S-Nitrosylation of AtSABP3 Antagonizes the Expression of Plant Immunity*

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Changes in cellular redox status are a well established response across phyla following pathogen challenge. In this context, 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 (Cys) 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, 2). Prominent among 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, 3). A conspicuous feature of this defense response is the synthesis of nitric oxide (NO),7 a key signal for numerous physiological processes in higher eukaryotes (4, 5), which cues the execution of host cells at sites of attempted pathogen infection (6) and drives the expression of a battery of redox-regulated defense genes (7, 8). However, the associated molecular mechanism(s) by which NO orchestrates these diverse cellular responses remains to be determined.

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 (9). Recently, a number of S-nitrosylated proteins have been identified in Arabidopsis (10, 13) and this redox-based post-translational modification shown to regulate the function of a small number of these plant proteins in vitro (10–12). However, a potential endogenous role for S-nitrosylation in the regulation of protein function remains to be demonstrated.

Emerging data suggests that SNO turnover may constitute a key mechanism to control cellular SNO levels. The addition of an NO moiety to the antioxidant tripeptide glutathione forms S-nitrosoglutathione (GSNO), which may function as a mobile reservoir of NO bioactivity. An enzyme has recently been uncovered that metabolizes this molecule, governing cellular SNO levels (14). Whereas this GSNO reductase (GSNOR) activity is highly specific for GSNO, it controls the cellular levels of both GSNO and SNO proteins in yeast and mice. Mutations in an Arabidopsis thaliana GSNOR (AtGSNOR1) impact cellular SNO homeostasis and disable multiple modes of plant disease resistance (15), consistent with a central role for SNOs in the regulation of the plant defense response (16).

Here we show that S-nitrosylation of A. thaliana salicylic acid-binding protein 3 (AtSABP3) at cysteine (Cys) 280, during the establishment of plant disease resistance, suppresses both binding of the immune activator, salicylic acid (SA), and the carbonic anhydrase (CA) activity of this protein. Our data suggests that inhibition of AtSABP3 CA function by S-nitrosylation could contribute to a negative feedback loop that modulates the plant defense response. These findings provide a molecular link between the accumulation of NO during

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7 The abbreviations used are: NO, nitric oxide; SNO, S-nitrosothiol; AtSABP3, A. thaliana SA-binding protein 3; LC/MS-MS, liquid chromatography/mass spectrometry/mass spectrometry; T-DNA, transferred DNA; SA, salicylic acid; CA, carbonic anhydrase; GSNO, S-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase.
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attempted pathogen infection and the expression of plant disease resistance.

EXPERIMENTAL PROCEDURES

Detection of Protein S-Nitrosylation—PstDC3000(avrB) challenged Arabidopsis leaf tissue was frozen in liquid nitrogen at the stated times post-inoculation. Samples were ground to a fine powder under liquid nitrogen and proteins extracted as previously described (10). Protein concentrations were determined according to Bradford (34), with bovine serum albumin as standard. The biotinylation of S-nitrosylated proteins was undertaken essentially as described (17). Biotin-labeled proteins were purified by incubation with streptavidin-agarose and bound proteins eluted with elution buffer (20 mM Hepes, pH 7.7, 100 mM NaCl, 1 mM EDTA and 100 mM 2-β mercaptoethanol). Proteins were resolved by SDS-PAGE (35) and the resulting gels stained with either Coomassie Blue or silver staining technique (17). Quantification of endogenous levels of SNO-AtSABP3 during the development of disease resistance was determined as described previously (36). PstDC3000(avrB) challenged cells were lysed and subject to immunoprecipitation using an antibody against AtSABP3. Subsequently, the level of SNO formation was determined using the well established 2,3-diaminonaphthalene assay (37), although we found this was not as sensitive as the biotin switch procedure.

Analysis by LC-MS/MS—Liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) experiments were carried out on a Thermo LTQ linear trap instrument equipped with a Thermo microelectrospray source and a Thermo Surveyor pump and autosample device (Thermo Electron Corporation, San Jose, CA). LC/MS/MS analyses were undertaken by an in-house reverse phase chromatography on an 11-cm fused silica capillary column (100 μm inner diameter) packed with sunchrom 3 C18. The peptides were sequentially eluted from the high pressure liquid chromatography column with a gradient of 0 to 100% of buffer B (acetonitrile:water:acetic acid, 80:9.9:0.1) in buffer A (acetonitrile:water:acetic acid, 5:94.9:5.0:1) at a post-split flow rate of 100 nl/min. The electrospray source parameters were 2.25 KV of electrospray voltage, and 5 V of capillary voltage, the range of m/z was from 400 to 2000, normalized collision energy of MS/MS was 35%. The data were analyzed with Bioworks and Qual Browser.

Production of Recombinant AtSABP3 and Antibody Generation—The AtSABP3 RIKEN cDNA clone pda04430 (At3g01500) was cloned into Ncol and Xhol sites of pET32a (Merck Biosciences) and expressed in Escherichia coli (BL21). Recombinant AtSABP3 was affinity purified through nickel-nitrilotriacetic acid-agarose (Merck Biosciences) and used for antibody production.

Site-directed Mutagenesis and Analysis of Protein Structure—Site-directed mutagenesis of AtSABP3 was undertaken using QuikChange® II Site-directed Mutagenesis Kits (Stratagene Corporate). Plasmid pET32a-AtSABP3 was used as template and primers were designed utilizing the tools from Stratagene and synthesized by Sigma Genosys. The primers were: forward primer: C280S, 5’TCAATGTGGCCGAGTGAAGGGAGGCAGG-3’, reverse primer C280S, 5’TGGCTTCCGTCGTACATTCG-3’. Mutations were confirmed by sequencing. The structural model of AtSABP3 was generated using SWISS-MODEL (38) with the structure of pea CA as a template (Protein Data Bank code 1ekj).

SA Binding and CA Activity Assays—Standard SA-binding reactions were carried out as described previously (20). The reaction with 300 nm [14C]-labeled SA (20.5 Ci mmol; 1 Ci = 37 GBq; New England Biolabs) with or without unlabeled SA (10,000-fold molar excess) was carried out in 100 μl of assay buffer (30 mM sodium citrate, pH 6.3, 1 mM EDTA) for 1 h on ice. Unbound ligand was removed with a 1-ml Bio-Spin 6 (BioRad) column by centrifugation for 4 min at 1,000 × g. Bound [14C]SA was quantified by scintillation counting using 50 μl of filtrate. CA enzymatic activity assays of recombinant AtSABP3 was performed and recorded essentially as reported earlier (39) by using 20 mM Tris-HCl (pH 8.3) as buffer. Chloroplasts were isolated as previously described (20).

Plant Material and Pathogen Inoculations—Arabidopsis accession Col-0 and mutants derived from it were grown under 16 h of light at 22 °C and 8 h of darkness at 18 °C. Arabidopsis lines possessing a T-DNA insertion into AtSABP3 were obtained from the Salk T-DNA insertion collection (25), distributed by the Nottingham Arabidopsis Stock Center, UK. The atsabp3-1 line was complemented with a wild-type copy of AtSABP3 and also transformed with the C260S AtSABP3 mutant. PstDC3000 strains were grown, maintained, and utilized as described (40). To monitor bacterial growth in planta given Arabidopsis lines were inoculated with avirulent PstDC3000(avrB) at 105 colony forming units ml–1.

RESULTS

AtSABP3 Is S-Nitrosylated in Vivo and in Vitro—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 (17). Pseudomonas syringae pv. tomato (Pst) DC3000 expressing the avirulence gene, avrB (18), is recognized by the R protein RPM1 (18, 19), triggering the expression of race-specific disease resistance in Arabidopsis accession, Col-0. Challenge of this plant line with avirulent PstDC3000(avrB) modified the S-nitrosylation status of a series of proteins including A. thaliana SA-binding protein 3 (AtSABP3; At3g01500) (20). We had previously established that SNO formation and turnover regulates both the biosynthesis of and signaling by the plant immune activator, SA, impacting multiple modes of plant disease resistance (15). A change in the S-nitrosylation status of
AtSABP3, a homolog of one of a small number of SA-binding proteins (20–22), therefore, appeared to be of particular significance, although the function of SA binding is unknown. AtSABP3 also exhibits chloroplastic CA activity (20), 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 biotin switch technique. Ponceau staining is a control for protein loading. Amount of AtSABP3 was determined by immunoblot analysis of corresponding protein extracts in the absence of the biotin switch technique. Ponceau staining is a control for protein loading.

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 immunoprecipitated at various times post PstDC3000 (avrB) challenge and the extent of S-nitrosylation determined using the 2,3-diaminonaphthalene assay. This analysis suggested that SNO-AtSABP3 formation increased over time during the establishment of disease resistance, peaking at 8 h post-inoculation of PstDC3000 (avrB) (Fig. 1D). Also, analysis using the 2,3-diaminonaphthalene assay did not appear to be as sensitive for determining the SNO status of AtSABP3 as the biotin switch procedure utilized in Fig. 1C. However, we did not generate the biotin switch data to make a direct comparison.

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 typically used in tests for in vitro S-nitrosylation (10–13) and SNO-AtSABP3 formation was monitored using the 2,3-diaminonaphthalene 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 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 in vitro.

Identification of Site of AtSABP3 S-Nitrosylation—To identify the target site(s) of S-nitrosylation we carried out LC-MS/MS analysis of GSNO-treated AtSABP3. This tentatively identified Cys$^{220}$ 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. 2, A and B). This approach robustly identified Cys$^{220}$ as the sole site of S-nitrosylation, confirming our previous LC-MS/MS analysis. Furthermore, Cys$^{220}$ is embedded within a canonical acid-base
motif (23), 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 (24), we utilized this structure as a template to model the hypothetical three-dimensional conformation of the Arabidopsis AtSABP3 (Fig. 2C), to provide us with structural insight into the spatial disposition of Cys280. 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. 2D).

**S-Nitrosylation Impacts AtSABP3 Carbonic Anhydrase Activity**—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 dithiothreitol. Also, an alternative NO donor, S-nitroso-N-acetylpenicillamine, strongly reduced SABP3 CA activity, confirming this effect was NO-dependent (Fig. 3B). Furthermore, this inhibition could be reversed in the presence of dithiothreitol. Together, these results imply that NO through S-nitrosylation of AtSABP3 may function to negatively regulate its CA activity. These findings prompted us to examine chloroplastic CA activity during the defense response. At 8 h post-inoculation of *Pst* DC3000(*avrB*), the time at which we found AtSABP3 to exhibit maximum S-nitrosylation, CA activity was significantly reduced (Fig. 3C). Thus, chloroplastic CA activity is modulated during the establishment of plant disease resistance. Next we determined this activity in *atgsnor1-1* and *atgsnor1-3* plants (15). The reduction of CA activity was decreased in *atgsnor1-1* plants but increased in the *atgsnor1-3* mutant line (Fig. 3C). Collectively, our findings provide a direct molecular link between S-nitrosylation of AtSABP3 and CA activity. However, it is possible that other mechanisms may also contribute to the regulation of AtSABP3 activity.

To investigate the potential role of Cys280 in AtSABP3 function, we carried out site-directed mutagenesis to replace this
We next explored if CA activity, suggesting these functions are independent (20). Identified and the binding of this small molecule does not affect amino acid residues required for SA binding have not yet been negatively regulates the CA activity of AtSABP3. To monitor the impact of was incubated with 1 mM GSNO prior to the determination of sylation of AtSABP3 significantly decreased SA binding. We responding recombinant protein in residue with serine (Ser) and subsequently synthesized the cor-

FIGURE 3. The CA activity of AtSABP3 is negatively regulated by S-nitrosylation of Cys280. A, recombinant AtSABP3 was incubated with the given concentrations of GSNO, GSH, and dithiothreitol (DTT) and the CA activity of this protein determined. B, the CA activity of recombinant AtSABP3 was determined following exposure to the NO donor S-nitroso-N-acetylpenicillamine (SNAP). C, relative chloroplastic CA activity measured mulated with an antibody against biotin. Equal protein loading of individual samples was determined by immunoblotting with an antibody against AtSABP3. E, the CA activity of recombinant wild-type AtSABP3 and the C280S mutant derivative were determined as described above. For A, B, and E, error bars represent 95% confidence limits. These experiments were repeated at least twice with similar results.

residue with serine (Ser) and subsequently synthesized the corresponding recombinant protein in E. coli. As expected, C280S AtSABP3 could not be S-nitrosylated (Fig. 3D), further confirming Cys280 as the site of S-nitrosylation. We then assessed the CA activity associated with this protein. The C280S derivative 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 Cys280 in CA activity (24). Nevertheless, our data imply that structural changes at this position may compromise CA activity, consistent with our findings that S-nitrosylation at Cys280 negatively regulates the CA activity of AtSABP3.

Role of AtSABP3 in Disease Resistance—AtSABP3 has previously been shown to bind SA with high affinity (20). 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 (20). We next explored if S-nitrosylation of Cys280 could also modulate the CA binding activity of AtSABP3. This activity was assessed in the presence of [14C]SA with or without excess unlabeled SA. To monitor the impact of S-nitrosylation, AtSABP3 was incubated with 1 mM GSNO prior to the determination of [14C]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 [14C]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 [14C]SA binding. Collectively, these data imply that S-nitrosylation of Cys280 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 (25). The atsabp3-1 and atsabp3-2 T-DNA lines were challenged with PstDC3000 (avrB) and pathogen growth monitored over time. Both atsabp3 alleles supported more PstDC3000 (avrB) growth compared with wild-type (Fig. 4C). Thus, AtSABP3 is required for a full defense response against this bacteri-
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) (15). In contrast, atgsnor1-1 plants show decreased levels of SNO-AtSABP3, increased CA activity, accelerated defense responses, and display resistance against ordinarily virulent pathogens (15). 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 (31).

Although the exogenous addition of numerous pharmacological agents has suggested NO regulates a wide variety of responses (5–7) 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 (9, 23). 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 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 (32). 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 R gene-mediated disease resistance (11). 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 (9, 33).

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