PNAS
www.pnas.org

Supplementary Information Appendix

A role for S-nitrosylation of the SUMO-conjugating enzyme, SCE1, in plant immunity

Michael J. Skelly, Saad I. Malik, Thierry Le Bihan, Yuan Bo, Jihong Jiang, Steven H. Spoel, and Gary J. Loake

Corresponding author: Gary J. Loake
Email: gloake@ed.ac.uk

This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1900052116

Supplementary Information Appendix

SI Appendix Materials and Methods

Plant growth conditions and pathogen inoculations

Arabidopsis plants were grown in a 16 hour photoperiod in controlled growth rooms at 65% humidity and 22°C unless otherwise stated. Seeds were stratified at 4-8°C in the dark for 2 days before moving to growth rooms. Plants were grown in a soil mix composed of peat moss, vermiculite and sand at a ratio of 4:1:1 respectively, and illumination was provided by general fluorescent tube lighting at an intensity of 70-100 μmol $m^{-2}sec^{-1}$. For plants grown under sterile conditions, seeds were sterilized by washing in 100% ethanol for 2 mins before incubating in 50% household bleach for 20 mins. After removal of bleach, seeds were washed at least 3 times with sterile H_2O before use. For growth in liquid media, sterilized seeds were stratified in sterile H_2O before adding to half strength Murashige and Skoog (½ MS) media (1) containing 1% (w/v) sucrose at pH 5.8. Flasks were shaken gently under the same light and temperature conditions as above to prevent clumping of seedlings. For bacterial growth assays, plants were inoculated with 10^5 colony-forming units per ml (cfu/ml), for analysis of gene expression, plants were inoculated with 10^6 cfu/ml, and for protein assays, plants were inoculated with 10^7 cfu/ml *Pst* or *Pst* (*avrB*). Four-week old plants were inoculated by needle-less syringe infiltration of the abaxial leaf surface. Single leaf discs from individual plants formed biological replicates.

Isolation and treatment of protoplasts

Protoplasts were isolated from 3-5 week old plants using the described tape-*Arabidopsis*

sandwich protocol (2). Protoplasts were counted using a haemocytometer and necessary dilutions were made for equivalent loading. All treatments were carried out on freshly isolated protoplasts on the same day. Heat-shock and GSNO treatments were performed on 1 ml of suspended protoplasts in 6-well tissue culture plates. Samples were then transferred to 15 ml Falcon tubes and centrifuged at 200 g for 2 mins to pellet the protoplasts. Supernatant was removed and pellets were frozen in liquid nitrogen then stored at -80°C until protein extraction and western blot analysis.

Recombinant protein expression and purification

Full-length coding sequences of SAE1a, SAE2 and SCE1, and the coding sequence for mature SUMO1 were amplified from *Arabidopsis* cDNA with the addition of *SphI* and *BglII* restriction sites (all primers used are listed in Table S1). PCR products were cloned into the pQE70 expression vector (Qiagen) by *SphI* and *BglII* digestion and after verification of correct sequences were transformed into the M15 (pRep4) *E. coli* strain. Site-directed mutagenesis of SCE1 Cys residues was performed using the QuikChange II XL kit (Agilent Technologies) according to manufacturers' instructions and mutations confirmed by sequencing. Primers used are listed in Table S1 with the altered codons underlined. Protein expression was induced in bacterial cultures with the addition of 1 mM IPTG and after 3-6 hours cells were pelleted by centrifugation at 6000g for 15 mins. Supernatant was removed and pellets were stored at -80°C until cell lysis and protein extraction. Cells were resuspended in lysis buffer [50 mM $KHPO_4$ pH 8, 300 mM NaCl, 10 mM Imidazole, 1 mg/ml lysozyme, 25 U/ml Benzonase nuclease, 0.1% Triton-X-100, 10 mM β-mercaptoethanol, 50 μg/ml N-tosyl-L-phenylalaninyl-chloromethylketone

(TPCK), 50 μg/ml N-alpha-tosyl-L-lysinyl-chloromethylketone (TLCK), 0.5 mM phenylmethanesulfonyl fluoride (PMSF)] and incubated with gentle rocking at room temperature for 30 mins. The lysate was then centrifuged at 4°C at 15,000 rpm for 15 mins and the supernatant collected. 6xHis-tagged proteins were then purified by gravity-flow method using HisPur Cobalt Resin (Thermo Scientific) according to the manufacturers' instructions. Immediately after elution, proteins were dialyzed against appropriate buffers using Slide-A-Lyzer dialysis cassettes (Thermo Scientific) to remove excess imidazole.

Biotin-switch and mass spectrometry analysis

S-nitrosylation of proteins was analysed by following the BST essentially as described (3), with the means of detection specified in the relevant figure legends. All excess exogenous NO-donors were removed by desalting using either Zeba (Thermo Scientific) or Micro Bio-Spin P6 (Biorad) columns before the BST. Where indicated –Asc refers to the omission of the ascorbate step in the biotin-switch procedure serving as a negative control. For MS analysis following the BST, biotinylated proteins were separated by SDS-PAGE and Coomasie stained. Protein bands were excised from the gels and subjected to tryptic and/or proteinase-K digests. LC-MS/MS (liquid chromatography/mass spectrometry/mass spectrometry) was carried out on an on-line system consisting of a Agilent 1200 binary HPLC system (Agilent Technologies, UK) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher, UK). MS^2 were analyzed using Mascot (Matrix sciences, UK) and targeted MS^3 were manually interpreted.

in vitro SUMOylation assays

Analysis of SUMO-SCE1 thioester formation was adapted from a described protocol (4). All proteins were dialyzed against reaction buffer (50 mM Tris-HCl, 100 mM NaCl and 10 mM $MgCl_2$). Reactions were set up in 20 µl volumes of reaction buffer containing 0.5 µM SAE1a, 0.5 µM SAE2, 1 µM SCE1 and 2.5 µM SUMO1 and kept on ice. Thioester formation was started by adding an ATP-regenerating mix (2 mM ATP, 10 mM creatine phosphate disodium salt, 3.5 U/ml creatine kinase and 6 U/ml inorganic pyrophosphatase) (5) and incubating samples at 30°C for the stated times. Reactions were stopped with the addition of SDS-PAGE sample buffer without DTT and samples were heated at 70°C for 10 mins before SDS-PAGE and western blot against SCE1. Formation of poly-SUMO chains *in vitro* was analyzed by setting up the same reactions as for the thioester assay but incubating the samples at 30°C for longer times, stated in each figure, and DTT was added to the sample buffer before SDS-PAGE and western blot against SUMO1. SUMOylation of *Sc*PCNA was analysed by mixing 8 µg of SUMO1, 1 µg each of SAE1a and SAE1b, 2 µg of SAE2, 2 µg of SCE1 and 4 µg of *Sc*PCNA. The reaction was started by adding 5 mM ATP and incubated at 25 °C for 4 hrs. Any excess NO-donors or controls from pretreatments of SCE1 were removed by desalting before setting up reactions. All *in vitro* assays using human SUMO machinery proteins were performed as described (5).

Generation of transgenic plants

For the *SCE1* and *SCE1(C139S)* constructs, the coding sequences of the relevant genes were amplified from the previously cloned plasmids for recombinant protein expression

with the addition of the nucleotide sequence CACC at the 5' end, necessary for TOPO® cloning (Life Technologies). The PCR products were gel-purified and cloned into the pENTR™/D-TOPO® vector according to the manufacturers' instructions. Subsequently, positive clones were verified by sequencing and digested with *Mlu*I prior to Gateway® cloning into the pEarleyGate 202 vector (6) by LR reaction (Life Technologies). Recombinant clones were selected and the coding sequences were verified as in-frame with both the CaMV *35S* promoter and the N-terminal FLAG sequence contained within the pEarleyGate 202 plasmid by sequencing. These plasmids were then used to transform *Agrobacterium tumifaciens* strain GV3101 (pMP90) (7). After selection of positive clones carrying the transgenes, approximately 6-week old flowering plants were transformed using the floral-dip method (8). Transformants were selected by spraying 10-day old seedlings with 120 μg/L BASTA at least three times. Further confirmation of transformation was performed by western blot against FLAG. Segregation analysis of BASTA resistance in the T_2 generation was carried out under sterile conditions using plates with ½ MS media containing 10 μg/ml glufosinate ammonium.

Protein extraction from plant tissue and protoplasts

Leaf tissue was weighed for equal loading, ground in liquid nitrogen then thawed in extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 and 0.2% Nonidet P-40) freshly supplemented with protease inhibitors (50 μg/ml TPCK, 50 μg/ml TLCK, and 0.5 mM PMSF) (9). Samples were centrifuged at 13,000 rpm at 4°C for 15 mins to remove plant debris and the supernatant was collected. Protein was extracted from protoplasts by thawing frozen pellets in the same extraction buffer

and vortexing before centrifugation. For analysis of *in vivo* SUMOylation, 2.5 mM *N*-Ethylmaleimide (NEM) was added to the extraction buffer to inactivate SUMO proteases.

SDS-PAGE and western blots

For SDS-PAGE, protein samples were added to a 4X stock of sample buffer to give a final concentration of 50 mM Tris-HCl pH 6.8, 2% SDS, 0.02% bromophenol blue and 10% glycerol with or without 50 mM DTT and heated at 70°C for 10 mins before separating on gels of appropriate polyacrylamide percentage. For Coomassie Blue staining, gels were washed in H_2O before incubating in staining solution (0.25% Brilliant Blue R, 40% methanol, 7% acetic acid) for 30 mins. Gels were then destained overnight in destaining solution (40% methanol, 10% acetic acid) and photographed. Immunoprecipitation of FLAG-SCE1 was performed using ANTI-FLAG M2 affinity gel (Sigma) according to the manufacturer's instructions. For western blots, proteins were transferred on to nitrocellulose membranes and stained with Ponceau S (0.1% Ponceau S, 5% acetic acid) for 1 min then rinsed in H_2O to remove background staining. Photographs were taken before the stain was removed by washing in PBS + 0.1% tween (PBS-T). Membranes were blocked for 1 hour at room temperature using 5% dried skimmed milk in PBS-T before antibody incubation. Primary antibodies (anti-SUMO1/2, Abcam ab5316; anti-SCE1, Abcam ab98965; anti-SUMO2, Ron Hay laboratory produced in house (University of Dundee); anti-His, Cell Signaling Technologies 2366; anti-biotin, Cell Signaling Technologies 7075; anti-FLAG, Sigma F3165) used are indicated in the figure legends and were incubated either at 4°C overnight or for 1-2 hours at room temperature. Appropriate secondary antibodies (Cell Signaling

Technologies) coupled to horseradish peroxidase (HRP) were used to detect bands with SuperSignal West Pico/Dura Chemiluminescent Substrate (Thermo Scientific) and exposure to X-ray films.

Gene expression analysis

Total RNA from *Arabidopsis* leaf tissue was isolated with a plant RNA isolation kit (Agilent Technologies). 1 μg RNA was used to synthesize cDNA using Omniscript RT (Qiagen) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green I Master, in a LightCycler 480 system (Roche). Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (10) using *UBQ5* as an internal reference gene. All primers used are listed in Table S1.

Measurement of salicylic acid

Salicylic acid levels were quantified by high-performance liquid chromatography as previously described (11).

Statistics

All statistical analyses were performed using GraphPad software. No statistical methods were used to predetermine sample sizes, nor were any methods of randomization used. All experiments were repeated a minimum of two times with similar results.

SI Appendix References

1. MURASHIGE T & SKOOG F (1962) A REVISED MEDIUM FOR RAPID GROWTH AND BIO ASSAYS WITH TOBACCO TISSUE CULTURES. *Physiologia Plantarum* 15(3):473-497.
2. Wu FH, *et al.* (2009) Tape-Arabidopsis Sandwich - a simpler Arabidopsis protoplast isolation method. *Plant Methods* 5:16.
3. Jaffrey SR & Snyder SH (2001) The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE* 2001(86):pl1.
4. Alontaga AY, Bobkova E, & Chen Y (2012) Biochemical analysis of protein SUMOylation. *Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.]* Chapter 10:Unit10.29.
5. Tatham MH, *et al.* (2001) Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *Journal of Biological Chemistry* 276(38):35368-35374.
6. Earley K, *et al.* (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant Journal* 45(4):616-629.
7. Koncz C & Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of agrobacterium binary vector. *Molecular & General Genetics* 204(3):383-396.
8. Clough SJ & Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* 16(6):735-743.

9. Spoel SH, *et al.* (2009) Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* 137(5):860-872.

10. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402-408.

11. Aboul-Soud MAM, Cook K, & Loake GJ (2004) Measurement of Salicylic Acid by a High-Performance Liquid Chromatography Procedure Based on Ion-Exchange. (Chromatographia), pp 129-133.

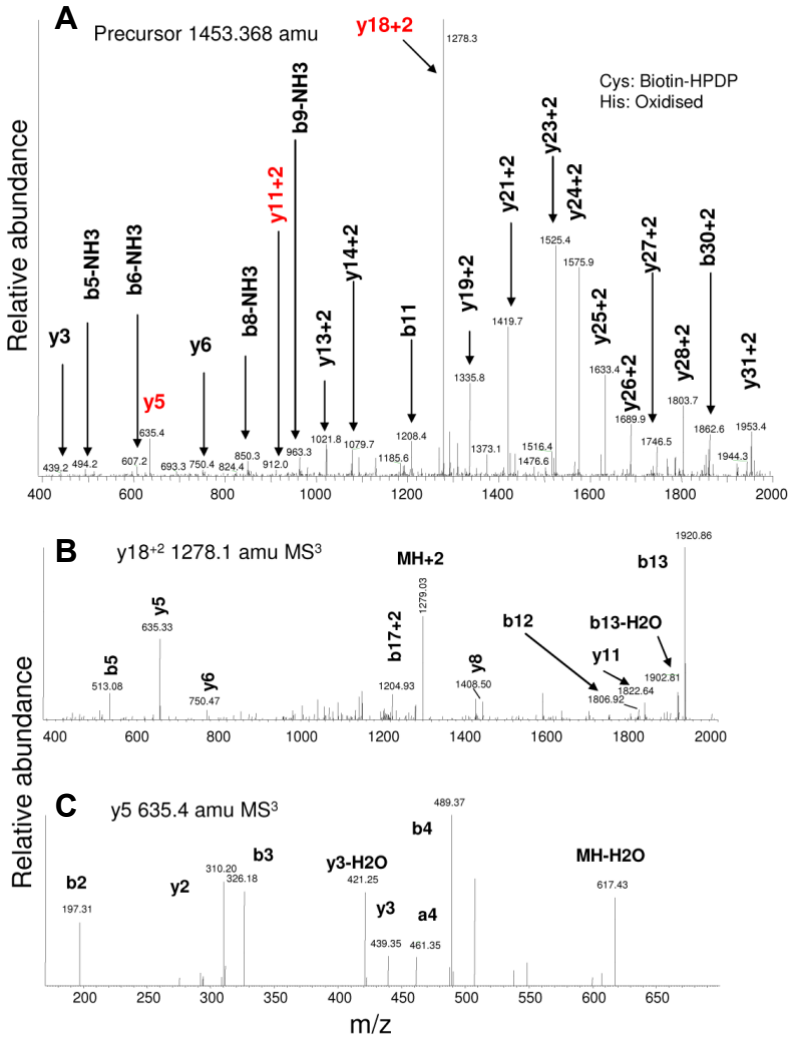

Fig S1. LC-MS/MS analysis of SCE1 S-nitrosylation at Cys139

Recombinant SCE1 samples were incubated with 200 μM GSNO and subjected to the BST before tryptic digest and LC-MS/MS analysis. **(A)** MS^2 spectra of a 3+ peptide at 1453.3680 amu was obtained after a tryptic digest of biotinylated samples containing 8ug/ul protein. The sequence assignment was QILVGIQDLLDTPNPADPAQTDGYHLFCQDPVEYK, with Cys139 blocked with biotin-HPDP. The mascot score for the assignment was 102 and the error mass was 0.0078 Da. The fragmentation pattern assignment was also shown and 2 selected MS^3 were also performed on **(B)** the specific fragment on y18+2 at 1278.1 amu, and **(C)** y5 at

635.4 amu. The MS^3 assignment was performed manually without the use of MASCOT program.

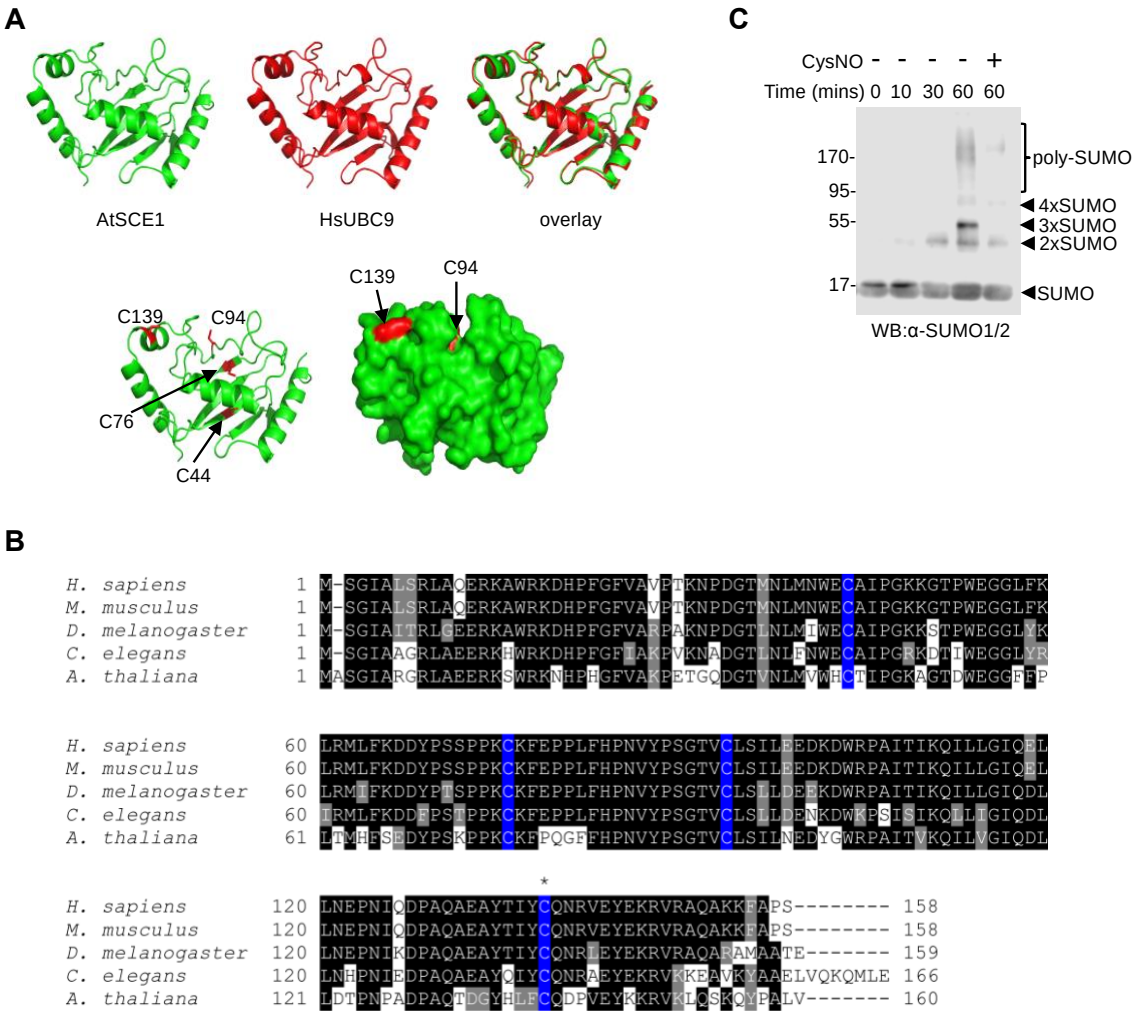

Fig S2. Structure and sequence of *Arabidopsis* SCE1 and homologues

(A) Cartoon representation showing the high structural similarity of the predicted structure of *Arabidopsis* SCE1 and the crystal structure of human Ubc9 (PDB code 1U9B). Predicted locations of the four cysteine residues of SCE1 are shown in red in cartoon and surface representations. Protein structures were viewed and analysed using PyMOL software (PyMOL Molecular Graphics System, Version 1.5.0.5 Schrödinger, LLC). The predicted structure of *Arabidopsis* SCE1 was modelled using SWISS-MODEL. **(B)** Sequences of SUMO E2 enzymes from various species were aligned using ClustalW2 software. All cysteines are shown in blue shaded boxes with the equivalent

residues to SCE1 Cys139 marked with an asterisk. **(C)** SCE1 was incubated with or without 500 μM CysNO for 20 mins. before addition to *in vitro* SUMOylation reactions, which were incubated at 30°C for the stated times. SUMO1 species were detected by western blot against SUMO1/2.

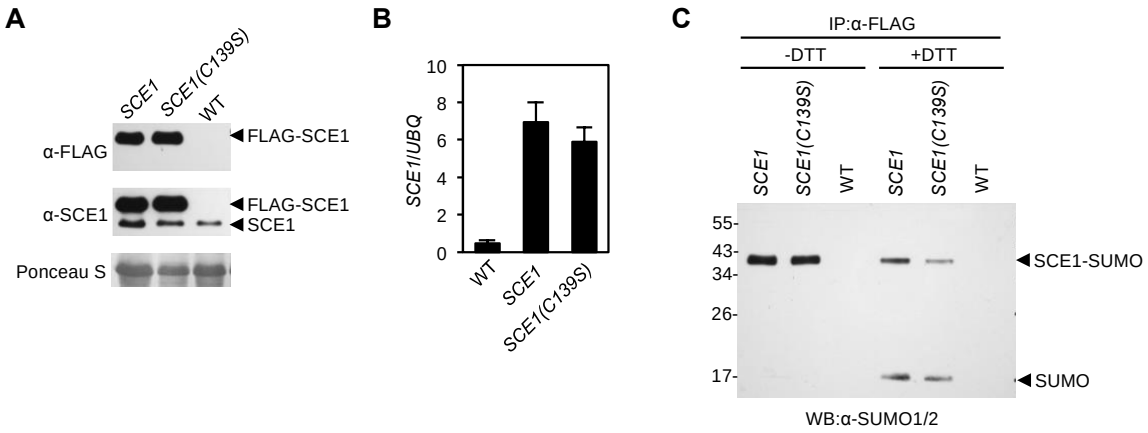

Fig S3. Expression and thioester activity of FLAG-SCE1 in transgenic *Arabidopsis*

(A) Plant protein extracts were analysed by western blot using the stated antibodies. Ponceau S staining of the large subunit of Rubisco is included as a loading control. **(B)** The expression of *SCE1* was analysed using qPCR and normalized against the constitutively expressed *UBQ5*. Data points represent mean ± SD (n=3) of three independent biological samples. **(C)** Protein extracts were subjected to anti-FLAG immunoprecipitation before analysis by non-reducing (-DTT) or reducing (+DTT) SDS-PAGE and western blot against SUMO1/2. WT plants were included as a negative control for the anti-FLAG immunoprecipitation.

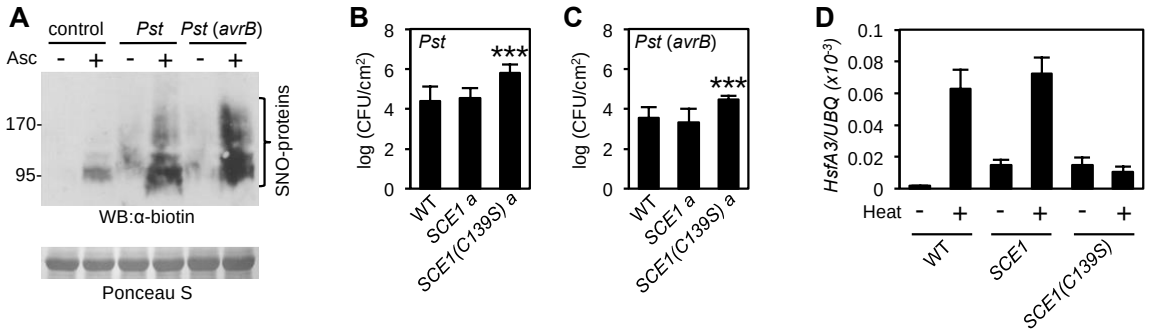

Fig S4. Cys139 of SCE1 is required for resistance to *Pst* DC3000 and heat-stress induced gene expression

(A) Protein extracts from WT untreated, or inoculated with 10^7 cfu/ml *Pst*DC3000 or *Pst*DC3000(*avrB*) (6 hpi) plants were subjected to the BST before non-reducing SDS-PAGE and western blot against biotin. Equal loading was confirmed by Ponceau S staining of Rubisco. **(B, C)** Plants were inoculated with 10^5 cfu/ml **(B)** *Pst* DC3000 or **(C)** *Pst* DC3000 (*avrB*) and leaf discs were assayed for bacterial growth at 3 dpi. Data points represent mean ± SD (n=6 biological replicates), with asterisks indicating significant difference from WT (Student's *t* test, $P < 0.05$). (**D)** Seedlings were incubated at 22°C (-) or 37°C (+) for 1h before expression of *HsfA3* was analysed using qPCR and normalized against the constitutively expressed *UBQ5*. Data points represent mean ± SD (n=3).

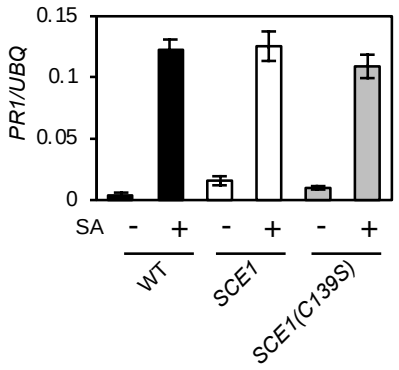

Fig S5. Exogenous SA application restores *PR1* expression in *SCE1(C139S)* plants

Plants of the indicated genotypes were sprayed with either 0.5 mM SA or H_2O and leaf tissue was harvested 12h post-spraying. Expression of *PR1* was analysed using qPCR and normalized against the constitutively expressed *UBQ5*. Data points represent mean ± SD (n=3).

PCR product	Purpose	Primer sequences
SAE1a	recombinant protein expression	**F** - ACATGCATGCACGGAGAAGAGCTTACCGAGC **R** - GAAGATCTAGAGGTAAAAGAGTCGGAAATG
SAE2	recombinant protein expression	**F** - ACATGCATGCCTACGCAACAACAGCAATCCG **R** - GAAGATCTTTCAACTCTTATCTTCTTTTTGCTCACC
SCE1	recombinant protein expression	**F** - ACGCATGCCTAGTGGAATCGCTCGTGGTCG **R** - GAAGATCTGACAAGAGCAGGATACTGCT
SUMO1	recombinant protein expression	**F** - ACGGATCCTCTGCAAACCAGGAGGAAGA **R** - ATCTGCAGGCCACCAGTCTGATGGAGCA
SCE1-C44S	mutagenesis	**F** - CTAATGGTGTGGCATAGCACTATACCTGGTAAA **R** - TTTACCAGGTATAGTGCTATGCCACACCATTAG
SCE1-C76S	mutagenesis	**F** - AGCAAACCTCCGAAAAGTAAATTTCCACAAGGG **R** - CCCTTGTGGAAATTTACTTTTCGGAGGTTTGCT
SCE1-C94S	mutagenesis	**F** - CCATCTGGAACTGTCAGTCTCTCTATCCTTAAC **R** - GTTAAGGATAGAGAGACTGACAGTTCCAGATGG
SCE1-C139S	mutagenesis	**F** - GGTTATCATCTCTTCAGTCAGGATCCAGTTGAG **R** - CTCAACTGGATCCTGACTGAAGAGATGATAACC
SCE1	TOPO cloning	**F** - CACCATGGCTAGTGGAATC **R** - TTAGACAAGAGCAGGATACTG
SCE1	qPCR	**F** - ATGGTGTGGCATTGCACTATAC **R** - GTGGAAAAACCCTTGTGGAA
PR-1	qPCR	**F** - CTAAGGGTTCACAACCAGGC **R** - AAGGCCCACCAGAGTGTATG
HsfA3	qPCR	**F** - TTCGCTAACGAGGCTTTCC **R** – CCTCAGTAGGTGACCCTT
UBQ5	qPCR	**F** - CCAAGCCGAAGAAGATCAAG **R** – ACTCCTTCCTCAAACGCTGA

Table S1. List of oligonucleotides used.