Running Head: Nitrosoproteomic analysis of S-nitrosylated proteins

Corresponding author: Jianru Zuo

Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

Tel: (+8610)6480 6585
Fax: (+8610)6480 6595
E-mail: jrzuo@genetics.ac.cn

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Site-specific Nitrosoproteomic Identification of Endogenously

S-Nitrosylated Proteins in Arabidopsis

Jiliang Hu, Xiahe Huang, Lichao Chen, Xuwu Sun, Congming Lu, Lixin Zhang, Yingchun
Wang and Jianru Zuo*

State Key Laboratory of Plant Genomics and National Plant Gene Research Center,
Institute of Genetics and Developmental Biology, Chinese Academy of Science, Beijing
100101, China (J.H., L.C. and J.Z.); Graduate University of Chinese Academy of Sciences,
Beijing 100049, China (J.H. and L.C.); State Key Laboratory of Molecular Developmental
Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences,
Beijing 100101, China (X.H. and Y.W.); Institute of Botany, Chinese Academy of Sciences,
Beijing 100093, China (X.S., C.L. and L.Z.)

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*Corresponding author e-mail: jrzuo@genetics.ac.cn.

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ABSTRACT

Nitric oxide (NO) regulates multiple developmental events and stress responses in plants. A major biologically active species of NO is S-nitrosoglutathione (GSNO) that is irreversibly degraded by GSNO reductase (GSNOR). The major physiological effect of NO is protein S-nitrosylation, a redox-based posttranslational modification mechanism by covalently linking a NO molecule to a cysteine thiol. However, little is known about the mechanisms of S-nitrosylation-regulated signaling, partly due to limited S-nitrosylated proteins being identified. In this study, we identified 1,195 endogenously S-nitrosylated peptides in 926 proteins from the Arabidopsis (Arabidopsis thaliana) by a site-specific nitrosoproteomic approach, which, up to date, is the largest dataset of S-nitrosylated proteins among all organisms. Consensus sequence analysis of these peptides identified several motifs that contain acidic, but not basic, amino acid residues flanking the S-nitrosylated cysteine residues. These S-nitrosylated proteins are involved in a wide range of biological processes and are significantly enriched in chlorophyll metabolism, photosynthesis, carbohydrate metabolism, and stress responses. Consistently, the gsnor1-3 mutant shows the decreased chlorophyll content and altered photosynthetic properties, suggesting that S-nitrosylation is an important regulatory mechanism in these processes. These results have provided valuable resources and new clues to the studies on S-nitrosylation-regulated signaling in plants.

Key words: nitric oxide, S-nitrosylation, S-nitrosoproteome, chlorophyll metabolism, photosynthesis
INTRODUCTION

Nitric oxide (NO), a gaseous signaling molecule, plays important regulatory roles in higher plants, including seed dormancy and germination, root development and hypocotyl elongation, floral transition, senescence and cell death, phytohormone signaling and responses to abiotic and biotic stresses (He et al., 2004; Besson-Bard et al., 2008; Hong et al., 2008; Neill et al., 2008; Leitner et al., 2009; Feng et al., 2013). S-nitrosoglutathione (GSNO) is a major biologically active form of reactive nitrogen species (RNS), and functions as a primary NO donor. The endogenous GSNO homeostasis is highly dynamic, and the GSNO level is negatively regulated by GSNO reductase (GSNOR), an evolutionally conserved enzyme catalyzing irreversibly degrading GSNO (Liu et al., 2001). Mutations in the GSNOR gene cause the elevated GSNO level and consequently severe abnormalities under physiological and pathological conditions in various species (Liu et al., 2004; Feechan et al., 2005; Que et al., 2005; Lee et al., 2008; Chen et al., 2009; Moore et al., 2009; Kwon et al., 2012).

In Arabidopsis (Arabidopsis thaliana), GSNOR1 is a single copy gene and the enzymatic activity of the encoded protein has been biochemically characterized (Sakamoto et al., 2002). Genetic studies revealed that the gsnor1-1 and gsnor1-2 mutants are gain-of-function mutations with the increased GSNO reductase activity and decreased cellular S-nitrosothiol (SNO) level. Conversely, gsnor1-3 is a loss-of-function mutant with a significantly increased S-nitrosothiol level (Feechan et al., 2005). The defense responses mediated by distinct resistance (R) genes are significantly impaired in the gsnor1-3 mutant, and GSNOR1 functions as a positive regulator of salicylic acid-regulated signaling network in the defense response (Feechan et al., 2005). In a genetic screen for thermotolerance-defective mutants, the sensitive to hot temperatures 5 (hot5) mutant was characterized to have decreased heat-acclimation and hot5 was shown to be allelic to gsnor1, indicating the importance of GSNOR1-regulated NO homeostasis in the regulation
of the abiotic stress response (Lee et al., 2008). In an independent genetic screen for the
oxidative stress-related mutants, the paraquat resistant 2 (par2) mutant was also identified
to be allelic to gsnor1, which showed an anti-cell death phenotype and multiple
developmental defects, revealing the critical role of GSNOR1/HOT5/PAR2 in the regulation
of oxidative stress-induced cell death (Chen et al., 2009). Similar to gsnor1-3, the hot5 and
par2 allelic mutants also accumulate the significantly increased level of NO. As a result of
this defect, these gsnor1/hot5/par2 mutants show a pleiotropic phenotype, with severe
developmental abnormalities in both reproductive and vegetative stages (Lee et al., 2008;
Chen et al., 2009; Kwon et al., 2012). These studies highlight the critical role of
GSNOR1/HOT5/PAR2-modulated NO homeostasis in diverse physiological processes,
including in plant growth and development as well as in responses to both biotic and abiotic
stresses. However, little is known about the underpinning molecular mechanisms of the
NO-modulated signaling in various physiological processes.

A major physiological effect of NO is executed by protein S-nitrosylation, a reversible
posttranslational modification by covalent addition of a NO molecule onto a cysteine thiol
to form S-nitrosothiol (SNO) (Jaffrey et al., 2001; Stamler et al., 2001). S-nitrosothiols are
dynamically labile in response to the intracellular redox status, allowing protein
S-nitrosylation as a highly sensitive mechanism in the regulation of cellular signaling
(Stamler et al., 2001; Hess et al., 2005). Emerging evidence indicates that S-nitrosylation
regulates the function of the modified proteins by various mechanisms, including the
enzymatic activity, stability, subcellular localization, three-dimensional conformation
changes, protein-protein interaction and ligand binding (Hess et al., 2005; Wang et al., 2006;
Astier et al., 2011; Gupta, 2011; Hess and Stamler, 2012). In Arabidopsis, S-nitrosylation
has been shown as an important mechanism in regulating the stress responses. The activity
of methionine adenosyltransferase 1 (MAT1), which catalyzes S-adenosylmethionine
synthesis, was shown to be inhibited by S-nitrosylation (Lindermayr et al., 2006).
S-nitrosylation negatively regulates the activity of a peroxynitrite detoxification enzyme
peroxiredoxin II E (PrxII E) and an NADPH oxidase, thereby modulating the oxidative
stress in the defense response (Romero-Puertas et al., 2007; Yun et al., 2011). Moreover,
S-nitrosylation has also been shown to regulate the conformational changes of NPR1
(NONEXPRESSER OF PR GENES 1), a master regulator of the defense response, and the
activity of SABP3 (SALICYLIC ACID BINDING PROTEIN 3), a key enzyme for
salicylic acid biosynthesis (Tada et al., 2008; Wang et al., 2009). In addition,
S-nitrosylation of TIR1 and AHP1, two key signaling components of the auxin and
cytokinin pathways, respectively, plays an important role in regulating respective
phytohormone signaling (Terrile et al., 2012; Feng et al., 2013). These studies illustrate the
importance of S-nitrosylation in the regulation of diverse physiological processes in plants.

S-nitrosylation has been considered as one of the most important posttranslational
modification mechanisms (Lane et al., 2001; Stamler et al., 2001; Hess et al., 2005). A
growing number of S-nitrosylated proteins have been identified using the proteomic
approach. Up to date, the S-nitrosoproteomic studies have identified more than 2,200
S-nitrosylated proteins, covering more than 4,100 S-nitrosylated cysteine residues. Of those
S-nitrosylated proteins, more than 95% were identified from mammals (Lee et al., 2012).
Several proteomic studies in Arabidopsis identified a number of S-nitrosylated proteins
(Lindermayr et al., 2005; Romero-Puertas et al., 2008; Palmieri et al., 2010; Fares et al.,
2011; Puyaubert et al., 2014). In GSNO-treated cell suspension cultures and NO-treated
leaves derived from Arabidopsis, 63 and 52 S-nitrosylated proteins were identified, which
are involved in the stress responses, redox-related, cytoskeleton, metabolic proteins and
cellular signaling (Lindermayr et al., 2005). In an independent study, 16 S-nitrosylated
proteins were identified from Arabidopsis seedlings undergoing the hypersensitive response
(HR) (Romero-Puertas et al., 2008). In another independent analysis, 46 S-nitrosylated
proteins were identified from cultured Arabidopsis suspension cells (Fares et al., 2011). In a
more specific analysis, 11 mitochondria proteins were identified to be S-nitrosylated and/or
glutathionylated (Palmieri et al., 2010). More recently, 62 endogenously S-nitrosylated
proteins were identified from Arabidopsis seedlings (Puyaubert et al., 2014). Notably, a large number of the S-nitrosylated proteins are repeatedly identified in these analyses, thus confirming the validation of each study. Because of the labile nature of S-nitrosylation, most of the S-nitrosoproteomic studies used the protein samples treated with NO donors or the protein extracts prepared from NO donor-treated cells or tissues. The Arabidopsis gsnor1-3 mutants accumulate excessive amount of NO (Feechan et al., 2005; Lee et al., 2008; Chen et al., 2009), and the identification of S-nitrosylated proteins in gsnor1-3 should depict a more comprehensive map of S-nitrosoproteome in Arabidopsis, and also provide important clues on the molecular basis of the pleiotropic phenotype of the mutant.

Because of the labile and dynamic nature of protein S-nitrosylation, large-scale identification of endogenously S-nitrosylated proteins remains technically challenging. At present, two major methods for identification of S-nitrosoproteome are shotgun and site-specific nitrosoproteomic analysis, both of which are based on biotin-switch method and mass spectrometry (Jaffrey et al., 2001; Hao et al., 2006; Torta et al., 2008). In shotgun analysis, S-nitrosylated proteins were first biotinylated, enriched by affinity-chromatography and then identified by mass spectrometry. Although the method is relatively simple, the number of S-nitrosylated proteins identified by shotgun proteomics is often few due to various technical limitations (Torta et al., 2008). The identification capacity of nitrosoproteomics was greatly improved by the site-specific strategy, in which biotinylated proteins were first digested by trypsin and the enriched peptides were then characterized by mass spectrometry (Hao et al., 2006; Chen et al., 2010). Moreover, S-nitrosylated cysteine residues can also be identified from site-specific nitrosoproteomic analysis.

In this study, we performed a large-scale site-specific proteomic analysis of endogenously S-nitrosylated proteins in Arabidopsis wild type and gsnor1-3 seedlings, and have identified 1,195 endogenously S-nitrosylated peptides in 926 proteins from the model plant species, representing the largest dataset thus far reported in any organisms and
providing important resources for future studies on S-nitrosylation-regulated signaling in plants.
RESULTS AND DISCUSSION

Identification of S-nitrosylated proteins in gsnor1-3 by shotgun proteomics

GSNOR1 is a key enzyme that irreversibly degrades GSNO, and mutations in the highly conserved GSNOR1 gene (gsnor1-3) cause a remarkably increased level of NO in Arabidopsis (Feechan et al., 2005; Lee et al., 2008; Chen et al., 2009). Because the major biological activity of NO is protein S-nitrosylation, we reasoned that the increased RNS level should cause a greater degree of S-nitrosylation of the proteome. To test this hypothesis, we performed a biotin-switch assay to analyze the level of S-nitrosylated proteins in wild type (Col-0) and gsnor1-3 (in the Col-0 background) seedlings. Total proteins prepared from leaves of Col-0 and gsnor1-3 seedlings were subjected to the biotin-switch assay as described (Jaffrey and Snyder, 2001). The biotinylated proteins, in which the S-nitrosylated cysteine residue of a protein was labeled by a biotin, were then analyzed by immunoblotting using an anti-biotin antibody. We found that a higher level of S-nitrosylated proteins was detected in gsnor1-3 than that in Col-0 seedlings (see Supplemental Fig. S1 online), suggesting that the increased RNS level in gsnor1-3 causes a greater degree of protein S-nitrosylation.

To characterize S-nitrosylated proteins, we first performed a pilot experiment by using the shotgun proteomic approach to identify biotinylated proteins enriched and purified from gsnor1-3 seedlings by the biotin-switch methods. Total proteins were prepared from 15-day-old gsnor1-3 seedlings and then subjected to the biotin-switch assay (see Supplemental Fig. S2A online). The biotinylated proteins were enriched and purified by affinity purification using streptavidin agarose beads and then separated by SDS-PAGE (see Supplemental Fig. S2B online). The sliced gels were digested with trypsin and the digested peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a linear ion trap quadrupole (LTQ) mass spectrometer. The collected data were used for the search in the NCBI Arabidopsis Protein Database (see Supplemental
Proteins with more than two matched unique peptides ($P < 0.05$) were reported as acceptable identifications. In two independent experiments, we have identified 306 putative S-nitrosylated proteins (see Supplemental Table S1 online). In several previous S-nitrosoproteomic studies, 169 putative S-nitrosylated proteins were identified from Arabidopsis under various experimental conditions (Lindermayr et al., 2005; Romero-Puertas et al., 2008; Palmieri et al., 2010; Fares et al., 2011; Puyaubert et al., 2014). Of those, 27.78% (85/306) unique S-nitrosylated proteins identified in this study were reported in previous S-nitrosoproteomic studies (see Supplemental Fig. S2C and Supplemental Table S1 online).

In particular, three previously characterized S-nitrosylated proteins, SABP3 (also known as carbonic anhydrase1, CA1) (Wang et al., 2009), fructose 1,6-bisphosphate aldolase 6 (FBA6) (van der Linde et al., 2011) and ascorbate peroxidase 1 (APX1) (Correa-Aragunde et al., 2013) were identified in our S-nitrosoproteomic analysis (see Supplemental Table S1 online). S-nitrosylation of SABP3 suppresses its binding with salicylic acid and the enzymatic activity during HR, whereas S-nitrosylation of FBA6 negatively regulates its enzymatic activity (Wang et al., 2009; van der Linde et al., 2011).

**Site-specific identification of S-nitrosylated proteins in Col-0 and gsnor1-3**

An advantage of the shotgun proteomic method is relatively less tedious. However, the method can only detect proteins containing potentially S-nitrosylated cysteine residues, but is incapable of precisely identifying the S-nitrosylated cysteine residues in a target protein. To more precisely identify S-nitrosylated proteins and their modified cysteine residues, we used a site-specific proteomics approach to characterize S-nitrosylated peptides. In this method, the biotinylated proteins were first subjected to tryptic digestion before biotin-affinity purification, and the biotinylated peptides were then affinity-purified for further analysis (Hao et al., 2006; Chen et al., 2010). By using this method, we analyzed and identified S-nitrosylated peptides from Col-0 and gsnor1-3 seedlings. Total proteins from...
15-day-old seedlings were prepared for the biotin-switch assay with minor modifications (see MATERIALS and METHODS) and the biotinylated proteins were subjected to in-solution tryptic digestion. Biotinylated peptides were enriched and purified by affinity.
purification using streptavidin agarose beads and then analyzed by LC-MS/MS using an LTQ Orbitrap Elite mass spectrometer (Fig. 1A). A false discovery rate (FDR) of < 0.01 was applied as the criteria for S-nitrosylated peptides identification. To improve the reliability, peptide samples of the negative controls (without treatment of sodium ascorbate) were also analyzed by LC-MS/MS and biotinylated peptides identified in the negative controls were excluded from the dataset. In four independent experiments, we have identified 3,726 biotinylated peptides and 1,137 non-biotinylated peptides in the samples treated with sodium ascorbate (see Supplemental Tables S2 and S3 online), and the non-biotinylated peptides was 23.4% of total peptides identified in four experiments. In all four experiments, various numbers of peptides were identified from the negative controls (not treated by sodium ascorbate; see Supplemental Tables S2 and S4 online), which were excluded from the final dataset. By excluding the peptides with hits in the negative controls, 865 and 938 unique peptides were identified in Col-0 and gsnor1-3, respectively, and these peptides were mapped to 697 endogenously S-nitrosylated proteins in Col-0 and 765 proteins in the gsnor1-3 mutant, respectively (Fig. 1B and 1C). Consistent with higher levels of NO and S-nitrosylated proteins in gsnor1-3 (see Supplemental Fig. S1 online), more S-nitrosylated peptides were identified from gsnor1-3 than that from Col-0. Combined these two sets of data together, 1,195 S-nitrosocysteine-containing peptides in 926 endogenously S-nitrosylated proteins were identified in this study (Fig. 1B and 1C; see Supplemental Tables S2, S5, S6 and S7 online). Up to date, 1,011 S-nitrosylated peptides in 647 proteins identified from various mouse tissues was the largest reported dataset of S-nitrosylated proteins (Doulias et al., 2013). The 1,195 S-nitrosylated peptides identified in Arabidopsis in this study represent the largest dataset of S-nitrosoproteome among all organisms thus far characterized. Among these S-nitrosylated peptides, 608 targets were identified in both Col-0 and gsnor1-3, covering 70.3% and 64.8% of S-nitrosylated peptides in Col-0 and gsnor1-3, respectively (Fig. 1B and Supplemental Table S5 online). Among these S-nitrosylated
proteins identified by this site-specific proteomics, 536 proteins were identified both in
Col-0 and gsnor1-3 mutant, covering 76.9% and 70.1% of S-nitrosylated proteins in Col-0
and gsnor1-3, respectively (Fig. 1C and Supplemental Tables S5, S6 and S7 online).

Notably, 161 and 229 S-nitrosylated proteins specific to Col-0 and gsnor1-3,
respectively, were identified by the site-specific nitrosoproteomic analysis (see
Supplemental Tables S8 and S9 online), suggesting that the gsnor1-3 mutation has a
profound effect on the nitrosoproteome. It is reasonable to presume that the increased
number of S-nitrosylated proteins in gsnor1-3 is caused by an elevated NO level in the
mutant. However, the identification of the relatively large number of S-nitrosylated proteins
specific to Col-0, which contains significantly lower NO level compared to gsnor1-3, is
somewhat unexpected (161 S-nitrosylated proteins; see Supplemental Table S8 online),
since the elevated intracellular NO level has been considered as a key for S-nitrosylation of
target proteins (Lane et al., 2001; Stamler et al., 2001; Hess et al., 2005). This observation
suggests that the specificity of S-nitrosylation is regulated by more complex mechanisms
than previously recognized, in which the intracellular NO level may not positively regulate
S-nitrosylation in some cases.

We found that 169 S-nitrosylated proteins identified by our shotgun proteomic
analysis were also identified by the site-specific analysis, covering 55.2% (169/306) of 306
S-nitrosylated proteins identified by the shotgun analysis (Fig. 1D; see also Supplemental
Tables S1 and S5 online), strongly supporting the validation of the results obtained by the
shotgun proteomic analysis. Comparing with these previous S-nitrosoproteomic studies in
Arabidopsis (Lindermayr et al., 2005; Romero-Puertas et al., 2008; Palmieri et al., 2010;
Fares et al., 2011; Puyaubert et al., 2014), we found that 42.6% (72/169) of those
previously reported S-nitrosylated protein were also identified in our site-specific analysis,
including the above-mentioned SABP3, FBA6, and APX1 (see Supplemental Table S5
online). For example, Cys173 of FBA6 was previously characterized as S-nitrosylation site
(van der Linde et al., 2011), and this site was also identified by our site-specific proteomic
analysis (Fig. 1E and Supplemental Table S5 online). In summary, 7.9% (73/926) of S-nitrosylated proteins identified in this site-specific analysis were reported in previous proteomic or functional studies (Fig. 1F and Supplemental Table S5 online).

We noticed that several well characterized S-nitrosylated proteins, including MAT1, PrxII E, NPR1, NADPH oxidase, TIR1 and AHP1 (Lindermayr et al., 2006; Romero-Puertas et al., 2007; Tada et al., 2008; Yun et al., 2011; Terrile et al., 2012; Feng et al., 2013), were not detected in our S-nitrosoproteomic analysis. This was possibly caused by the relatively low abundance of these proteins, the detection limits of our study and different conditions used in those studies.

Identification of the consensus sequences of S-nitrosylated peptides
Previous studies suggested that consensus sequence of S-nitrosylated cysteine residues are flanked by acidic and basic amino acid residues to form an acid-base motif that is susceptible to protein S-nitrosylation (Stamler et al., 1997; Greco et al., 2006; Seth and Stamler, 2011). Due to the limited number of the S-nitrosylated peptides being identified, the consensus sequences for protein S-nitrosylation remains poorly defined. To get more insights into the possible underlying determinants of protein S-nitrosylation, we analyzed the characteristics of flanking sequences of all 1,195 S-nitrosylated cysteine residues identified in this site-specific proteomic analysis using Motif-X algorithm (Schwartz and Gygi, 2005).

All identified 1,195 S-nitrosylated peptides were subjected to consensus sequence analysis. We found that the sequence of EXC (where X indicates any amino acid residues) was a putative consensus sequence with the highest statistical significance ($P < 1 \times 10^{-12}$), which matched more than 140 S-nitrosylated peptides identified in this study (see Fig. 2A and Supplemental Table S5 online). Other identified consensus sequences includes EC, CD, CE, CXXE, CXD, CXE, DXXC, DC, and EXXXC (Fig. 2A and Supplemental Table S5 online). Among these putative S-nitrosylation motifs, the probability of the presence of E was significantly higher than that of D. Consistent with these predictions, these putative motifs
were found in the S-nitrosylated proteins identified in previous studies. For example, Rubisco (ribulose bisphosphate carboxylase/oxygenase) activase (RCA), which was identified as a S-nitrosylated protein by several S-nitrosoproteomic studies (Lindermayr et al.,...
S-nitrosylated at Cys<sup>451</sup> flanking by two acidic E/D residues in this study, which matched both the EXC and CXD motifs (Fig. 2B and Supplemental Table S5 online). Similarly, several previously reported S-nitrosylated proteins were also identified in this study to be S-nitrosylated at the EXC motif (Fig. 2C and Supplemental Table S5 online).

Notably, in all the predicted S-nitrosylation motifs with P < 1 X 10<sup>-4</sup>, acidic amino acid residues were found to be present in positions from -4 to +3 and preferably in positions from -2 to +1 (Fig. 2A). However, basic amino acid residues did not appear to be essential for the S-nitrosylation motif as previously proposed (Stamler et al., 1997; Greco et al., 2006; Seth and Stamler, 2011). The deduced S-nitrosylation motifs in this study are based on a larger dataset than previous studies, which should be statistically more reliable. Therefore, we propose that the maintenance of a local acidic environment may play an important role for S-nitrosylation of a target cysteine residue.

**Functional classification of S-nitrosylated proteins in Arabidopsis**

To explore the regulatory roles of S-nitrosylation in specific biological processes, we performed a Gene Ontology (GO) analysis to functionally classify these S-nitrosylated proteins identified by site-specific proteomics using agriGO software (Du et al., 2010). S-nitrosylated proteins in Col-0 (697 proteins), gsnor1-3 mutant (765 proteins), and the combined dataset (926 proteins) were subjected to GO biological process analysis separately and similar distributions were observed from these three groups (Fig. 3A). We found that these 926 S-nitrosylated proteins covered a wide range of GO biological processes, including metabolism (28.96%), abiotic and biotic stresses (16.99%), cell organization (5.26%), development (5.10%), transport (2.99%), energy process (1.63%), signaling transduction (1.73%), transcription (0.95%), and other processes (36.40%) (Fig. 3A). GO biological process analysis of S-nitrosylated proteins identified by shotgun proteomics also showed a similar pattern (see Supplemental Fig. S2D online).
Among these S-nitrosylated proteins annotated with known functions, the most enriched category was related to various metabolic processes in both experiments shotgun (25.87%) and site-specific proteomics (28.96%) (Fig. 3A and Supplemental Fig. S2D).
online). This is possibly due to the relatively high abundance of these proteins and also implies that the basal metabolism is actively regulated by S-nitrosylation (see also below). Moreover, a large number of proteins involved in the stress responses were identified to be S-nitrosylated. Among these proteins, several reactive oxygen species (ROS) scavenging enzymes, including three catalases (CAT1, 2, 3), an ascorbate peroxidase (APX1), two dehydroascorbate reductases (DHAR1 and 2), monodehydroascorbate reductase 6 (MDAR6) and superoxide dismutase 2 (SOD2) were identified as S-nitrosylated proteins (see Supplemental Tables S1 and S5 online), supporting the notion of active interactions between ROS- and RNS-mediated signaling pathways. Multiple proteins involved in regulating the cellular redox status and NO metabolism are also identified to be S-nitrosylated, including SAHH2 (S-adenosyl-l-homocysteine hydrolase 2), two nitrilases (NIT1 and 2), nitrite reductase 1 (NIR1), γ-glutamylcysteine synthetase (GSH1) and glutathione synthetase 2 (GSH2) (see Supplemental Tables S1 and S5 online), suggesting the involvement of feedback regulation in these processes. Remarkably, multiple mutant alleles in GSH1, which encodes a rate-limiting enzyme in GSH (a precursor of GSNO) biosynthesis, have been identified in various genetic screens, including cad2 (cadmium-sensitive 2), rml1 (root meristemless1), rax1 (regulator of APX2) and pad2 (phytoalexin-deficient 2) (Cobbett et al., 1998; Vernoux et al., 2000; Ball et al., 2004; Parisy et al., 2007), indicating that GSH1 functions in diverse physiological processes, of which S-nitrosylation may play a critical role in regulating its activity. Several proteins involved in regulating the stress responses, defense response and cell death have also been identified as the candidates of S-nitrosylation, including ACCELERATED CELL DEATH 2 (ACD2), MOSAIC DEATH 1 (MOD1), metacaspase 4 (MC4), cyclophilin CYP20-3 (also known as ROC4), eukaryotic translation initiation factor 5A-2 (eIF5A-2/FBR12), protein arginine methyltransferase 5 (PRMT5 or also known as SKB1), MAP kinase 4 (MPK4), glyceraldehyde-3-phosphate dehydrogenase C1 (GAPC1) (Mou et al., 2000; Pružinská et al., 2005; Yao and Greenberg, 2006; Feng et al., 2007; Dominguez-Solis et al., 2008;
Together, the identification of S-nitrosylated proteins involved in these distinctive physiological and developmental processes is consistent with the pleiotropic phenotypes of the gsnor1 mutants (Feechan et al., 2005; Lee et al., 2008; Chen et al., 2009; Kwon et al., 2012).

GO subcellular component analysis revealed that the S-nitrosylated proteins showed a similar distribution patterns in Col-0 and gsnor1-3 as well as in the combined dataset of these two genotypes of plants (Fig. 3B). The combined dataset of 926 S-nitrosylated proteins were significantly enriched in the chloroplast/plastid (26.03%), followed by the membrane (8.33%), cytosol (7.96%), nucleus (7.02%), mitochondria (3.82%), extracellular component (2.55%) and cell wall (1.34%) (Fig. 3B). GO subcellular component analysis of S-nitrosylated proteins identified by shotgun proteomics also revealed a similar distribution pattern (see Supplemental Fig. S2E online). The most abundant fraction was found to be localized in the chloroplast/plastid, suggesting that the physiological activities associated with the photosynthetic organelle are extensively regulated by S-nitrosylation. Whereas photosynthesis is a series of oxidoreduction-based reactions, the utilization of the redox-based S-nitrosylation as a major regulatory means is physiologically relevant. Moreover, because the chloroplast is a major site for basal metabolism of plant cells, this result is also consistent with the analysis by GO biological processes, in which the largest number of the identified S-nitrosylated proteins was related to metabolism (Fig. 3A and Supplemental Fig. S2D; see also below).

The similar distribution patterns between wild type and gsnor1-3 in both biological processes and subcellular localization were somewhat unexpected, considering the pleiotropic phenotype of gsnor1-3. We speculate that most of the identified S-nitrosylated proteins are relatively abundant in plant cells, whereas the relatively low abundance signaling molecules were not detected, such as NPR1, TIR1, and APH1, as highlighted above, which are expected to play more important roles in determining the gsnor1-3
phenotype. In addition, the difference between wild type and gsnor1-3 is more likely reflected by different levels of S-nitrosylation of the target proteins, but rather than the status being nitrosylated or denitrosylated. In agreement with these notions, NPR1, TIR1 and AHP1 all showed altered levels of S-nitrosylation in gsnor1-3 compared to wild type (Tada et al., 2008; Terrile et al., 2012; Feng et al., 2013), and several enzymes in chlorophyll metabolism and photosynthesis also had an increased S-nitrosylation level in gsnor1-3 (see below for details).

Enriched S-nitrosylated proteins in the chlorophyll metabolism pathway

GO categorization of S-nitrosylated proteins revealed that a large number of these proteins are involved in chlorophyll metabolism and the related pathways (see Supplemental Tables S1 and S5). In Arabidopsis, 124 proteins were annotated to be related to chlorophyll metabolism, of which 34 proteins (27.42%) were identified as S-nitrosylated proteins in this nitrosoproteomic analysis. Whereas most of these proteins are annotated as related to chlorophyll metabolism with unknown biochemical functions, 14 S-nitrosylated proteins were found to be enzymes directly or indirectly involved in the chlorophyll metabolic pathway (Fig. 4 and Supplemental Tables S1 and S5). These enzymes are directly or indirectly involved in various steps or branches of chlorophyll biosynthesis and degradation.

POR (protochlorophyllide oxidoreductase) and CAO (chlorophyllide a oxygenase) are two key enzymes in the chlorophyll biosynthesis. POR catalyzes light-dependent reduction of protochlorophyllide a to chlorophyllide a, which is then converted into chlorophyllide b by CAO (Armstrong et al., 1995; Tanaka et al., 1998; Espineda et al., 1999; Reinbothe et al., 2010). Among three POR proteins in Arabidopsis (A, B, and C), PORB represents the major activity of the POR enzyme (Armstrong et al., 1995; Masuda and Takamiya, 2004). DCA1 (DE-REGULATED CAO ACCUMULATION 1; an ATP-dependent Clp protease ATP-binding subunit ClpC or CLPC1) was found to negatively regulate the stability of
22 CAO (Nakagawara et al., 2007). During chlorophyll degradation, RCCR (RED 437 CHLOROPHYLL CATABOLITE REDUCTASE; also known as ACD2) is a key enzyme, and mutations in ACD2 cause severe abnormalities in development and cell death.

**Figure 4. S-nitrosylated proteins in the chlorophyll metabolism and the related pathways**

Top: a diagram of chlorophyll metabolism and the related pathways. S-nitrosylated enzymes or proteins are highlighted in red with accession numbers (see Supplemental Tables S1 and S5 online for detailed annotation of these proteins). Other enzymes or proteins in the pathway are not shown.

Bottom: other S-nitrosylated proteins annotated as related to chlorophyll metabolism. S-nitrosylated proteins identified by site-specific proteomics were marked by red asterisks.

6 CAO (Nakagawara et al., 2007). During chlorophyll degradation, RCCR (RED 437 CHLOROPHYLL CATABOLITE REDUCTASE; also known as ACD2) is a key enzyme, and mutations in ACD2 cause severe abnormalities in development and cell death.
(Pružinská et al., 2005; Yao and Greenberg, 2006). Therefore, both chlorophyll biosynthesis and chlorophyll degradation are potentially regulated by protein S-nitrosylation, suggesting that NO plays a role in the regulation of chlorophyll homeostasis.

By the use of the biotin-switch assay, we found that POR and DCA proteins are indeed S-nitrosylated in vivo in both Col-0 and gsnor1-3 mutant seedlings, and the accumulation of the S-nitrosylated POR and DCA proteins was significantly increased in gsnor1-3 compared with Col-0 (Fig. 5A and 5B). To explore the possible physiological significance of S-nitrosylation on chlorophyll metabolism, we analyzed the chlorophyll level in gsnor1-3 in comparison with wild type plants. Compared to that of wild-type (Col-0) seedlings, gsnor1-3 leaves showed a yellowish phenotype. Consistently, the chlorophyll level in the gsnor1-3 seedlings was significantly reduced compared to that in wild type (Fig. 5C). A similar result was also obtained in a previous study on the hot5 mutant allele (Lee et al., 2008). Together, these results suggest that the increased level of NO level is correlated to the reduced chlorophyll level, which is likely involved in the S-nitrosylation of enzymes in the chlorophyll metabolism and the related pathways.

**Enriched S-nitrosylated proteins in the photosynthesis system**

We also identified 26 S-nitrosylated proteins that are directly involved in photosynthesis (Fig. 6 and Supplemental Tables S1 and S5). Among these S-nitrosylated proteins, 9 are involved in the light-dependent reaction of photosynthesis, including 5 components of photosystem II (PSII; PsbP-1, PsbP-2, DEG2, PsbO-2, and PsbTn), three components of photosystem I (PSI; PsaC, PsaD-1 and PsaD-2) and ATPA (ATP synthase subunit α), suggesting that key components of light-dependent reaction are extensively regulated by S-nitrosylation.

In the photosynthetic dark reaction or the Calvin cycle, 17 key enzymes were identified to be S-nitrosylated. Remarkably, the key enzymes in all three phases of the
Calvin cycle (carbon fixation, reduction and the regeneration of ribulose) were identified to be S-nitrosylated (Fig. 6). In the carbon fixation reaction, three subunits of the Rubisco

**Figure 5. Regulation of chlorophyll metabolism by S-nitrosylation**

(A) and (B) Analysis of S-nitrosylation of POR (A) and DCA proteins (B) in Col-0 and gsnorl-3 seedlings by in vivo biotin-switch method. The blots were detected by anti-POR (A) and anti-DCA (B) antibodies, respectively. Asc: sodium ascorbate; SNO: S-nitrosylated proteins.

(C) Top: Two-week-old Col-0 and gsnorl-3 seedlings grown in soil. Bar, 1 cm. Bottom: Analysis of the chlorophyll content of the seedlings shown at the top. The data presented are mean values of three independent repeats. Error bars indicate S.D.; ** indicates $P < 0.01$ (Student’s t-test).
complex (RBCS1A, RBCS3B and RBCL) and RCA (Rubisco activase) were S-nitrosylated. Moreover, phosphoglycerate kinase (PGK), which phosphorylates 3-phospho-D-glycerate to form 1, 3-bisphospho-D-glycerate, was also S-nitrosylated. During the reduction reaction,
three GAPDH members GAPA1, GAPA2 and GAPB were found to be S-nitrosylated. Other identified S-nitrosylated proteins involved in the Calvin cycle included TIM, CTIMC, FBA1, FBA2, HCEF1, FINS1, SBPASE, EMB3119 and PRK (Fig. 6; see Supplemental Tables S1 and S5 for detailed annotation of these proteins). These S-nitrosylated proteins covered nine of all fourteen steps in the Calvin cycle.

Our S-nitrosoproteomic analysis is supported by the observations that Rubisco proteins of Kalanchoe pinnata and Brassica juncea are modified by S-nitrosylation (Abat et al., 2008; Abat and Deswal, 2009). To further validate these results, we performed a biotin-switch assay on five proteins PsbO, RBCS1A, RCA, FINS1 and PRK, which are important components in PSII and the Calvin cycle, respectively. We found that all these proteins were S-nitrosylated in vivo (Fig. 7A through 7E) and the level of the S-nitrosylated FINS1 and PRK proteins was significantly increased in gsnor1-3 compared to that in Col-0 (Fig. 7D and 7E). Taken together, these results suggest that the photosynthesis system, especially key components of the Calvin cycle, is subjected to extensive regulation by S-nitrosylation.

Altered photosynthetic properties in gsnor1-3
To evaluate the potential regulatory roles of S-nitrosylation on photosynthesis, we measured the chlorophyll fluorescence parameters of Col-0 and gsnor1-3 seedlings by the pulse amplitude modulated (PAM) method. No significant difference in the PSII maximum photochemical efficiency (represented as Fv/Fm, where Fv and Fm are the variable fluorescence and the maximum fluorescence, respectively) was observed between Col-0 and gsnor1-3 seedlings (Fig. 7F and Supplemental Fig. S3A and S3B online). However, the quantum efficiency of PSII photochemistry (ΦPSII) and photochemical quenching (qP) were significantly increased in gsnor1-3 under relatively low light intensity (Fig. 7G and 7H; see also Supplemental Fig. S3C and S3D online), whereas the non-photochemical quenching (NPQ) was repressed in the gsnor1-3 mutant (Fig. 7I and Supplemental Fig.
S3E), suggesting that the photosynthetic properties are affected by the gsnor1-3 mutation.

Presumably, the PSI antenna becomes smaller compared to the PSI antenna in the mutant.
as the reduction in the chlorophyll contents has more profound effect on PSII (Andersson et al., 2001; Jenny et al., 2003; Kovács et al., 2006).

The significant enrichment of S-nitrosylated proteins in chlorophyll metabolism and photosynthesis implies an important role of NO in regulation of these physiological processes. Correlated to this observation, the chlorophyll level is reduced, whereas the photosynthetic activity is affected in gsnor1-3. Consistently, when treated with the NO donor sodium nitroprusside, barley leaves show the increased accumulation of several photosynthesis-related proteins and the enhanced effective quantum yield of PSII (ΦPSII) in the light (Zhang et al., 2006).

Several proteomic analyses have identified 169 unique S-nitrosylated proteins in Arabidopsis (Lindermayr et al., 2005; Romero-Puertas et al., 2008; Palmieri et al., 2010; Fares et al., 2011; Puyaubert et al., 2014). In this study, we identified 926 unique endogenously S-nitrosylated proteins with 1,195 S-nitrosylation sites in Arabidopsis, which, up to date, is the largest database of S-nitrosylated proteins among all organisms. Of these 926 proteins, 72 have been identified as S-nitrosylated proteins by independent proteomic studies (Lindermayr et al., 2005; Romero-Puertas et al., 2008; Palmieri et al., 2010; Fares et al., 2011; Puyaubert et al., 2014), ten additional proteins were characterized to be S-nitrosylated in planta, including SABP3 (Wang et al., 2009), APX1 (Correa-Aragunde et al., 2013), FBA6 (van der Linde et al., 2011), POR, DCA, PsbO, RBCS1A, RCA, FIN1 and PRK (see Fig. 5 and 7). Together, more than 8.2% (76/926) of the identified targets were confirmed as S-nitrosylated proteins in vivo. Most S-nitrosylated proteins identified in this study have not been reported by previous studies and are involved in diverse biological processes. Therefore, the data generated by this study will provide valuable clues for future studies on these physiological and developmental processes regulated by NO. In particular, we found that a number of proteins in chlorophyll metabolism and photosynthesis are candidates of S-nitrosylation, thereby revealing a potentially important mechanism linking these two physiologically fundamental processes with redox signals.
MATERIALS AND METHODS

Plant materials and growth conditions
Arabidopsis wild-type accession Columbia-0 (Col-0) and gsnor1-3 mutant (Feechan et al., 2005) were used in this study. For nitrosoproteomic analysis of S-nitrosylated proteins, analysis of protein S-nitrosylation by in vivo biotin-switch method, chlorophyll content and chlorophyll fluorescence measurement, the Col-0 and gsnor1-3 mutant was grown under continuous white light (100 μmol m⁻² s⁻¹) at 22 °C in soil.

Identification of S-nitrosylated proteins by shotgun LC-MS/MS analysis
The biotinylation and purification of S-nitrosylated proteins from two-week-old Arabidopsis seedlings were performed as described (Jaffrey and Snyder, 2001; Feng et al., 2013). The biotinylated proteins (the S-nitrosylated proteins) were recovered by incubating with elution buffer (20 mM Hepes-NaOH pH 7.7, 100 mM NaCl, 1 mM EDTA, 100 mM β-Mercaptoethanol). The purified S-nitrosylated proteins were subjected to immunoblotting using various antibodies (see below) or for nitrosoproteomic analysis.

Purified S-nitrosylated proteins were separated on 10% SDS-PAGE. The gel was stained with Coomassie blue R350 and was cut into 8 slices along the sample migration direction, and each slice contained proteins with different molecular masses. The gel slices were washed three times with Milli-Q water, cut into small pieces and then incubated with 25 mM NH₄HCO₃ in 50% acetonitrile with gentle shaking at room temperature until the blue color disappeared completely. The gel pieces were then incubated with 100% acetonitrile until the gel pieces become white, dried completely under vacuum. The dried sample was dissolved in 25 mM NH₄HCO₃ containing 10 mM dithiothreitol and incubated at 56 °C for 1 h, and iodoacetamide (IAM) was added at a final concentration of 55 mM.
followed by incubation at room temperature for 45 min in the dark. Afterward, gel pieces were dehydrated by 50% acetonitrile and 100% acetonitrile, and then completely dried under vacuum. The sample was digested by trypsin (0.01 mg/ml; Promega, cat #: V5111) at 37 °C overnight. The digested peptides were extracted by incubating with 5% trifluoroacetic acid (TFA) at 40 °C for 1 h with ultrasonic processing for 3 min and extracted again by 50% acetonitrile containing 2.5% TFA at 30 °C for 1 h. The combined peptide extracts were evaporated by lyophilization and suspended in 0.1% TFA solution containing 2% acetonitrile.

The digested peptides from each of the eight gel slices were individually analyzed by nano-LC-MS/MS using linear ion trap quadrupole (LTQ) mass spectrometer (Thermo Finnigan, USA). Five microliters of each sample were loaded onto C18 column (300 μm i.d. × 15 cm, PepMap300 C18, Dionex) equilibrated in 95% solvent A (2% acetonitrile, 0.1% formic acid) and 5% solvent B (80% acetonitrile, 0.1% formic acid). Peptides were eluted by a series of linear gradients of solvent A to solvent B (0 to 5 min, 5% B; 5 to 85 min, gradient from 5 to 50% B; 85 to 95 min, gradient from 50 to 95% B; 95 to 125 min, hold at 95% B; 125 to 126 min, gradient from 95 to 5% B; 126 to 136 min, hold at 5% B) at a flow rate of 0.3 mL/min and then subjected to MS/MS analysis. The LTQ Linear ion trap mass spectrometer was operated with the following parameters: spray voltage was 2.2 kV, capillary temperature was 160 °C, full scan m/z range was 350–1600 and normalized collision energy was 35%. The most intense ten ions in each full scan were selected for MS/MS.

Raw data of eight gel slices were used for a search against Arabidopsis protein database (www.ncbi.nlm.nih.gov/guide/proteins/; Version, Aug 22, 2012; 914,357 sequences) with the taxonomy restriction to “Arabidopsis thaliana”. The BioWorks TurboSequest software (version 3.2, Thermo Finnigan) was used for the database searching using the following parameters: the enzyme was set to trypsin, one missed cleavage was allowed, carbamidomethylation of cysteine as the fixed modification, oxidation of
methionine as the variable modification, mass tolerances for peptides and fragment ions were set to 1.0 Da and 0.5 Da, respectively. The search results were filtered using the following parameters: false discovery rate (FDR) < 0.05; Xcorr ≥ 1.5 when charge = 1; Xcorr ≥ 2.5 when charge = 2; Xcorr ≥ 3.5 when charge > 3; ΔCn ≥ 0.1 when charge = 1; ΔCn ≥ 0.1 when charge = 2; ΔCn ≥ 0.1 when charge > 3; Removing peptides from decoy database was processed as well. Proteins with more than two matched unique peptides were reported as acceptable identifications.

The GO categorization of S-nitrosylated proteins were performed using gene ontology (GO) annotation module (The Arabidopsis Information Resource, TAIR; http://www.arabidopsis.org) and agriGO software (Du et al., 2010).

Identification of in vivo S-nitrosylated peptides by site-specific S-nitrosoproteomic analysis

For the identification of S-nitrosylated peptides, the biotinylation of S-nitrosylated proteins was carried out by using S-alkylating labeling strategy as described (Chen et al., 2010) with modifications, followed by tryptic proteolysis and affinity-purification of the biotinylated peptides. The lysates prepared from ~1 g fresh plant samples were blocked by S-alkylation with 200 mM IAM at 37 °C for 2 h and the S-nitrosylated cysteine residues were biotinylated by incubating with HENS buffer (250 mM Hepes pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS) containing 1 mM sodium ascorbate and 4 mM biotin-maleimide (Sigma-Aldrich) at room temperature for 2 h in the dark. After tryptic digestion, NeutrAvidin agarose resins (Thermo Scientific) was used for purification of the S-nitrosylated peptides by affinity chromatography. The purified peptides were desalted by C18 Zip-tip (Millipore) and then subjected to LC-MS/MS analysis.

For mass spectrometric analysis, purified biotinylated peptides were resuspended in 0.1% FA and analyzed by LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) coupled online to an Easy-nLC 1000 (Thermo Fisher Scientific) in the data-dependent
mode. The peptides were then separated by reverse phase LC with a 75 μm (ID) × 150 mm (length) analytical column packed with C18 particles of 5 μm diameter. The mobile phases for the LC contains buffer A (2 % ACN, 0.1 % FA) and buffer B (98 % ACN, 0.1 % FA), a linear gradient of buffer B from 3%-30% for 90 min was used for the separation. All MS measurements were performed in the positive ion mode. Precursor ions were measured in the Orbitrap analyzer at 240,000 resolution (at 400 m/z) and a target value of 10^6 ions. The twenty most intense ions from each MS scan were isolated, fragmented, and measured in the linear ion trap. The CID normalized collision energy was set to 35.

The data obtained from LC-MS/MS analysis was analyzed using a pre-release version of Thermo Scientific Proteome Discoverer™ software version 1.3. The UniProt proteome sequences for *Arabidopsis thaliana* (including canonical and isoforms) were used for the database searching and the mass tolerance was set to 0.05 Da. The maximum number of missed cleavages was set at two, the minimum peptide length was set at six amino acids and the maximum peptide length was set at 144 amino acids. The false discovery rate was set at 0.01 for peptide and protein identifications. Cysteine biotinylation (451.200 Da), cysteine carbamidomethylation, and methionine oxidation were included in the search as the variable modifications. Peptides identified in negative controls (without ascorbate treatment) were excluded. The mass spectrometry data have been deposited to the PeptideAtlas database (http://www.peptideatlas.org; accession number: PASS00638).

Prediction of consensus sequences of S-nitrosylated peptides was performed by the Motif-X algorithm (Schwartz and Gygi, 2005). The GO categorization of S-nitrosylated proteins were performed as described above.

**Immunoblotting analysis**

Immunoblotting was performed as previously described (Feng et al., 2013). Primary antibodies used in this study are: anti-biotin antibody (Cell Signaling Technology, cat #: 7075), anti-POR antibody (Agrisera, cat #: AS05067), anti-DCA antibody (Agrisera, cat #:...
AS01001), anti-PsbO antibody (Agrisera, cat #: AS05092), anti-RBCS1A antibody (Agrisera, cat #: AS07222), anti-RCA antibody (Agrisera, cat #: AS10700), anti-FINS1 antibody (Agrisera, cat #: AS04043), anti-PRK antibody (Agrisera, cat #: AS07257), and anti-α-tubulin antibody (Sigma-Aldrich cat #: T5168). The HRP-conjugated goat anti-rabbit IgG antibody (cat #: IH-0011) and HRP-conjugated goat anti-mouse IgG antibody (cat #: IH-0031) were purchased from Beijing Dingguo Changsheng Biotechnology.

Chlorophyll content and chlorophyll fluorescence analysis

The measurement of chlorophyll content was performed as described previously (Feng et al., 2007). Chlorophyll fluorescence parameters were measured using a IMAGING PAM chlorophyll fluorometer (Walz), and the measurement was carried out in a dark room with stable ambient conditions as previously described (Sun et al., 2010).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ACD2/RCCR (AT4G37000), ACO3 (AT2G05710), AHP1 (AT3G21510), APX1 (AT1G07890), ATP4 (ATCG00120), CA1/SABP3 (AT3G01500), CAO (AT1G44446), CAT1 (AT1G20630), CAT2 (AT4G35090), CAT3 (AT1G20620), CDC48 (AT3G09840), CH42 (AT4G18480), CHL12 (AT5G45930), CLB4 (AT5G60600), CSY4 (AT2G44350), CTIMC (AT3G55440), CYSC1 (AT3G61440), DCA1/CLPC1 (AT5G50920), DEG2 (AT2G47940), DHAR1 (AT1G19570), DHAR2 (AT1G75270), DXR (AT5G62790), eIF5A-2/FBR12 (AT1G26630), EMB3119 (AT3G04790), FBA1 (AT2G21330), FBA2 (AT4G38970), FBA6 (AT2G36460), FINS1 (AT1G43670), GAPA1 (AT3G26650), GAPA2 (AT1G12900), GAPB (AT1G42970), GAPC1 (AT3G04120), GSA2 (AT3G48730), GSH1/CAD2/RML1/RAX1/PAD2 (AT4G23100), GSH2 (AT5G27380), GSNO1/HOT5/PAR2 (AT5G43940), HCEF1 (AT3G54050), HEMC (AT5G08280), HEME2 (AT2G40490), IPP1 (AT5G16440), IPP2
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The increased accumulation of S-nitrosylated proteins in gsnor1-3.

Supplemental Figure S2. Identification of S-nitrosylated proteins in gsnor1-3 by shotgun proteomics.

Supplemental Figure S3. Analysis of chlorophyll fluorescence of Col-0 and gsnor1-3 leaves under different light intensities.

Supplemental Table S1. A list of S-nitrosylated proteins identified by shotgun S-nitrosoproteomic analysis.

Supplemental Table S2. Site-specific identification of S-nitrosylated peptides in Col-0 and gsnor1-3.

Supplemental Table S3. A list of peptides without nitrosocysteine identified in site-specific proteomics.

Supplemental Table S4. A list of peptides identified in the negative controls.

Supplemental Table S5. A list of S-nitrosylated proteins identified by site-specific
proteomic analysis.

**Supplemental Table S6.** Detailed information of S-nitrosylated proteins identified in Col-0 by site-specific proteomics.

**Supplemental Table S7.** Detailed information of S-nitrosylated proteins identified in *gsnor1-3* by site-specific proteomics.

**Supplemental Table S8.** A list of S-nitrosylated proteins specifically identified in Col-0 by site-specific proteomic analysis.

**Supplemental Table S9.** A list of S-nitrosylated proteins specifically identified in *gsnor1-3* by site-specific proteomic analysis.

**ACKNOWLEDGEMENTS**

We thank Dr. Gary Loake for providing the *gsnor1-3* seeds.
FIGURE LEGENDS

Figure 1. Site-specific identification of endogenously S-nitrosylated proteins in Arabidopsis

(A) Schematic illustration of the procedure of site-specific S-nitrosoproteomic analysis.
(B) The S-nitrosylated peptides identified by site-specific proteomics. The peptides identified in both Col-0 and gsnor1-3 plants are shown by a Venn diagram.
(C) The S-nitrosylated proteins identified by site-specific proteomics. The proteins identified in both Col-0 and gsnor1-3 plants are shown by a Venn diagram.
(D) Comparison of the S-nitrosylated proteins identified by shotgun proteomics and site-specific proteomics. The proteins identified in both experiments are shown by a Venn diagram.
(E) Mass spectrometric identification of Cys\textsuperscript{173} of FBA6 as an S-nitrosylated residue by site-specific proteomics.
(F) Comparison of S-nitrosylated proteins identified in previous studies (Lindermayr et al., 2005; Romero-Puertas et al., 2008; Palmieri et al., 2010; Fares et al., 2011; Puyaubert et al., 2014) and this study.

Figure 2. Analysis of the consensus sequences of S-nitrosylated peptides

(A) The putative consensus sequences of the S-nitrosylation motifs derived from the analysis of 1,195 S-nitrosylated peptides. The probability of each putative motif is given below the peptide sequences.
(B) Mass spectrometric identification of Cys\textsuperscript{451} of RCA as an S-nitrosylated residue by site-specific proteomics.
(C) Sequence alignment of the S-nitrosylated peptides containing an EXC motif (highlighted by arrows on the top). The accession numbers and the names of proteins containing these peptides are given at the left side. The sequences shown are tryptic fragments identified by
mass spectrometry. See Supplemental Table S5 online for detailed annotation of these proteins).

**Figure 3. Functional categorization of S-nitrosylated proteins identified by**
**site-specific proteomics**

(A) Functional categorization of S-nitrosylated proteins by biological processes.

(B) Functional categorization of S-nitrosylated proteins by subcellular components.

S-nitrosylated proteins identified by site-specific proteomics from Col-0, gsnor1-3, and the combined dataset were analyzed separately.

**Figure 4. S-nitrosylated proteins in the chlorophyll metabolism and the related pathways**

Top: a diagram of chlorophyll metabolism and the related pathways. S-nitrosylated enzymes or proteins are highlighted in red with accession numbers (see Supplemental Tables S1 and S5 online for detailed annotation of these proteins). Other enzymes or proteins in the pathway are not shown.

Bottom: other S-nitrosylated proteins annotated as related to chlorophyll metabolism.

S-nitrosylated proteins identified by site-specific proteomics were marked by red asterisks.

**Figure 5. Regulation of chlorophyll metabolism by S-nitrosylation**

(A) and (B) Analysis of S-nitrosylation of POR (A) and DCA proteins (B) in Col-0 and gsnor1-3 seedlings by *in vivo* biotin-switch method. The blots were detected by anti-POR (A) and anti-DCA (B) antibodies, respectively. Asc: sodium ascorbate; SNO: S-nitrosylated proteins.

(C) Top: Two-week-old Col-0 and gsnor1-3 seedlings grown in soil. Bar, 1 cm. Bottom: Analysis of the chlorophyll content of the seedlings shown at the top. The data presented are mean values of three independent repeats. Error bars indicate S.D.; **indicates *P* < 0.01.
Figure 6. S-nitrosylated proteins involved in photosynthesis
Top and middle: diagrams of the light reactions and the Calvin cycle (the dark reaction) of photosynthesis, respectively. S-nitrosylated enzymes or proteins are highlighted in red with accession numbers (see Supplemental Tables S1 and S5 online for detailed annotation of these proteins). Other enzymes or proteins in the pathway are not shown.
Bottom: other S-nitrosylated proteins annotated as related to photosynthesis.
S-nitrosylated proteins identified by site-specific proteomics were marked by red asterisks.

Figure 7. Regulation of photosynthesis by S-nitrosylation
(A) through (E) Analysis of S-nitrosylation of PsbO (A), RBCS1A (B), RCA (C), FINS1 (D) and PRK (E) proteins in Col-0 and gsnor1-3 seedlings using the indicated antibodies as described in Fig. 5A.
(F) through (I) Analysis of chlorophyll fluorescence of Col-0 and gsnor1-3 seedlings. Seedlings were grown in 100 μmol m⁻² s⁻¹ for 15 days and dark-adapted for 20 min before the measurement. Fv/Fm: maximal photochemical efficiency of photosystem II (PSII); ΦPSII: actual PSII efficiency; qP: photochemical quenching; NPQ: non-photochemical quenching. The data presented are as mean values of three independent experiments. Error bars indicate S.D.
Supplemental Figure S1. The increased accumulation of S-nitrosylated proteins in gsnor1-3.

(A) Analysis of the total S-nitrosylated protein level of Col-0 and gsnor1-3 seedlings by immunoblotting. Asc: sodium ascorbate; SNO: S-nitrosylated protein.

(B) Quantitative analysis of the total S-nitrosylated protein level of Col-0 and gsnor1-3 seedlings. The results presented are as mean values of three independent repeats. Error bars indicate S.D.; **indicates $P < 0.01$ (Student’s $t$-test).
Supplemental Figure S2. Identification of S-nitrosylated proteins in gsnor1-3 by shotgun proteomics.

(A) Schematic illustration of shotgun S-nitrosoproteomic analysis.

(B) SDS-PAGE separation of enriched S-nitrosylated proteins in gsnor1-3 for shotgun proteomics. Asc: sodium ascorbate; SNO: S-nitrosylated protein.

(C) Comparison of S-nitrosylated proteins identified in previous studies and this study by shotgun S-nitrosoproteomic analysis.

(D) Functional categorization of S-nitrosylated proteins by biological processes.

(E) Functional categorization of S-nitrosylated proteins by subcellular components.
**Supplemental Figure S3.** Analysis of chlorophyll fluorescence of Col-0 and *gsnor1-3* leaves under different light intensities.

(A) Two-week-old Col-0 and *gsnor1-3* seedlings

(B) through (E) Analysis of the chlorophyll fluorescence in 2-week-old Col-0 and *gsnor1-3* seedlings. Fv/Fm: maximal photochemical efficiency of photosystem II (PSII); ΦPSII: actual PSII efficiency; qP: photochemical quenching; NPQ: non-photochemical quenching.
Supplemental Table S1. A list of S-nitrosylated proteins identified by shotgun S-nitrosoproteomic analysis.

Supplemental Table S1 is presented as a separated Excel file.

Supplemental Table S2. Site-specific identification of S-nitrosylated peptides in Col-0 and gsnor1-3.

<table>
<thead>
<tr>
<th></th>
<th>Exp #1 Asc (+)</th>
<th>Exp #1 Asc (-)</th>
<th>Exp #2 Asc (+)</th>
<th>Exp #2 Asc (-)</th>
<th>Exp #3 Asc (+)</th>
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*The total numbers of the unique biotinylated peptides identified from the four experiments excluded those identified in the negative controls. Numbers inside parenthesis indicate non-biotinylated peptides identified in each sample.

Asc: sodium ascorbate.

Supplemental Table S3. A list of peptides without nitrosocysteine identified in site-specific proteomics.

Supplemental Table S4. A list of peptides identified in the negative controls.

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**Supplemental Table S9.** A list of S-nitrosylated proteins specifically identified in *gsnor1-3* by site-specific proteomic analysis.

**Supplemental Tables S1, S3 through S9** are presented as separated Excel files.


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