NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants

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Salicylic acid (SA) is a plant immune signal produced after pathogen challenge to induce systemic acquired resistance. It is the only major plant hormone for which the receptor has not been firmly identified. Systemic acquired resistance in Arabidopsis requires the transcription cofactor nonexpressor of PR genes 1 (NPR1), the degradation of which acts as a molecular switch. Here we show that the NPR1 paralogues NPR3 and NPR4 are SA receptors that bind SA with different affinities. NPR3 and NPR4 function as adaptors of the Cullin 3 ubiquitin E3 ligase to mediate NPR1 degradation in an SA-regulated manner. Accordingly, the Arabidopsis npr3 npr4 double mutant accumulates higher levels of NPR1, and is insensitive to induction of systemic acquired resistance. Moreover, this mutant is defective in pathogen effecter-triggered programmed cell death and immunity. Our study reveals the mechanism of SA perception in determining cell death and survival in response to pathogen challenge.

After pathogen challenge, host cells have to make a life-and-death decision to fend off infection. Recognition of a pathogen effector by a host resistance protein can lead to effector-triggered immunity (ETI), characterized by rapid programmed cell death (PCD) known as the hypersensitive response1. The clearly defined boundary of the hypersensitive response indicates the presence of a mechanism that controls cell death and survival. Despite intense studies of plant mutants defective in controlling the spread of PCD2, the regulatory mechanism still remains a mystery.

Localized PCD can induce systemic acquired resistance (SAR) through the production of the immune signal, salicylic acid (SA)3. SA triggers global transcriptional reprogramming and resistance to a broad spectrum of pathogens. The receptor for SA has been sought after for many years, mainly through biochemical purification of SA-binding proteins4-6. However, genetic data for these SA-binding proteins, which include a catalase, a chloroplast carbonic anhydrase, and a methyl SA esterase, suggest that none of them functions as a bona fide SA receptor. By contrast, genetic studies of SA-insensitive mutants have strongly suggested that NPR1, which contains a BTB (bric à brac, tramtrack, broad-complex) domain, an ankyrin repeat domain and a nuclear localization sequence, is a potential SA receptor7. However, the NPR1 protein does not have considerable SA binding activity under different test conditions (Supplementary Fig. 2).

Instead of direct binding, SA has been shown to control the nuclear translocation of NPR1 through cellular redox changes8. In the absence of pathogen challenge, NPR1 is retained in the cytoplasm as an oligomer through redox-sensitive intermolecular disulfide bonds. After induction, these disulfide bonds are reduced, releasing NPR1 monomers into the nucleus, where NPR1 acts as a cofactor for transcription factors, such as TGAs, to induce defence-related genes. In the absence of a functional NPR1 protein, SA-induced transcriptional reprogramming is almost completely blocked.

The presence of a BTB domain in NPR1 suggests that, like other BTB domain-containing proteins, it may interact with Cullin 3 (CUL3) E3 ligase and mediate substrate degradation9. However, our research led to the surprising finding that the NPR1 protein itself is degraded by the proteasome. Although NPR1 is degraded in the nucleus of resting cells to dampen basal expression of defence genes, it is phosphorylated after immune activation at an IkB-like phosphodegron motif, ubiquitylated by a CUL3 E3 ligase, and degraded to sustain maximum levels of target gene expression probably through accelerated recycling of the transcription initiation complex8. Blocking NPR1 degradation by mutating the IkB-like phosphodegron in NPR1 or the two CUL3 genes (cul3a cul3b) in Arabidopsis led to increased basal resistance, but insensitivity to SAR induction. Therefore, nuclear accumulation of NPR1 is needed for basal defence gene expression and resistance, whereas its subsequent turnover is required for establishing SAR.

NPR3 and NPR4 are CUL3 adaptors for NPR1 degradation

In a search for the adaptor proteins of the CUL3 E3 ligase that specifically target NPR1 for degradation, we considered its paralogues, NPR3 and NPR4, as possible candidates, because both contain the BTB domain as well as an extra protein–protein interaction domain (ankyrin repeat) (Supplementary Fig. 3), which are typical for CUL3 substrate adaptors8. More importantly, despite their sequence similarities to NPR1, the npr3 npr4 double mutant has the opposite phenotype of npr1 in that it exhibits enhanced disease resistance10, a phenotype reminiscent of the cul3a cul3b mutant11.

To test our hypothesis that NPR3 and NPR4 are CUL3 adaptors for NPR1 degradation, we examined the accumulation of NPR1 protein in wild-type, npr3, npr4 and npr3 npr4 mutant plants. NPR1 protein levels were higher in the npr4 and npr3 npr4 mutants than in the wild type in the absence of exogenous SA, and increased faster in the npr3, npr4 and npr3 npr4 mutants compared with wild type in response to SA treatment (Fig. 1a). The effects of npr3 and npr4 on NPR1 were probably post-transcriptional, as NPR1 transcripts were not increased in these mutants (Supplementary Fig. 4). To prove our hypothesis

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Figure 1 | NPR3 and NPR4 mediate degradation of NPR1. a, NPR1 protein levels in wild type (WT), npr3, npr4 and npr3 npr4 (npr34) plants treated with 0.5 mM SA. The NPR1 level (shown at bottom) was determined on the basis of the ratio of the NPR1 band intensity to that of the non-specific band (asterisk). b, GST–NPR1 degradation in extracts from wild-type or npr3 npr4 double mutant (npr34), with or without (~) MG115 or with recombinant His–MBP–NPR3 and His–MBP–NPR4 proteins (NPR3,4). c, In vitro pull-down assay of GST–CUL3A and NPR3–HA and NPR4–HA. d, Co-immunoprecipitation of NPR1–GFP and CUL3 in npr1 and npr1 npr3 npr4 (npr134) plants.

Further, we performed in vitro degradation experiments using purified recombinant glutathione S-transferase (GST)-tagged NPR1 protein. We found that after 15 min of incubation, the recombinant NPR1 protein was degraded in the wild-type plant extract, but not in npr3 npr4 (Fig. 1b). The addition of purified recombinant NPR3 and NPR4 proteins tagged with histidine (His) and maltose binding protein (MBP) to the extract complemented the mutant phenotype, supporting a role of NPR3 and NPR4 in mediating NPR1 degradation. This degradation is probably through the proteasome, as application of the proteasome inhibitor MG115 stabilized the protein (Fig. 1b). To demonstrate further that NPR3 and NPR4 act as adaptors for the CUL3 E3 ligase, we first performed pull-down experiments using in vitro translated haemagglutinin (HA)-tagged NPR3 (NPR3–HA) and NPR4–HA. We found that CUL3 could pull down NPR3 and NPR4, with NPR4 showing a stronger interaction (Fig. 1c). Then we performed a co-immunoprecipitation assay using transgenic plants constitutively expressing NPR1–green fluorescent protein (GFP) in npr1 and npr1 npr3 npr4 mutants. We found that the amount of the endogenous CUL3 protein pulled down by NPR1–GFP was significantly reduced in the npr1 npr3 npr4 triple mutant compared with the npr1 single mutant (Fig. 1d), indicating that the NPR1–GFP interaction with CUL3 requires NPR3 and NPR4. These results further support our hypothesis that NPR4 and NPR3 are CUL3 adaptors for the degradation of NPR1 before and after SA induction, respectively (Fig. 1a).

SA affects NPR1–NPR3 and NPR1–NPR4 interactions

Proteasome-mediated protein degradation has a crucial role in regulating plant hormone receptors15. In some of these cases, the hormones act as a molecular glue to enable the formation of the receptor complex15,16, which includes the substrate adaptor for the E3 ligase and the corresponding substrate. Our data show that proteasome-mediated degradation of NPR1 is also involved in SA signalling9, although a different E3 ligase (CUL3, instead of CUL1) is used.

To test the possibility that SA is part of the NPR1–NPR3/4 complex, we performed a yeast two-hybrid (Y2H) assay. Using NPR3 as bait and NPR1 as prey, little growth was observed on plates without SA (Fig. 2a). However, yeast growth was observed on plates supplemented with 100 μM SA or with the functional analogue of SA 2,6-dichloroisonicotinic acid (INA)13, but not on plates with 4-hydroxybenzoic acid (4-HBA)17, which cannot induce SAR. Interestingly, although SA promoted the NPR1–NPR3 interaction, it disrupted the interaction between NPR1 and NPR4 (Fig. 2b). Moreover, NPR3 and NPR4 could form both homodimers and heterodimers with each other in the presence of SA and INA, but not 4-HBA (Fig. 2c). This suggests that NPR3 and NPR4 not only control NPR1 stability, but also self-regulate. Because NPR1 did not form homodimers with or without SA and interacted with NPR2 independently of SA (Supplementary Fig. 5), we focused on the regulatory roles of NPR3 and NPR4.

To validate the Y2H data further, we performed in vitro pull-down assay. As shown in Fig. 2d, using the GST–NPR3 protein, we were able to pull down His–MBP–NPR1 only in the presence of SA. By contrast, GST–NPR4 could pull down His–MBP–NPR1 only in the absence of SA, indicating that the NPR1–NPR4 interaction was disrupted by SA.
NPR3 and NPR4 bind SA with different affinities

Both the Y2H and the in vitro pull-down results strongly suggest that SA directly binds to NPR3 and NPR4 to control their interactions with NPR1. To prove that NPR3 and NPR4 are SA receptors, we measured their SA-binding activities using [3H]-SA. We found that both GST-tagged NPR3 and NPR4 recombinant proteins bound [3H]-SA (Fig. 3a, b and Supplementary Fig. 2a). Next, we assessed whether active or inactive SA analogues could compete with [3H]-SA to bind to GST–NPR3 and GST–NPR4. The active SAR inducer 5-chlorosalicylic acid and INABA reduced the binding of [3H]-SA to GST–NPR3 and GST–NPR4, whereas 4-HBA had little effect (Fig. 3a, b). To assess the binding affinity of NPR3 and NPR4, we performed saturation binding experiments. Whereas NPR4 had a classical saturation curve (Fig. 3c), NPR3 binding could be saturated even with 1,000 nM [3H]-SA, indicating that NPR3 has a lower affinity than NPR4. Accordingly, the binding of SA to NPR3 was slower than NPR4 (Supplementary Fig. 6). Next, we analysed the saturation binding data with GraphPad Prism using different models, and found that the model ‘one site—specific binding with Hill slope’ is significantly better than the other models, which indicates that there are several binding sites or fractions in NPR3 and NPR4. The dissociation constant (Kd) for NPR4 was 46.2 ± 2.35 nM (mean ± s.e.m.) with a Hill coefficient (n) of 0.830 ± 0.0314. To check the cooperativity of different binding sites, we carried out dissociation experiments by the addition of 1 mM non-radioactive labelled SA (cold SA) or by infinite dilution. The dissociation curves (Fig. 3d) indicate that NPR4 has several SA-binding sites, and the lack of overlap between the two curves suggests negative cooperativity between these binding sites (the first binding reduces the affinity for subsequent binding). The Kd value for NPR3 (981 nM, Supplementary Fig. 7) was significantly higher than 100 nM, which made saturation binding an inappropriate way to calculate the Kd. Therefore, we performed a homologous competitive binding assay (Fig. 3e). The half-maximum inhibitory concentration (IC50) was calculated to be 1.811 nM (log(IC50) = 3.26 ± 0.0901) with a Hill coefficient of 0.554 ± 0.0612. Through these analyses, we demonstrated that NPR3 and NPR4 bind SA specifically and with different affinities.

To examine the receptor complex further, we performed gel filtration analysis on the purified recombinant NPR4 protein—the receptor with the higher affinity to SA. Because the recombinant NPR4 protein spontaneously oligomerized in vitro in the absence of a reducing agent, our analysis focused on samples pretreated with 100 μM dithiothreitol (DTT) followed by dialysis against 5 mM DTT. We discovered that NPR4 was present in an estimated tetrameric form, which was competent in binding to SA (Fig. 3f). Notably, SA binding did not change the gel filtration elution profile of the protein. Further experiments are required to investigate how SA affects the receptor complexes to make them either more accessible (that is, NPR3 binding to NPR1) or less accessible (that is, NPR4 binding to NPR1) for substrate binding.

The npr3 npr4 double mutant is defective in SAR and ETI

To understand the biological significance of NPR3/4-mediated degradation of NPR1, a positive regulator of SAR, we first performed SAR tests in the npr3, npr4 and npr3 npr4 mutants using Pseudomonas syringae pv. maculicola ES4326 (Psm ES4326). Consistent with a previous report11, there was a significant reduction in Psm ES4326 growth in the npr3 npr4 double mutant without SAR induction (Fig. 4a). However, even after SAR induction by local inoculation of avirulent Psm ES4326/avrRpt2, no further reduction in growth of virulent Psm ES4326 in systemic tissue was observed in the npr3 npr4 double mutant. To a lesser degree, SAR was also defective in the npr3 single mutant. Thus, stabilization of NPR1 protein in the npr3 and npr3 npr4 mutants rendered these plants insensitive to SAR induction. This phenotype is similar to that observed in the cul3a cul3b double mutant12, validating the role of NPR3 and NPR4 in CUL3-mediated degradation of NPR1 and SAR.

On the basis of our knowledge that SAR and ETI are two distinct defence strategies, with the former promoting cell survival and the latter triggering PCD, we then tested the npr mutants for ETI using Pseudomonas strains expressing different effectors. Surprisingly, we found that the npr3 npr4 mutant failed to undergo PCD (Fig. 4b and Supplementary Fig. 8a) as quantified by ion leakage (Fig. 4c), and was compromised in resistance triggered by the effectors (Fig. 4d). The same phenotypes were observed in different mutant alleles of npr3, npr4 and npr3 npr4 (Supplementary Fig. 8b). The ETI deficiency observed in npr3 npr4 is probably caused by the increased accumulation of NPR1, because this phenotype was suppressed in the npr1 npr3 npr4 triple mutant.

To observe NPR1 turnover in response to pathogen challenge in situ, we inoculated Psm ES4326/avrRpt2 in the 35S:NPR1(C82A)-GFP transgenic plant, in which NPR1 is constitutively localized in the nucleus (Fig. 4e)14. Eleven hours after inoculation, some cells showed increased chlorophyll leakage (Fig. 4f, red) with overlapping accumulation of phenolic compounds (Fig. 4e, larger green spots) indicative of PCD (Fig. 4g, yellow), whereas other cells were still intact. The NPR1(C82A)-GFP fluorescence was markedly reduced inside the inoculated region (Fig. 4g, h). Notably, the NPR1(C82A)-GFP fluorescence level was the highest in the cells surrounding the hypersensitive
level of NPR1. This is crucial because SA-deficient plants, eds5 (ref. 17), ics1 (also known as eds16)23 and the nahG transgenic line expressing an SA-degrading enzyme19, are impaired in maintaining NPR1 homeostasis (Supplementary Fig. 9), resulting in enhanced disease susceptibility (Supplementary Fig. 1a, b). After challenge by pathogens that trigger ETI, SA levels increase both locally and systemically20 to form a concentration gradient from the infection site21,22. Previous research has shown that high levels of SA facilitate PCD3,14, and the spread of PCD may be controlled by the activities of proteins such as LSD1 (a zinc-finger protein) and AtRboh D (an NADPH oxidase). Because the npr3 npr4 double mutant can no longer undergo PCD in response to pathogen effectors, we propose that NPR1, which over-accumulates in the npr3 npr4 mutant, can act as a negative regulator of PCD. Our finding is in line with a previous report suggesting that NPR1 suppresses hypersensitive response25. In support of this function of NPR1, the NPR1–GFP signal is the lowest inside the developing hypersensitive response (Fig. 4e–h) owing to CUL3–NPR3–mediated degradation of NPR1 (Supplementary Fig. 1c). In neighbouring cells, the lower SA level limits NPR1–NPR3 interaction, enabling NPR1 to accumulate in the margin of the hypersensitive response to restrict the spread of PCD and establish SAR (Supplementary Fig. 1d).

METHODS SUMMARY

Protein analysis was carried out as previously described30. Total NPR1 protein was detected by an antibody against NPR1. For the in vitro degradation assay, the purified recombinant GST–NPR1 was incubated with plant extracts and detected by an anti-GST antibody (GE Healthcare). The in vitro pull-down assays for CUL3A, NPR3 and NPR4 were performed using purified recombinant GST–CUL3A and in vitro translated NPR3–HA and NPR4–HA, which were detected by an anti-HA antibody (GenScript). Purified recombinant GST–NPR3 and GST–NPR4 proteins retained on the glutathione agarose beads were used to pull down purified Hu–MBP–NPR1. Bound Hu–MBP–NPR1 was detected by western blot using an anti-His antibody (GenScript). For co-immunoprecipitation, the immunoprecipitation was performed using an anti-GFP antibody (Abcam) and the western blot using an anti-CUL3A antibody30 and an anti-GFP antibody (Clontech). Y2H assays were carried out using the Matchmaker system (Clontech). The interactions were determined by yeast growth on selective medium (SD media lacking Trp, Leu and His, plus 3 mM 3-amino triazole) with or without 100 µM SA, INA or 4-HBA. The SA-binding assays were performed as described30 with modifications using purified recombinant GST–NPR3 or GST–NPR4 and [3H]SA (American Radiolabelled Chemicals). Pathogen infection and ion leakage assay using Psm ES4326 with or without avrRpt2 were carried out as previously described30.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 30 November 2011; accepted 26 April 2012. Published online 16 May 2012.


Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank Y. Zhang for sharing the npr3, npr4, npr3 npr4 and npr1 npr3 npr4 mutants; J. Song for providing the NPR3 and NPR4 Y2H constructs; Z. Mou for providing the data on the NPR1-GFP protein levels in the nrt6 transgenic plants; P. Zhou for discussion of the work and for critiquing the manuscript. This work was supported by the Hargitt Fellowship (to Z.Q.F.), grants GM069594-05 (to X.D.), CA10734 (to N.Z.), T32GM008268-23 (to J.R.), Grants-in-Aid for Scientific Research (no. 23120520) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y.T.), and the Royal Society UF090321 (to S.H.S.), N.Z. is a Howard Hughes Medical Institute investigator and X.D. is a Howard Hughes Medical Institute-Gordon and Betty Moore Foundation Investigator.

Author Contributions Z.Q.F., S.Y., A.S., R.M., and S.H.S. conceived and discovered that NPR3 and NPR4 mediate NPR1 degradation, Z.Q.F., A.S., and S.Y. found that SA regulates the interactions between the NPR proteins. S.Y., W.W., and A.S. found that NPR3 and NPR4 can bind SA with different affinities. J.R. and N.Z. showed that purified NPR4 recombinant protein exists as a tetramer, which is competent in SA binding. Z.Q.F., W.W., and R.M., demonstrated that the npr3 and npr4 single and double mutants are impaired in ETI and SAR, N.O. and Y.T. observed in situ accumulation of NPR1-GFP in response to Pto ES4326 AvrPtoB2. S.H.S. provided data on the detection of NPR1-GFP protein in eds5 and ssc1 plants. X.D. designed the research and, together with Z.F., S.Y., W.W., S.H.S., and R.M., wrote the manuscript.

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**METHODS**

_Arabidopsis thaliana_ mutants and transgenic lines. *Arabidopsis thaliana* mutants (in ecotype Col-0) np-3-1, np-3-1, np-3-1 np-4-3, np-1 np-3-1 np-3-3, np-3-2 (SALK_034055), np-2-4 (SALK_094660) and np-3-2 np-4-2 were provided by Y. Zhang. **35S:NPR1-GFP** was introduced into the np-1 np-2 np-3 np-4-3 background by crossing the 35S:NPR1-GFP transgenic plants (in np-1-2) with the np-3-1 np-4-3 plants. Homozygous plants were selected by genotyping.

**Co-immunoprecipitation assay.** Three-week-old plant sample was collected and ground in liquid N₂. Protein was extracted in the extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Trition X-100, 0.2% Nonidet P-40, and inhibitors: 50 µg/mL N-[N-tosyl-L-phenylalanyl] chloromethyl ketone (TPCK), 50 µg/mL N-[N-tosyl-L-leucine chloromethyl ketone (TLCK), 0.6 mM phenylmethylsulfonyl fluoride (PMSF) and 40 µM MG115). The extracts were then pre-cleared with 50 µL of Dynabeads Protein G (Invitrogen). After 1 h of incubation with an anti-GFP antibody (Abcam) was added to the extracts and incubated for 2 h, 50 µL of magnetic Dynabeads were added to the samples and incubated for another hour with gentle rocking. The magnetic Dynabeads were then washed three times using the protein extraction buffer, and bound proteins were eluted by heating the magnetic beads in the SDS sample buffer containing 100 mM DTT for 10 min. The NPR1–GFP and CUL3 proteins were detected by western blotting using an anti-GFP antibody (Clonech) and an anti-CUL3 antibody respectively.

**Pathogen infection.** To test for the hypersensitive response, the avirulent pathogen _Pmm_ ES4326 carrying arrvP12 (attenuation (D)4060 = 0.02) or arrvP11 (D)4060 = 0.01) and _Pst_ carrying arrvP77 (D)4060 = 0.01) were infiltrated into 3–4-week-old leaves. Cell death was recorded 2–3 days after the infiltration. Ion leakage was recorded over time as described.

**Imaging of NPR1–GFP in infection site.** The **35S:NPR1:C2tau**–GFP plants were inoculated with _Pmm_ ES4326 carrying arrvP12 (D)4060 = 0.02) and incubated for 11 h. Leaf tissues were mounted in 10% glycerol and viewed with a BIOR Guerrero microscope (Keyence) BZ 9000 fluorescence microscope. The GFP signal is monitored with an excitation wavelength of 472.5 nm and a bandpass 502.5 to 537.5 nm emission filter. Red chlorophyll was viewed with an excitation wavelength of 540 nm and a bandpass 573 to 613 nm emission filter. To obtain wide-field view (2 × 2 pictures), image stitching was performed by BZ-II Image Analysis Application. Experiments were repeated eight times.

**Molecular cloning of NPR1s.** The coding regions of _NPR1s_, _2, 3, 4 and 6_ were amplification with PrimeSTAR HS DNA polymerase (Takara) using specific primers containing Gateway attB sites (Supplementary Table 1), and then cloned into the pDONR207 vector entry vector using the BP Clonase (Invitrogen) to create the NPR1 entry clones. After verification, each of the clones was mobilized using the LR Clonase (Invitrogen) into the Gateway destination vectors pDEST-GK7 and pDEST-GADY7 for yeast transformation, respectively. The protein expression vectors pDEST15 (Invitrogen) and pDEST-HisMBP (Addgene plasmid 11085) were used for making the GST and His–MBP fusions, respectively.

**Detection of the NPR1 protein.** Four-week-old plants were sprayed with 0.5 mM SA and collected at different time points. Total protein was extracted in a buffer containing 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 and 40 µM MG115 (ref. 30). The homogenates were centrifuged twice at 13,500g for 15 min each. Protein was denatured in the SDS sample buffer containing 100 mM DTT at 75 °C for 10 min, and western blot analysis was performed using an antibody against NPR1 (ref. 16).

**Quantitative real-time PCR.** Total RNA was extracted from 4-week-old control and SA-treated plants at the indicated time points using TRizol Reagent (Invitrogen). Genomic DNA was eliminated by treatment of the RNA with 2 U TURBO DNA-free (Ambion). cDNA was synthesized using the Superscript III Reverse Transcript kit (Invitrogen) and analysed by quantitative real-time PCR using the FastStart Universal SYBR Green Master (Roche) kit (Roche) with gene-specific primers for NPR1 and ubiquitin (Supplementary Table 1).

**In vitro degradation assay.** The NPR1 degradation assay was performed as described. Leaves from wild-type or np-3-1 np-4-3 double mutant plants were ground in liquid N₂ and resuspended in the proteolysis buffer containing 25 mM Tris- HCl, pH 7.5, 10 mM MgCl₂, 10 mM NaCl, 10 mM ATP and 5 mM DTT. After centrifugation, the supernatants were mixed with the GST–NPR1 protein purified from _Escherichia coli_ and incubated at room temperature for 15 min. The reactions were stopped by adding the SDS sample buffer containing 100 mM DTT and incubated at 75 °C for 10 min. The level of GST–NPR1 protein was analysed by western blotting using an anti-GST antibody (GE Healthcare).

**In vitro pull-down assay.** The coding sequence of _CUL3A_ was cloned into the GST vector pGEX-KG-2 (GE Healthcare) for expression in _E. coli_ BL21 (DE3). The coding sequences of NPR3 and NPR4 were cloned into pCMX-P2 for in vitro translation using TNT Quick Coupled Transcription/Translation System (Promega) to produce the HA-tagged NPR3 and NPR4 proteins. The purified GST–CUL3A protein was bound to the glutathione agarose beads, incubated with the HA-tagged NPR3 or NPR4 protein, and washed three times with EB buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% dimethyl sulfoxide, 20 mM DTT and 0.1% NP40). The HA-tagged NPR3 or NPR4 protein bound to GST–CUL3A protein was detected by western blot analysis using an anti-HA antibody (GenScript). Recombinant His–MBP-tagged NPR1 and GST-tagged NPR3 and NPR4 protein performed in _E. coli_. The recombinant His–MBP-tagged NPR1 protein was purified using the Ni-NTA resin (Qiagen). GST-tagged NPR3 and NPR4 proteins were purified using glutathione beads and retained on the beads to pull down purified His–MBP–NPR1 protein with or without 100 µM SA in a buffer containing 50 mM Tris-HCl, pH 6.8, 100 mM NaCl and 0.1% NP40. After washing three times, bound His–MBP–NPR1 was eluted by heating the glutathione beads at 95 °C for 10 min in the SDS sample buffer containing 100 mM DTT and detected by western blot analysis using an anti-His antibody (GenScript). Equal loadings were verified by staining the membrane with Ponceau S.

**Yeast two-hybrid assay.** The _Saccharomyces cerevisiae_ yeast strains AH109 and Y187 were transformed with pGADT7_NPR1, 3, 4, and pGBKT7 NPR1, 3, 4, respectively, according to the Clontech yeast transformation protocol. Yeast strains were grown on SD–Trp–Leu plates, and then fresh single colonies were grown for 1 day in the SD–Trp–Leu liquid media. Interactions between bait and prey were detected on the selective media: SD–Trp–Leu–His (control), SD–Trp–Leu–His with 100 µM sodium salicylate (SA), 100 µM INA, or 100 µM 4-HBA. All of the SD–Trp–Leu–His selective media also contained 3 mM 3-aminoatrazole.

**SA-binding assay.** The SA-binding assay was performed as described with modifications. The GST–NPR1 and GST–NPR4 proteins were expressed in _E. coli_ C41 and purified using Pierce Glutathione Magnetic Beads (Thermo). The protein–bound beads were incubated in 100 µL buffer containing 50 mM sodium citrate, pH 6.3, 1.5 mM EDTA and [³⁵S]SA (American Radiolabeled Chemicals, specific activity 30 Ci mmol⁻¹). The beads were washed twice, resuspended in 100 µL H₂O, mixed with 6 mL Ultima Gold Cocktails (PerkinElmer) and counted using the LC6000SC liquid scintillation counter (Beckman Instruments). The non-specific binding was determined in the presence of 1 mM unlabelled SA. The data were analysed using GraphPad Prism 5.

**Gel filtration analyses of NPR4.** NPR4 was overexpressed as a GST-fusion protein in insect cells and purified by glutathione affinity chromatography in the presence of 100 mM DTT. After TEV cleavage, NPR4 was further purified by anion exchange chromatography and dialysed against a buffer containing 20 mM Tris-HCl, pH 8.0, 200 mM NaCl and 5 mM DTT. After concentration, 0.5–1 mg NPR4 was analysed in the same buffer with and without 10 μM SA as indicated by size exclusion chromatography on a Superdex 200 gel filtration column. Co-elution of SA and NPR4 was monitored by [³⁵S]SA, which was preixed with the NPR4 protein before injection.

**REFERENCES**


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