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Citation for published version:

Skelly, M, Malik, S, Le Bihan, T, Bo, Y, Jiang, J, Spoel, S & Loake, G 2019, 'A role for S-nitrosylation of the SUMO-conjugating enzyme SCE1 in plant immunity', *Proceedings of the National Academy of Sciences (PNAS)*, vol. 116, no. 34, pp. 17090-17095. <https://doi.org/10.1073/pnas.1900052116>

Digital Object Identifier (DOI):

[10.1073/pnas.1900052116](https://doi.org/10.1073/pnas.1900052116)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Proceedings of the National Academy of Sciences (PNAS)

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

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ABSTRACT

SUMOylation, the covalent attachment of the small ubiquitin-like modifier (SUMO) to target proteins is emerging as a key modulator of eukaryotic immune function. In plants, a SUMO1/2-dependent process has been proposed to control the deployment of host defence responses. However, the molecular mechanism underpinning this activity remains to be determined. Here we show that increasing NO levels following pathogen recognition promote S-nitrosylation of the *Arabidopsis* SUMO E2 enzyme, SCE1, at Cys139. The SUMO-conjugating activities of both SCE1 and its human homologue, UBC9, were inhibited following this modification. Accordingly, mutation of Cys139 resulted in increased levels of SUMO1/2 conjugates, disabled immune responses and **enhanced** pathogen susceptibility. Our findings imply that S-nitrosylation of SCE1 at Cys139 enables NO bioactivity to drive immune activation by relieving SUMO1/2-mediated suppression. The control of global SUMOylation is predominantly thought to occur at the level of each substrate via complex local machineries. Our findings uncover a novel, parallel and complementary mechanism by suggesting that total SUMO conjugation may also be regulated directly by SNO formation at SCE1 Cys139. This Cys is evolutionary conserved and specifically S-nitrosylated in UBC9, implying this immune-related regulatory process might be conserved across phylogenetic kingdoms.

SIGNIFICANCE STATEMENT

S-nitrosylation, the addition of a nitric oxide (NO) moiety to a reactive protein cysteine (Cys) thiol to form an S-nitrosothiol (SNO) is emerging as a pivotal redox-based, post-translational modification (PTM) during plant immune function. However, the Cys target sites of NO bioactivity and the associated consequences on cellular signalling are not well defined. Our findings suggest that S-nitrosylation of small ubiquitin-like modifier (SUMO) conjugating enzyme 1 (SCE1) at Cys139 controls SUMOylation, a protein-based PTM that negatively regulates plant immunity through conjugation of SUMO1/2. This Cys is evolutionary conserved and specifically S-nitrosylated in the human homologue, UBC9, implying this mechanism might be conserved across phylogenetic kingdoms.

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INTRODUCTION

The production of nitric oxide (NO) is a conspicuous feature of immune responses in complex eukaryotes (1, 2). In this context, S-nitrosylation, the addition of an NO moiety to a protein cysteine (Cys) thiol to form an S-nitrosothiol (SNO), is thought to be a major route to regulate protein function (3-5). In combination with reactive oxygen intermediates (ROIs), NO regulates the hypersensitive response (HR) (3, 6), a programmed execution of plant cells at sites of attempted infection(7) and the expression of a suite of immune-related genes (8-10). However, the underpinning molecular mechanisms are not well understood.

The small ubiquitin-like modifier (SUMO) is present in all eukaryotes and is essential for viability (11, 12). SUMO is conjugated to target proteins via a pathway analogous to ubiquitylation, involving E1 and E2 enzymes as well as E3 ligases. The SUMO activating enzyme (E1) is a heterodimeric complex and forms a high-energy thioester bond with the C-terminal carboxyl group of SUMO. Next, SUMO is transferred to the SUMO conjugating enzyme (E2), which catalyzes the conjugation of SUMO to its targets. SUMO ligases (E3) enhance the efficiency of conjugation and may contribute to target specificity but are not required for SUMO conjugation *in vitro* (13). SUMOylation has been implicated in plant immunity by virtue of its function in HR control (14) and signalling integral to salicylic acid (SA), a major immune activator (15-17). Either loss of function of the SUMO E3 ligase, SAP and Miz 1 (SIZ1), or knockdown of SUMO1 and SUMO2, the two major stress-responsive SUMO isoforms, results in constitutive SA-dependent gene expression and increased pathogen resistance (16, 17). Furthermore, immune phenotypes of *siz1* mutants are dependent on the immune receptor SNC1 (18). Collectively these studies suggest that in the

absence of pathogen challenge, global SUMOylation mediated by SUMO1/2 negatively regulates plant immunity.

Despite the central role of SUMOylation in both plant and animal cell biology, there is currently little insight into the regulatory processes underpinning this post-translational modification. Here we show that following pathogen recognition, the *Arabidopsis* SUMO E2 enzyme, SUMO conjugating enzyme 1 (SCE1), is S-nitrosylated at a highly conserved cysteine, Cys139. We show that this site-specific modification inhibits the SUMO-conjugating activity of both SCE1 and its human homologue UBC9, suggesting that this might constitute an evolutionary conserved mechanism of regulating levels of SUMO conjugates in cells. Furthermore, expression of mutant SCE1(C139S) in *Arabidopsis* results in elevated levels of SUMOylated proteins after pathogen infection, compromised immune gene activation and increased disease susceptibility. These data therefore suggest that after immune activation, increasing NO levels are, in part, transduced into immune responses by inhibiting global conjugation of SUMO1/2 through S-nitrosylation of SCE1.

RESULTS

S-nitrosylation of SUMO E2 enzymes inhibits SUMOylation *in vitro*

To determine if NO might in part sculpt the plant immune response by regulating SUMOylation, protoplasts were isolated from wild-type (WT) *Arabidopsis* plants and used to monitor the potential impact of the natural NO donor, S-nitrosoglutathione (GSNO), on this modification following heat shock. This is a well-established method to increase SUMO2/3 or SUMO1/2 conjugates in human or plant cells, respectively (19, 20). This SUMOylation response was also observed in protoplasts after exposure to 37°C for 15 minutes, with a concurrent decrease in levels of free SUMO (Fig. 1A). Pretreatment of protoplasts with 1 mM GSNO inhibited this response and also reduced levels of SUMO conjugates under resting conditions, suggesting that SUMOylation is inhibited by GSNO. Since NO can modulate protein activity by S-nitrosylation, we hypothesized that components of the SUMOylation machinery might be targeted by this post-translational modification. The sole *Arabidopsis* SUMO E2 conjugating enzyme, SCE1, possesses four cysteine residues, including its active site Cys94 that could potentially be targets of S-nitrosylation. To test this, we employed the biotin-switch technique (BST), which specifically replaces protein SNOs with a biotin label (21). Purified, recombinant SCE1 was efficiently S-nitrosylated by GSNO in a concentration-dependent manner *in vitro*. Further, another natural NO donor, CysNO, also S-nitrosylated SCE1 (Fig. 1B). Informatively, this modification could be reversed by dithiothreitol (DTT), consistent with S-nitrosylation of SCE1 on a given Cys thiol. Mutagenesis of the Cys residues within SCE1, established that only mutation of Cys139 prevented S-nitrosylation by GSNO and subsequent detection by BST (Fig. 1B). This finding was further confirmed by mass-spectrometry (SI Appendix, fig. S1).

Structural modelling of SCE1 based on its human homologue UBC9 revealed that Cys139 is likely solvent-exposed and therefore accessible for modification (SI Appendix, fig. S2A). This contrasts with Cys44 and Cys76 which are both located in the interior of the protein structure with their side chains orientated inwards, and Cys94, which sits within the active-site cleft. Importantly, the cysteine corresponding to *Arabidopsis* Cys139 is highly conserved in various higher eukaryotes (SI Appendix, fig. S2B), suggesting it may have a functional role. To test this, the corresponding residue (Cys138) in human UBC9 was mutated and the protein subjected to BST analysis. Similar to SCE1, UBC9 was specifically S-nitrosylated at Cys138 (Fig. 1D).

After identifying Cys139 of SCE1 and Cys138 of Ubc9 as sites of S-nitrosylation *in vitro*, we sought to uncover the effect of these modifications on enzymatic activity. By reconstituting the *Arabidopsis* SUMO machinery *in vitro*, the formation of poly-SUMO1 chains was used as a read out of E2 activity and revealed that both WT and C139S forms of SCE1 are equally capable of rapidly forming SUMO1-chains (Fig. 1E). Therefore, it appears that mutation of Cys139 does not affect enzyme activity *in vitro*. However, pretreatment of WT SCE1 with GSNO, inhibited its SUMO-conjugating activity (Fig. 1E). Importantly, this effect was not observed with GSNO pretreatment of the C139S protein, suggesting specific modification of Cys139 inhibits SCE1 activity (Fig. 1E). We also confirmed that SUMO chain formation was inhibited by pretreating SCE1 with another NO donor, CysNO (SI Appendix, fig. S2C). We next established if S-nitrosylation of UBC9 affected its SUMO-conjugating activity by monitoring the *in vitro* formation of poly-SUMO2 chains using the reconstituted human SUMO machinery (22). Similar to SCE1, only GSNO pretreatment of WT, but not C138S UBC9, inhibited SUMO conjugating activity (Fig. 1F).

SUMO first forms a thioester with the E1 heterodimer before it is transferred to the active site of the E2, also establishing a thioester linkage. Subsequently, SUMO is conjugated to its target substrate forming an isopeptide bond. Thus, inhibition of SCE1 by S-nitrosylation of Cys139 can occur at either SUMO-SCE1 thioester formation or the transfer of SUMO from SCE1 to the given target. To discriminate between these two alternative possibilities, we performed *in vitro* SCE1-SUMO1 thioester formation assays. Both WT and C139S SCE1 proteins were equally capable of forming thioester bonds with SUMO1 and this reaction was unaffected by pretreatment with GSNO (Fig. 1G). This suggests that the inhibition of poly-SUMO1 chain formation by GSNO observed in Fig. 1E did not result from inhibition of SCE1-SUMO1 thioester formation. We next determined if SNO formation at Cys139 might interfere with SUMO1 transfer to a given target. To explore this, we performed *in vitro* SUMOylation reactions using *Saccharomyces cerevisiae* proliferating cell nuclear antigen (ScPCNA), a model substrate (23). Similar to poly-SUMO chain formation, the SUMOylation of ScPCNA was inhibited by GSNO pretreatment of SCE1 (Fig 1H). Collectively, these data suggest that mutation of SCE1 Cys139 does not affect the activity of this enzyme and significantly, S-nitrosylation of this redox-active residue blunts SCE1 function by inhibiting the ability of SCE1 to transfer SUMO to its substrates, rather than interfering with SCE1-SUMO thioester formation.

S-nitrosylation of SCE1 Cys139 inhibits SUMOylation *in vivo*

Next, we determined if SCE1 is subjected to S-nitrosylation *in vivo* by generating transgenic plants expressing either FLAG epitope-tagged WT, or C139S SCE1. Expression was confirmed as comparable between these lines (SI Appendix, fig. S3A and S3B) and SUMO1/2 co-immunoprecipitated with both FLAG-SCE1 and FLAG-

C139S at the expected SCE1-SUMO thioester molecular weight, suggesting these proteins are active *in vivo* (SI Appendix, fig. S3C). We tested if SCE1 expressed in plants could be S-nitrosylated by GSNO by subjecting protein extracts to BST analysis, followed by isolation of S-nitrosylated proteins, with their subsequent analysis by western blotting employing an anti-FLAG antibody, to detect the possible presence of FLAG-SCE1 among these protein SNOs. Very little SNO-SCE1 was detected under basal conditions but pre-incubating the extracts with 1 mM GSNO resulted in S-nitrosylation of SCE1 (Fig. 2A) suggesting that FLAG-SCE1 expressed *in vivo* can be S-nitrosylated.

Global SNO levels are increased in *Arabidopsis* upon pathogen recognition (24, 25), so we performed the BST on plants challenged with either the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, or an avirulent strain expressing AvrB, recognized by the RPM1 resistance protein in WT Col-0 plants (26). Low levels of SNO-SCE1 were detected in non-inoculated plants (Fig. 2B) while SNO-SCE1 levels were increased at 6 hours post inoculation (hpi) with either *Pst* DC3000 or especially *Pst* DC3000 (*avrB*), suggesting that S-nitrosylation of SCE1 is enhanced in response to pathogen challenge (Fig. 2B). Importantly, SCE1(C139S) was not S-nitrosylated in response to attempted pathogen ingress (Fig. 2B), implying that Cys139 is also the site of SNO formation *in vivo*. Further, total SNO levels increased significantly at 6 hpi following challenge with same *Pst* DC3000 strains (SI Appendix, fig. S4A). Collectively, these data suggest that following pathogen recognition, global SNO levels are increased, promoting S-nitrosylation of SCE1 at Cys139.

Since SCE1(C139S) is insensitive to S-nitrosylation and this modification was shown to inhibit SUMO-conjugating activity *in vitro*, we next examined the impact of SCE1(C139S) expression on global SUMOylation levels in either the absence or

presence of pathogen challenge. Consistent with a previous report (16), *Pst* DC3000 inoculation had no observable effect on global SUMOylation in leaves of WT plants, with similar results observed for *SCE1* expressing plants (Fig. 2C). Strikingly, in the *SCE1(C139S)* line, SUMO conjugate levels were increased after challenge with either *Pst* DC3000 or *Pst* DC3000 (*avrB*) (Fig. 2C). In a similar fashion, *SCE1(C139S)* plants also exhibited increased and prolonged SUMOylation following heat shock, which also results in rapid NO synthesis (27) (Fig. 2D). Thus, S-nitrosylation of Cys139 following engagement of the pathogen-triggered nitrosative burst, may be required to suppress *SCE1* activity and by extension SUMOylation, during plant immune function.

Cys139 of *SCE1* is required for immunity and stress-induced gene expression.

After establishing that S-nitrosylation of *SCE1* at Cys139 is driven by attempted pathogen infection, we next explored the biological consequences of this redox-based modification on plant disease resistance. Bacterial growth assays revealed that compared to WT and *SCE1* expressing plants, which both showed similar levels of pathogen growth, *SCE1(C139S)* plants were more susceptible to infection by *Pst* DC3000 (Fig. 3A). Similarly, the *SCE1(C139S)* line exhibited increased growth of *Pst* DC3000 (*avrB*) (Fig. 3B). Additional independent transgenic lines also showed similar results (SI Appendix, fig. S4B, S4C). However, the difference in bacterial titre between *Pst* DC300 and *Pst* DC3000 (*avrB*) was similar between *SCE1* and *SCE1(C139S)* expressing plants, suggesting RPM1 mediated disease resistance may not be impacted. In aggregate, these data imply that SNO formation at Cys139 of *SCE1* is required for full basal disease resistance. To uncover the molecular basis of these observations, the expression of the SA marker gene, *PR1*, was monitored after *Pst* DC3000 inoculation. [As expected, *PR1* expression was induced in WT plants and more](#)

pertinently, *SCE1* plants at 12 hpi (Fig. 3C). In contrast, *PR1* expression was compromised in *SCE1(C139S)* plants (Fig. 3C). Furthermore, *Pst* DC3000 (*avrB*)-induced *PR1* expression was also reduced at 12 hpi in *SCE1(C139S)* plants (Fig 3D).

Since pathogen-induced gene expression was compromised in *SCE1(C139S)* plants that also displayed higher levels of SUMO conjugation, we tested whether similar links between SUMO conjugate levels and transcriptional responses might exist in response to heat shock. It is well-established that heat stress induces NO levels in plants (28) so we monitored the expression of the heat-stress marker gene *HsfA3* in seedlings after exposure to 37°C for 1h. *HsfA3* expression was induced to similar levels in WT and *SCE1* plants but was not induced in *SCE1(C139S)* plants (SI Appendix, fig. S4D) suggesting that signalling through C139 of *SCE1* is also required for optimal transcriptional responses to heat stress.

S-nitrosylation of *SCE1* impacts plant immunity

Next, we tested SA-induced immunity in *SCE1* and *SCE1(C139S)* plants. As expected, pre-treatment of WT plants with SA resulted in dramatically less growth of *Pst* DC3000 compared to mock treated lines (Fig. 4A). In agreement with previous experiments, mock-treated *SCE1(C139S)* plants showed significantly higher levels of *Pst* DC3000 growth compared to mock treated WT and *SCE1* plants (Fig. 4A). However, SA treatment of both *SCE1* and *SCE1(C139S)* plants reduced the titre of *Pst* DC3000 to similar levels (Fig. 4A). To confirm that exogenous SA treatment rescued immunity in *SCE1(C139S)* plants, we monitored SA-induced *PR1* expression. SA-induced *PR1* expression reached a similar level in WT, *SCE1* and *SCE1(C139S)* plants (SI Appendix, fig. S5). Therefore, to establish if the disease-susceptible phenotype of *SCE1(C139S)* plants was attributed to reduced endogenous SA accumulation, we

challenged with *Pst* DC3000 to induce accumulation of this metabolite and subsequently determined its concentration. SA accumulated to a lesser extent in *SCE1(C139S)* plants compared to *SCE1* plants in response to both *Pst* DC3000 and *Pst* DC3000 (*avrB*) (Fig 4B). Collectively, these data suggest that S-nitrosylation of *SCE1* at C139 is required for maximal SA accumulation and associated disease resistance. Thus, our findings support a model in which attempted pathogen infection promotes increasing levels of NO, leading to S-nitrosylation of *SCE1* at Cys139. This serves to limit SUMO1/2 conjugation by *SCE1* enabling both the accumulation of SA and maximal activation of SA-dependent defence gene expression (Fig. 4C).

DISCUSSION

SUMO conjugation has been implicated in a plethora of regulatory systems across eukaryotes, including human disease pathways (29). The control of global SUMOylation integral to cellular signalling is currently thought to occur predominantly at the level of each protein target via local regulatory mechanisms, rather than by direct modulation of the core SUMOylation machinery by PTMs (30). However, acetylation or SUMOylation of UBC9 is thought to enable discrimination between individual target substrates (31, 32). Conversely, the redox active small molecule, hydrogen peroxide, has been shown to reduce total SUMOylation, by driving the formation of disulphide bonds between SUMO E1 and E2 enzymes (33). These modifications, however, have not yet been linked to cellular signalling. Our findings suggest that changes in global SUMOylation, which underpin plant immune function, may result from direct regulation of the SUMOylation apparatus, by SNO formation at Cys139 of SCE1. This means of controlling SUMO conjugation may also serve to limit SUMOylation of proteins involved in heat-stress signalling and thus may be a widespread means of transcriptional regulation in general. Significantly, this Cys is evolutionary conserved and specifically S-nitrosylated in the corresponding human enzyme, UBC9, modulating its activity. Therefore, this mechanism might be conserved between plants and animals thereby providing a potential target for either future agrochemical or pharmaceutical intervention, respectively.

The effect of S-nitrosylation on enzymatic activity can typically be directly mediated through modification of active site Cys residues (4). However, our findings suggest that Cys139 is the only S-nitrosylation site of SCE1 both *in vitro* and *in vivo*. In a mutational study of *S. cerevisiae* Ubc9, residues close to this area were shown to be important for Smt3p-Smt3p conjugate formation (34). Similar to S-nitrosylation of

SCE1 at Cys139, these same mutations did not have any effect on Ubc9-Smt3p thioester formation. The fact that S-nitrosylation of SCE1 at Cys139 does not affect SUMO thioester formation suggests that it does not interfere with binding to the E1 complex. This is not surprising since a well-defined region of the Ubc9 N-terminal has been identified as the binding site for E1:E2 noncovalent interactions (34-37) and Cys139 is located at a distant site near the C-terminus. Although there are currently no structures available for components of the *Arabidopsis* SUMOylation machinery, data from the structure of human Ubc9 in complex with the SUMO substrate RanGAP1 revealed that residues close to Cys138 on the same α -helix are important for interaction with RanGAP1 (38). Mutation of a conserved tyrosine to phenylalanine (Y134F) dramatically reduced the ability of Ubc9 to conjugate SUMO to RanGAP1 suggesting this residue plays an important role. This tyrosine is conserved in SCE1 (Tyr135) and its side chain is predicted to occupy a similar position in Ubc9. A possible mechanism for S-nitrosylation of Cys139 to inhibit SUMOylation is therefore by interfering with interactions between Tyr135 and substrate proteins.

The means by which SUMOylation by SUMO1/2 regulates plant immunity are now beginning to emerge. Loss of SIZ1 function results in elevated SA levels, constitutive activation of *PR* gene expression and increased resistance to *Pst* DC3000 (16). These phenotypes were reverted to WT by expression of the bacterial salicylate hydroxylase NahG, which degrades SA, suggesting that the phenotypes of *siz1* mutant plants are due to the elevated levels of SA. More recently, evidence has emerged that SIZ1-mediated SUMOylation of TPR1 inhibits its transcriptional repressor activity (39). Since TPR1 functions together with SNC1 to activate plant immunity (40), it appears that the TPR1/SNC1 complex is a central node of SUMO-mediated immune signalling in plants (18, 39, 41). Interestingly, studies also suggest that SUMO1/2 conjugation

regulated by SUMO proteases might have a positive role in SA signalling. Mutation of the SUMO proteases OVERLY TOLERANT TO SALT1 and -2 (OTS1/2) was shown to increase SA levels in plants leading to constitutive activation of SA signalling pathways (42). Similarly, the SUMO protease EARLY IN SHORT DAYS 4 (ESD4) has also been shown to accumulate SA (43). Therefore, SUMOylation by SUMO1/2 appears to have both positive and negative effects on SA-mediated immunity. Indeed, SUMOylation can both positively and negatively regulate immune responses in animals depending on the substrate proteins affected (44). Therefore, the balance between which substrates are SUMOylated at a given time under certain cellular conditions can have diverse effects on signalling at the organism level. To further elucidate the complex roles of SUMOylation in plant immunity, proteins that are SUMOylated after immune activation must be identified and the effect of their modification studied. Interestingly, the central regulator of SA-mediated immunity, the transcription coactivator NONEXPRESSOR OF PATHOGENESIS RELATED 1 (NPR1) has recently been identified as a SUMO substrate (15). Modification of NPR1 by SUMO3 appears to be regulate its stability and binding to cognate transcription factors promoting immunity. In Arabidopsis, SUMO3 is expressed at lower levels and appears to be conjugated to far less proteins than SUMO1/2 (17, 20). Furthermore, patterns of SUMO3 conjugation do not appear to be affected by various cellular stresses that increase conjugation of SUMO1/2 (20). However, SUMO3 expression is strongly induced by SA suggesting a key role in SA-mediated immunity (17). Thus, SUMO3 conjugation is also a key regulatory mechanism in plant immunity. Nonetheless, the fact that suppression by SUMO1/2 conjugation appears to be required to prevent the induction of immune function (17) suggests that defence responses are constitutively primed and ready for rapid deployment. Thus, our proposed model may constitute a molecular mechanism

by which the nitrosative burst associated with attempted pathogen ingress is perceived and translated into immune activation.

MATERIALS AND METHODS

Detailed descriptions of materials and methods used including plant growth conditions, pathogen inoculations, protoplast isolation, protein analyses, gene expression analyses and statistics are provided in SI Materials and Methods. All primers used are listed in Table S1.

ACKNOWLEDGEMENTS

We thank Ron Hay and Ellis Jaffray (University of Dundee, UK) for providing the human proteins and constructs used in this study. This work was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) studentship to M.J.S. (BB/F017073/1), a Higher Education Commission Pakistan scholarship to S.I.M., a Royal Society Research Fellowship (UF090321) and research grant to S.H.S. (Rg110495) and BBSRC research grant BB/DO11809/1 to G.J.L.

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FIGURE LEGENDS

Figure 1. S-nitrosylation of SUMO E2 enzymes inhibits SUMOylation *in vitro*

(A) WT protoplasts were pretreated with or without 1 mM GSNO before 15 min incubation at either 22°C or 37°C. Protein extracts were analysed by western blot against SUMO1/2 and S5a (loading control). **(B)** Purified recombinant SCE1 was subjected to the stated treatments before the BST. Total SCE1 was detected by Coomassie staining, while SNO-SCE1 was detected by western blot against biotin. **(C)** Each cysteine mutant form of SCE1 was incubated with either 100 µM GSH or GSNO as in (B). **(D)** Purified recombinant UBC9 was incubated with or without 500 µM GSNO before the BST. Biotinylated protein (SNO-UBC9) was enriched by streptavidin pulldown before detection by western blot against His-tag. The omission of ascorbate (-Asc) serves as a negative control for the BST. **(E)** The stated proteins were incubated with or without 500 µM GSNO 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. **(F)** Proteins were incubated as in (E) before adding to *in vitro* SUMOylation reactions and incubating at 37°C for the stated times. before western blot against SUMO2. **(G)** Proteins were incubated as in (E), then added to a reaction mix containing the E1 heterodimer and SUMO1, before the addition of ATP and incubation at 30°C for the stated times. The SCE1-SUMO1 thioester was observed by western blot against SCE1. **(H)** SCE1 was pretreated with the stated concentrations of GSNO before adding to reaction mixtures. SUMOylated forms of PCNA were visualized by western blot against SUMO1/2.

Figure 2. S-nitrosylation of SCE1 Cys139 inhibits SUMOylation *in vivo*

(A) Protein extracts from *35S::FLAG-SCE1* plants were subjected to the BST with or without pre-incubation with 1 mM GSNO. Biotinylated proteins were enriched by Streptavidin affinity pull-down before western blot against FLAG. **(B)** Protein extracts from control, or plants inoculated with 10^7 cfu/ml *Pst* DC3000 or *Pst* DC3000 (*avrB*) (6 hpi) were subjected to the BST and both SNO-SCE1 and Total SCE1 were detected by western blot against FLAG. **(C)** Plants were inoculated as in (B), and leaf tissue collected at 6 hpi. Protein extracts were then analysed by western blot against SUMO1/2. Ponceau S staining of the large subunit of Rubisco indicates equal loading. **(D)** Liquid-grown seedlings of the stated lines were exposed to 37°C for the indicated times and analysed as in (C).

Figure 3. Cys139 of SCE1 is required for resistance to *Pst* DC3000

(A, B) Plants were inoculated with 10^5 cfu/ml **(A)** *Pst* DC3000 or **(B)** *Pst* DC3000 (*avrB*) and leaf discs were assayed for bacterial growth at 3 dpi. Data points represent mean \pm SD (n=6 biological replicates), with asterisks indicating significant difference from WT (Student's *t* test, $P < 0.05$). **(C)** Plants were inoculated with 10^6 cfu/ml *Pst* DC3000 and leaf tissue was harvested at the stated times. The expression of *PR1* was analysed using qPCR and normalized against the constitutively expressed *UBQ5*. Data points represent mean \pm SD (n=3) of three independent biological samples while asterisks represent significant differences between the indicated samples (Student's *t* test, * $P < 0.05$, *** $P < 0.0001$). **(D)** Plants were inoculated with 10^6 cfu/ml *Pst* DC3000 (*avrB*) and *PR1* expression analysed as in (C).

Figure 4. S-nitrosylation of SCE1 impacts plant immunity

(A) Plants were sprayed with either 0.5 mM SA or H₂O and inoculated with 10⁵ cfu/ml *Pst* DC3000 24 hours post-spraying. Leaf discs were then assayed for bacterial growth at 3 dpi. Data points represent mean ± SD (n=6 biological replicates), with letters indicating significant differences between samples (Tukey Kramer ANOVA; α = 0.05).

(B) Plants were inoculated with 10⁶ cfu/ml *Pst* DC3000 or *Pst* DC3000 (*avrB*) and SA levels were measured after 24 hours. Data points represent mean ± SD (n=3 biological replicates) with asterisks representing significant differences between the indicated samples (Student's *t* test, *P* < 0.01).

(C) In pathogen unchallenged plant cells, SCE1-dependent conjugation of SUMO1/2 contributes to the repression of *PR1* gene expression in part through limiting SA levels. In pathogen challenged cells, increasing NO levels associated with attempted pathogen infection promote the S-nitrosylation of SCE1 at Cys139. This inhibits the SUMO-conjugating activity of SCE1 and reduces global SUMOylation, which in turn allows accumulation of SA, relieves repression of *PR1* gene expression and contributes to the activation of immunity.