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# Supplemental Data

# Proteasome-Mediated Turnover of the Transcription Coactivator NPR1 Plays Dual Roles in Regulating Plant Immunity

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## Supplemental Experimental Procedures

### Chemical induction and pathogen infection

*Arabidopsis thaliana* Col-0, transgenic, and mutant plants were sown in soil (Metro Mix 200, Grace-Sierra, Milpitas, CA). After two weeks plants were transferred to individual pots and grown for an additional two weeks. Subsequently, plants were transferred to culture plates with the roots submerged in water, 0.5 mM SA, 100 μM cycloheximide (Sigma), or 100 μM of either MG115 or MG132 (Sigma). Alternatively, 12-day-old MS-grown seedlings were submerged in solutions containing 100 μM cycloheximide and 5 μM dexamethasone (Sigma).

The virulent bacterial leaf pathogen *Psm* ES4326 and the avirulent pathogen *Pst* DC3000/*avrRpt2* were grown overnight in liquid King's B medium. Bacterial cells were collected by centrifugation and diluted in 10 mM $MgCl_2$. For protein and mRNA analysis in systemic tissue, half leaves were infiltrated with *Pst* DC3000/*avrRpt2* ($OD_{600}$= 0.02) before the other halves were collected and frozen in liquid nitrogen until further analysis. To induce SAR, two lower leaves were pressure-infiltrated with *Pst* DC3000/*avrRpt2* ($OD_{600}$= 0.02). Three days later two upper leaves were challenged with *Psm* ES4326 ($OD_{600}$= 0.001). *In planta* growth of *Psm* ES4326 was determined three days after infection as described previously (Wang et al., 2006). All experiments were repeated three times with similar results.

### Mutagenesis of NPR1, plant transformation, and mutant isolation

The codons for Ser11/15 of NPR1 in pRTL2ΔN-mGFPS65T/*35S::NPR1-GFP* (Kinkema et al., 2000) were replaced with those encoding either alanine or aspartate, using a Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutations were confirmed by DNA sequencing. The *35S::npr1Ser-GFP* fragment was excised with *PstI* and cloned into the corresponding site of pGREENII0029 (Hellens et al., 2000) or pCB302, electroporated into *Agrobacterium tumefaciens* strain GV3101(pMP90), and transformed into *npr1-2* plants by floral dipping (Bent, 2000). Transgenic plants were selected on MS medium supplemented with kanamycin or

glufosinate ammonium. Independent transgenic plants with comparable transgene expression levels were selected for further analysis.

Homozygous *cul3a* (SALK_046638) and *cul3b* (SALK_098014) T-DNA insertion mutants were isolated from the SALK collection (Alonso et al., 2003), the *wrky38* mutant (SAIL_749_B02) from the SAIL collection (Sessions et al., 2002), and the *wrky62* mutant (SM_3_38820) from the EXOTIC collection (www.jic.ac.uk/science/cdb/exotic). Single mutants were crossed with each other and with *npr1-1* to yield double and triple mutants. The pCB302 plasmid carrying *35S::NPR1-GFP* (Mou et al., 2003) was used to transform the *cul3a cul3b* mutant. Trangenic plants were selected on soil by spraying with Basta (glufosinate ammonium, dilution 1:500). *35S::NPR1-GFP (in cop9)* plants were generated by crossing *35S::NPR1-GFP (in WT)* with heterozygous mutant *cop9-1* plants. Transgenic *35S::NPR1-GFP (in npr1-1)*, *35S::npr1-nls-GFP (in npr1-1)*, and *35S::NPR1-GR (in npr1-3)* plants were previously described (Kinkema et al., 2000; Mou et al., 2003).

**Protein analysis**

Protein extraction was performed by homogenizing leaf tissue in extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.2% Nonidet P-40, and inhibitors: 50 μg/ml TPCK, 50 μg/ml TLCK, 0.6 mM PMSF, 40 μM MG115, and Sigma phosphatase inhibitor cocktail; for analysis of ubiquitinylated NPR1 10 mM iodoacetamide was included). Homogenates were centrifuged (14,000 *g*) for 15 min at 4ºC. For immunoprecipitation, protein extracts were centrifuged twice, filtered through a 0.2 μm filter (Nalgene), and pre-cleared with protein A agarose beads (Upstate) for 1 hour. For co-immunoprecipitation analysis anti-CUL3 serum (Dieterle et al., 2005), anti-COP9 (Santa Cruz), anti-CSN4 (Affiniti), anti-CSN5 (Affiniti), and anti-GFP (AbCam) antibodies were added to the extracts (1:100 - 1:1000) and incubated for 2 hours with gentle rocking. Next, protein A beads were added (50 μl/ml) and incubated with gentle rocking for another hour. Beads were collected by brief centrifugation (6,000 rpm) and washed 5 times with extraction buffer. Proteins were eluted by boiling in 1X SDS sample buffer.

For Western blot analysis SDS sample buffer was added to the protein extracts from a 4 X stock solution and supplemented with or without 50 mM DTT. Protein samples were heated to 70°C for 10 min, separated on SDS-PAGE gels, and transferred to nitrocellulose membranes. Western blots were probed with anti-NPR1 (Mou et al., 2003; Tada et al., 2008), anti-pS11/15 (see below), anti-phosho Ser/Thr (Clonetech), anti-GFP (Clonetech), anti-CUL3, anti-Ubiquitin (FK-2, Upstate) anti-CAS (Han et al., 2003), or anti-TUB (Sigma).

A phospho-protein enrichment kit (Clontech) was used to column-purify phospho-proteins according to the manufacturer's protocol. The eluted phospho-proteins were further concentrated using StrataClean resin (Stratagene, CA).

**Phospho-antibody production**

The anti-pS11/15 antibody was produced by Proteintech Group (Chicago, IL). A phosphorylated NPR1 peptide (DGFAD S[$PO_3$] YEI S[$PO_3$] STSFVC) was synthesized and conjugated to KLH. The peptide was used to immunize rabbits. Antiserum was

purified in two affinity purification steps. First, the antiserum was purified using a phospho-peptide column. Second, the partly purified antibody was cross-absorbed with the non-phosphorylated peptide to eliminate any anti-non-phospho-peptide antibodies. The titer and specificity of the purified anti-pS11/15 antibody was tested by ELISA and Western blot analysis.

***In vitro* degradation assays**

Cell-free degradation assays were carried out essentially as described previously (Osterlund et al., 2000). Three-week-old plants were ground in liquid nitrogen and resuspended in proteolysis buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM NaCl, 10 mM ATP, and with or without 5 mM DTT). Protein extracts were centrifuged (14,000 $g$) for 10 min at 4°C and incubated at room temperature. Degradation reactions were terminated by addition of SDS sample buffer and incubation at 70°C for 10 min. Inhibitor studies were carried out for 2 hours in the presence or absence of MG115 (40 μM), MG132 (40 μM), PMSF (4 mM), Leupeptin (40 μM), or the solvent DMSO (0.2%).

***In vitro* NPR1-TGA interaction assay**

Plants were sprayed with 0.5 mM SA. After 16 hours total protein was extracted and NPR1-GFP immunoprecipitated as described above. TGA2 transcription factor was synthesized from a pET28a plasmid in rabbit reticulocyte lysates in the presence of $^{35}S$-labeled methionine, using a TNT Quick Coupled Transcription/ Translation kit (Promega). Lysates containing TGA factors were then incubated for 2 hours with the immunoprecipitated plant proteins in interaction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, and inhibitors: 50 μg/ml TPCK, 50 μg/ml TLCK, 0.6 mM PMSF, 40 μM MG115, and Sigma phosphatase inhibitor cocktail). Subsequently, the immunoprecipitates were washed 5 times with interaction buffer and analyzed by Western blotting and gel autoradiography.

**RNA analysis**

RNA extraction, electrophoresis, and hybridization to gene-specific probes were performed as described previously (Cao et al., 1994; Spoel et al., 2003). To synthesize cDNA, mRNA (2 μg) was incubated with oligo(dT) primer (2.5 μM), dNTPs (0.5 mM), DTT (5 mM), 1 X First-Strand Buffer (Invitrogen), and 200 units of Reverse Transcriptase (Invitrogen SuperScript III). The mixture was incubated at 50°C for 1 hour, followed by 70°C for 15 min. Real-time PCR was carried out on 20-times diluted cDNA using the Quantitect SYBR Green PCR kit (Qiagen) and gene-specific primers in a LightCycler (Roche) according to the manufacturer's protocols.

## Supplemental Figures

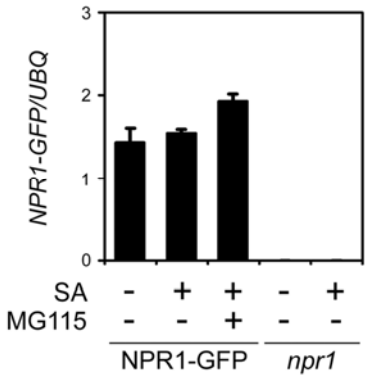


**Figure S1. The *35S::NPR1-GFP* transgene is expressed constitutively independent of SA or MG115 treatment.** *35S::NPR1-GFP* and *npr1-2* plants were treated with (+) or without (-) 0.5 mM SA and 100 μM MG115 for 28 hours. Subsequently, the expression of *NPR1-GFP* was analyzed using qPCR and normalized with constitutively expressed *UBQ*. Error bars represent SD ($n$ = 3).

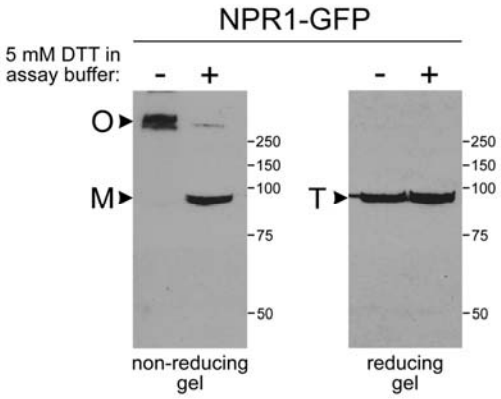


**Figure S2. DTT in the proteolysis assay buffer reduces the NPR1-GFP oligomer to monomer.**
Total protein was extracted from *35S::NPR1-GFP* (in *npr1-1*) plants in proteolysis buffer supplemented with (+) or without (-) 5 mM DTT. Subsequently, proteins were analyzed by non-reducing SDS-PAGE (left panel) to examine the relative levels of oligomer (O) and monomer (M), as well as by reducing SDS-PAGE (right panel) to detect the total level of NPR1-GFP. NPR1-GFP was visualized by Western blotting using an anti-GFP antibody. Molecular weight standards are indicated.

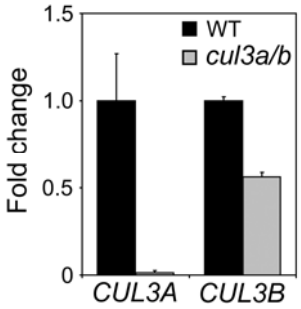


**Figure S3. Gene expression of *CUL3A* and *CUL3B* in the *cul3a cul3b* double mutant.**
Gene expression was analyzed using qPCR, normalized with constitutively expressed *UBQ*, and shown as fold change relative to the wild type. Error bars represent SD ($n$ = 3).

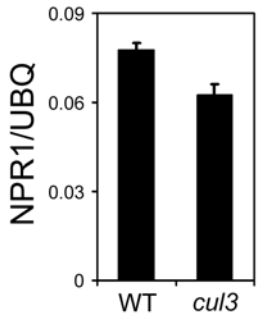


**Figure S4. NPR1 mRNA levels are reduced in the *cul3a cul3b* mutant.**
Gene expression of *NPR1* in wild-type (WT) and *cul3a cul3b* (*cul3*) plants was analyzed using qPCR and normalized with constitutively expressed *UBQ*. Error bars represent SD ($n$ = 3).

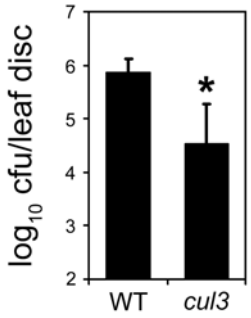


**Figure S5. Mutant *cul3*a *cul3b* plants show constitutive resistance.**
Virulent *Psm* ES4326 was inoculated in the leaves of wild-type (WT) and *cul3a cul3b* (*cul3*) plants. After three days the growth of *Psm* ES4326 was assessed. Cfu, colony-forming units. Error bars represent 95% confidence limits ($n$ = 8). Asterisk indicates a statistically significant difference compared with the control treatment (Tukey–Kramer ANOVA test; $\alpha = 0.05$, $n$ = 8).

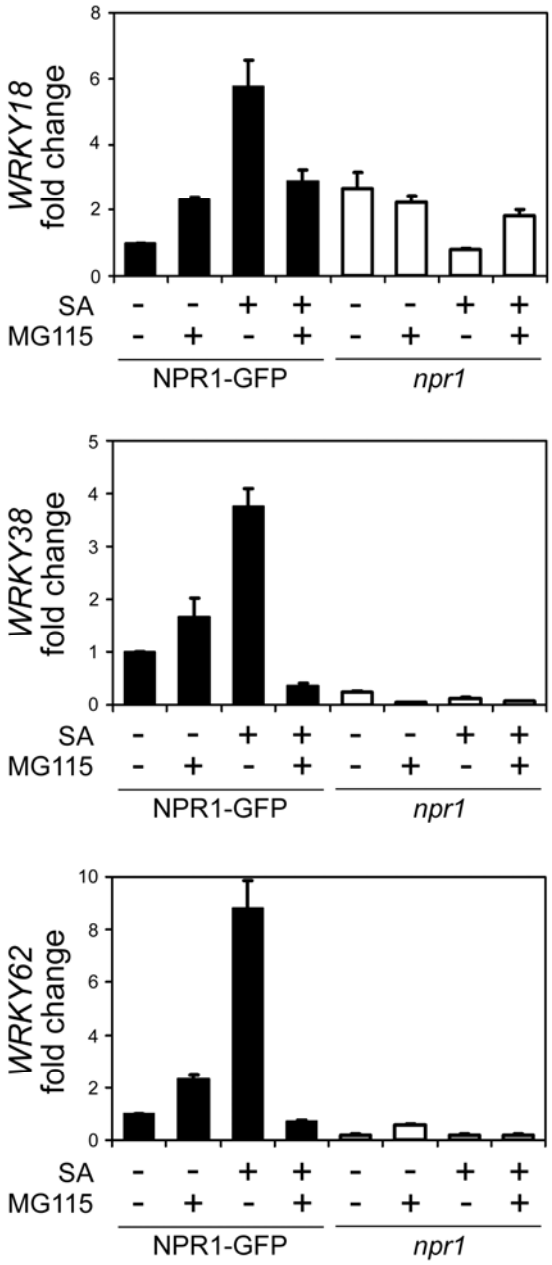


**Figure S6. NPR1 and the proteasome are required for SA-induced *WRKY* gene transcription.**

*35S::NPR1-GFP* and *npr1* plants were treated with (+) or without (-) 0.5 mM SA and/or 100 μM MG115 for 28 hours. Subsequently, the expression of *WRKY18*, *WRKY38*, and *WRKY62* was analyzed using qPCR. Data is presented as fold induction compared to untreated *35S::NPR1-GFP* plants. Error bars represent SD ($n$ = 3).

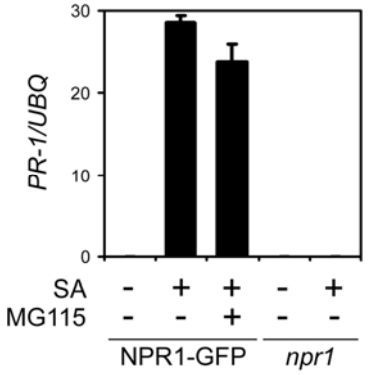


**Figure S7. The proteasome is partially required for *PR-1* gene expression.**
*35S::NPR1-GFP* and *npr1-2* plants were treated with (+) or without (-) 0.5 mM SA and 100 μM MG115 for 28 hours. Subsequently, the expression of *PR-1* was analyzed using qPCR and normalized with constitutively expressed *UBQ*. Error bars represent SD (*n* = 3).

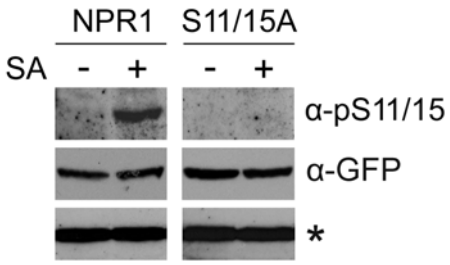


**Figure S8. Specificity of the anti-pSer11/15 antibody.**
*35S::NPR1-GFP* and *35S::npr1S11/15-GFP* plants were treated for 24 hours with water (-) or 0.5 mM SA (+). Total protein was extracted and analyzed by SDS-PAGE in the presence of DTT followed by Western blotting using anti-pS11/15 and anti-GFP antibodies. A non-specific band (*) confirmed equal loading.

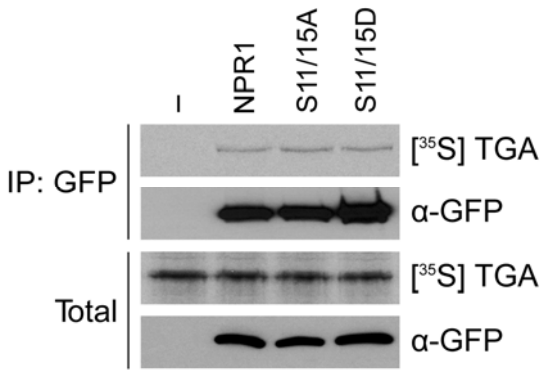


**Figure S9. The phosphorylation status of NPR1 does not affect interaction with TGA transcription factors.**
*35S::NPR1-GFP*, *35S::npr1S11/15-GFP*, *35S::npr1S11/15D-GFP* plants were treated for 16 hours with 0.5 mM SA. As a control, untransformed plants (-) were treated similarly. Total protein was extracted and immunoprecipitated (IP) with antibody against GFP. Immunoprecipitated proteins were incubated with *in vitro* translated radioactive [$^{35}$S]TGA protein for 2 hours, washed, and analyzed by autoradiography and Western blotting.

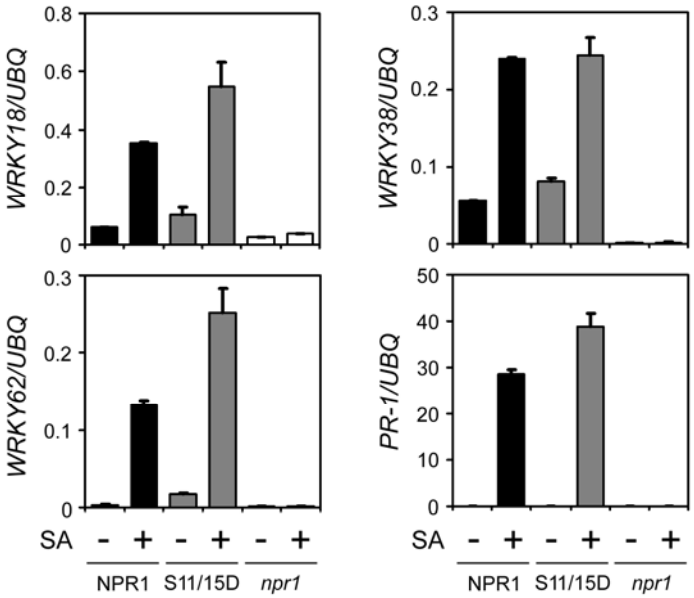


**Figure S10. Basal and SA-induced defense gene expression in *35S::npr1S11/15D-GFP* plants.**
*35S::NPR1-GFP*, *35S::npr1S11/15D-GFP*, and *npr1-2* plants were treated with (+) or without (-) 0.5 mM SA for 28 hours. Subsequently, gene expression of *WRKY18*, *WRKY38*, *WRKY62*, and *PR-1* was analyzed using qPCR and normalized with constitutively expressed *UBQ*. Error bars represent SD ($n$ = 3).

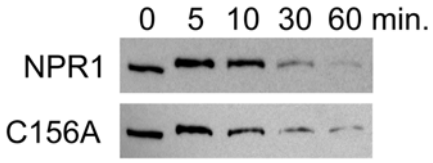


**Figure S11. The C156A mutation does not affect NPR1 degradation.**
Total protein was extracted from *35S::NPR1-GFP* and *35S::npr1C156A-GFP* plants in a buffer supporting proteolytic activity. Extracts were incubated at room temperature for the time points indicated. Subsequently, proteins were analyzed by SDS-PAGE in the presence of DTT followed by Western blotting using an anti-GFP antibody.

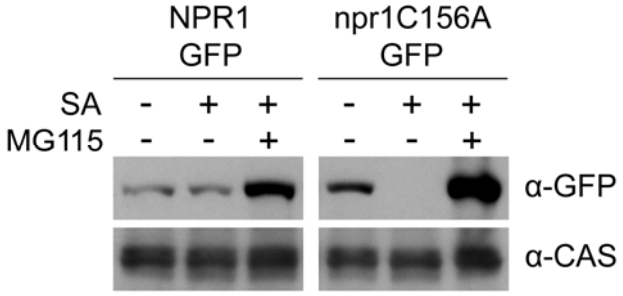


**Figure S12. SA-induced instability of the npr1C156A-GFP protein is rescued by proteasome inhibitor.**
*35S::NPR1-GFP* and *35S::npr1C156A-GFP* plants were placed in 6-well plates containing water. Subsequently, the leaves were sprayed with water or 0.5 mM SA. After 24 hours plants were transferred to new 6-well plates containing fresh water or 100 μM MG115. After an additional 24 hours total protein was analyzed by SDS-PAGE in the presence of DTT followed by Western blotting using an anti-GFP antibody. An antibody against a constitutively expressed calcium-sensing receptor (CAS) was used to confirm equal loading.

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