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Supplemental Data

Chloroplastic Protein NRIP1 Mediates Innate Immune Receptor Recognition of a Viral Effector

Jeffrey L. Caplan, Padmavathi Mamillapalli, Tessa M. Burch-Smith, Kirk Czymmek, and S.P. Dinesh-Kumar

Supplemental Experimental Procedures

Plasmid constructs

Plasmids gN-TAP, gN-Citrine, gN-YN, gN(TIR)-TAP, p50-U1-3xHA, p50-U1-Ob-3xHA, p50-Cerulean, GUS-YC are described in (Burch-Smith et al., 2007). Citrine and the C-terminal 255 bases of Citrine were cloned into 3' end of p50 to generate the p50-Citrine and p50-YC constructs respectively. To generate cDNA NRIP1-Cerulean constructs under the native promoter, the p50 fragment was removed from our p50-Cerulean construct in pYL400 (Burch-Smith et. al., 2007). The coding sequence of *NRIP1* was amplified by polymerase chain reaction (PCR) and inserted in its place, resulting in 35S-NRIP1-Cerulean. The 35S promoter was replaced with the endogenous PCR amplified 1kb NRIP1 promoter to create NIP-Cerulean under its native promoter (pJC59). To generate genomic NRIP1-Cerulean constructs used for transient expression, the PCR amplified 1 kb NRIP1 promoter was ligated to *NRIP1's* genomic region containing all exons and introns. Cerulean, fused to the 3' end sequence of *NRIP1*, was then cloned into this intermediate plasmid (SPDK1012). To create TRV2-*NRIP1(FL)*, 836 bp of the NRIP1 was PCR amplified from NRIP1 cDNA and cloned into pYL156

(Liu et al., 2002a). TRV2-*NRIP1(3')* clone was generated by cloning PCR amplified 417 bp of the 3' untranslated region of *NRIP1* into pYL156. The primer pair 5'-TCT AGA ATG GCT GCT ATA ACT TCA GTT AGC G-3' and 5'- CTC GAG TCA GTT CCA GAA AAT ATG CCA AAA AAA GGC-3' were used to amplify *NbSTR14* from *N. benthamiana* cDNA and cloned into TRV2 cut with XbaI-XhoI to generate TRV2-*NbSTR14*. To generate GST-NRIP1, the NRIP1 coding sequence was amplified from *N. benthamiana* cDNA with primers containing *attB1-attB2* sites. To generate GST-NRIP1(C145S) mutant, a mutation was added with the primer 5'-ATA GTT GGG T**C**C CAG TTG GGG AAG A-3' The PCR products were cloned into pDEST15 using Gateway (Invitrogen). To generate TIR bait plasmid, TIR domain containing sequence from N cDNA was cloned into LexA vector, pTBS1 (Liu et al., 2002b). All constructs described above were confirmed by DNA sequencing.

Cloning *NRIP1* from *N. benthamiana*

The primer pair 5'-ACA AAG AGC TGA TCA TCT TTT CAA AAT C-3' and 5'-CGG CTC GAG GCA ATA TTT ATT TCA TCA-3' was used to amplify the full mRNA sequence from *N. benthamiana* cDNA and the full genomic sequence from *N. benthamiana* genomic DNA by PCR. The *NRIP1* promoter and terminator sequences were amplified from EcoRV and DraI GenomeWalker^{TM} (Clonetech) *N. benthamiana* libraries by nested PCR and cloned into the TOPO 2.1 vector (Invitrogen). Primer sequences will be available upon request.

Creation of transgenic NRIP1-Cerulean lines

N- and non-*N*-containing *N. benthamiana* plants were transformed with NRIP1-Cerulean under the control of its native promoter using leaf-disc transformation method (Horsch et al., 1985). Transformants were selected on 100mg/liter of kanamycin. PCR analyses were performed to confirm the presence of transformed construct in T0 plants. The transformants were selfed to obtain T1 and then T2 homozygous plants.

Yeast two-hybrid assay

A tomato cDNA library in pJG4-5 (Liu et al., 2002b) was transformed into EGY48/pSH18-34 yeast containing the TIR bait plasmid. Interacting proteins were selected on synthetic complete (SC) plates lacking uracil, histidine, tryptophan, and leucine. TIR interactors were selected after 2 to 3 days of incubation at 30^{o} C. Positive interactors were re-transformed into yeast strains carrying LexA-BS4 (TIR), LexA-N(TIR), LexA-N(NBS), LexA-N(LRR), LexA-N(TIR-NBS), LexA-N or LexA-p50 and tested for the ability to turn on *LEU2* reporter gene.

Expression of NRIP1 proteins in *E. coli*

BL21 CodonPlus® *E. coli* (Stratagene) cells transformed with GST-NRIP1 and GST-NRIP1(C145S) constructs were grown in 250 ml liquid cultures with appropriate antibiotics.. Expression was induced for 3 hours with 1 mM IPTG and cells were resuspended in 50 mM NaCl, 50 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 5% glycerol, 1% Triton X-100, 0.1% ß-mercaptoethanol, 1 mM PMSF, and 1X Complete® protease inhibitors (Roche). Cells were sonicated for 40 seconds three

times, debris was spun down, and 100 µl of glutathione-sepharose beads (Amersham Biosciences) was added to supernatant. The mixture was tumbled for 2 hours at 4°C and washed three times with lysis buffer. Proteins were eluted with 20mM glutathione and 50mM Tris/HCl pH 9.0 buffer. The glutathione was removed by dialysis overnight in Tris/HC1 pH 9.0.

Supplemental References

Burch-Smith, T. M., Schiff, M., Caplan, J. L., Tsao, J., Czymmek, K., and Dinesh-Kumar, S. P. (2007). A Novel Role for the TIR Domain in Association with Pathogen-Derived Elicitors. PLoS Biol *5*, e68.

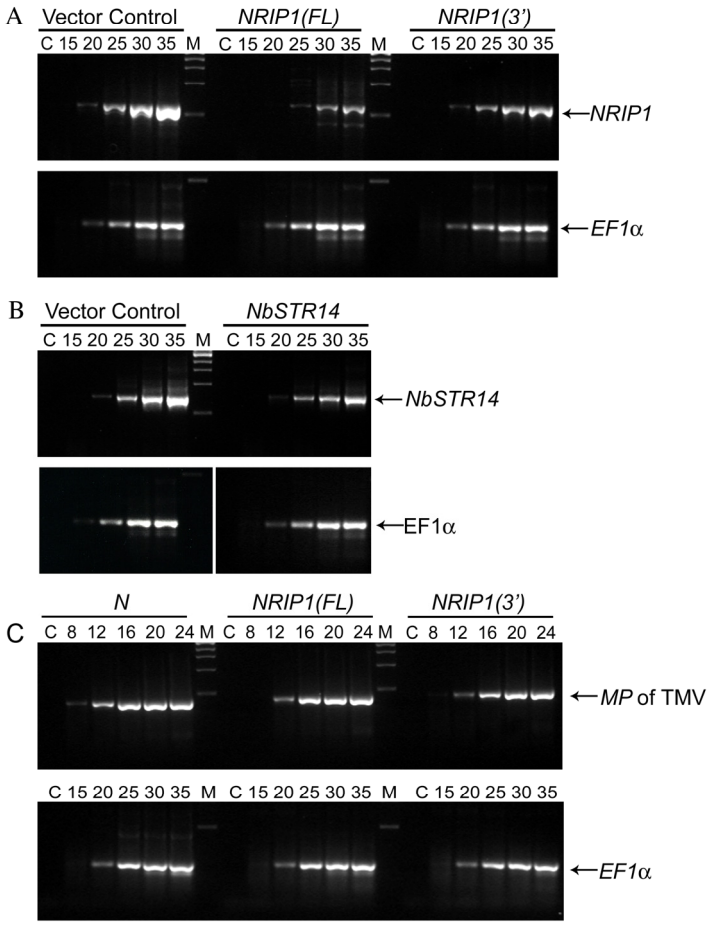
Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. (1985). A simple and general method for transferring genes into plants. Science *227*, 1229-1231.

Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S. P. (2002a). Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J *30*, 415-429.

Liu, Y., Schiff, M., Serino, G., Deng, X.-W., and Dinesh-Kumar, S. P. (2002b). Role of SCF ubiquitin-ligase and the COP9 signalosome in the *N* gene-mediated resistance response to tobacco mosaic virus. Plant Cell *14*, 1483-1496.

Supplemental Figures

Supplementary Figure 1
Caplan et al., 10/11/07



D

Experiment	*NRIP1(FL)*	*NRIP1(3')*	*N*	*STR14*	Vector Control
6/29/2007	4/4	N.D.	4/4	N.D	0/4
7/13/2007	6/6	3/3	6/6	0/6	0/6
8/24/2007	6/6	3/6	6/6	0/6	0/6
9/5/2007	5/5	5/5	5/5	0/6	0/6

Figure S1. Quantification of silencing data

(A) Semi-quantitative RT-PCR using tissue derived from VIGS vector control (left), NRIP1(FL)-silenced (center), and NRIP1(3')-silenced (right) plants were used to determine the degree of *NRIP1* silencing. Samples were collected at 15, 20, 25, 30, and 35 PCR cycles. C is the no RT control and M is the marker. The lower panel is the internal quantity control, EF-1α.

(B) The silencing efficiency of *NbSTR14* was determined by semi-quantitative RT-PCR using tissue derived from VIGS vector control (left) and NbSTR14-silenced (right) plants. Samples were collected at 15, 20, 25, 30, and 35 PCR cycles. C is the no RT control and M is the marker. The lower panel is the internal quantity control, EF-1α.

(C) The level of TMV movement was examined with semi-quantitative RT-PCR of the upper leaves of the *N*-silenced positive control (left), NRIP1(FL)-silenced (center), and NRIP1(3')-silenced (right) plants. Samples were collected at 8, 12, 16, 20, and 24 PCR cycles. C is the no RT control and M is the marker. The lower panel is the internal quantity control, EF-1α. Samples were collected at 15, 20, 25, 30, and 35 PCR cycles.

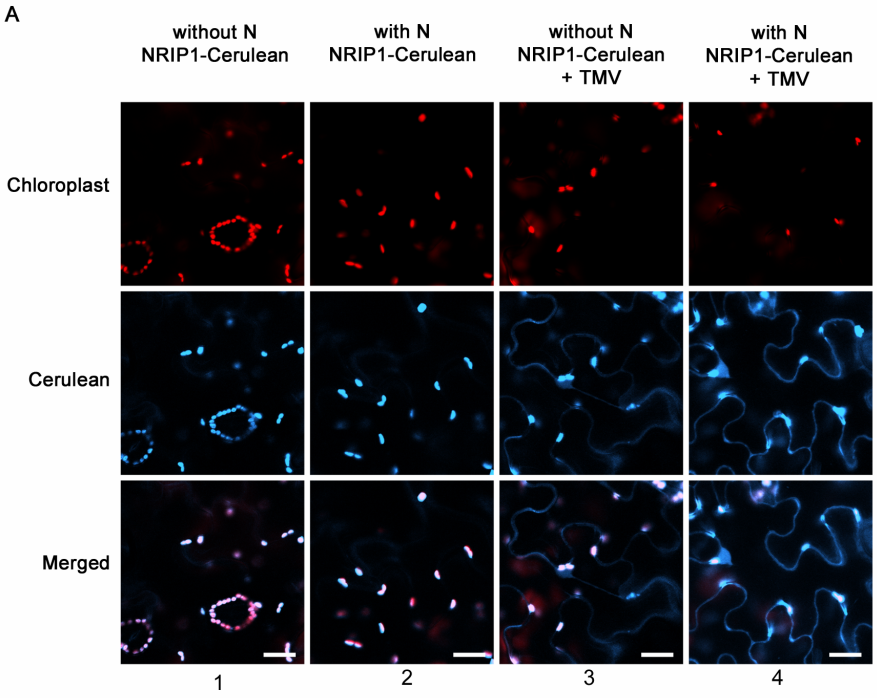
(D) Loss of *N*-mediated resistance to TMV. The number of plants that showed a loss of resistance of TMV that was indicated by movement of TMV-GFP to the upper leaves. Data was collected for four independent biological replicates.

Supplementary Figure 2
Caplan et al., 12/10/07

Figure S2. Amino acid sequence of NRIP1

The amino acid sequence of *N. benthamiana* NRIP1 is compared to related proteins radish DIN1 (RsDIN1), tobacco DIN1 (NtDIN1), cucurbits OP1 (CsOP1) and *Arabidopsis thaliana* SEN1 (AtSEN1). A line is drawn over the chloroplast-targeting signal. The catalytic domain is boxed. Alignment was performed with ClustalW and identical and similar residues highlighted with BoxShade program (http://www.ch.embnet.org/software/BOX_form.html).

Supplementary Figure 3
Caplan et al., 12/10/07



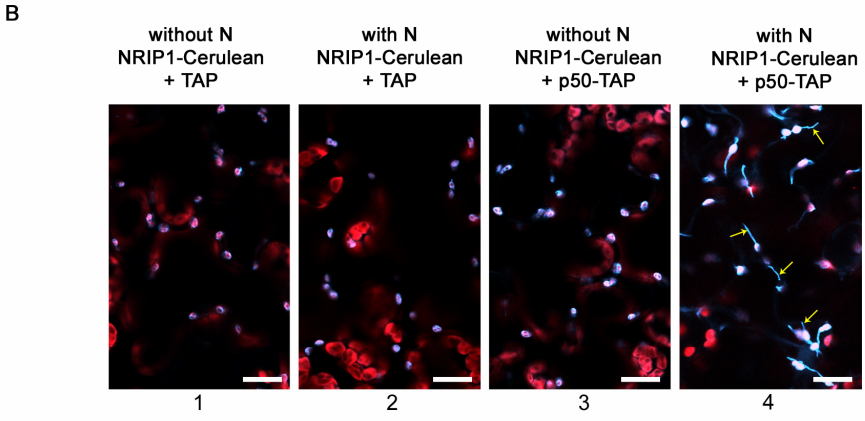


Figure S3. NRIP1 localization is TMV dependent

(A) NRIP1-Cerulean localizes to the chloroplasts in uninfected non-*N*-containing (column 1) and *N*-containing (column 2) NRIP1-cerulean transgenic plants. NRIP1-cerulean localizes to cytoplasm and nucleus in addition to chloroplasts in the presence of TMV in non-*N*-containing (column 3) or *N*-containing (column 4) NRIP1-Cerulean transgenic plants. Scale bar is 20 μm.

(B) Thread-like stromules (yellow arrows) were strongly induced in the presence of p50-TAP in NRIP1-Cerulean *N*-containing transgenic plants (column 4) but not in the presence of TAP alone (column 2). A strong increase in the quantity of stromules was not observed in NRIP1-Cerulean plants without *N* that expressed p50-TAP (column 3) or TAP (column 1). Scale bar is 20 μm.

Supplementary Figure 4
Caplan et al., 12/10/07

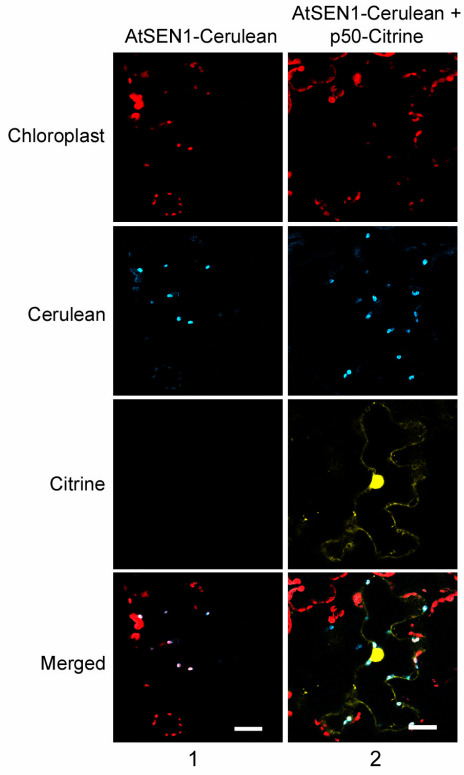


Figure S4. p50 does not alter the localization of AtSEN1

AtSEN1-Cerulean was expressed in wild-type *N. benthamiana* plants. AtSEN1-Cerulean alone colocalized to the red autoflourescence of chloroplasts (column 1). AtSEN1-Cerulean remained localized only to the chloroplasts in the presence of p50-U1-Citrine (column 2). Scale bar is 20 μm.

Supplementary Figure 5
Caplan et al., 12/10/07

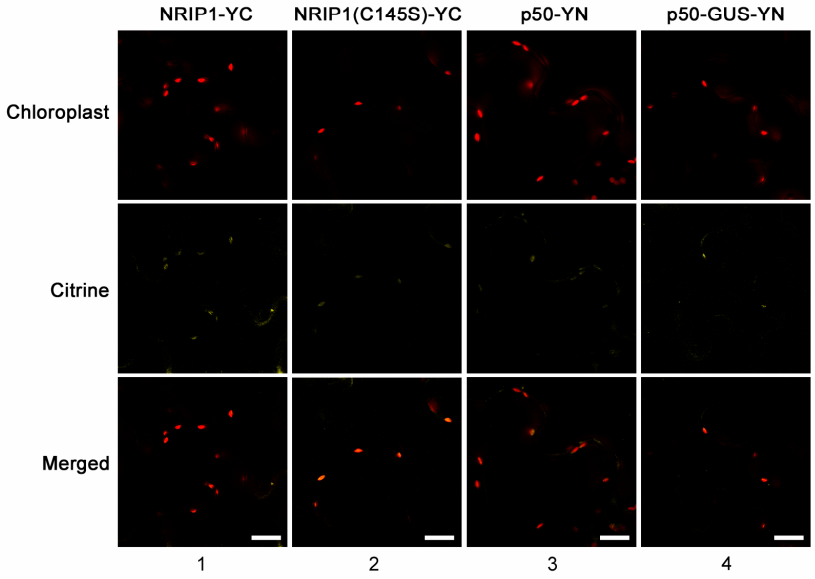
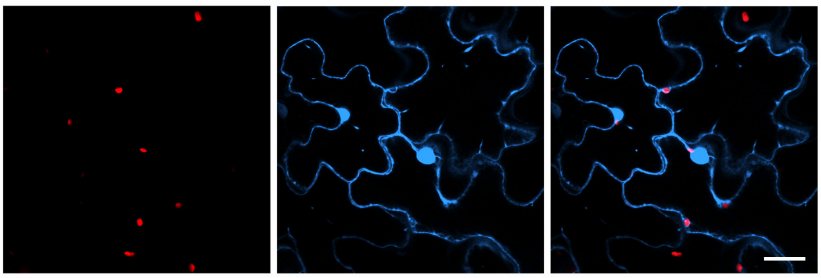


Figure S5. Controls of constructs used in BiFC assay

To determine if the constructs used in BiFC assays can result in fluorescence when expressed separately, NRIP1-YC (column 1), NRIP1(C145S)-YC (column 2), p50-YN (column 3), and GUS-YN (column 4) were expressed individually. Images were taken using comparable confocal settings used in BiFC assays. All YN constructs were detectable by Western blot analysis (data not shown). Scale bar is 20 μm.

Supplementary Figure 6
Caplan et al., 12/10/07



Chloroplast Cerulean Merged

Figure S6. NRIP1 lacking the signal peptide constitutively localizes to the cytoplasm and nucleus

NRIP1(-SP)-Cerulean lacks the chloroplast signal peptide. Expression of NRIP1(-SP)-Cerulean in wild-type *N. benthamiana* plants localized constitutively to the cytoplasm and nucleus. Colocalization with red autoflourescence of chloroplasts was not observed. Scale bar is 20 μm.