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S-nitrosylation of AtSABP3 regulates the
expression of plant disease resistance

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Changes in cellular redox status are a well established response across phyla following pathogen challenge. The synthesis of nitric oxide (NO) is a conspicuous feature of plants responding to attempted microbial infection and this redox-based regulator underpins the development of plant immunity. However, the associated molecular mechanism(s) have not been defined. Here we show that NO accretion during the nitrosative burst promotes increasing S-nitrosylation of the *Arabidopsis thaliana* salicylic acid binding protein 3 (AtSABP3) at cysteine (C)280, suppressing both binding of the immune activator, salicylic acid (SA) and the carbonic anhydrase (CA) activity of this protein. The CA function of AtSABP3 is required for the expression of resistance in the host against attempted pathogen infection. Therefore, inhibition of AtSABP3 CA function by S-nitrosylation could contribute to a negative feedback loop that modulates the plant defense response. Thus, AtSABP3 is one of the first targets for S-nitrosylation in plants for which the biological function of this redox-based post-translational modification has been uncovered. These data provide a molecular connection between the changes in NO levels triggered by attempted pathogen infection and the expression of disease resistance.

Plants have evolved a complex series of integrated defense systems in response to microbial colonization (1). Prominent amongst these is a repertoire of resistance (*R*) gene products, which recognize either directly or indirectly pathogen effector proteins, triggering a battery of protective mechanisms (2). A conspicuous feature of this defense response is the synthesis of nitric oxide (NO), a key signal for numerous physiological processes in higher eukaryotes (3), which cues the execution of host cells at sites of attempted pathogen infection (4) and drives the expression of a battery of redox-regulated defense genes (5,6).

S-nitrosylation, the addition of a NO moiety to a specific cysteine thiol, to form an S-nitrosothiol (SNO), has emerged as a principal mechanism by which NO orchestrates cellular functions in
animals (7). Recently, a number of S-nitrosylated proteins have been identified in *Arabidopsis* (8,9) and this redox-based post-translational modification shown to regulate the function of a small number of these plant proteins *in vitro* (8-11). However, a potential endogenous role for S-nitrosylation in the regulation of protein function remains to be demonstrated. Mutations in an *Arabidopsis thaliana* S-nitrosoglutathione reductase (*AtGSNOR1*), however, impact cellular SNO homeostasis and influence multiple modes of plant disease resistance (12), consistent with a central role for SNOs in the plant defense response.

To identify protein targets for S-nitrosylation during the development of plant disease resistance we employed the biotin-switch method, a well established technique to monitor SNO formation in animals, which has recently been applied successfully in plants (13). *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 expressing the avirulence gene, *avrB* (14), is recognized by the R protein RPM1 (15), triggering the expression of race specific disease resistance in *Arabidopsis* accession, Col-0. Challenge of this plant line with avirulent *Pst*DC3000(*avrB*) modified the S-nitrosylation status of a series of proteins (table S1).

We had previously established that SNO formation and turnover regulates both the biosynthesis of and signaling by the plant immune activator, salicylic acid (SA), impacting multiple modes of plant disease resistance (12). A change in the S-nitrosylation status of *Arabidopsis thaliana* SA-binding Protein 3 (*AtSABP3; At3g01500*) (16), a homolog of one of a small number of SA-binding proteins (16U18), therefore, appeared to be of particular significance, although the function of SA binding is unknown. *AtSABP3* also exhibits chloroplastic carbonic anhydrase (CA) activity (16), catalyzing the reversible hydration of CO$_2$ to HCO$_3^-$.

To confirm increased S-nitrosylation of *AtSABP3* during the defense response, protein extracts from inoculated *Arabidopsis* leaves were subjected to the S-nitrosylation biotin switch procedure.
and S-nitrosylated proteins purified using streptavidin agarose. Subsequent western blot analysis with an antibody raised against AtSABP3 verified enhanced S-nitrosylation of this protein in response to an avirulent but not a virulent strain of *Pst* DC3000 (Fig. 1A). Congruent immunoblot analysis of AtSABP3 protein levels revealed no significant change in their relative abundance at 3 hours post inoculation (hpi) with either virulent or avirulent strains of *Pst* DC3000 (Fig. 1B), suggesting that enhanced SNO-AtSABP3 levels reflected increased S-nitrosylation.

We next determined the extent of SABP3 S-nitrosylation in the *Arabidopsis atgsnor1-3* and *atgsnor1-1* mutants, which exhibit either increased or decreased levels of cellular SNOs, respectively (12). SNO-AtSABP3 levels were less conspicuous in *atgsnor1-1* plants and more pronounced in the *atgsnor1-3* line relative to wild-type (Fig. 1C), implying that AtGSNOR1 governs the S-nitrosylation status of AtSABP3 during the defense response. To monitor the profile of SNO-AtSABP3 formation during the establishment of disease resistance, AtSABP3 was immuno-precipitated at various times post *Pst* DC3000(*avrB*) challenge and the extent of S-nitrosylation determined using the 2,3-diaminonaphthalene (DAN) assay. This analysis suggested that SNO-AtSABP3 formation increased over time during the establishment of disease resistance, peaking at 8 hpi of *Pst* DC3000(*avrB*) (Fig. 1D).

We also investigated whether S-nitrosylation of AtSABP3 could occur *in vitro*. Recombinant AtSABP3 synthesized in *Escherichia coli* was incubated with a series of GSNO concentrations and SNO-AtSABP3 formation monitored with the S-nitrosylation biotin-switch assay. This analysis revealed that AtSABP3 was S-nitrosylated *in vitro* (Fig. 1E). Furthermore, increasing levels of GSNO promoted SNO-AtSABP3 formation, suggesting S-nitrosylation of AtSABP3 was GSNO concentration dependent. The absence of SNO-AtSABP3 in samples treated with glutathione devoid of NO (GSH) demonstrated the specificity of this post-translational modification. Furthermore, the addition of dithiothreitol (DTT) strikingly reduced the level of
SNO-AtSABP3 formation, which is consistent with the presence of a reversible thiol modification. Collectively, these experiments showed that AtSABP3 was specifically S-nitrosylated \textit{in vitro}.

To identify the target site(s) of S-nitrosylation we carried out liquid chromatography / mass spectrometry / mass spectrometry (LC-MS/MS) analysis of GSNO-treated AtSABP3. This tentatively identified C280 as the site of S-nitrosylation, however, the intensity of MS/MS spectra obtained was low (data not shown), probably due to the labile nature of SNO formation during sample preparation for this analysis. We therefore carried out LC/MS/MS of S-biotinylated peptides of AtSABP3 following the S-nitrosylation biotin switch assay (Fig.2A and B). This approach also identified C280 as the sole site of S-nitrosylation. Furthermore, C280 is embedded within a canonical acid-base motif (7), which additionally defines this amino acid as a potential target site for SNO formation. As the three-dimensional structure of a pea CA has been determined (19), we utilized this structure as a template to model the hypothetical three-dimensional conformation of the \textit{Arabidopsis} AtSABP3, to provide us with structural insight into the spatial disposition of C280. This residue was present as a solvent exposed free amino acid at the base of a structural pocket, consistent with it being a target for S-nitrosylation (Fig. 2C).

We investigated the potential biological significance of this post-translational modification by assessing its possible impact on the CA activity of AtSABP3. We found that exposure of AtSABP3 to GSNO but not GSH resulted in a dramatic, concentration-dependent decrease in CA activity (Fig. 3A). Furthermore, this inhibition could be reversed by DTT. Also, an alternative NO donor, S-Nitroso-N-acetylpenicillamine (SNAP), strikingly reduced SABP3 CA activity and this inhibition could be reversed in the presence of DTT (Fig. 3B). Together, these results imply that S-nitrosylation of AtSABP3 may function to negatively regulate its CA activity. These findings prompted us to examine the profile of chloroplastic CA activity during the defense
response. This activity was increased at 4 hpi of \textit{PstDC3000(avrB)}, however, by 8 hpi it was significantly reduced (Fig. 3C). Thus, chloroplastic CA activity is modulated during the establishment of plant disease resistance. Next we determined this activity in \textit{atgsnor1-1} and \textit{atgsnor1-3} plants (12). The reduction of CA activity was decreased in \textit{atgsnor1-1} plants but increased in the \textit{atgsnor1-3} mutant line (Fig. 3D). Collectively, our findings provide a direct molecular link between S-nitrosylation of AtSABP3 and CA activity.

To investigate the potential role of C280 in AtSABP3 function, we carried out site-directed mutagenesis to replace this residue with serine (S) and subsequently synthesized the corresponding recombinant protein in \textit{E. coli}. As expected, C280S AtSABP3 could not be S-nitrosylated (Fig. 3D), further confirming C280 as the site of S-nitrosylation. We then assessed the CA activity associated with this AtSABP derivative. The C280S mutant was found to be strikingly reduced in CA activity (Fig. 3E). This was unexpected because this residue is some distance from the active site and previous structure-function studies of this enzyme have not uncovered a significant role for C280 in CA activity (19). Nevertheless, our data imply that structural changes at this position may compromise CA activity, consistent with our findings that S-nitrosylation at C280 negatively regulates the CA activity of AtSABP3.

AtSABP3 has previously been shown to bind SA with high affinity, distinguishing between biologically active and inactive analogs (16). However, amino acid residues required for SA binding have not yet been identified and the binding of this small molecule does not affect CA activity, suggesting these functions are independent (16). We next explored if S-nitrosylation of C280 could also modulate the SA binding activity of AtSABP3. This activity was assessed in the presence of $[^{14}\text{C}]$SA with or without excess unlabelled SA. To monitor the impact of S-nitrosylation, AtSABP3 was incubated with 1mM GSNO prior to the determination of $[^{14}\text{C}]$SA-binding (Fig. 4A). Our results suggested that S-nitrosylation of AtSABP3 significantly decreased
SA binding. We also investigated the binding of this small molecule in the C280S AtSABP3 mutant. In the absence of GSNO exposure, this mutant derivative bound a similar amount of \[^{14}\text{C}]SA to wild-type AtSABP3, revealing that C280S AtSABP3 retains full SA-binding capacity (Fig. 4B). Furthermore, exposure of C280S AtSABP3 to GSNO did not impact \[^{14}\text{C}]SA binding. Collectively, these data imply that S-nitrosylation of C280 AtSABP3 reduces SA binding. Thus, increasing S-nitrosylation of AtSABP3 during the defense response may reduce both the SA binding ability and CA activity of AtSABP3.

To determine a possible role for AtSABP3 in the establishment of disease resistance in Arabidopsis, we identified two independent atsabp3 loss-of-function alleles from the Salk Institute transferred DNA (T-DNA) insertion collection (20). The atsabp3-1 and atsabp3-2 T-DNA lines were challenged with PstDC3000(\textit{avrB}) and pathogen growth monitored over time. Both atsabp3 alleles supported more PstDC3000(\textit{avrB}) growth compared to wild-type (Fig. 4C). Thus, AtSABP3 is required for a full defense response against this bacterial strain. To assess whether CA activity is required for AtSABP3-dependent disease resistance, we utilized an atsabp3-2 Arabidopsis line containing either a 35S::AtSABP3 transgene, which complements the CA function of AtSABP3 or a 35S::C280S AtSABP3 construct that does not. No increased susceptibility to PstDC3000(\textit{avrB}) was observed in the atsabp3-2 Arabidopsis line containing a 35S::AtSABP3 transgene (Fig. 4D). In contrast, PstDC3000(\textit{avrB}) growth was increased in atsabp3-2 plants possessing a 35S::C280S AtSABP3 construct relative to wild-type but to a level less than that observed in atsabp3-2 plants (Fig. 4D). Taken together, these data suggest that the CA activity of AtSABP3 contributes to resistance against PstDC3000(\textit{avrB}). Furthermore, the atgsnor1-3 line is significantly more susceptible to PstDC3000(\textit{avrB}) (12) than atsabp3-2 plants, suggesting other defense signaling proteins maybe negatively regulated by S-nitrosylation.
Tobacco SABP3 has previously been shown to be required for R gene-dependent defense responses (16). Moreover, a potato homolog of this gene was found to be quickly suppressed during the development of late blight disease and silencing SABP3 in *Nicotiana benthamiana* resulted in increased pathogen growth (21). Our data, in addition to these findings, supports a role for CA activity in disease resistance. CA enzymes are evolutionary conserved across phyla and are thought to play a key role in lipolysis (22). In multiple plant species, CA function is required for lipid biosynthesis within the chloroplast, possibly due to its interaction with acetyl-CoA carboxylase and the enzymes of the fatty acid synthase complex, where it may efficiently “channel” carbon into fatty acid (22). This role for the CA activity of *AtSABP3* may be particularly pertinent within the context of the plant defense response because lipid-based signals are thought to be fundamental to the development of disease resistance (23,24).

Collectively, our findings suggest that increasing S-nitrosylation of AtSABP3 during the progression of the nitrosative burst may blunt its cognate CA activity, reducing fatty acid biosynthesis in the chloroplast and thereby diminishing the transient production of lipid-based defense cues. Formation of SNO-AtSABP3 might therefore govern a negative feedback-loop that serves to dampen defense signaling. Consistent with this hypothesis, *atgsnor1-3* plants exhibit increased levels of SNO-AtSABP3, decreased CA activity, diminished and delayed defense responses and are compromised in resistance against *PstDC3000*(*avrB*) (12). In contrast, *atgsnor1-1* plants show decreased levels of SNO-AtSABP3, increased CA activity, accelerated defense responses and display resistance against ordinarily virulent pathogens (12). The prompt engagement of a negative feedback-loop, reported here, parallels recent findings in the signaling network that underpins perception of the lipid-based defense signal, jasmonate-isoleucine (25).

While the exogenous addition of numerous pharmacological agents has suggested NO regulates a wide variety of responses (4,5,26) the *in planta* molecular detail underpinning these processes
remains to be established. S-nitrosylation has emerged as a principal mechanism by which NO exerts biological effects in animal systems, with a large variety of proteins reported as targets for this key post-translational modification (7). By demonstrating that SNO-AtSABP3 formation governs the engagement of a negative feedback loop during the defense response, we provide molecular characterization of a possible \textit{in vivo} function for S-nitrosylation, suggesting this process may also represent a fundamental regulatory process in plants. Furthermore, recent findings imply that S-nitrosylation of NPR1, a key transcriptional regulator of defense gene expression, promotes the oligomerization of this protein, preventing its translocation to the nucleus, which consequently suppresses the development of plant immunity (27). Also, S-nitrosylation of peroxiredoxin II E has lately been proposed to promote the accretion of peroxynitrite leading to increased tyrosine nitration, which might help drive the programmed execution of directly challenged plant cells, a routine feature of \textit{R} gene-mediated disease resistance (9). Thus, SNO formation may target multiple nodes of the plant defense signaling network resulting in a variety of regulatory permutations that collectively optimize cellular responses. This is reminiscent of the NF-κB immune signaling network in animals, where the activity of manifold components is controlled by S-nitrosylation (7).
References and notes


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**Figure legends**

**Fig. 1.** AtSABP3 can be S-nitrosylated by NO in vivo and in vitro. (A) Protein extracts were made from *Arabidopsis* Col-0 leaves infiltrated with \(10^7\) colony forming units (cfu) ml\(^{-1}\) of either virulent *PstDC3000* or avirulent *PstDC3000(avrB)* at the indicated hours post inoculation (hpi). These extracts were subjected to the S-nitrosylation biotin-switch assay and subsequently immunoblotted with an antibody raised against AtSABP3. (B) Level of AtSABP3 accumulation at 3 hpi of either virulent *PstDC3000* or avirulent *PstDC3000(avrB)* at \(10^7\) cfu ml\(^{-1}\). Amount of AtSABP3 determined by immunoblot analysis of corresponding protein extracts in the absence of the biotin-switch technique. Ponceau staining is a control for protein loading. (C) The S-nitrosylation status of AtSABP3 determined in wild-type Col-0, *atgsnor1-3* and *atgsnor1-1* plants. Protein extracts from leaves infiltrated with \(10^7\) cfu ml\(^{-1}\) *PstDC3000(avrB)* at the indicated time points were subjected to the S-nitrosylation biotin-switch assay, subsequently immunoblotted with an antibody against AtSABP3 and the resulting blot quantified. (D) Leaves of *Arabidopsis* were challenged with *PstDC3000(avrB)* at \(10^7\) cfu ml\(^{-1}\) and samples taken at the given hpi, lysates were immunoprecipitated with an AtSABP3 antibody and the extent of S-nitrosylation quantified using the DAN assay. (E) S-nitrosylation of recombinant AtSABP3 in vitro. Recombinant AtSABP3 was incubated with the stated concentrations of GSNO, GSH or DDT and subsequently subjected to the S-nitrosylation biotin-switch assay. An immunoblot with an antibody against AtSABP3 was employed to check equal protein loading. Error bars represent 95% confidence limits. These experiments were repeated at least twice with similar results.
**Fig. 2.** The target site for S-nitrosylation of AtSABP3 is identified as C280 by mass spectrometry. (A) MS/MS spectra of the biotinylated tripeptide C*ER (C*: biotin-HPDP derivitized cysteine, +428), both single (418.66_2+) and double charged (835.58_1+) ions are indicated by arrows. (B) MS/MS spectra of biotinylated tripeptide C*ER. The b2 (661.42) and b2-H2O (643.47) ions indicate a mass shift (+428) was present due to the Cys-N-[6(biotinamido)hexyl]-3'(2'-pyridyldithio) propionamide (HPDP-biotin) adduct. Two fragment ions 429.32 and 463.40 indicated by arrows were derived from biotin-HPDP. (C) Structural model of AtSABP3 showing the position of C280 in blue, C277 is in green. (D) Close-up of AtSABP3 structural model revealing that C280 is located within a solvent exposed pocket. The structure of the GRCE S-nitrosylation motif is superimposed. Yellow indicates the sulfur atom on C280. Blue, red and green colors indicate nitrogen, oxygen and carbon, respectively.

**Fig. 3.** The CA activity of AtSABP3 is negatively regulated by S-nitrosylation of C280. (A) Recombinant AtSABP3 was incubated with the given concentrations of GSNO, GSH and DTT and the CA activity of this protein determined using the Wilbur and Anderson method. (B) The CA activity of recombinant AtSABP3 was determined as above following exposure to the NO donor SNAP. (C) Chloroplastic CA activity determined at the given times following challenge of wild-type Col-0 plants with 10^7 cfu ml^-1 PstDC3000(avrB). (D) Chloroplastic CA activity measured in the stated plant genotypes at 8 hpi of PstDC3000(avrB). (E) Recombinant wild-type AtSABP3 and the C280S mutant derivative were incubated with either 100 µM GSNO or GSH and subsequently these proteins were subjected to the S-nitrosylation biotin-switch technique and subsequently immunoblotted with an antibody against biotin. Equal protein loading of individual samples was determined by immunoblotting with an antibody against AtSABP3. (F) The CA activity of recombinant wild-type AtSABP3 and the C280S mutant derivative were determined as
described above. Error bars represent 95% confidence limits. These experiments were repeated at least twice with similar results.

**Fig. 4.** S-nitrosylation of AtSABP3 blunts SA binding and the CA activity of AtSABP3 is required for disease resistance. (A) The affinity of wild-type AtSABP3 for SA was determined using 300 nM $^{14}$C labeled SA (20.5 Ci mmol; 1 Ci = 37 GBq) in the presence or absence of unlabeled SA (10,000-fold molar excess) or 100 µM GSNO. Following removal of unbound ligand, bound $^{14}$C-SA was quantified by scintillation counting. (B) The affinity of the C280S AtSABP3 mutant for $^{14}$C labeled SA in the presence or absence of excess unlabeled SA and 100 µM GSNO was undertaken as described above. (C) Growth of *PstDC3000*(avrB) in the indicated plant lines at 5 (white bar) and 7 (black bar) days post inoculation. (D) Growth of *PstDC3000*(avrB) in the indicated transgenic *Arabidopsis* plant lines. Error bars represent 95% confidence limits. These experiments were repeated at least twice with similar results.
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