Hyalanoperonospora arabidopsidis* race Emoy2 or Cala2. Plants were phenotyped 7 d after inoculation. (a–c) Full resistance was observed in Col-0 and Ws-2 wild-type (WT); full susceptibility was observed in Col-0_*eds1* (*eds1-2*) and Ws-2_*eds1* (*eds1-1*); an intermediate phenotype was observed in Col-0_*nrg1* (*Col-0_nrg1-nrg1b*) and Ws-2_*nrg1* (Ws-2_*nrg1a-nrg1b*). Ler-0 and Oy-0 were used as susceptible controls for Cala2 and Emoy2 infections, respectively. Significant differences between WT and *nrg1* mutant plants are indicated (*t*-test): *, $P < 0.05$. Data are expressed as spores per plant. (d) Cotyledons of Ws-2 WT or *nrg1a-nrg1b* infected with Cala2 were stained with trypan blue to visualize hyphal growth. Images were taken with a microscope and annotated using the software 'Affinity Photo'. Hyphae are coloured in red. The original photographs are shown at the top.

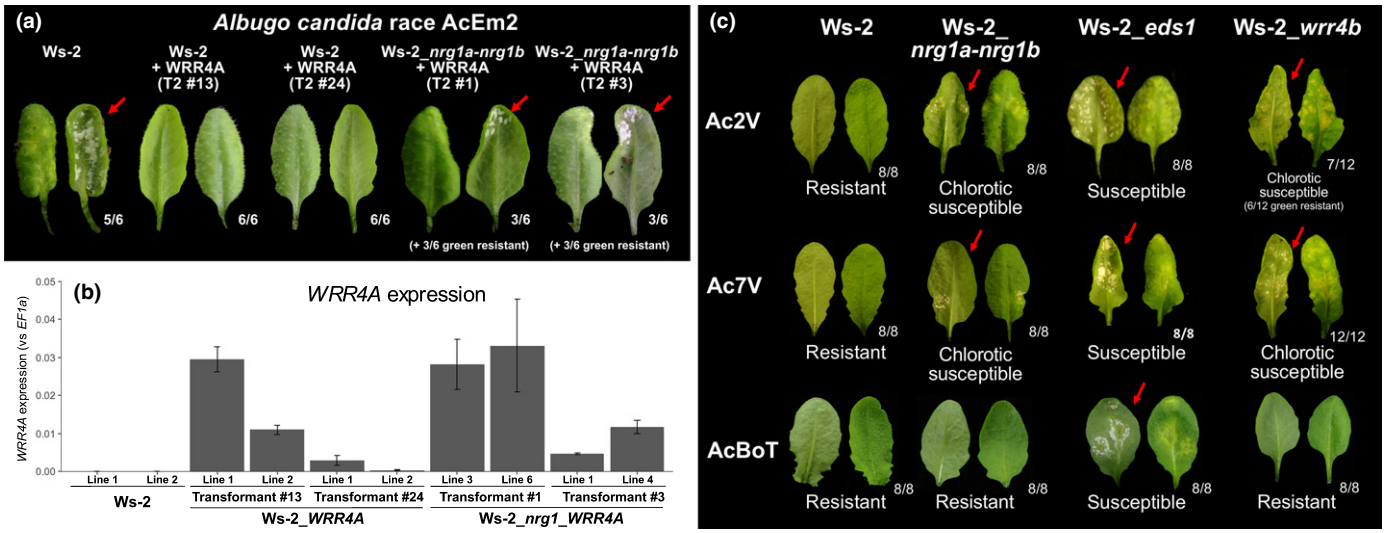

Fig. 5 *nrg1* mutants are partially compromised in resistance to white rust. Three- to five-wk-old plants were spray inoculated with *Albugo candida*. Plants were phenotyped at 12 d after inoculation. Abaxial and adaxial photographs of the same leaf are shown. Numbers indicate the number of individual plants showing a similar phenotype from the number of plants tested. (a) Two independent lines for Ws-2 wild-type (WT) and two independent lines for Ws-2_*nrg1a-nrg1b* were tested for *Albugo candida* race AcEm2 resistance. Six plants were tested for each line. Ws-2 is susceptible, whereas *WRR4A* transgenic lines are resistant. Ws-2_*nrg1a-nrg1b* expressing *WRR4A* is still partially susceptible to AcEm2. The Oy-0 allele of *WRR4A* was used here. (b) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was conducted in *WRR4A* transgenic lines. Two lines were tested for each independent transformant. Error bars indicate ± SE of three technical replicates. *WRR4A* is expressed in these lines at a higher level than in the fully resistant Col-0 WT. (c) Eight or 12 plants for each genotype were tested with *Albugo candida* races Ac2V, Ac7V and AcBoT. Ws-2_*eds1* (*eds1-1*) is fully susceptible to *Albugo candida* races Ac2V and Ac7V, whereas Ws-2_WT is resistant. Ws-2_*nrg1a-nrg1b* and Ws-2_*wrr4b* are partially susceptible to Ac2V and Ac7V. AcBoT resistance is not affected in Ws-2_*nrg1a-nrg1b* and Ws-2_*wrr4b* mutants, whereas Ws-2_*eds1* is partially susceptible. Red arrows indicate the presence of white pustules, resulting from the production of zoospores. T2, line from the second generation after transformation. #, reference number of the independent transformant line.

NRG1 is required for *SOC3/CHS1*- and *CSA1/CHS3*-induced HR in *N. benthamiana*

CSA1 and *CHS3* are two adjacent TIR-NLR encoding genes from Arabidopsis. CHS3 carries an integrated C-terminal LIM domain. *chs3-2D* is a gain-of-function allele that confers a *CSA1*-dependent autoimmune phenotype in Arabidopsis (Xu *et al.*, 2015). Similarly, SOC3 and CHS1 are a TIR-NLR/TIR-NB (TIR, NB-ARC but no LRR) pair encoded by two adjacent genes in Arabidopsis. They interact to modulate immunity (Zhang *et al.*, 2016). We transiently expressed *CSA1* and *chs3-2D* or *SOC3* and *CHS1* in leaves of *N. benthamiana* and the *Nb_nrg1* mutant. We found that *CSA1* and *chs3-2D* can induce HR in *N. benthamiana*, but not in *Nb_nrg1* (Fig. 6). We also observed an HR triggered by SOC3 and CHS1 alone or in combination, which is lost in the absence of NRG1. Although the CHS1/SOC3 and CHS3/CSA1 phenotypes are weak in WT, they never induce HR in *Nb_nrg1*. The CC domain of the CC-NLR MLA from barley and the D505V 'autoimmune' allele of the CC-NLR RPM1 from Arabidopsis activate HR in *N. benthamiana* (Gaoa *et al.*, 2011; Maekawa *et al.*, 2011). Transient expression of these alleles results in HR in both *N. benthamiana* and *Nb_nrg1*. We tested the expression of CHS1 and SOC3 and found that the proteins are still expressed in Nb_*nrg1* (Fig. S6). As CHS3-2D and CSA1 are not tagged, we tested the expression of their mRNA and found that they were also expressed in Nb_*nrg1*. We conclude that NRG1 is required for the HR initiated by the TIR-NLR pair CSA1-CHS3 and the TIR-NLR/TIR-NB pair SOC3-CHS1, but not by the CC-NLRs MLA7 and RPM1 in *N. benthamiana*.

Discussion

NRG1 is a conserved clade of RPW8-NLR required for TIR-NLR signalling

NRG1 was originally identified in *N. benthamiana* as required for resistance mediated by the TIR-NLR N (Peart *et al.*, 2005). *NRG1* is broadly conserved within angiosperms, forming the so-called NRG1 clade (Collier *et al.*, 2011). NRG1 could be a helper clade for many NLR sensors. Interestingly, there is a correlation of NRG1 and TIR-NLR occurrence within plant genomes; the NRG1 lineage is missing in monocots and Lamiales, which also lack TIR-NLRs (Collier *et al.*, 2011). However, the NLRome of *Amborella trichopoda*, an early-diverging lineage among angiosperms, contains both TIR-NLRs and NRG1 (Shao *et al.*, 2016). This observation suggests that NRG1 could be specifically required for all TIR-NLR-mediated immunity. To test this hypothesis, we mutated *NRG1* from *N. benthamiana* and Arabidopsis and tested for loss or reduction of TIR-NLR function. *Nicotiana benthamiana* and Arabidopsis carry one and two copies of *NRG1*, respectively (*NbNRG1* and *AtNRG1A/AtNRG1B*). We found that *NbNRG1* is required for the HR triggered by the TIR-NLR pairs SOC3/CHS1 and CSA1/CHS3-2D

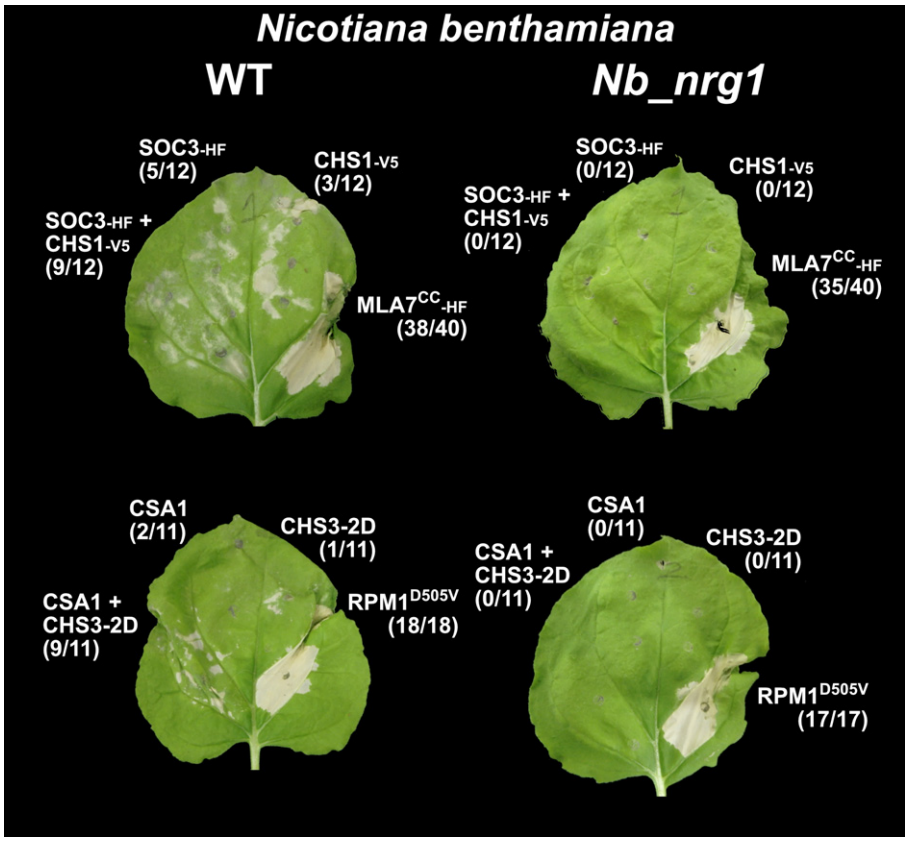

Fig. 6 CSA1/CHS3 and SOC3/CHS1 induce an NRG1-dependent hypersensitive response (HR) in *Nicotiana benthamiana.* Four-wk-old *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* strain GV3101 at an optical density at 600 nm (OD_{600}) = 0.4. Genes are expressed under the control of the *35S* promoter and the *Ocs* terminator. Photographs were taken at 5 d post-inoculation (dpi). Both SOC3/CHS1 and CSA1/CHS3 pairs induce an HR in the wild-type (WT), but not in the *nrg1* mutant. SOC3, CHS1, CSA1 and CHS3-2D sometimes give an HR on their own. The CC domain of MLA7 from barley and the $RPM1^{D505V}$ auto-active allele were used as non-TIR-NLR controls. The numbers indicate the number of HRs observed from the number of infiltrations for a given combination. Even weak HRs were considered. For instance, on WT plants in this figure, SOC3, CHS1, SOC3 + CHS1, CSA1 + CHS3-2D and CHS3-2D were considered to be positive for HR, whereas CSA1 was considered to be negative.

(Fig. 6). Signalling of the CC-NLRs MLA7 and RPM1 still results in an HR in *Nb_nrg1* that is indistinguishable from that of WT. In Arabidopsis, CHS3 signals via NRG1, but not CHS1, which signals via ADR1 (Wu *et al.*, 2018). It is possible that NbADR1 is too diverged from the three Arabidopsis ADR1 alleles to support CHS1 function and complement the loss of NRG1.

In Arabidopsis accession Ws-2, transgenic expression of *WRR4A* confers *NRG1*-dependent resistance to the white rust race AcEm2 (Fig. 5a). Moreover, resistance to *A. candida* races Ac2V and Ac7V is strongly reduced in an *nrg1-nrg1b* double mutant (Fig. 5c). This resistance is mediated, at least in part, by the TIR-NLR WRR4B, suggesting that the WRR4B paralogue of WRR4A can also signal via NRG1. We also found that resistance mediated by the TIR-NLRs RPP1, RPP2 and RPP4 is partially dependent on NRG1 in Arabidopsis (Fig. 4). However, the requirement of NRG1 by RPP1, RPP2 and RPP4 for downy mildew resistance, although significant, is weak. In addition, RPP4 function was not affected in an independent experiment (Wu *et al.*, 2018). Thus, other helpers, such as ADR1 family proteins, already reported to contribute to RPP4 function (Bonardi *et al.*, 2011), also probably contribute to RPP1, RPP2 and RPP4 function.

Using the Pf0-1 system, we found that the HR induced by RRS1/RPS4 and RRS1B/RPS4B is fully dependent on NRG1 in Arabidopsis (Fig. 1a). Surprisingly, we observed a weak reduction in the HR induced by AvrRpt2 (via CC-NLR RPS2) and AvrRpm1 (via CC-NLR RPM1). The HR induced by AvrPphB (via CC-NLR RPS5) is unaltered in Col-0_*nrg1a-nrg1b*. It has been reported previously that another CC-NLR, Rx2 from potato, also requires NRG1 redundantly with ADR1 to confer resistance to *Potato virus X* (Collier *et al.*, 2011).

NRG1 is indeed required for full function of all tested TIR-NLRs. However, the function of some CC-NLRs is also compromised in the absence of NRG1.

Disease resistance is possible without HR

Despite the absence of AvrRps4-induced HR, RRS1/RPS4 still confers bacterial resistance in a Ws-2_*nrg1a-nrg1b* background (Figs 1, 2). HR is a form of cell death associated with plant resistance, and is strongly correlated with the activation of a host R protein by a pathogen avirulence factor (Morel & Dangl, 1997). For instance, the recognized effectors AvrRpm1 and AvrRpt2 trigger both HR and bacterial resistance in Arabidopsis as a result of recognition by the R proteins RPM1 and RPS2 (Mackey *et al.*, 2002). However, not all avirulence factors induce HR. For example, the effectors HopZ5 and HopPsyA from *P. syringae* pv. *actinidiae* and *P. syringae* pv. *syringae* induce an HR-independent resistance in Arabidopsis accession Col-0 (Gassmann, 2005; Jayaraman *et al.*, 2017). Similarly, the HR induction by the CC-NLR Rx is not required to confer extreme resistance against *Potato virus X* (Bendahmane *et al.*, 1999). In addition, a genetic screen revealed a *dnd1* (*defence no death 1*) mutant of Arabidopsis accession Col-0 which lacks the AvrRps2-induced HR, but still resists *P. syringae* expressing AvrRpt2 (Yu *et al.*, 1998). *DND1*

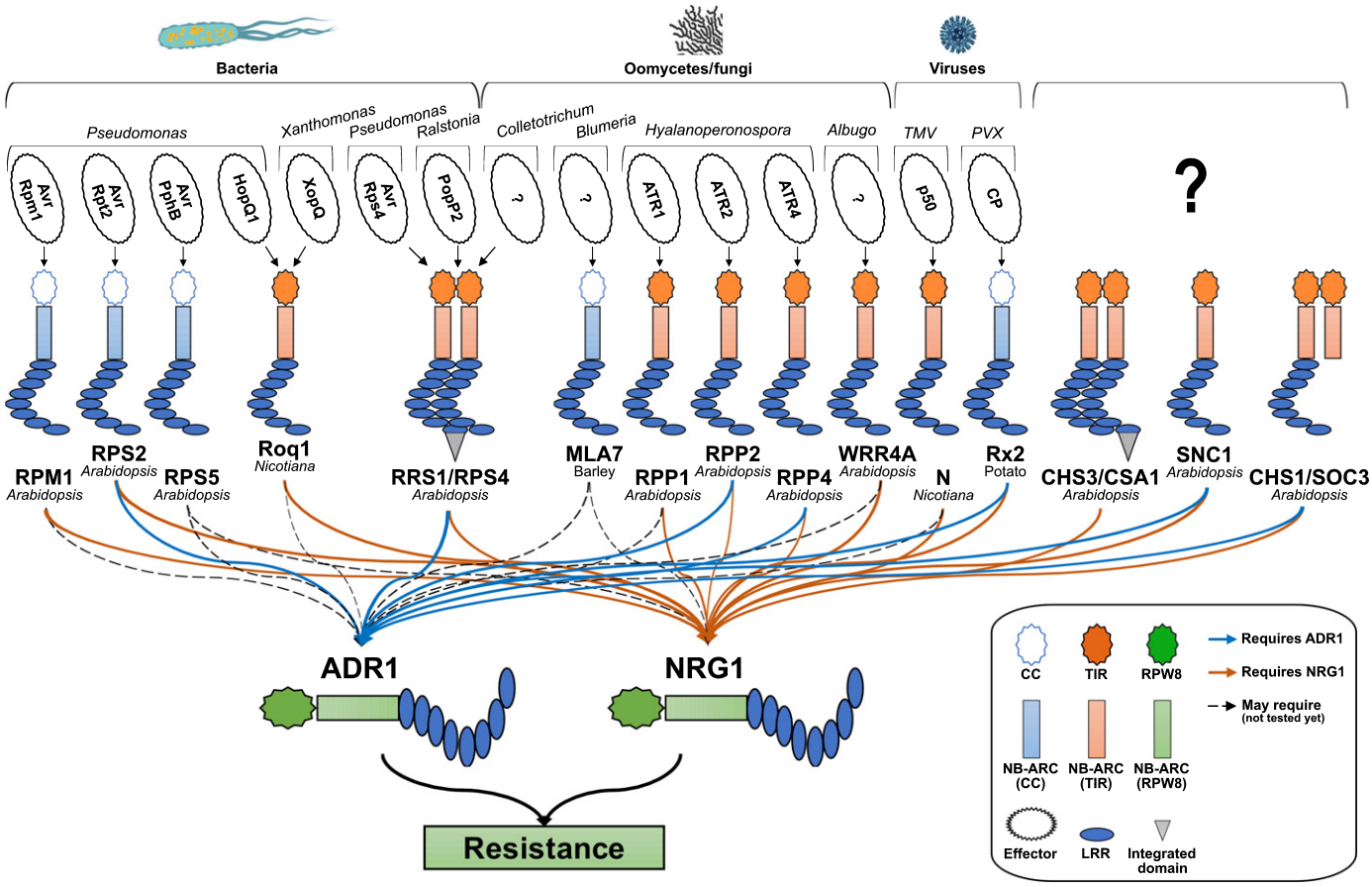

Fig. 7 CC-NLRs and TIR-NLRs can signal via NRG1 and/or ADR1. NLRs are immune surveillance devices. Specific activation by pathogen effectors triggers resistance. For full response, they require RPW8-containing proteins NRG1 and ADR1. All the TIR-NLRs can signal via NRG1 and some can also signal via ADR1. CC-NLRs signal via ADR1 and partially via NRG1. Some NLRs may signal independently of ADR1 and NRG1. ADR1 and NRG1 are conserved within Angiosperms, suggesting that this model can be applied for all flowering plants. RPM1, RPS2, RRS1-RPS4, RPP1, RPP2, RPP4, WRR4A, CSA1/CHS3 and SOC3/CHS1 signal via NRG1 (this article). RPS2, RPP2 and RPP4 signal via ADR1 (Bonardi *et al.*, 2011). RPP4, RRS1, but not CHS3, signal via ADR1 (Dong *et al.*, 2016). Roq1 signals via NRG1 (Brendolise *et al.*, 2018). Roq1 and RPP1 signal via NRG1 (Qi *et al.*, 2018). N signals via NRG1 (Peart *et al.*, 2005). Rx2 signals via both NRG1 and ADR1 (Collier *et al.*, 2011). SNC1 signals via both ADR1 and NRG1, and CHS3 signals specifically via NRG1 (Wu *et al.*, 2018). TMV, *Tobacco mosaic virus*; PVX, *Potato virus X*.

encodes for a putative cyclic nucleotide-gated Ca^{2+}, K^+ and/or Na^+ channel (Clough *et al.*, 2000). Here, we found that NRG1 is required for HR, but not for resistance, mediated by the TIR-NLR pair RRS1/RPS4. It has been reported previously that AvrRps4 activates cell death from the cytoplasm and resistance from the nucleus, and that these two functions are independent (Heidrich *et al.*, 2011). Whether NRG1 plays a specific role in this cytoplasmic cell death pathway is unknown. Moreover, RPP1-mediated HR in *N. benthamiana* fully requires NbNRG1 (Brendolise *et al.*, 2018; Qi *et al.*, 2018), whereas RPP1 function is partially retained in Ws-2_*nrg1a-nrg1b* (Fig. 4). The uncoupling of HR and resistance highlights the ambiguous relationship between these two defence outputs.

By contrast, NRG1 is required for Roq1-mediated HR and Roq1-dependent bacterial resistance in *N. benthamiana* (Qi *et al.*, 2018). We speculate that ADR1 proteins, from the sister clade of NRG1, might complement the loss of NRG1s for some TIR-NLRs, e.g. RRS1/RPS4 and RPP1, but not Roq1.

TIR-NLRs activate in parallel an SA-dependent pathway and an NRG1-dependent pathway that results in HR

ADR1 proteins are required for RPS2/AvrRpt2-induced SA production in Arabidopsis (Bonardi *et al.*, 2011). We tested whether the same is true for NRG1 proteins. We measured the induction of SA and the expression of the SA biosynthetic gene *ICS1* and the SA-responsive gene *PR1* in Ws-2_*nrg1a-nrg1b* (Dong, 2004). In this assay, we compared induction after infiltration of Pf0-1_AvrRps4^{WT} (inducing both PTI and effector-triggered immunity (ETI) via RRS1/RPS4 and RRS1B/RPS4B) and infiltration of Pf0-1_AvrRps4^{KRVY} (inducing PTI only) (Figs 3, S4). In Ws-2_*rrs1-rps4-rps4b* and Ws-2_*eds1*, *ICS1* and *PR1* expression are not induced by AvrRps4 or its inactive allele AvrRps4^{KRKY}. However, AvrRps4 specifically enhances *ICS1* and *PR1* expression in both WT and *nrg1a-nrg1b* mutant lines. This is consistent with the production of SA, which is maintained at similar levels after induction in both WT and *nrg1a-nrg1b*

(Fig. 3b). Although NRG1 is required for RRS1/RPS4-mediated HR, it is dispensable for SA pathway activation. Unlike ADR1, NRG1 is not involved in SA regulation during ETI. Retention of SA production on RRS1/RPS4 activation could explain the WT-like bacterial resistance in Ws-2_*nrg1a-nrg1b,* despite the loss of HR. These results indicate that the SA-dependent pathway and NRG1-dependent pathway (resulting in HR) can be activated in parallel by TIR-NLRs. Of the genes differentially regulated during XopQ-induced ETI (via the NRG1-dependent TIR-NLR Roq1), 80% depend on NRG1 (Qi *et al.*, 2018). Some of these genes are involved in photosynthesis, RNA processing and protein degradation. Thus, it is likely that NRG1 regulates response pathways in addition to HR. By contrast, as *c.* 20% of the ETI genes are still normally regulated in *nrg1* (Qi *et al.*, 2018), SA signalling is probably not the only immune response still active in *nrg1* mutants.

Some TIR-NLRs signal via NRG1s only, whereas some signal via both ADR1s and NRG1s

RPW8-NLRs are a monophyletic anciently diverged clade. They do not show extensive diversification or expansion, and are conserved in angiosperms, gymnosperms and bryophytes (Zhong & Cheng, 2016). RPW8-NLRs diverged into ADR1 and NRG1 subclades before the monocot/dicot separation (Collier *et al.*, 2011). ADR1s have been characterized in Arabidopsis as helpers for the R proteins RPP2, RPP4 and RPS2 (Bonardi *et al.*, 2011). In addition, an *adr1-adr1-l1-adr-l2* (*adr1-triple*) mutant can partially suppress the autoimmune phenotype of *slh1-9*, which is an Arabidopsis line with an auto-active allele of *RRS1* (Dong *et al.*, 2016). Thus, RPS2, RPP2, RPP4 and RRS1/RPS4 can signal via ADR1s. Here, we found that full RPP2-, RPP4- and RRS1/RPS4-mediated immune responses require NRG1s. Therefore, these three immune receptors signal via both ADR1s and NRG1s.

Unlike *slh1-9*, the *chs3-1* autoimmune phenotype is totally independent of ADR1s (Dong *et al.*, 2016). Here, we found that CHS3-induced HR requires *NbNRG1*. Moreover, the *chs3-2D* autoimmune phenotype is completely lost in an *nrg1a-nrg1b-nrg1c* mutant of Arabidopsis (Wu *et al.*, 2018). Thus, CHS3 signals only via NRG1s.

Conversely, RPS2-mediated macroscopic HR does not require NRG1 (Fig. 1a), but it requires ADR1 (Bonardi *et al.*, 2011). Thus, RPS2 mainly signals via ADR1. It also partially requires NRG1, as indicated by a reduction in ion leakage in the absence of NRG1 (Fig 1b).

AvrRpm1-induced macroscopic HR in Arabidopsis is still active in the *adr1*-triple (Bonardi *et al.*, 2011) and *nrg1a-nrg1b* mutant backgrounds (Fig. 1a). RPM1 either signals independently of ADR1 and NRG1, or can achieve full function dependent on either ADR1s or NRG1s, like the CC-NLR Rx2 (Collier *et al.*, 2011).

In conclusion, some R proteins signal via NRG1 only (e.g. CHS3) and some via both ADR1 and NRG1 (e.g. RRS1/RPS4, RPP2, RPP4, RPS2, Rx2, CHS1, SNC1) (Fig. 7). Some others may signal via ADR1 only. In a companion paper in this issue of *New Phytologist*, an *adr1-adr1-L1-adr1-L2-nrg1a-nrg1b-nrg1c* sextuple mutant was characterized and showed redundancy between NRG1s and ADR1s for TIR-NLR function (Wu *et al.*, 2018) . Unravelling the mechanism of RPW8 (Xiao *et al.*, 2001; Chae *et al.*, 2014) and RPW8-containing NLRs will push forward our understanding of the plant immune system and may ultimately be applied to deploy wisely genetic resistance in crops.

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Author contributions

BC, PD and JDGJ designed the research. BC, P-MN, D-SK, VC, AR, YY and PD performed the experiments. BC and JDGJ wrote the manuscript.

ORCID

Baptiste Castel https://orcid.org/0000-0002-2722-0228
Volkan Cevik https://orcid.org/0000-0002-3545-3179
Pingtao Ding https://orcid.org/0000-0002-3535-6053
Jonathan D. G. Jones https://orcid.org/0000-0002-4953-261X
Dae-Sung Kim https://orcid.org/0000-0002-4579-2094
Pok-Man Ngou https://orcid.org/0000-0002-0760-1058
Amey Redkar https://orcid.org/0000-0001-5171-8061

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Supporting Information

Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 *NbADR1* sequence and phylogenetic tree of RPW8-NLRs (NB-ARC domain) from Arabidopsis and *Nicotiana benthamiana.*

Fig. S2 CRISPR enables the generation of *nrg1* mutants in plants.

Fig. S3 Cell death assay using *Pseudomonas syringae* strain DC3000.

Fig. S4 Induction of *ICS1* and *PR1* on Pf0-1 infiltration.

Fig. S5 Ws-2_*nrg1a-nrg1b* mutants are partially compromised in resistance to RPP1-mediated resistance to Cala2.

Fig. S6 Expression of CHS1/SOC3 and CHS3/CSA1 in *Nicotiana benthamiana.*

Methods S1 sgRNA cloning, plasmids used in this article, *NRG1* cloning, Golden Gate cloning protocol, USER cloning protocol, Gibson Assembly protocol.

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