

NbRD21 protease controls receptor kinase homeostasis in *Nicotiana benthamiana*

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Summary

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Received: 22 April 2026

Accepted: 10 June 2026

New Phytologist (2026) 251: 2741–2752

doi: 10.1111/nph.71407

Key words: immunity, proteolysis, *Pseudomonas syringae*, receptor homeostasis, receptor kinase.

- RD21-like proteases are papain-like cysteine proteases with a C-terminal granulin domain that are abundant and ubiquitous in angiosperms and have often been implicated in immunity. We previously found that the activity of RD21 in *Nicotiana benthamiana* (NbRD21) is suppressed during infection with *Pseudomonas syringae*. Here, we studied the role of NbRD21 in immunity and proteome processing.
- NbRD21 was disrupted by genome editing and *rd21* mutants were subjected to disease assays and shotgun proteomics. Dipeptide substrate zLR-AMC was used in protease assays and agroinfiltration was used to transiently express NbRD21 and candidate substrates.
- Genome-edited lines lacking NbRD21 develop normally but have drastically reduced zLRase activity and are significantly more susceptible to *P. syringae*. Shotgun proteomics revealed an increased accumulation of c. 20 diverse receptor-like kinases (RLKs) in untreated *rd21* knock-out lines, but their transcript levels are unaltered when compared to wild-type plants. 35S-driven GFP-tagged RLKs accumulate more upon transient expression in *rd21* plants than in wild-type plants.
- These data indicate that NbRD21 post-translationally controls RLK homeostasis, either by directly degrading RLKs or indirectly by regulating endocytic RLK recycling.

Introduction

Responsive-to-Desiccation-21 (RD21)-like proteases are ubiquitous in angiosperm plants. These papain-like cysteine proteases (PLCPs) are typified by having a C-terminal granulin domain and are amongst the most active and abundant PLCPs in vegetative tissues. RD21-like proteases accumulate in the vacuole, lytic vesicles, ER-bodies, and in the apoplast (reviewed by Huang & van der Hoorn, 2025). Orthologs of Arabidopsis RD21 (At1g47128) are C14 in tomato, CP1A in maize, oryzain in rice, and triticain in wheat. We previously also identified the granulin-containing NbC14 in *Nicotiana benthamiana* (Kaschani *et al.*, 2010; Bozkurt *et al.*, 2011), but this protease belongs to PLCP subfamily-4 instead of subfamily-1 (Richau *et al.*, 2012) and is therefore not an RD21 ortholog. NbC14 has therefore been appropriately renamed into NbCP14 (Paireder *et al.*, 2016), since it is orthologous to NCP14, which is involved in developmental cell death during embryo development (Zhao *et al.*, 2013).

Subfamily-1 RD21-like proteases are frequently associated with abiotic and biotic stresses. The *RD21* gene is abundantly

transcribed in non-stressed plants and is further induced upon drought and osmotic stress, hence the name responsive-to-desiccation (Koizumi *et al.*, 1993). RD21 proteases are also implicated in immunity and are often targeted by pathogen-secreted effectors that inhibit RD21 or trigger its degradation or mislocalisation (reviewed in Huang & van der Hoorn, 2025). Tomato C14, for instance, is inhibited by cystatin-like EpiC inhibitors secreted by *Phytophthora infestans* (Kaschani *et al.*, 2010) and by chagasin-like CIP1 produced by *Pseudomonas syringae* (Shindo *et al.*, 2016), whilst the RD21 ortholog in maize (CP1) is inhibited by Pit2 (Misas-Villamil *et al.*, 2019) and rice OsRD21 is inhibited by MoErs1 (Liu *et al.*, 2024). Consistent with immune proteases being suppressed by pathogens, *rd21* mutant Arabidopsis lines are more susceptible to *Botrytis cinerea* (Shindo *et al.*, 2012), and mutant wheat lines are more susceptible to wheat yellow mosaic virus (Liu *et al.*, 2023). However, how RD21-like proteases act in immunity has not yet been resolved.

We recently studied the suppression of apoplastic hydrolases of *Nicotiana benthamiana* (Nb) during infection by *Pseudomonas*

syringae (*P₃*) pathovar *tomato* DC3000 (*Pto*DC3000) lacking Type-III effector HopQ1-1, which is pathogenic on *N. benthamiana* (Wei *et al.*, 2007). Activity-based proteomics on apoplastic fluids revealed 60 apoplastic hydrolases that were suppressed upon infection (Sueldo *et al.*, 2024). *Nb*RD21 (NbL08g19980) was one of the strongly suppressed hydrolases during infection. Here, we studied whether *Nb*RD21 acts in immunity by taking advantage of the versatility of the *Nb*/*P₃* pathosystem.

Materials and Methods

Plants

Nicotiana benthamiana Domin (LAB strain) was grown in a growth chamber at room temperature with a 16-h light cycle and 60% humidity and watered on alternating days.

Molecular cloning

Used primers and plasmids are summarised in Supporting Information Tables S1 and S2, respectively. *Nb*RD21^{C161A} was created by site-directed mutagenesis using pPB034 (Sueldo *et al.*, 2024) as a template. Overlapping primers incorporating the desired mutations were designed, and the fragments amplified by PCR using Q5 Polymerase (New England Biolabs, Ipswich, MA, USA). DpnI was used to digest the methylated template. To generate binary constructs expressing C-terminally tagged RLKs, the Cf-4 sequence of pJK227 was replaced by the coding sequences of *Nb*FER2 and *Nb*BAM1 amplified from cDNA obtained from *N. benthamiana* leaf tissue via Gibson assembly and transformed into *Escherichia coli* DH10β. Sanger sequencing was used to verify the constructs before transformation into *Agrobacterium tumefaciens* GV3101 (pMP90) and selection for kanamycin and gentamicin resistance.

Total extract (TE) isolation

Leaf discs (1 cm diameter) were punched from leaves and flash-frozen in liquid nitrogen. Leaf tissue was ground to a powder with a plastic pestle and resuspended in PBS (1 : 3 w : v). Samples were centrifuged at 14 000 *g* for 10 min at 4°C and either used immediately or stored at −20°C.

Western blot analysis

Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes using the *Trans*-blot Turbo transfer system (BioRad). Membranes were blocked with 5% milk in PBS-T (PBS pH 6.1, 0.1% Tween-20). Membranes were rinsed in PBS-T before the addition of primary antibody, and between the addition of primary antibody (Kaschani *et al.*, 2010) and secondary antibody. Membranes were finally washed in PBS. Antibodies were incubated with agitation for at least 1 h at room temperature or overnight at 4°C. Chemiluminescence was measured using an ImageQuant LAS 4000.

Genome editing

Cas9 vector pJK309, containing an *in planta* NPTII selection cassette, 2x35S-driven Cas9 module, and a multiple cloning site, was generated by combining pICSL4723, pICH47751 (Addgene plasmid #48002), pICH41766 (Addgene plasmid #48018) (Weber *et al.*, 2011), pICSL11024 (Addgene plasmid #51144), pICH47742::2x35S-5'UTR-hCas9(STOP)-NOST (Belhaj *et al.*, 2013; Addgene plasmid #49771) in a BsaI GoldenGate reaction. Two independent sgRNA sequences targeting NbL08g19980 (Table S2) were cloned into the Cas9 vector pJK309 to produce pPJ64. *Agrobacterium* cultures were grown in LB media (kanamycin 50 µg ml^{−1} and gentamicin 10 µg ml^{−1}) overnight at 28°C. The next day, cultures were centrifuged at 4000 *g* for 10 min at room temperature and resuspended in agroinfiltration buffer (10 mM MgCl₂, 10 mM MES, 150 µM acetosyringone) to a final OD₆₀₀ of 0.1. Four-week-old *N. benthamiana* plants were infiltrated and 3 d later, leaf discs were punched from infiltrated leaves, incubated in distilled water for 1 h and soaked in 10% bleach for 10 min. Leaf discs were incubated on shooting media without antibiotics for 3 d before transferring onto media containing kanamycin. Shoots were transferred to fresh shooting media every 1–2 wk until transplantation into rooting media from week 6. Once roots were established, plantlets were potted and grown in the growth chamber. Seeds of heterozygous primary transformants were sown and genotyped.

Genotyping

Genomic DNA was extracted from three plants of each genotype by crushing a leaf disc in extraction solution (1 : 10 GOWARDS, TEBY). Specific PCR products were generated using the primers described in Table S1, designed to amplify the sgRNA-targeted regions. PCR products were separated on 1% agarose gel at 90 V to check successful amplification. DNA was purified by ethanol precipitation, the DNA concentration was measured using a nanodrop spectrophotometer, and samples were sent for sequencing.

zLRase assay

Cysteine protease activity was measured using the fluorogenic substrate Z-Leu-Arg-7-amino-4-methylcoumarin (zLR-AMC, R&D Systems, Minneapolis, MN, USA). TE was mixed with 80 µM zLR-AMC in a black 96-well plate in the presence of 10 mM TCEP and 50 mM sodium acetate, pH 5, at a final reaction volume of 100 µl. Fluorescence at 380 nm excitation/460 nm emission was recorded in a plate reader (Tecan Infinite M200) at 20-s intervals for 10–60 min. Activity was calculated by the change in fluorescence over time. *P*-values were determined by *t*-test or one-way ANOVA with Dunnett correction.

Protease activity profiling

Activity-based labelling of PLCPs was performed as described previously (Richau *et al.*, 2012), but now with STR1, a

fluorescent E-64 synthesised by WuXi AppTec. TE samples were incubated with 1 μ M STR1 in the presence of 50 mM sodium acetate pH 6.0 and 5 mM DTT in the dark for 3 h at room temperature with agitation. Samples were precipitated with ice-cold acetone and resuspended in 2 $\times$ SDS gel loading buffer (200 mM Tris-Cl pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol) before heating at 95°C for 5 mins. The proteins were separated on 12% acrylamide gels at 100 V for 2.5 h. The labelled proteins were detected by imaging the gel using the Typhoon scanner with Cy3 settings. Coomassie stain was used to check equal protein loading.

Disease assays

Bacterial growth assays were performed as described previously (Buscaill *et al.*, 2021). *Pto*DC3000(Δ hQ) was grown in LB media at 28°C for 24 h, centrifuged at 3500 g for 10 min at room temperature and resuspended in water at OD₆₀₀ = 0.0001. Six plants per genotype and three leaves per plant were syringe-infiltrated. Three days later, three 1 cm diameter leaf discs were taken per plant, surface sterilised with 15% hydrogen peroxide and washed in MilliQ water. Leaf discs were placed into Eppendorf tubes with 3 mm diameter metal beads and 1 ml MilliQ water and lysed in tissue lyser at 30 hertz per second for 5 min. Leaf samples were plated on LB agar plates containing cetrimide (10 μ g ml⁻¹), fusidic acid (10 μ g ml⁻¹) and cefaloridine (50 μ g ml⁻¹) in a 10-fold serial dilution and incubated at 28°C. Two days later, colonies were counted and converted to colony-forming units (CFU) per cm² of leaf surface. *P*-values were calculated using two-way ANOVA including an interaction factor and *post hoc* Dunnett's test to compare bacterial growth in each *rd21* mutant to wild-type plants, accounting for variation resulting from different replicate experiments.

Proteome analysis

TE was isolated from wild-type and *rd21* mutant *N. benthamiana* plants in *n* = 3 replicates. Protein concentration was measured using the DC assay (BioRad). TE samples contained 200 μ g protein in 100 μ l. Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific Waltham, MA, USA) was added to samples at 1 : 100 to prevent proteolytic degradation. Samples were carbamidomethylated by reduction with 5 mM DTT for 30 min at 37°C followed by alkylation with 20 mM 2-chloroacetamide for 30 min at room temperature. Proteins were precipitated using methanol and chloroform (Wessel & Flügge, 1984), pellets were air-dried and proteins were eluted by sonicating in 6 M urea. Proteins were digested with trypsin/LysC at 1 : 40 enzyme : proteome for 3 h at 37°C. Samples were then diluted in 50 mM Tris-HCl pH 8 (until < 1 M urea) and incubated overnight at 37°C with shaking. The pH of the samples was adjusted to < 3 with heptafluorobutyric acid (HFBA) and 100 μ g protein, the sample was desalted on stage tips (Bond Elut OMIX C18, Agilent A57003100) first with 50% acetonitrile, then with 1% and 0.1% HFBA. The next day, peptides were eluted in 0.1% formic acid and 75% acetonitrile before being dried for 2 h (SpeedVac).

LC-MS/MS analysis

Each sample was analysed on an Orbitrap Fusion Lumos instrument (Thermo Fisher Scientific) that was coupled to an EASY-nLC 1200 liquid chromatography (LC) system (Thermo Fisher Scientific). The LC was operated in the one-column mode. The analytical column was a fused silica capillary (75 μ m $\times$ 44 cm) with an integrated fritted emitter (15 μ m; CoAnn Technologies, Richland, WA, USA) packed in-house with Reprosil-Pur 120 C18-AQ 1.9 μ m resin (Dr Maisch). The analytical column was encased by a column oven (Sonation) and attached to a nanospray flex ion source (Thermo Fisher Scientific). The column oven temperature was adjusted to 50°C during data acquisition. The LC was equipped with two mobile phases: solvent A (0.1% formic acid, FA, in water) and solvent B (0.1% FA, 19.9% water and 80% acetonitrile, ACN). All solvents were of UPLC grade (Honeywell, Charlotte, NC, USA). Peptides were directly loaded onto the analytical column with a maximum flow rate that would not exceed the set pressure limit of 980 bar (usually *c.* 0.6–1.0 μ l min⁻¹). Peptides were subsequently separated on the analytical column by running a gradient of solvent A and solvent B.

Peptide and Protein Identification using MaxQuant

RAW spectra were submitted to an Andromeda (Cox *et al.*, 2011) search in MAXQUANT (v.2.5.2.0) using the default settings (Cox & Mann, 2008). Label-free quantification and match between runs were activated (Cox *et al.*, 2014). The MS/MS spectra data were searched against the *N. benthamiana* (NbLab360.v103.gff3.CDS.fasta.AA.fasta, 45 730 entries). All searches included a contaminants database search (as implemented in MaxQuant, 245 entries). The contaminants database contains known MS contaminants and was included to estimate the level of contamination. Andromeda searches allowed oxidation of methionine residues (16 Da) and acetylation of the protein N-terminus (42 Da) as dynamic modification. Carbamidomethylation on cysteine (57 Da) was set as a static modification. Enzyme specificity was set to 'Trypsin/P specific'. The instrument type in Andromeda searches was set to Orbitrap, and the precursor mass tolerance was set to $\pm$ 20 ppm (first search) and $\pm$ 4.5 ppm (main search). The MS/MS match tolerance was set to $\pm$ 0.5 Da. The peptide spectrum match FDR and the protein FDR were set to 0.01 (based on the target-decoy approach). Minimum peptide length was 8 amino acids, and maximum amino acid length was 25. For protein quantification, unique and razor peptides were allowed. Modified peptides were allowed for quantification. The minimum score for modified peptides was 40. Label-free protein quantification was switched on, and unique and razor peptides were considered for quantification with a minimum ratio count of 2. Retention times were recalibrated based on the built-in non-linear time-rescaling algorithm. MS/MS identifications were transferred between LC-MS/MS runs with the 'match between runs' option, in which the maximal match time window was set to 0.7 min and the alignment time window set to 20 min. The quantification is based on the 'value at maximum' of the

extracted ion current. At least two quantitation events were required for a quantifiable protein. Further analysis and filtering of the results were done in Perseus v1.6.10.0. (Tyanova *et al.*, 2016). Comparison of protein group quantities (relative quantification) between different MS runs is based solely on the label-free quantification (LFQ) as calculated by MaxQuant, the MaxLFQ algorithm (Cox *et al.*, 2014).

Proteomics analysis

MS data were searched against the LAB360 proteome database (Ranawaka *et al.*, 2023) and a contaminants database (implemented in MAXQUANT (v.2.0.2.0, Cox *et al.*, 2011)). Data analysis, filtering and statistics of the MAXQUANT output received from ACE were completed using the Perseus computer software (v.2.0.3.0, Tyanova *et al.*, 2016). Missing values were imputed in order to calculate a volcano plot (wild-type – knockout). Categorical groups were compared by performing a Student's *t*-test (FDR = 0.05; S0 = 0.1, 250 randomisations). The volcano plot was annotated in Perseus and edited in Inkscape. Significant hits were functionally annotated using information from the following webpages: BLAST, InterPro, Pfam, UniProt, and TAIR. Principal component analysis (PCA) was carried out using TBtools-II (Chen *et al.*, 2023).

Detection of RLK-GFP proteins

For quantifying GFP fluorescence, six plants per genotype were agroinfiltrated with *NbBAM1* and *NbFER2*. Leaves were collected at 3 d post-infiltration and scanned for fluorescence with an Amersham Typhoon using filters Cy2 (GFP) and Cy5 (red fluorescence).

RT-PCR

To measure the expression of PRRs in wild-type (WT) *N. benthamiana* and *rd21* mutants, similar age *N. benthamiana* leaves (c. 100 mg) were harvested for Vazyme FastPure Universal Plant Total RNA Isolation Kit (#RC411-01) based RNA extraction, following the manufacturer's protocol. Next, cDNA synthesis was performed using the SuperScript IV First Strand Synthesis System (#18091050) kit. Primers used in RT-qPCR are provided in Table S1. *Nbelf4A-14* (NbL17g05310.1) was used as an internal reference gene. qPCR reactions were performed on the StepOne-Plus Real-Time PCR System (#15360337) using PowerUP SYBR Green Master Mix (#4368577) in a MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (#4346906), following the manufacturer's protocol. Relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ method.

Transient cGFP/sRFP expression

To examine the expression of cGFP and sRFP in the WT and *rd21* mutants, at least four plants were agroinfiltrated. At 3 d postinfiltration, fluorescence signals were scanned using an Amersham Typhoon scanner with Cy2 (GFP) and Cy3 (RFP) filters.

Confocal microscopy

Transiently expressed *NbBAM1*-GFP proteins were visualised in abaxial epidermal cells of WT and *rd21* mutant *N. benthamiana* leaves using a Zeiss LSM 880 confocal microscope with a C-Apochromat 40 $\times$ /1.2 W Korr FCS M27 water-immersive objective 4 d post infiltration. GFP fluorescence was excited with 2% laser power at 488 nm and emission detected with a PMT detector at 493 to 598 nm. For z-stacks, 16-bit images were captured at a slice interval of 1.31 μ m and Z-projections were made using Fiji image analysis software.

Results

Transient *NbRD21* overexpression increases protease activity and immunity

To investigate if *NbRD21* contributes to plant immunity by overcoming its suppression, we first transiently overexpressed *NbRD21* and its catalytic mutant (*NbRD21*^{C161A}) by agroinfiltration. Western blot analysis of leaves transiently overexpressing WT *NbRD21* with anti-C14 antibody (Kaschani *et al.*, 2010) revealed a 36 kDa signal that represents intermediate *NbRD21*, consisting of the catalytic domain and the granulin domain, and a 26 kDa mature *NbRD21*, consisting of only the catalytic domain (Fig. 1a). Transient expression of the *NbRD21*^{C161A} mutant caused a high accumulation of the intermediate isoform and a low accumulation of the mature isoform (Fig. 1a). These data are consistent with the catalytic mutant of Arabidopsis *AtRD21* expressed by agroinfiltration (Gu *et al.*, 2012) and demonstrate that *NbRD21* also removes the granulin domain autocatalytically. The prodomain of *NbRD21*^{C161A}, however, is still removed, consistent with the removal of the prodomain of *AtRD21* by other proteases (Yamada *et al.*, 2001).

To demonstrate which isoform is active, we custom-synthesised a fluorescent activity-based probe STR1, consisting of the covalent PLCP inhibitor E-64 coupled to a Bodipy fluorophore (Fig. 1b, Huang *et al.*, 2026). This probe is identical to the previously used MV201 (Richau *et al.*, 2012), except that STR1 lacks the azide minitag on the Bodipy fluorophore. Labelling of total extracts of leaves transiently overexpressing *NbRD21* with 2 μ M STR1, followed by separation on SDS-PAGE and detection by fluorescence scanning, revealed that both the intermediate and mature isoforms of *NbRD21* are fluorescently labelled (Fig. 1c), indicating that they are both active proteases. Preincubation with 10 μ M E-64 suppresses labelling. The intermediate and mature isoforms of *NbRD21*^{C161A}, however, are not labelled, consistent with being inactive and lacking the catalytic cysteine that would be labelled by STR1 (Fig. 1c).

To further demonstrate that the *NbRD21* is active and *NbRD21*^{C161A} is not, we used commercially available zLR-AMC, a Leu-Arg dipeptide linked to the 7-amino-4-methyl coumarin (AMC) fluorophore (Fig. 1d). Incubation of total leaf extracts with zLR-AMC revealed that *NbRD21* overexpression strongly increased zLRase activity (Fig. 1e). By contrast, extracts

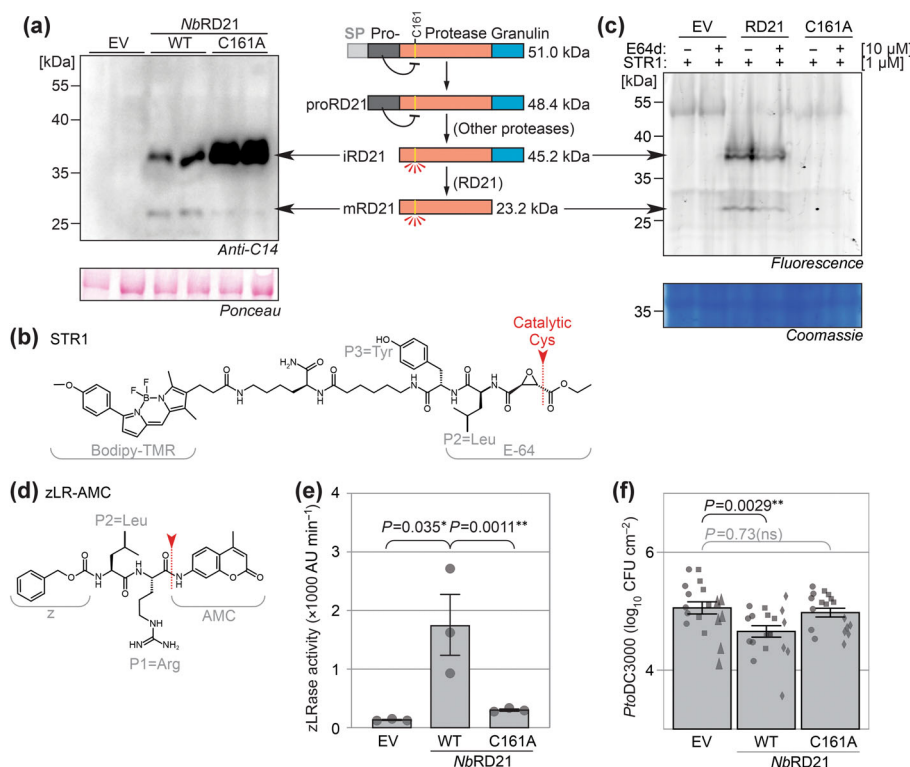


Fig. 1 Activity-dependent immunity upon *NbRD21* overexpression. (a) The *NbRD21* catalytic mutant accumulates in agroinfiltrated *Nicotiana benthamiana* leaves. *NbRD21* and its catalytic mutant (C161A) and the empty vector (EV) control were co-expressed with silencing inhibitor P19 by agroinfiltration and total extracts were isolated at 5 dpi, separated by SDS-PAGE and analysed by western blotting with α -C14 antibody and Ponceau staining. (b) Molecular structure of the activity-based probe STR1 probe for papain-like cysteine proteases (PLCPs). STR1 consists of the E-64 warhead linked to a Bodipy-TMR fluorophore. (c) Both intermediate and mature *NbRD21* are labelled with STR1. *NbRD21* and its catalytic mutant (C161A) and the empty vector (EV) control were co-expressed with silencing inhibitor P19 by agroinfiltration, and total extracts were isolated at 5 dpi, and preincubated with 10 μ M E-64d for 1 h and then labelled with 1 μ M STR1 for 3 h. Samples were separated on protein gel and detected by in-gel fluorescence scanning with Cy2 settings at PMT700. (d) Molecular structure of the PLCP substrate zLR-AMC. (e) *NbRD21* has zLRase activity. Total extracts from plants transiently expressing wild-type or the C161A mutant of *NbRD21* or an empty vector (EV) control were isolated at 5 dpi and incubated with 40 μ M zLR-AMC in the presence of 2.5 mM DTT and sodium acetate, pH 5. Fluorescence was measured at 380ex/460em at 15 min at room temperature in a plate reader. Error bars represent mean $\pm$ SE of $n = 3$ replicates. Statistical significance was calculated with the Student's t -test; *, $P < 0.05$; **, $P < 0.01$. (f) *NbRD21* requires catalytic activity for its immune function. *NbRD21*, its catalytic site C161A mutant, and the EV control were transiently co-expressed by agroinfiltration in 4-wk-old *N. benthamiana* with silencing inhibitor P19. Two days later, the same leaves were infiltrated with 10^6 CFU ml⁻¹ *PtoDC3000*. Bacterial growth in colony-forming units (CFU) was measured at 3 d after *PtoDC3000* inoculation. Error bars represent SE of $n = 6$ replicates of three experiments. P -values were calculated by two-way ANOVA followed by *post-hoc* comparison using the Dunnett test. **, $P < 0.01$; dpi, days post inoculation; ns, non-significant.

containing *NbRD21*^{C161A} had zLRase activity similar to the empty vector (EV) control (Fig. 1e).

Having established that *NbRD21* overexpression increases STR1 labelling and zLRase activity, and *NbRD21*^{C161A} does not, we next monitored the growth of WT *PtoDC3000* in these agroinfiltrated tissues through the 'agromonas' assay (Buscaill *et al.*, 2021). At 2 d after agroinfiltration, leaves were infiltrated with WT *PtoDC3000* and the CFU were determined 3 d later. *NbRD21* overexpression caused a significant reduction in bacterial growth, in contrast to *NbRD21*^{C161A} which displayed similar susceptibility to the EV control (Fig. 1f). Thus, *NbRD21* overexpression increases immunity to WT *PtoDC3000* in an activity-dependent manner, consistent with being an immune protease.

We next tested bacterial growth of the Δ *hopQ1-1* mutant of *PtoDC3000*, which is pathogenic on *N. benthamiana* because it

lacks HopQ1-1, the Type-III effector that is recognised in *N. benthamiana* by Roq1 (Wei *et al.*, 2007). Remarkably, bacterial growth of *PtoDC3000*(Δ *hQ*) is indistinguishable between leaves overexpressing *NbRD21* and the EV control (Fig. S1a). We also tested bacterial growth of pv *tabaci* 6605 (*Pta6605*) and pv *syngae* B728a (*PsyB728a*) and found that bacterial growth was indistinguishable between leaves overexpressing *NbRD21* and the EV control (Fig. S1b,c). Thus, *NbRD21* suppresses the growth of WT *PtoDC3000*, which is recognised by Roq1, but not of *P. syngae* strains that cause disease.

Gene-edited *rd21* mutants have reduced protease activity and increased susceptibility

To investigate if *NbRD21* is also required for immunity, we disrupted the open reading frame of *NbRD21* (NbL08g19980) in

N. benthamiana with CRISPR/Cas9 genome editing. We selected two independent homozygous lines with truncated gene products: the *rd21-1* line has a 4-base pair deletion, and the *rd21-2* line has a 1 base pair insertion (Fig. 2a). We also sequenced two potential off-site targets of the used sgRNAs, but found that two RD21-like genes (NbL04g16800 and NbL17g14770) were unaltered in the *rd21* mutants (Fig. S2).

Activity-based labelling of total extracts with STR1 demonstrated that the fluorescent 36 kDa signal is absent in the mutants (Fig. 2b), indicating that this signal is caused by intermediate NbRD21. *In vivo* labelling by infiltrating STR1 into leaves also revealed that the 36 kDa signal is absent in both *rd21* mutants (Fig. 2c). The zLRase activity is also significantly reduced in total leaf extracts of *rd21* mutants when compared to the WT control (Fig. 2d), demonstrating that NbRD21 caused the major zLRase activity in total extracts. Despite lacking NbRD21, the *rd21* mutant plants have no macroscopic developmental or growth phenotype (Fig. 2e). Infection assays revealed that *rd21* plants are slightly but significantly more susceptible to WT *PtoDC3000* (Fig. 2f), consistent with reduced growth of *PtoDC3000* upon NbRD21 overexpression (Fig. 1f).

Proteomic analysis of *rd21* mutants

To identify changes in the proteome of *rd21* mutants, we performed shotgun proteomics on total leaf extracts of both *rd21*

lines and the WT control and annotated the spectra to the LAB360 proteome (Ranawaka *et al.*, 2023). A total of 2779 proteins of *N. benthamiana* were detected and PCA analysis revealed that the *rd21* mutants are distinct from WT samples (Fig. S3). We therefore combined these *rd21* samples for a statistical analysis to identify differentially accumulating proteins. We identified 15 proteins that accumulate significantly less in the *rd21* mutants, including NbRD21 itself (Fig. 3a,b, Table S3). Interestingly, four Rab GTPases also accumulate significantly less in the *rd21* mutants: Rab1 (NbL10g13430), Rab2 (NbL19g08250), Rab11 (NbL14g13650), and Rab8a (NbL08g02890) (Fig. 3c).

Amongst the 49 proteins that accumulate significantly more in both *rd21* mutants (Table S3) are six receptor-like kinases (RLKs) of which four have extracellular leucine-rich repeats (LRRs): BAM1 (Barely-Any-Meristem-1, NbL19g02860), BRI1 (Brassinosteroid Insensitive-1, NbL15g00030), BIR1 (BAK1-Interacting-RLK-1, NbL09g13380), HAK1 (Herbivory-associated Kinase-1, NbL05g07580), and two have extracellular malectin-like domains: FER1 (Feronia-1, NbL05g07500) and FER2 (Feronia-2, NbL19g15210) (Fig. 3d). Other proteins that accumulate significantly more in the *rd21* mutants are two vacuolar sorting receptors (VSR1 (NbL08g14990) and VSR3 (NbL17g22450), Fig. 3e) and various hydrolases including a vacuolar processing enzyme (VPE, NbL08g18900), subtilisin SBT1.7 (NbL16g14350), and two serine carboxy peptidases-like

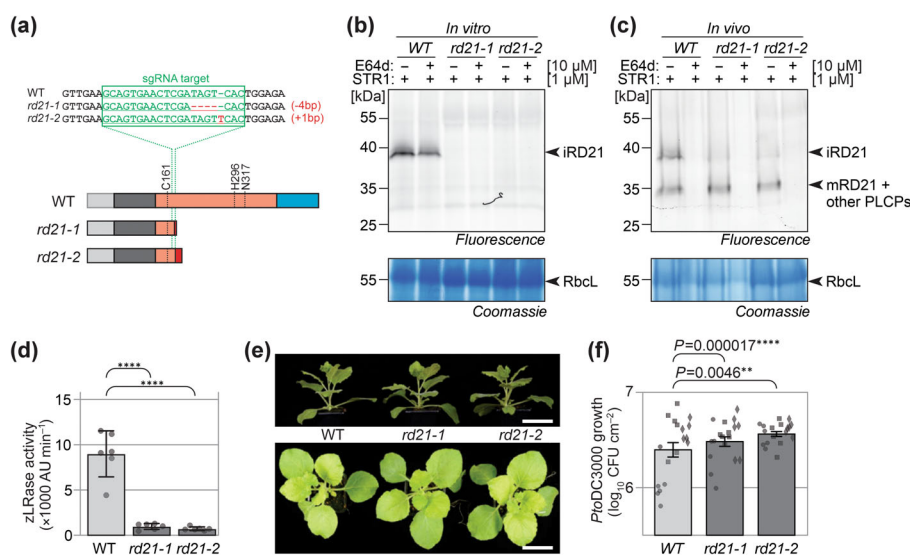


Fig. 2 *rd21* mutant plants have reduced zLRase activity and increased susceptibility. (a) Two *rd21* mutants contain frameshift-inducing indels in the sgRNA-targeted region. *Nicotiana benthamiana* mutants *rd21-1* and *rd21-2* carry a 4 bp deletion and a 1 bp insertion, respectively. (b) Reduced *in vitro* papain-like cysteine proteases (PLCP) labelling in both *rd21* mutants. Leaves from 4-wk old plants were injected with 10 μ M E-64d or Mock and incubated in the dark for 1 hr and then extracts were made and labelled with 1 μ M STR1 for 2 h. Proteins were separated by SDS-PAGE and scanned for fluorescence or stained with Coomassie. (c) Reduced *in vivo* PLCP labelling in both *rd21* mutants. Leaves of 4-wk old plants were injected with 10 μ M E-64d or Mock and incubated in the dark for 1 h and then injected with 1 μ M STR1 and incubated for 2 hrs. Leaf extracts were generated, separated by SDS-PAGE and scanned for fluorescence and stained with Coomassie. (d) Both *rd21* knockouts have significantly reduced zLRase activity. Protease activity was measured in total leaf extracts using 40 μ M zLR-AMC. Error bars represent SE of *n* = 6 replicates. *P*-values were determined using one-way ANOVA and Dunnett's multiple comparisons test. ****, *P* < 0.0001. (e) Both *rd21* mutants have no growth or developmental phenotype. Shown are representative photos of 4-wk-old plants. Bar, 7 cm. (f) *PtoDC3000* growth is increased in *rd21* knockout lines. Leaves of wild-type (WT) and *rd21* knockout plants were infiltrated with wild-type *PtoDC3000* at OD₆₀₀ = 0.0001. Bacterial growth in colony-forming units (CFU) was measured at 3 dpi. Bars represent SE of *n* = 6 replicates of three experiments. *P*-values were calculated by two-way ANOVA followed by *post-hoc* comparison using the Dunnett test. **, *P* < 0.01; ****, *P* < 0.0001; dpi, days post inoculation.

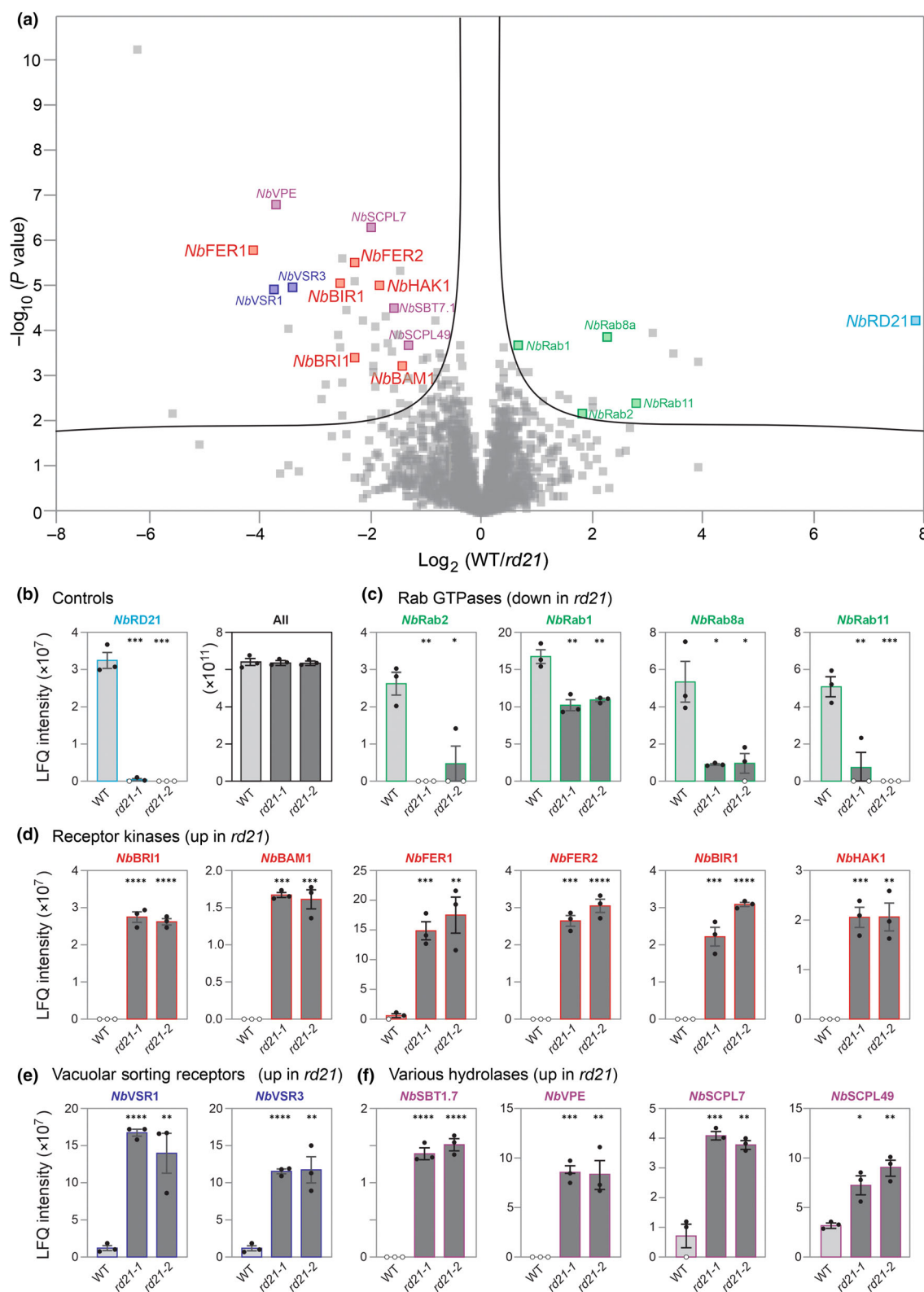


Fig. 3 Differential protein accumulation in *rd21* knockout plants. (a) Volcano plot of MS data showing $\log_2$ of the fold-change in label-free quantification (LFQ) intensity between wild-type and both *rd21* knockout lines against the significance ($-\log_{10}(P\text{-value})$) of this difference. All data points above the black line have significant *P*-values (*t*-test, FDR = 0.05, SO = 0.1). (b–f) Bar charts showing raw LFQ intensities in wild-type (WT) and both knockout (*rd21-1* and *rd21-2*) plants. Open dots indicate the absence of a detectable MS signal. All in (B) is the sum of the intensities of all detected peptides of *Nicotiana benthamiana* proteins. Error bars represent SE of *n* = 3 samples. Student's *t*-test compared to WT values: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

proteins (SCPL49 (NbL05g22050) and SCPL7 (NbL18g14840), Fig. 3f; Table S3).

To test if increased RLK levels in *rd21* plants are associated with higher transcript levels of the corresponding RLK-encoding genes, we performed quantitative RT-PCR on these plants with primers specific for *FER1*, *BRI1*, *BAM1*, and *HAK1*, using *eIF4A* as a control. This RT-qPCR experiment demonstrated that transcript levels of these four RLKs are not different in the *rd21* mutants (Fig. S4), indicating that the higher RLK accumulation is a post-translational process.

To investigate the higher accumulation of RLKs in *rd21* mutants further, we searched for all detected RLKs and evaluated their differential LFQ intensity. Besides the six RLKs discussed above, we identified 16 additional RLKs in the proteomics dataset. Three RLKs accumulated to similar LFQ intensities in *rd21* mutants when compared to WT plants (Fig. S5), but the remaining 13 RLKs seem to accumulate more in the *rd21* mutants, although for 10 RLKs this is not statistically significant because they were not detected in sufficient samples (Fig. S6). RLKs that accumulate more in *rd21* plants, as well as the RLKs that have that tendency, carry very different ectodomains and belong to different RLK subfamilies assigned in the metaRLK database (Zhang *et al.*, 2026, Fig. S7). Likewise, the three RLKs that accumulate to similar levels are unrelated: two RLKs have different LRR ectodomains and the other RLK carries a lectin-G ectodomain (Fig. S7). In conclusion, although protein accumulation is unchanged for three RLKs, most RLKs accumulate higher in *rd21* plants, indicating that increased protein levels are common for diverse RLKs in *rd21* mutants.

Transient expression confirms increased RLK accumulation in *rd21* mutants

To confirm the increased accumulation of RLKs in the *rd21* mutants, we transiently expressed *NbBAM1*-GFP and *NbFER2*-GFP driven by a constitutive 35S promoter in the *rd21* mutants and WT control plants and monitored their accumulation by measuring GFP fluorescence. GFP fluorescence was detected for *NbBAM1*-GFP but not for *NbFER2*-GFP (Fig. 4a). *NbBAM1*-GFP fluorescence was significantly increased in both *rd21* mutants (Fig. 4a,b), consistent with the higher accumulation of *NbBAM1* in *rd21* plants in the proteomics experiment. Confocal imaging showed that GFP fluorescence of *NbBAM1*-GFP accumulates in the plasma membrane and in endosome-like vesicles, but this subcellular localisation does not seem to be different between WT and *rd21* mutants (Fig. 4c).

Transient expression of *NbFER2*-GFP, however, did not produce sufficient GFP fluorescence (Fig. 4a) but triggered cell death instead (Fig. 4d). We therefore measured the reduction of leaf autofluorescence in the red spectrum as a quantitative readout for cell death (Xi *et al.*, 2021). These measurements demonstrated that *NbFER2*-GFP significantly reduces red autofluorescence in both *rd21* mutants when compared to the WT control plants (Fig. 4e). Since cell death is a functional readout of *NbFER2*-GFP, these data are consistent with the increased accumulation of this receptor in *rd21* plants.

To test if higher transient expression in *rd21* mutants is not caused by increased *Agrobacterium* susceptibility, we expressed cytoplasmic GFP (cGFP) and secreted RFP (sRFP) (Dodds *et al.*, 2025) in WT and *rd21* plants and monitored their expression by measuring fluorescence. This experiment revealed that transient GFP expression causes fluorescence that is not significantly different in the *rd21* mutant compared to WT plants (Fig. S8A). Likewise, the transient expression of secreted RFP also caused fluorescence that is similar between WT and *rd21* plants (Fig. S8B). Thus, the increased levels of *BAM1*-GFP fluorescence and *FER2*-GFP-induced cell death cannot be caused by increased transient expression in *rd21* mutants but involve a post-translational process that affects specific proteins.

Discussion

Protease activity assays established that *NbRD21* is responsible for most zLRase activity in leaf extracts. *NbRD21* overexpression suppresses the growth of *PtoDC3000*, and this requires *NbRD21* activity. However, *NbRD21* overexpression did not suppress bacterial growth of virulent *P. syringae* strains. Conversely, *rd21* plants are significantly more susceptible to *PtoDC3000*. Proteomics uncovered that *rd21* mutants accumulate more hydrolases, vacuolar sorting receptors, and receptor kinases, whilst four Rab proteins accumulate less in *rd21* plants. Further proteome studies indicated that most, but not all, RLKs accumulate in *rd21* plants, and RT-PCR experiments indicated that this is not due to higher transcript levels of RLK-encoding genes. Transient expression of two of these RLKs indicates that they indeed accumulate more in *rd21* mutants. This higher accumulation is not caused by general improved transient expression in *rd21* plants because similar fluorescence levels were produced upon transient expression of cytoplasmic GFP or secreted RFP.

The observation that *NbRD21* overexpression only caused phenotypes with avirulent *PtoDC3000* and not with the virulent *PtoDC3000*(*ΔhQ*), *Pta6605* or *PsyB728a* is intriguing. The dependence of the disease phenotype in *PtoDC3000* on the Type-III effector HopQ1-1 might suggest that HopQ1-1 is a substrate of *NbRD21*, but this is unlikely, as HopQ1-1 is a cytoplasmic effector (Li *et al.*, 2013), whereas RD21 is mostly in the endomembrane system (Ondzighi *et al.*, 2008; Lampl *et al.*, 2013). Rather, we believe that the phenotype can be explained by the fact that HopQ1-1 triggers the effector-triggered immune (ETI) response that includes the release of the vacuolar content into the apoplast upon the fusion of the vacuolar and plasma membranes (Hatsugai *et al.*, 2009). During ETI, *PtoDC3000* will be exposed to large quantities of *NbRD21* released from the vacuole, and this may directly or indirectly reduce bacterial growth. The absence of disease phenotypes with virulent strains might be explained by the fact that *NbRD21* concentrations in the apoplast are lower than in the vacuole, and these *NbRD21* levels might be sufficiently suppressed during infection by CIP1 and other PLCP inhibitors (Shindo *et al.*, 2016; Sueldo *et al.*, 2024).

The *rd21* mutants showed a drastically reduced zLRase activity in total extracts, indicating that *NbRD21* is responsible for this

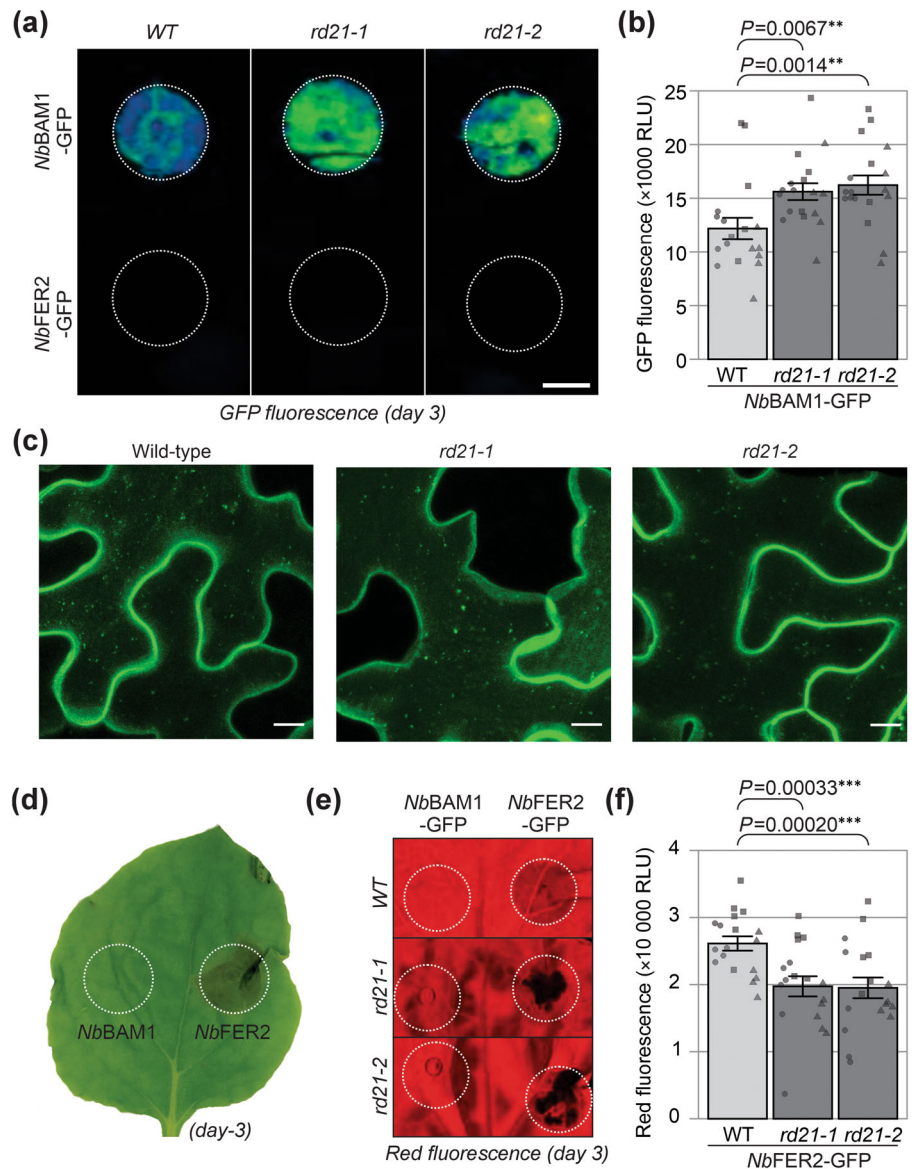


Fig. 4 *NbBAM1* and *NbFER2* levels are increased in *rd21* plants. (a) GFP fluorescence upon transient expression of *NbBAM1*-GFP and *NbFER2*-GFP of *Nicotiana benthamiana*. Shown are agroinfiltrated regions expressing *NbBAM1*-GFP (top) and *NbFER2*-GFP (bottom). Bar, 1 cm. (b) Quantified GFP fluorescence upon transient expression of *NbBAM1*-GFP. Error bars represent SE of $n = 6$ replicates of three experiments. P -values were calculated by two-way ANOVA followed by *post-hoc* comparison using the Dunnett test. **, $P < 0.01$. (c) Z-projection of confocal images of transiently expressed *NbBAM1*-GFP in various genotypes at day 4. Bar, 10 μm . (d) Cell death triggered by transient expression of *NbFER2*-GFP at day 3. (e) Red autofluorescence upon transient expression of *NbBAM1*-GFP and *NbFER2*-GFP. (f) Quantified red autofluorescence upon transient expression of *NbFER2*-GFP. (a–f) *NbBAM1*-GFP and *NbFER2*-GFP were transiently expressed in WT and *rd21* plants and images were taken 3 d later. Error bars represent SE of $n = 6$ replicates over three experiments. P -values were calculated by two-way ANOVA followed by *post-hoc* comparison using the Dunnett test. ***, $P < 0.001$.

activity. This zLRase activity probably comes from *NbRD21* directly because zLR-AMC is a good substrate for purified *NbRD21* *in vitro* (Paireder *et al.*, 2017; named *NbCysP6* there). Indeed, zLRase activity is also strongly increased upon overexpressing *NbRD21* but not *NbRD21*^{CI61A}. The decreased RbcL degradation during activity-based labelling of leaf extracts is consistent with the fact that RD21 is a major protease activity in *N. benthamiana*. In fact, RbcL degradation in *Arabidopsis* leaf extracts was even stronger in the presence of SDS, which activates latent RD21 (Gu *et al.*, 2012). The diversity of substrates is consistent with *NbRD21* being a general protease, rather than a specific processing enzyme. Indeed, purified *NbRD21* can completely degrade various IgG antibodies *in vitro* and Proteomics Identification of Cleavage Sites revealed that *NbRD21* has a broad substrate range, only preferring hydrophobic residues at the P2 position (Paireder *et al.*, 2017). Despite these strong biochemical phenotypes, *rd21* mutants grow indistinguishably from

WT *N. benthamiana*, which is similar to the absence of growth phenotypes in *rd21* mutants of *Arabidopsis thaliana* (Shindo *et al.*, 2012).

Our analysis of leaf proteomes, however, revealed several proteins that accumulate differentially in *rd21* plants. Perseus analysis of LFQ values revealed 49 proteins accumulating more in the *rd21* mutants, which could be direct and indirect *NbRD21* substrates, or result from mechanisms attempting to compensate for *NbRD21* loss. However, none of the hydrolases accumulating in the *rd21* mutants is predicted to replace the broad protease specificity of *NbRD21*. Rather, we detect the accumulation of specific processing enzymes such as VPE, which cleaves after Asn(N) and Asp(D) residues (Yamada *et al.*, 2020), and putative phytaspase SBT1.7, which seems to cleave after Asp(D) residues (Buscaill *et al.*, 2024). Interestingly, VPEs and SBT1.7 might accumulate in response to reduced *NbRD21* activity. Prodomain removal of RD21 proteases is catalysed by other proteases that are present in

leaf extracts (Yamada *et al.*, 2001). Although VPEs were proposed to be responsible because of Asp/Asn residues at the prodomain junction (Yamada *et al.*, 2001), quadruple *vpe* mutants showed unaltered RD21 activation (Gu *et al.*, 2012), indicating that other proteases might act redundantly with VPEs in RD21 activation. Interestingly, SBT1.7 is a phytaspase that cleaves after Asp(D) residues and might act redundantly with VPE in *NbRD21* activation. Thus, both VPE and SBT1.7 might be upregulated in an attempt to activate *NbRD21* in the *rd21* mutant. This activation by Asp-specific proteases is similar to the activation of papain-like Rcr3 protease of tomato by subtilase P69B, cleaving at Asp(D) residues after the prodomain (Paulus *et al.*, 2020). Like Rcr3, *NbRD21* also carries an Asp(D) at this position.

Proteome analysis revealed an increased accumulation of six RLKs in *rd21* plants. Further analysis of the proteomics data indicated that another 13 RLKs accumulate higher in *rd21* plants, whilst three RLKs accumulate equally well in *rd21* plants. These RLKs carry various ectodomains and belong to different subfamilies. RT-PCR analysis showed that the increased accumulation of FER1, BRI1, BAM1, and HAK1 is not associated with increased expression of genes encoding these RLKs.

These data suggest that *NbRD21* is involved in RLK homeostasis, either by directly degrading these proteins or by regulating their protein levels otherwise. Indeed, the higher accumulation of transiently expressed *NbBAM1*-GFP confirms the increased RLK accumulation. The fact that *NbBAM1*-GFP is expressed from a constitutive 35S promoter and still differentially accumulates in *rd21* mutants implies that increased *NbBAM1* accumulation is not caused by differential gene expression but by post-translational regulation. Likewise, although the *NbBAM1* gene carries an intron, the 35S:*BAM1*-GFP construct does not carry this intron, excluding the possibility that differential accumulation is caused by differential splicing. The absence of detectable GFP fluorescence with transiently expressed *NbFER2*-GFP is probably caused by the cell death that is triggered by *NbFER2* expression, which is associated with pleiotropic phenotypes described for Feronia mutants in Arabidopsis (Malivert & Hamant, 2023). Cell death might only be triggered at high FER2 levels reached upon agroinfiltration because *rd21* plants have no phenotypes despite increased FER2 levels. Although the cause of this cell death is yet unclear, this cell death response is a functional readout of *NbFER2* activity and the increased cell death in the *rd21* mutants, therefore, implies that also *NbFER2*-GFP accumulates to higher levels in the *rd21* mutant.

NbRD21 might decrease RLK receptor levels by degrading them directly, or indirectly, by regulating RLK homeostasis, either before delivery to the plasma membrane, or after. Receptors that are not folded in the ER are selected by ER quality control and degraded through ER-associated protein degradation. It is possible that *NbRD21* facilitates the degradation of misfolded receptors. However, *NbRD21* can also act in receptor-mediated endocytosis (RME), where receptors are sorted at the plasma membrane (PM) into vesicles which travel through the endosomal system and are either recycled back to the PM or taken to the vacuole for degradation (Claus *et al.*, 2018). A role of *NbRD21*

in RME is supported by the increased accumulation of vacuolar sorting receptors VSR1 and VSR3 in the *rd21* mutants and the reduced levels of Rab proteins involved in vesicle fusion in the *rd21* mutants. Furthermore, *AtRD21* also accumulates in the trans-Golgi network and in lytic vesicles (Ondizighi *et al.*, 2008), and cysteine protease inhibitor E-64 caused the accumulation of endosomes in starving BY-2 tobacco cell cultures (Yamada *et al.*, 2005), suggesting that a PLCP like RD21 might facilitate endosomal trafficking to the vacuole. We did indeed detect *NbBAM1*-GFP in vesicles, but its subcellular localisation is not altered in the *rd21* mutants.

The absence of developmental phenotypes and disease phenotypes with virulent *P. syringae* strains is remarkable, given the increased accumulation of *c.* 20 diverse RLKs in noninfected *rd21* plants. There are several explanations for this. First, BIR1 is a negative regulator of RLKs (Liu *et al.*, 2016, 2023) and its increased accumulation might cancel out increased RLK activities. Second and similarly, the effect of increased RLK activity may be cancelled out by the reduced immunity described in *rd21* mutants (reviewed in Huang & van der Hoorn, 2025). Third, increased RLK activities might have been cancelled out by Type-III effectors of *P. syringae*, many of which effectively suppress RLK activities (Block & Alfano, 2011). But besides cancelling out increased RLK activity, it is also possible that the increased RLK protein levels represent a larger proportion of RLKs that are no longer functional and are destined for degradation, originating either from the ER or the PM. *NbRD21* might facilitate the removal of these nonfunctional RLKs, either directly, or indirectly.

Taken together, we have established important new tools in elucidating the functions of RD21-like proteases in plants and used these to discover their involvement in receptor homeostasis in plants. These *rd21* mutants and other materials and protocols will be instrumental for future research into receptor kinases and the roles of RD21 proteases.

Acknowledgements

We sincerely thank Ursula Pyzio for excellent plant care and Sarah Rodgers, Caroline O'Brien, and Patricia Bowman for technical support. This project was financially supported by Interdisciplinary DTC project DDT00060 AP01.40 (AG); H2020 project 760331 'Newcotiana' (PJ); BBSRC projects BB/S003193/1 (MS) and BB/Y00969X/1 (KZ); Chinese Academy of Sciences Scholarship (YL); and ERC project 101019324 (JH, TL, RALvdH).

Competing interests

None declared.

Author contributions

RALvdH conceived the project; AG, LE, MS and KZ performed most experiments with the help of YL, TL and JH; PVJ generated the *rd21* mutant; RT performed microscopy; TL performed RT-

PCR and RLK classification; TB and JK provided materials; and JH, FK and MK performed proteomic analysis; RALvdH wrote the manuscript with input from all authors.

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Data availability

The mass spectrometry proteomics data for the on-bead digestions have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno *et al.*, 2016) partner repository (<https://www.ebi.ac.uk/pride/archive/>) with the dataset identifier PXD063276.

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Supporting Information

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

Fig. S1 No differential bacterial growth in *rd21* mutants for virulent *P. syringae* strains.

Fig. S2 Unaltered off-targets of used sgRNAs.

Fig. S3 PCA analysis of the shotgun proteomics on the nine samples.

Fig. S4 Transcript levels of RLK-encoding genes.

Fig. S5 No increased accumulation of three RLKs in *rd21* plants.

Fig. S6 Thirteen additional RLKs seem to accumulate more in *rd21* mutants.

Fig. S7 Domain structure of the detected RLKs.

Fig. S8 No differential cytoplasmic GFP or secreted RFP accumulation in *rd21* mutants.

Table S1 Used oligonucleotides.

Table S2 Used plasmids.

Table S3 Proteomics data.

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