Letters

Helper NLR proteins NRC2a/b and NRC3 but not NRC1 are required for Pto-mediated cell death and resistance in *Nicotiana benthamiana*

Introduction

Plants defend against pathogens using both cell surface and intracellular immune receptors (Dodds & Rathjen, 2010; Win *et al.*, 2012). Plant cell surface receptors include receptor-like kinases (RLKs) and receptor-like proteins (RLPs), which respond to pathogen-derived apoplastic molecules (Boller & Felix, 2009; Thomma *et al.*, 2011). By contrast, plant intracellular immune receptors are typically nucleotide-binding leucine-rich repeat (NB-LRR or NLR) proteins, which respond to translocated effectors from a diversity of pathogens (Eitas & Dangl, 2010; Bonardi *et al.*, 2012). These receptors engage in microbial perception either by directly binding pathogen molecules or indirectly by sensing pathogen-induced perturbations (Win *et al.*, 2012). However, signaling events downstream of pathogen recognition remain poorly understood.

In addition to their role in microbial recognition, some NLR proteins contribute to signal transduction and/or amplification (Gabriels *et al.*, 2007; Bonardi *et al.*, 2011; Cesari *et al.*, 2014). An emerging model is that NLR proteins often function in pairs, with 'helper' proteins required for the activity of 'sensors' that mediate pathogen recognition (Bonardi *et al.*, 2011, 2012). Among previously reported NLR helpers, NRC1 (NB-LRR protein required for hypersensitive-response (HR)-associated cell death 1) stands out for having been reported as a signaling hub required for the cell death mediated by both cell surface immune receptors such as Cf-4, Cf-9, Ve1 and LeEix2, as well as intracellular immune receptors, namely Pto, Rx and Mi-1.2 (Gabriels *et al.*, 2006, 2007; Sueldo, 2014; Sueldo *et al.*, 2015). However, these studies did not take into account the *Nicotiana benthamiana* genome sequence, and it remains questionable whether NRC1 is indeed required for the reported phenotypes.

Functional analyses of NRC1 were performed using virus-induced gene silencing (VIGS) (Gabriels *et al.*, 2007), a method that is popular for genetic analyses in several plant systems, particularly the model solanaceous plant *N. benthamiana* (Burch-Smith *et al.*, 2004). However, interpretation of VIGS can be problematic as the experiment can result in off-target silencing (Senthil-Kumar & Mysore, 2011). In addition, heterologous gene fragments from other species (e.g. tomato) have been frequently used to silence homologs in *N. benthamiana*, particularly in studies that predate the sequencing of the *N. benthamiana* genome (Burton *et al.*, 2000; Liu *et al.*, 2002b; Lee *et al.*, 2003; Gabriels *et al.*, 2006, 2007; Senthil-Kumar *et al.*, 2007; Oh *et al.*, 2010). In the NRC1 study, a fragment of a tomato gene corresponding to the LRR domain was used for silencing in *N. benthamiana* (Gabriels *et al.*, 2007). Given that a draft genome sequence of *N. benthamiana* has been generated (Bombarely *et al.*, 2012) and silencing prediction tools have become available (Fernandez-Pozo *et al.*, 2015), we can now design better VIGS experiments and revisit previously published studies.

Two questions arise about the NRC1 study. First, is there a *NRC1* ortholog in *N. benthamiana*? Second, are the reported phenotypes caused by silencing of *NRC1* in *N. benthamiana*? In this study, we investigated *NRC1*-like genes in solanaceous plants using a combination of genome annotation, phylogenetics, gene silencing and genetic complementation experiments. We discovered three paralogs of *NRC1*, which we termed *NRC2a, NRC2b* and *NRC3*, are required for hypersensitive cell death and resistance mediated by Pto, but are not essential for the cell death triggered by Rx and Mi-1.2. *NRC2a/b* and *NRC3* weakly contribute to the hypersensitive cell death triggered by Cf-4. Our results highlight the importance of applying genetic complementation assays to validate gene function in RNA silencing experiments.

Materials and Methods

Identification of NRC homologs and phylogenetic analyses

Tomato NRC1 (Solyc01g090430) was used to identify homologs in the predicted protein databases (*Nicotiana benthamiana* Genome v0.4.4 predicted protein, Tomato proteins ITAG release 2.40, and Potato ITAG release 1 predicted proteins) on Solanaceae Genomics Network (SGN). Top hits of BLASTP search results were collected for further analyses. NRC2 homologs in potato were missing in Potato ITAG release 1 predicted proteins database. Therefore, two NRC2 sequences of potato identified in Potato PGSC DM v3.4 protein sequences were included in the analyses. The phylogenetic tree of NRC homologs was built using MEGA6-BETA2 (Tamura *et al.*, 2013) with Neighbor-joining and Maximum-likelihood methods with bootstrap values based on 1000 iterations. Chromosome assignments of NRC homologs were based on tomato and potato genomes.

Cloning of solanaceous NRC homologs

Cloning of tomato *NRC* homologs was performed with Gateway cloning kit (Invitrogen, Thermo Scientific, Waltham, MA, USA) following the manufacturer's instruction. Primer pairs used in the

cloning are listed as follows: *Sl*NRC1-F/R (5′-CACCATGG TTGATGTAGGGGTTGAATTTC-3′ and 5′-CTAAGAAGC TGTCTGTACATCAGAATC-3′), *Sl*NRC2-F/R (5′-CACCA TGGCGAACGTAGCAGTGGAATTTC-3′ and 5′-TCAGAG ATCAGGAGGGAATATGGAAAG-3′), and *Sl*NRC3-F/R (5′-CACCATGGCGGATGTAGCAGTAAAGTTCTTA-3′ and 5′-TTACAATCCAAGATCATGAGGGAAT-3′). The amplified fragments from tomato cDNA were cloned into pENTR/D-TOPO (Invitrogen) and then introduced into the pK7WG2 destination vector (Karimi *et al.*, 2002) by Gateway LR recombination enzyme (Invitrogen). *Nicotiana benthamiana NRC2a, NRC2b* and *NRC3* were amplified with the corresponding primer pairs (*Nb*NRC2a-F/R: 5′-CACCATGGCGAACGTTGCGGT GGAGTTTCTGG-3′ and 5′-TCAGAGATCGGGAGGGAAT ATAGAGAGCTT-3′; *Nb*NRC2b-F/R: 5′-ATGGCGAACG TTGCGGTGGA-3′ and 5′-AATTGGTCTCTAAGCTTAG AGATCGGGAGGGAATATAGAG-3′; *Nb*NRC3-F/R: 5′-A ATTGGTCTCTAATGGCAGATGCAGTAGTGAATTTTCT GGTG-3′ and 5′-ATTGGTCTCGAAGCTTACTGTGTGGC CTTGGATCCAGCTTC-3′) from cDNA and cloned into pCR8/GW/TOPO (Invitrogen) by TA cloning. The fragments were then used for further amplification and subcloning into pICH86988 with Golden Gate cloning (Weber *et al.*, 2011). The synthetic fragments of *NbNRC2a/b* and *NbNRC3* were designed manually to introduce synonymous substitution in every codon when possible, and the syntheses of these fragments were performed by Genewiz (South Plainfield, NJ, USA). The synthetic fragments were then subcloned into pICH86988 together with the remaining *NbNRC2a, NbNRC2b* or *NbNRC3* fragment to generate full-length *NbNRC* variants.

Virus-induced gene silencing (VIGS)

VIGS was performed in *N. benthamiana* as described by Liu *et al.* (2002a). Suspensions of *Agrobacterium tumefaciens* strain GV3101 harboring TRV RNA1 (pYL155) and TRV RNA2 (pYL279) (Liu *et al.*, 2002a) with corresponding fragments from indicated genes were mixed in a 2 : 1 ratio in infiltration buffer (10 mM MES (2-[*N*-morpholino]ethanesulfonic acid), 10 mM magnesium chloride ($MgCl_2$), and 150 μM acetosyringone, pH 5.6) to a final OD_{600} of 0.3. Two-week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* for VIGS assays, upper leaves were used 2–3 wk later for further agroinfiltrations. For silencing of *NRC* homologs in *N. benthamiana*, 5′ coding region of each gene (*NbNRC2a/b*, 1-429b; *NbNRC2c*, 1-426bp; *NbNRC3*, 1-444bp) were cloned into TRV RNA2 vector. For co-silencing of *NbNRC2a/b* and *NbNRC3*, the fragments were fused by overlap PCR and cloned into TRV RNA2 vector.

The following primers were designed for generating the TRV2-*SlNRC1* construct based on the *SlNRC1* fragment that was used for silencing by Gabriels *et al.* (2007): 5′-CACCTTAAAGTCATTC CGAAACATGTTGG-3′ and 5′-TCGAGAGAACATACTCAG TGCAGC-3′. The silencing constructs for *SGT1* and *SERK3* were described previously (Peart *et al.*, 2002; Heese *et al.*, 2007).

Transient gene expression and complementation assays

In planta transient agroinfiltration assays were performed according to methods described previously (Bos *et al.*, 2006). Four to five-week-old *N. benthamiana* plants (i.e. 2–3 wk after virus inoculation) were used to test for cell death in VIGSed leaves. The concentration of *A. tumefaciens* strains carrying the expression constructs were adjusted in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, and 150 μM acetosyringone, pH 5.6). The final concentrations of *A. tumefaciens* (OD_{600}) used in cell death assays in VIGSed leaves are indicated as follows: Pto, 0.6 (de Vries *et al.*, 2006); AvrPto, 0.1 (de Vries *et al.*, 2006); Cf-4, 0.4 (Liebrand *et al.*, 2012), AVR4, 0.4 (Van der Hoorn *et al.*, 2000); Rx, 0.2 (Tameling & Baulcombe, 2007); CP, 0.1 (Tameling & Baulcombe, 2007); Mi-1.2^{T557S}, 0.8 (Lukasik-Shreepaathy *et al.*, 2012), INF1, 0.3 (Bos *et al.*, 2006) and *Sl*NRC/*Nb*NRC variants, 0.6. The HR cell death phenotype was scored at 7 d post infiltration (dpi), according to a previously described scale, which was modified from zero (no HR observed) to seven (confluent necrosis) (Segretin *et al.*, 2014). To detect the accumulation of proteins in complementation assays, tomato and *N. benthamiana* NRCs are subcloned into pK7WGF2 or pICH86966 with N-terminal GFP fusion (Karimi *et al.*, 2002; Weber *et al.*, 2011). Three days after agroinfiltration in control or *NRC*-silenced leaves, total plant proteins were extracted and analyzed by western blot analyses according to previously described methods (Oh *et al.*, 2009). Commercial anti-GFP (Invitrogen) and anti-rabbit antibody conjugated to horseradish peroxidase (Sigma-Aldrich) were used as primary and secondary antibody.

RNA extraction and semi-quantitative RT-PCR

Plant total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). 2 μg RNA of each sample was subject to first strand cDNA synthesis using Ominiscript RT Kit. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using DreamTaq (Thermo Scientific) with 25 to 30 amplification cycles followed by electrophoresis with 2% agarose gel stained with Ethidium bromide. Primer pairs used in the PCR reaction are listed as follows: *NbNRC2a/b-RT-F/R* (5′-AGTGGATGAGAGTGTGGGTG-3′ and 5′-AAGCAGGGA TCTCAAAGCCT-3′), *NbNRC2c-RT-R/F* (5′-TCAAAACATGC CGTGTTCAT-3′ and 5′-CCTGCGGGTTTTGTACTGAT-3′) and *NbNRC3-RT-F/R* (5′-CCTCGAAAAGCTGAAGTTGG-3′ and 5′-TGTCCCCTAAACGCATTTTC-3′). Primers for internal control *NbEF1α* were as described previously (Segonzac *et al.*, 2011).

Bacteria growth assay

VIGS was used to silence *NRC2a/b* and *NRC3* in both wild type and *Pto/Prf* transgenic (R411B) *N. benthamiana* plants (Balmuth & Rathjen, 2007). Bacteria growth assay were performed as previously described with minor modifications (Balmuth & Rathjen, 2007). The *Pseudomonas syringae* DC3000 Δ*hopQ1-1* culture (Wei *et al.*, 2007) was adjusted to OD_{600} of 0.2 and then diluted 10 000-fold with 10 mM $MgCl_2$. Five-week-old

N. benthamiana with VIGS control or *NRC2a/b/3*-silencing were inoculated with the bacterial culture using needleless syringe. Four replicate plants were sampled using 0.33 cm^2 cork borer at each time points, and then the sample were homogenized in 10 mM $MgCl_2$ for serial dilution and plating. Experiments were repeated three times with similar results. Polyclonal anti-myc antibody A-14 (Santa Cruz Biotechnology, Dallas, TX, USA) was used for detecting accumulation of Prf:5myc.

Nomenclature

The nomenclatures of the genes mentioned in this article are based on the orthology to NRC1 and results of phylogenetic analysis as shown in Fig. 1. Species names are indicated as two-letter prefixes to the gene names; *Sl* is used for tomato (*Solanum lycopersicum*), *St* is used for potato (*Solanum tuberosum*) and *Nb* is used for *N. benthamiana*.

DNA sequences and accession numbers

DNA sequence data from this article can be found in the SGN or GenBank/EMBL databases under the following accession numbers: *Sl*NRC1 (Solyc01g090430, NP_001234202), *Sl*NRC2 (Solyc10g047320), *Sl*NRC3 (XP_004238948.1), *Nb*NRC2a (NbS-00018282), *Nb*NRC2b (NbS00026706), *Nb*NRC2c (NbS000-31134), *Nb*NRC3 (NbS00011087), *St*NRC3 (Sotub05g007690).

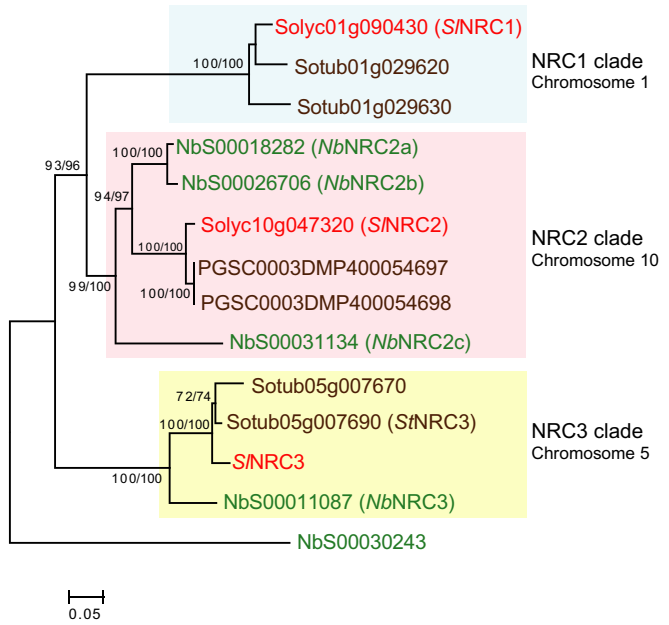

Fig. 1 Phylogenetic neighbor-joining (NJ) tree of NRC homologs in solanaceous plants. Top hits of BLASP search with tomato NRC1 (*Sl*NRC1) protein sequence were analyzed in MEGA6 to generate NJ and maximum-likelihood (ML) trees. Chromosome assignments are based on potato and tomato genome. Numbers at branches indicate bootstrap support values (1000 replicates) with NJ/ML methods at each node, and branch lengths indicate the evolutionary distance in amino acid substitution per site. Sequences from tomato (Solyc-), potato (Sotub-, PGSC-) and *Nicotiana benthamiana* (NbS-) are marked in red, brown and green, respectively.

Sequences of *SlNRC1*, *SlNRC2*, *SlNRC3* and *NbNRC3* were confirmed by cDNA sequencing, and are identical to the sequences in the database with accession numbers listed above. Sequence of *NbNRC2a and NbNRC2b* was re-annotated by sequencing the coding region and submitted to NCBI under accession numbers KT936525, KT936526.

Results and Discussion

NRC1 and related NLR proteins form a complex family in solanaceous plants

To identify putative homologs of NRC1 in *N. benthamiana*, potato, and tomato genomes, we performed a BLASTP (Altschul *et al.*, 1990) search against the predicted protein databases in SGN using the polypeptide sequence of tomato NRC1 (Solyc01g090430) as a query. Phylogenetic analyses and sequence comparisons of the top protein hits indicated that the NRC family is composed of at least three subclades (NRC1-3) belonging to clade CNL-14 as described by Andolfo *et al.* (2014). This NRC/CNL-14 clade is distinct from a previously described clade CC_R/CNL-RPW8, which includes helper NLRs ADR1 and NRG1 (Collier *et al.*, 2011; Andolfo *et al.*, 2014). Surprisingly, a *N. benthamiana* ortholog was missing in the NRC1 subclade and a tomato ortholog was also missing in the NRC3 subclade.

To determine whether the missing sequences are due to misannotation in the tomato and *N. benthamina* genomes, we searched all the available nucleotide and protein databases of *N. benthamiana* and tomato in SGN with representative NRC sequences. We failed to identify sequences that show high similarity to NRC1 in *N. benthamiana*, even after blast searches against scaffolds and contigs sequences in both SGN and the *N. benthamiana* genome database (http://www.benthgenome.com) (Naim *et al.*, 2012; Nakasugi *et al.*, 2014). We, therefore, concluded that NRC1 is probably missing in the *N. benthamiana* genome although it may have been somehow omitted from the assembly. By contrast, using TBLASTN searches, we detected a misannotated tomato gene in contig SL2.40ct02653 with high similarity to potato NRC3. Based on sequence comparisons, this gene has three exons and two introns; the first two exons were annotated as Solyc05g009630 whereas the third exon was missing in the annotation (Supporting Information Fig. S1a). To validate the sequence and expression of tomato *NRC3*, we designed primers based on our predicted full-length sequence and performed PCR using tomato cDNA and genomic DNA as template. We successfully amplified a fragment from genomic DNA and cDNA (Fig. S1b). The amplified cDNA fragment was cloned and sequenced. The identity between tomato NRC3 and potato NRC3 is 95%, consistent with our interpretation that the encoding gene is the *NRC3* ortholog in tomato (Fig. S1c).

Phylogenetic analyses that include the newly identified tomato NRC3 revealed that the sequences in the NRC family fall into three subclades that are supported by robust bootstrap values (Fig. 1). Pairwise comparisons indicated that protein sequences from the same subclade have at least 78% sequence identity (Figs 1, S2; Table S1). According to the genome information of potato and

tomato, sequences in these three clades are located on three different chromosomes (Fig. 1), consistent with the view that genes in the same NRC subclade are orthologous.

Silencing of NRC family members suppresses cell death mediated by Pto

We exploited the *N. benthamiana* genome sequence and associated gene silencing target prediction tool (SGN VIGS tool; http://vigs.solgenomics.net) to analyze the specificity of the *NRC1* VIGS fragment that was used in the *NRC1* VIGS experiments (Gabriels *et al.*, 2007). We found that the tomato *NRC1* (*SlNRC1*) fragment, which matches the LRR domain, would most probably target the *N. benthamiana* genes *NbNRC2a/b* and *NbNRC2c*, and possibly *NbNRC3*. Based on pairwise sequence comparisons, this *SlNRC1*-LRR fragment has 70–80% sequence identity to *NbNRC2a/b/c* and *NbNRC3* (Fig. S3). This prompted us to test the degree to which silencing of the individual *NRC2a/b*, *NRC2c* or *NRC3* genes could suppress the cell death mediated by different immune receptors. To design more specific constructs for silencing individual *NbNRC* paralogs, we analyzed the *NbNRC* sequences with the VIGS tool. The 5′ coding regions of each gene provided the highest specificity and were selected to design new gene silencing constructs. *Nicotiana benthamiana* plants were subjected to VIGS and challenged with the cell death triggered by immune receptors Pto, Rx, and Mi-1.2. Silencing of *NRC2a/b* or *NRC3* moderately but significantly reduced the cell death mediated by Pto but not Rx and Mi-1.2 (Fig. 2a). Semi-quantitative RT-PCR indicated that the VIGS constructs reduced the expression of the targeted gene with no detectable effects on the other paralogs (Fig. 2b).

Next, we combined the two *NRC2a/b* and *NRC3* fragments in one construct with the aim of obtaining more robust phenotypes. Interestingly, the double-silencing construct that targets both *NRC2a/b* and *NRC3* dramatically suppressed Pto-mediated cell death close to background levels (Fig. 2a,c). Rx and Mi-1.2-mediated cell death remained unaffected with single or double NRC-silencing constructs, whereas silencing SGT1 compromised Rx-, Mi-1.2 as well as Pto-mediated cell death (Fig. 2a). These results suggest that *NRC2a/b* and *NRC3* may be functionally redundant in Pto-mediated cell death.

Gabriels *et al.* (2006, 2007) reported that silencing in *N. benthamiana*, with a tomato *NRC1* fragment matching part of the LRR, reduced the cell death induced by AVR4 and INF1, which are recognized extracellularly by RLPs (Rivas & Thomas, 2005; Du *et al.*, 2015). We tested whether silencing of *NRC2a/b*, *NRC2c* or *NRC3* genes impairs cell death triggered by these proteins. Silencing of *NRC2a/b* and/or *NRC3* weakly reduced the Cf-4/AVR4 cell death but did not affect INF1-triggered cell death, whereas cell death from both Cf-4/AVR4 and INF1 were reduced in *SERK3* silencing (Fig. 2b).

Our *NbNRC* silencing experiments did not fully match the results of Gabriels *et al.* (2006, 2007) given that we did not observe effects on cell death mediated by Rx, Mi-1.2, and INF1 with any of the tested constructs (Fig. 2a). This prompted us to perform VIGS with the original fragment used in Gabriels *et al.* (2006, 2007) (Fig. S4, see also nucleotide alignment of this fragment with *NbNRCs* in Fig. S3). These VIGS experiments revealed moderate effects on Pto and Mi-1.2 mediated cell death but no detectable alteration of cell death mediated by Rx, Cf-4 and INF1 (Fig. S4). The discrepancy between our results and those of Gabriels *et al.* (2006, 2007) are striking but could still be due to differences in experimental set up and materials used in the experiments. To summarize, we observed robust reduction of Pto-mediated cell death after silencing *NRC2a/b* and *NRC3*, as a result we decided to focus on these genes in follow-up experiments.

Tomato NRC3 mediates Pto-induced cell death

To clarify which tomato NRC homologs are implicated in Pto-mediated cell death, we performed complementation experiments in *N. benthamiana* plants silenced for endogenous *NRC* genes (Figs 1, 2). Our experiment was motivated by the observation that the tomato NRC sequences are probably divergent enough from the *N. benthamiana* ones to be resilient to silencing. These experiments revealed that *SlNRC3* partially rescued Pto-elicited cell death, *SlNRC2* showed weak complementation activity but *SlNRC1* did not rescue Pto-mediated cell death (Fig. S5a). To test whether silencing affects the expression of *SlNRC1*, *SlNRC2*, and *SlNRC3*, we generated GFP-tagged *Sl*NRC1, *Sl*NRC2, and *Sl*NRC3 and assessed protein accumulation in *NRC2a/b/3* silenced leaves. Protein level of *Sl*NRC variants were reduced but were still detectable in *NRC2a/b/3*-silenced leaves (Fig. S5b). Based on these results and the observation that the *NRC1* ortholog is missing in *N. benthamiana*, we reasoned that *NRC1* is not the gene responsible for Pto-elicited hypersensitive response in *N. benthamiana*.

Genetic complementation with synthetic *NbNRC2a/b* and *NbNRC3* genes

We aimed to determine which *N. benthamiana NRC* homologs are implicated in the cell death elicited by Pto given that the complementation assays were performed with tomato *NRC* genes (Fig. S5). We focused on *NRC2a/b* and *NRC3*, as silencing of *NRC2c* did not yield reduction of cell death (Figs 2, S2). To achieve this, we generated a synthetic version of *NbNRC2a*, *NbNRC2b*, and *NbNRC3*, termed *NbNRC2a*^{syn}, *NbNRC2b*^{syn}, and *NbNRC3*^{syn}, with shuffled synonymous codon sequences and that should be divergent enough to evade VIGS (Figs 3a, S6a, S7a). Expression of *NbNRC2a*^{syn}, *NbNRC2b*^{syn}, or *NbNRC3*^{syn} in *NRC2a/b*/3-silenced *N. benthamiana* leaves rescued the cell death mediated by Pto, whereas the original versions of *NbNRC2a*, *NbNRC2b*, or *NbNRC3* gene failed to complement (Figs 3b, S6b, S7b). To confirm that the synthetic variants of *NbNRC2a*, *NbNRC2b*, and *NbNRC3* evade VIGS, we generated GFP-tagged *Nb*NRC2a, *Nb*NRC2b, and *Nb*NRC3 variants and assessed protein accumulation in *NRC2a/b/3* silenced leaves (Figs 3c, S6c, S7c). *Nb*NRC2a^{syn}, *Nb*NRC2b^{syn}, and *Nb*NRC3^{syn} accumulated to a similar level to control treatments whereas the original

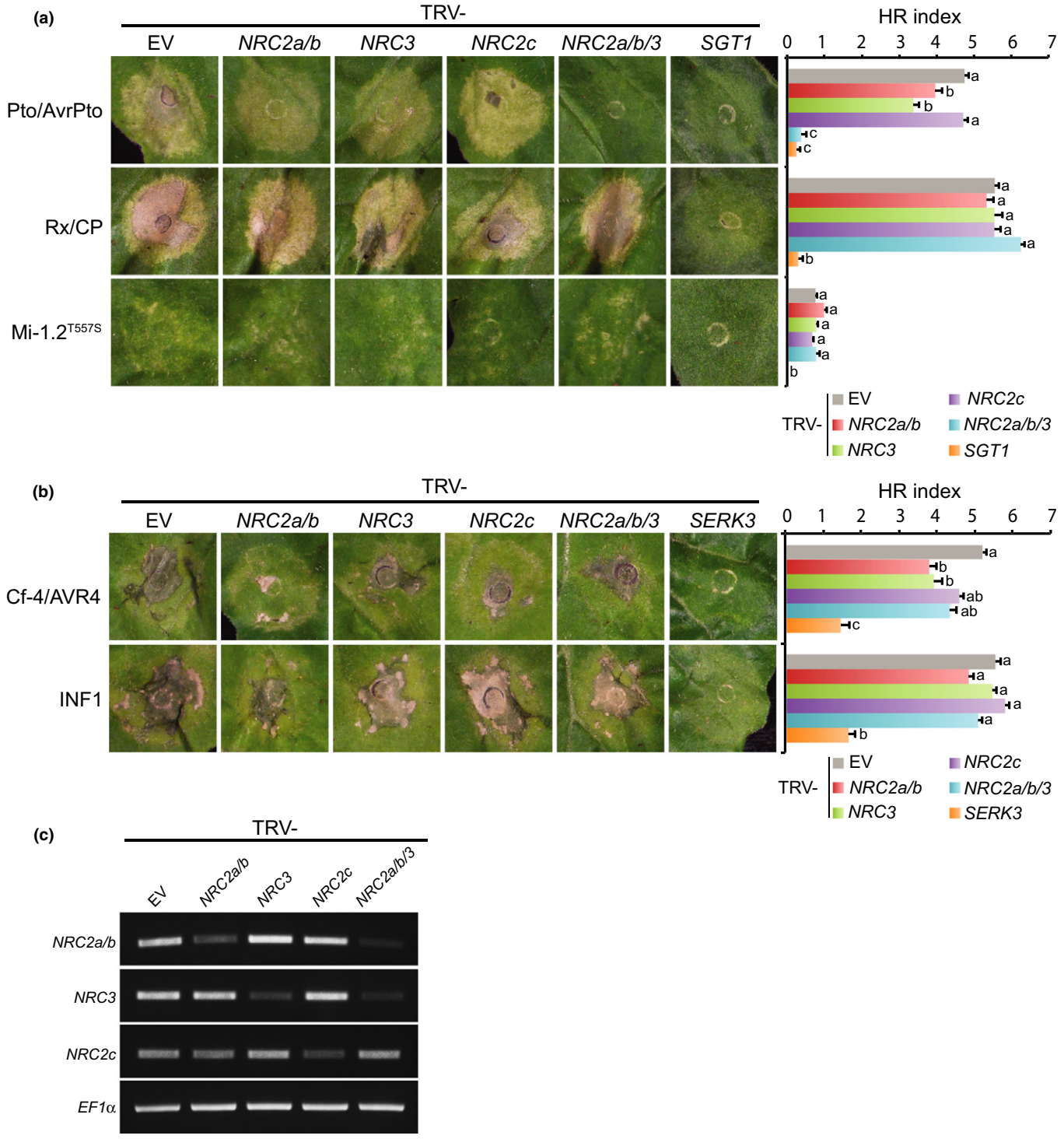

Fig. 2 Silencing of *Nicotiana benthamiana NRC* homologs suppress cell death mediated by Pto/AvrPto. (a, b) Immune receptors and corresponding AVR proteins, autoactive immune receptor (Mi-1.2^{T557S}), or elicitin (INF1) were transiently expressed in *N. benthamiana* leaves silenced with different *NRC* homologs. *SGT1* and *SERK3* silencing were used as control. The hypersensitive response (HR) results are presented with representative images. HR index was established at 7 d post infiltration (dpi). Bars represent mean + standard error (SE) of 24 infiltrations from one biological replicate. The different letters at the right of each columns indicate statistical significant differences based on ANOVA and Tukey's HSD test (*P*-value < 0.001). Experiments were performed at least three times with similar results. (c) Semi-quantitative RT-PCR of *NRC*-silenced *N. benthamiana* leaves. Leaves were collected 3 wk after virus inoculation. Elongation factor-1α (*EF1α*) was used as an internal control.

*Nb*NRC2a, *Nb*NRC2b, and *Nb*NRC3 were undetectable in silenced leaves, indicating that the shuffled codons enabled VIGS evasion (Figs 3c, S6c, S7c). These experiments clearly demonstrate that *NbNRC2a/b* and *NbNRC3* are the causal *N. benthamiana* genes that mediate hypersensitive death following Pto perception of AvrPto.

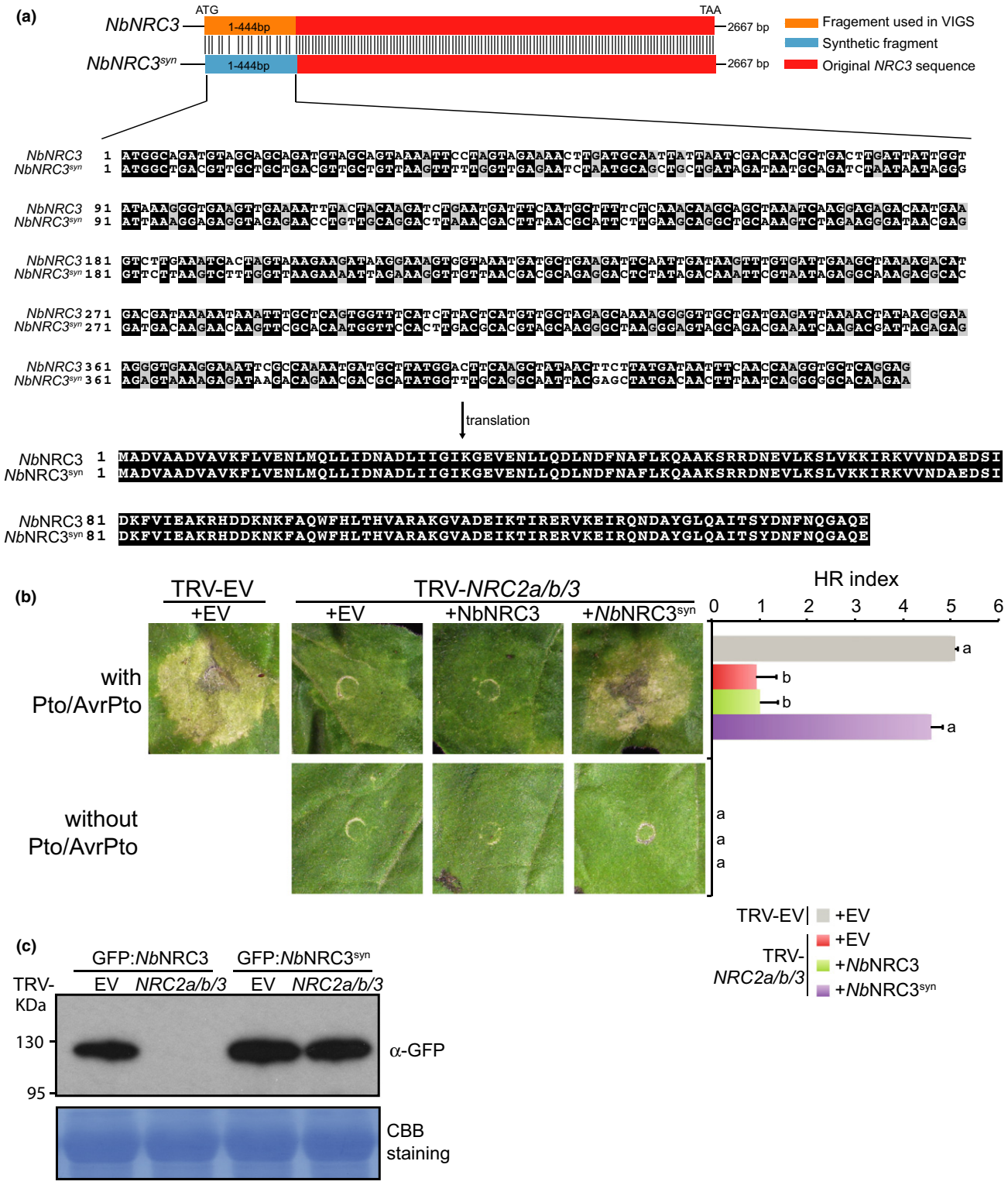

Fig. 3 Synthetic *Nb*NRC3 rescues Pto-mediated cell death in *NRC*-silenced *Nicotiana benthamiana*. (a) Schematic representation of experimental design, DNA and protein sequences of the synthetic region. Shuffled synonymous codons were introduced in the synthetic sequence (*NbNRC3*^{syn}) without changing the identity in protein sequence. (b) *Nb*NRC3^{syn} rescues the cell death of Pto/AvrPto. *Nb*NRC3 and *Nb*NRC3^{syn} were co-expressed with Pto/AvrPto in *NRC2a/b/3*-silenced *N. benthamiana* leaves. Expression of Pto/AvrPto in VIGS control (EV) and expression of *Nb*NRC3 and *Nb*NRC3^{syn} without Pto/AvrPto were used as control. Hypersensitive response (HR) index was established at 7 d post infiltration (dpi). Bars represent mean + SE of 14 infiltrations from one biological replicate. The different letters at the right of each columns indicate statistical significant differences based on ANOVA and Tukey's HSD test (*P*-value < 0.001). Experiments were performed at least three times with similar results. (c) Protein accumulation of NRC3 variants in VIGS control and *NRC2a/b/3*-silenced leaves. GFP is fused to NRC3 variants at N-terminal and transiently expressed in VIGS control and *NRC2a/b/3*-silenced *N. benthamiana*. Samples were collected at 3 dpi for Western blot analyses.

NRC2a/b and NRC3 are required for Pto-mediated resistance in *N. benthamiana*

In the previous study, Gabriels *et al.* (2007) showed that silencing of *NRC1* did not affect Pto-mediated resistance. Since we have now identified that *NRC2a/b* and *NRC3* are the genes required for Pto-mediated cell death, we decided to test whether silencing of *NRC2a/b* and *NRC3* compromise Pto-mediated resistance. *Pto/Prf* transgenic *N. benthamiana* has higher resistance to *P. syringae* DC3000 compared to wild type plants (Balmuth & Rathjen, 2007). Hence, we silenced *NRC2a/b* and *NRC3* in *Pto/Prf* transgenic *N. benthamiana* (R411B), and inoculated *P. syringae* DC3000 Δ*hopQ1-1* by syringe infiltration. Bacterial growth assay revealed that Pto-mediated resistance is compromised in *NRC2a/b* and *NRC3* silenced leaves (Fig. 4a), demonstrating that *NRC2a/b* and *NRC3* are required for Pto-mediated resistance. The results of western blot analyses indicated that the accumulation of Prf is not affect by *NRC2a/b* and *NRC3* silencing (Fig. 4b).

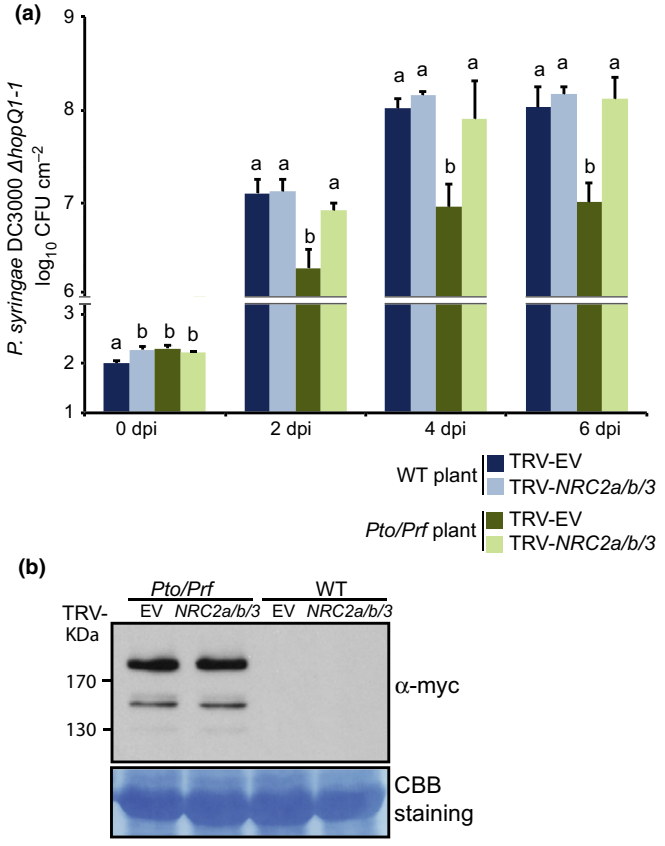

Fig. 4 Silencing of *NbNRC2a/b* and *NRC3* compromised Pto-mediated resistance. (a) Populations of *Pseudomonas syringae* DC3000 Δ*hopQ1-1* in VIGS control and *NRC2a/b/3*-silenced wild type (WT) or *Pto/Prf* transgenic (R411B) *Nicotiana benthamiana* were measured at 0, 2, 4, 6 d post inoculation (dpi). Error bars indicate + standard deviation (SD) of population from four replicates in one biological replicate. The different letters at the top of the columns indicate statistical significant differences based on ANOVA and Tukey's HSD test (P-value < 0.05). Experiments were performed three times with similar results. (b) Protein accumulation of Prf:5myc in VIGS control and *NRC2a/b/3*-silenced leaves. Leaves of wild type or *Pto/Prf* transgenic (R411B) *N. benthamiana* were collected 3 wk after virus inoculation. Accumulation of Prf:5myc was detected with α-myc antibody.

Conclusions

In summary, we revisited the role of NRC1 as a helper NLR protein and discovered that NRC2a/b and NRC3, rather than NRC1, are the causal proteins required for Pto-mediated cell death in *N. benthamiana*. Therefore, the previously proposed model of NRC1 as a signaling hub for multiple immune receptors postulated by Gabriels *et al.* (2007) needs to be revised. In fact, the *N. benthamiana* genome appears to lack an ortholog of tomato *NRC1* (Fig. 1). Furthermore, although NRC2a/b and NRC3 are required for the hypersensitive cell death induced by Pto, silencing of these genes did not affect the response elicited by Rx and Mi-1.2. The previous finding of Gabriels *et al.* (2007) that silencing of *NRC1* suppresses Rx and Mi-1.2 –mediated cell death may be due to other NRC1-like sequences in *N. benthamiana*. We did observe that *NRC* silencing reduced the cell death induced by Cf-4 as reported earlier (Gabriels *et al.*, 2007). However, the effect of *NRC2/NRC3* silencing is not as dramatic as in the case of Pto-mediated cell death (Fig. 2).

Our findings emphasize the importance of following RNA silencing experiments with genetic complementation assays to minimize the risk of misinterpreting data due to off-target effects (Kumar *et al.*, 2006; Jonchere & Bennett, 2013; Pliego *et al.*, 2013). Genetic complementation can be performed using genes from a different species or using a silencing-resilient synthetic version of the gene with shuffled codon sequences. We recommend that genetic complementation be applied to RNA silencing experiment whenever possible to avoid gene misidentification.

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Author contributions

C-H.W., K.B., T.O.B. and S.K. planned and designed the research. C-H.W. and M.S.B. performed experiments, C-H.W. and S.K. analyzed data and wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Cloning of tomato NRC3.

Fig. S2 Protein sequence alignment of NRC homologs of *N. benthamiana*.

Fig. S3 Pairwise alignment of *SlNRC1*-LRR fragment with *NbNRC2a* and *NbNRC3*.

Fig. S4 Virus-induced gene silencing (VIGS) assay with *SlNRC1*-LRR fragment.

Fig. S5 Tomato NRC3 mediates Pto-induced cell death in *N. benthamiana*.

Fig. S6 Synthetic *Nb*NRC2a rescues Pto-mediated cell death in *NRC*-silenced *N. benthamiana*.

Fig. S7 Synthetic *Nb*NRC2b rescues Pto-mediated cell death in *NRC*-silenced *N. benthamiana*.

Table S1 Pairwise protein sequences comparison of tomato and *N. benthamiana* NRC homologs

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Key words: cell death, *Nicotiana benthamiana*, NRC1 (NB-LRR protein required for HR-associated cell death 1), nucleotide-binding leucine-rich repeat protein, plant immunity, Pto.