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COP1 is modified by nitric oxide via S-nitrosylation

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Abbreviations

CAT3 Catalase 3
COP1 CONSTITUTIVELY PHOTOMORPHOGENIC 1
cPTIO 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
GSNO S-nitrosylated glutathione
GSNOR GSNO reductase
HY5 ELONGATED HYPOCOTYL 5
NO Nitric Oxide
SNO S-nitrosothiol
SNP Sodium nitroprusside
TRXh3 Thioredoxin-h3
TRXh5 Thioredoxin-h5

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Abstract

Protein S-nitrosylation, which is defined by the covalent attachment of NO to the thiol group of cysteine residues, is known to play critical roles in plant development and stress responses. NO promotes seedling photomorphogenesis and NO emission is enhanced by light. However, the function of protein S-nitrosylation in plant photomorphogenesis is essentially unknown. E3 ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) and transcription factor ELONGATED HYPOCOTYL 5 (HY5) antagonistically regulate seedling photomorphogenesis. COP1 inhibits plant photomorphogenesis by targeting photomorphogenic promoters like HY5 for 26S proteasome degradation. Here, we report that COP1 is S-nitrosylated in vitro. Mass spectrometry analyses revealed that two evolutionarily well conserved residues, cysteine 425 and cysteine 607, in the WD40 domain of COP1 are S-nitrosylated. S-nitrosylated glutathione (GSNO) is an important physiological NO donor for protein S-nitrosylation. The *gsnor1-3* mutant, which accumulates higher level of GSNO, accumulated higher HY5 levels than wildtype, indicating that COP1 activity is inhibited. Protein S-nitrosylation can be reversed by Thioredoxin-h5 (TRXh5) in plants. Indeed, COP1 interacts directly with TRXh5 and its close homolog TRXh3. Moreover, catalase 3 (CAT3) acts as a transnitrosylase that transfers NO to its target proteins like GSNO reductase (GSNOR). We found that CAT3 interacts with COP1 in plants. Taken together, our data indicate that the activity of COP1 is inhibited by NO via S-nitrosylation to promote photomorphogenesis.

Introduction

Nitric oxide (NO) regulates plant growth, development and stress response (Yu et al. 2014; Domingos et al. 2015; Feng et al. 2019; Kolbert et al. 2021). NO exerts its functions by modulating protein function or activity through posttranslational modifications including protein S-nitrosylation, tyrosine nitration and metal nitrosylation. Protein S-nitrosylation, which is defined by the covalent attachment of NO to the thiol group of cysteine residues to form S-nitrosothiol (-SNO), is the most important type of protein modification by NO (Feng et al. 2019; Ageeva-Kieferle et al. 2021). Certain proteins are S-nitrosylated in plants in response to different environment factors (Vanzo et al. 2016; Jain et al. 2018). As an

important environment factor light can induce NO production in plants, however, the function of S-nitrosylation in plant photomorphogenesis remains essentially unknown.

CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) is among the first-characterized negative regulators of plant photomorphogenesis (Deng et al. 1991). COP1 targets several photomorphogenic promoters including the bZIP transcription factor HY5 for degradation (Osterlund et al. 2000; Pacin et al. 2014). SUPPRESSOR OF PHYTOCHROME A-105 (SPA) proteins interact directly with COP1 and this COP1/SPA complex is considered as a functional E3 ubiquitin ligase (Hoecker 2017). To promote photomorphogenesis the activity of the E3 ubiquitin ligase COP1/SPA is inhibited by light via different mechanisms, including disruption of COP1-SPA interaction, exclusion of COP1 from the nucleus and degradation of some SPA proteins (Lian et al. 2011; Liu et al. 2011; Zheng et al. 2013; Sheerin et al. 2015; Lu et al. 2015; Chen et al. 2015; Hoecker 2017; Menon et al. 2016; Podolec and Ulm 2018). Several photoreceptors including UVR8, CRY1 and CRY2 can compete with some COP1 substrates for binding to the WD40 domain, thereby inhibiting of COP1 activity (Ponnu et al. 2019; Lau et al. 2019). Regulation of COP1 activity by protein modifications is less explored.

S-nitrosylated glutathione (GSNO) is an important natural and physiological NO donor (Kovacs and Lindermayr 2013). GSNO is degraded by GSNO reductase (GSNOR) and Arabidopsis *gsnor1* mutants accumulate high levels of both cellular GSNO and protein-SNO (Chen et al. 2009; Feechan et al. 2005). S-nitrosylatoin is reversible. Thioredoxin-h3 (TRXh3) and TRXh5 interact with and denitrosylate the S-nitrosylated proteins to regulate plant immunity (Kneeshaw et al. 2014). S-nitrosylation has long been considered as a nonenzymatic reaction in plants. Recently, catalase 3 (CAT3) was shown to act as a transnitrosylase that it transfers its bearing NO moiety to GSNOR in Arabidopsis (Chen et al. 2020). It would be interesting to test whether the transnitrosylase CAT3 has other protein substrates.

Plants exposed to light emit more NO than plants in darkness (Ageeva-Kieferle et al. 2021), suggesting that protein S-nitrosylation may play roles in light responses. Indeed, proteins involved in photosynthesis and chlorophyII metabolism are S-nitrosylated in *gsnor1-3* plants (Hu et al. 2015). However, it is to be tested whether proteins involved in plant

photomorphogenesis are S-nitrosylated. COP1 is critical player in several major photoreceptor-mediated light signaling pathways. In this work, we found that COP1 is S-nitrosylated at its two well conserved cysteine residues located in its WD40 domain being responsible for binding to many of its degradation targets like HY5. Through analysis of HY5 protein levels in *gsnor1-3*, which accumulates high levels of GSNO, we showed that COP1 activity is inhibited probably via S-nitrosylation.

Materials and methods

Plant material and growth conditions

The *gsnor1-3* and *cop1-4/35S:YFP-COP1* were of Columnbia ecotype background. For YFP-COP1 protein level assay, after being stratified in the dark at 4°C for 2 d, seedlings were grown at 23°C under continuous white-light field (3.6 μmol/m^2/s, measured by LI-250A Light Meter). For HY5 protein detection, seedlings were grown either in dark or under continuous white light 100 μmol/m^2/s at 23°C.

Protein expression and purification

The coding sequences of *COP1, COP1^{N282}* (aa 1-282) and *COP1^{C340}* (aa 336-675) were cloned into the MBP-tag expression vector pMAL-c5x, respectively. The expression vectors were transformed into *E.coil* strain BL21/DE3. Amylose magnetic beads were usedf for protein purifications.

Biotin switch assay

Protein S-nitrosylation was detected with a similar biotin switch method (Jaffrey and Snyder 2001). Purified proteins were precipitated and then exchanged for HEN buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine). Proteins were divided into two parts and treated with DTT (110 mM) or Cys-NO (110 mM) for 30 minutes in dark. The sample was precipitated with acetone, and resuspended in 250 μl blocking buffer (250 mM HEPES, 1 mM EDTA, pH7.7, 0.1 mM neocuproine, 5%SDS, 50 mM NEM) for 20 min at 50°C. Samples were precipitated with acetone. 50 μl biotin-HPDP (4 mM) and 25 μl sodium ascorbate (500 mM) were added to the precipitates and incubated at room temperature for 1 h in dark. The

reaction mixture was precipitated with acetone and then boiled at 95°C for 5 min in SDS buffer without reducing agent.

Mass Spectrometric analysis COP1-C340 protein charged by biotin-HPDP

The target bands of biotinylated proteins were cut from SDS-PAGE gels stained by coomassie blue. Samples were digested with trypsin (Promega) and dissolved in 0.1% formic acid. An Easy-nLC1200 system coupled online to a Q Exactive plus mass spectrometer (Thermo Scientific, Bremen, Germany) were supplied to analyze the above reaction products. Aliquots including peptides were conducted on a analytical column (C18, 20cm×75μm, 3μm) using a 1 h acetonitrile gradient in 0.1% formic acid at a flow rate of 600 nL/min. The mobile phase was 0.1% formic acid as solvent A and 80% acetonitrile containing 0.1% formic acid as solvent B with a linear gradient from 2% to 8% solvent B over 0 to 3 min, 8% to 20% over 3 to 39 min, 20% to 35% over 39 to 47 min, 35% to 100% over 47 to 48 min and hold for 12 min. MS analysis was performed using MS1 scans (350–1,800 m/z) with 70,000 resolution (AGC, 3e6 ions) and then followed by MS2 scans of up to 20 abundant multiply charged precursors in the MS1 spectrum fragmented by the higher energy collisional dissociation (HCD) with a normalized collision energy of 28. Capillary temperature was 275°C and spray voltage was 2100V. The HCD-MS2 spectra were analysed in 17,500 resolution (AGC, 1e5). The Peaks software was used to analyze the sequence of samples with a precursor ion mass tolerance of 10 ppm. The modifications include: Cysteine/+428.19 Da (charged with Biotin-HPDP), Carbamidomethyl (C), Deamidation (NQ), Oxidation (M). FDR≤1%.

Immunoblotting

For immunoblot analysis, proteins were separated by SDS-PAGE and transferred to PVDF membranes according to the manufacturer's instructions (Bio-Rad). The anti-GFP (Proteintech), anti-HY5, anti-MBP (Proteintech) and anti-biotin (CST) were used as primary antibodies. And anti-rabbit immunoglobulins (Proteintech) and anti-mouse immunoglobulins (Proteintech) were taken as secondary antibodies. Signals were detected using LumiBest ECL substrate solution kit (Share-bio).

Yeast two-hybrid Interaction assay

AD-containing plasmids and BD-containing plasmids were respectively transformed into yeast strain Y187 and GoldY2H using LiAc-mediated yeast transformation. Yeast growth assays were carried out on selective solid medium SD–Trp–Leu–His at 30°C.

Split-luciferase assay

Full length of *COP1* and *CAT3* were cloned into the pJW772/cLUC and pJW771/nLUC to generate cLUC-COP1 and nLUC-CAT3, respectively. Different combinations of the constructs were infiltrated into *N. benthamiana* leaves mediated by Agrobacterium tumefaciens. Plants were kept in darkness for two more days. D-luciferin (Promega) solution was then infiltrated into the leaves, and luciferase activity was recorded using a CCD camera (Tanon).

Results

COP1 contains a RING finger domain, coiled-coiled domain and a C-terminal WD40 domain (Figure 1A). Therefore, we directly tested whether COP1 is S-nitrosylated through biotin switch assays (Jaffrey and Snyder 2001). The purified recombinant MBP-COP1 expressed in E. coli can be S-nitrosylated by CysNO (Figure 1B). Furthermore, we performed biotin switch assays with two distinct COP1 fragments. Immunoblotting analyses after biotin switch revealed that $COP1^{336\text{-}675}$ fragment (WD40 domain) but not $COP1^{1\text{-}282}$ fragment was S-nitrosylated (Figure 1C). The $COP1^{336\text{-}675}$-SNO products were subjected to mass spectrometry analyses to identify the modified cysteine residues. In two independent experiments, both C425 and C607 of COP1 were identified (Figures 1D&E). Both C425 and C607 are evolutionary conserved ranging from unicellular algae to higher plants and mammals, suggesting that both cysteine residues are important for COP1 function (Figure S1).

To test whether NO regulates COP1 protein abundance, the *35S:YFP-COP1/cop1-4* seedlings were either treated with either NO donor sodium nitroprusside (SNP), or a NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Immunoblotting analyses showed that COP1 levels were not obviously altered by SNP or cPTIO (Figure S2), suggesting that S-nitrosylation does not regulate COP1 abundance. Next,

we tested whether NO regulates COP1 activity through immunoblotting analyses for HY5, which is a well-studied substrate of COP1 (Osterlund et al. 2000). Obviously, more HY5 can be detected in *gsnor1-3* mutant than in wildtype (WT) both in white light and in dark (Figures 2A&B). We noticed that the difference of HY5 levels in the two genotypes is more obvious in dark than in light, which is consistent with the notion that COP1 is inhibited by light via several other mechanisms (Figures 2A&B). Thus, the activity of COP1 is inhibited by NO via S-nitrosylation.

TRXh3 and TRXh5 act as a selective protein-SNO reductase (Kneeshaw et al. 2014). TRXh3 and TRXh5 interact with their substrate and reverses SNO modifications in Arabidopsis (Kneeshaw et al. 2014). We found that both TRXh3 and TRXh5 interact directly with COP1 (Figure 2C). Supporting this notion that both TRXh3 and TRXh5 interact with NPR1, which is regulated by NO through S-nitrosylation (Tada et al. 2008). Future work is needed to test whether the S-nitrosylation of COP1 is regulated by these Thioredoxin proteins. Moreover, COP1 interacts with a transnitrosylase CAT3 both in yeasts and tobacco leaves, suggesting that CAT3 may catalyze the S-nitrosylation of COP1 in plants (Figure 3A&B). Collectively, these observations suggest that the activity of COP1 in photomorphogenesis is regulated by NO through S-nitrosylation.

Discussion

Independent studies reported that plant photomorphogenesis is promoted by NO (Castillo et al. 2018; Beligni and Lamattina 2000; Lozano-Juste and Leon 2011; Bai et al. 2014; Domingos et al. 2015). Treatment of etiolated Arabidopsis seedling with NO leads to short hypocotyls, mimicking the effect of light (Castillo et al. 2018; Beligni and Lamattina 2000; Lozano-Juste and Leon 2011). HY5 protein levels were shown be tightly regulated by COP1 activity and to be inversely correlated with seedling hypocotyl length (Osterlund et al. 2000). The Arabidopsis *gsnor1-3* seedlings have short hypocotyls (Shi et al. 2015). Our finding that *gsnor1-3* accumulated higher HY5 levels is consistent with the effect of NO in seedling photomorphogenesis.

It should be noted that NO may regulate plant photomorphogenesis via different mechanism. NO represses Arabidopsis root growth and NO-specific scavenger 2-(4-carboxyphenylalanine) 4,4,5,5tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) can at least partially rescue root growth inhibition (Bai et al. 2014). The phytochrome interacting factor 3 (PIF3), which is an important transcription factor regulating plant photomorphogenesis, is involved in the inhibition of root growth by NO. The *PIF3ox* line was partially insensitive to NO and the *pif3-1* mutant was hypersensitivity to NO (Bai et al. 2014). Interestingly, NO promotes the accumulation of photoreceptor phyB protein levels (Bai et al. 2014). Evidence has been provided that etiolated seedlings emit NO and light promotes NO accumulation (Ageeva-Kieferle et al. 2021). The light-indued NO accumulation corresponds to histone acetylation (Ageeva-Kieferle et al. 2021). It is to be tested whether the NO-induced epigenic regulation mechanisms contribute to photomorphogenesis. We found that the key negative regulator of photomorphogenesis COP1 is post-translationally modified by NO via S-nitrosylation in vitro. Moreover, COP1 interacts with two known denitrosylases TRXh3 and TRXh5 and with a transnitrosylase CAT3 in yeasts and plant cells. Thus, our work revealed a potentially unrecognized mechanism for NO in the regulation of plant photomorphogenesis.

Since NO can promote plant photomorphogenesis via distinct mechanisms, the short seedling hypocotyl phenotype of *gsnor1* mutants is likely a combination of diverse effects of over-accumulated GSNO on plant photomorphogenesis. Nevertheless, the short stature of *gsnor1* mutant is largely suppressed by *cat3*, suggesting that S-nitrosylation may play a major role in the regulation of plant development in the *gsnor1* mutant (Chen et al. 2020).

S-nitrosylation in plants could be transient. Currently, biotin switch method is routinely used to detect protein S-nitrosylation. It is still a major challenge to detect protein S-nitrosylation in plants (Feng et al. 2019). We could not successfully detect S-nitrosylated COP1 in *35S:YFP-COP1/cop1-4* transgenic plants after intensive trials. This could be due to the low expression of YFP-COP1 and only a small fraction of total COP1 is S-nitrosylated. C425 and C607 of COP1 were detected to be S-nitrosylated. Notably, Arabidopsis COP1 contains twenty cysteine residues in total. It is possible that some other cysteine residues of COP1 are S-nitrosylated in plants under certain conditions. We recently showed that C509

of COP1 is important for its function(Zhang et al. 2023). And C509 of COP1 is located on the surface of its WD40 domain (Lau et al. 2019; Zhang et al. 2023), suggesting that it could be readily S-nitrosylated from the structural point of view. Future work is needed to investigate the contribution of C425 and C607 to COP1 S-nitrosylation in plants.

S-nitrosylation regulates protein activities by various mechanisms, including stability, biochemical activity, conformation change, subcellular localization, and protein–protein interaction (Feng et al. 2019) . Treatment of seedlings with NO donor SNP or NO scavenger cPTIO did not alter COP1 protein levels (Figure S2). The exact mechanism for the inhibition of COP1 activity by NO awaits further investigations.

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AUTHOR CONTRIBUTIONS

L.L. and X.C. designed the research; Q.Z. and B.T. performed the experiments; Q.Z., X.C., B.C., R. Y. and L.L. analyzed data; B.C. provided analytical tools. Q.Z., X.C., R.Y. and L.L. wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data of this manuscript is available upon reasonable request.

Figure legends

Figure 1. COP1 is S-nitrosylated in vitro.

(a) Schematic diagrams of COP1 functional domains. $COP1^{1-282}$ contains Ring finger (Ring) and Coiled-coil (CC) domains. $COP1^{336-675}$ contains WD40 domain. The arrowheads indicate the estimated position of C425 and C607. (b,c) Detection of S-nitrosylated full length COP1 (b) and COP1 fragments (c) by immunoblotting. Purified MBP-COP1 recombinant protein

was treated with CysNO or DTT (negative control) and then subjected to biotin-switch. The S-nitrosylated protein was detected by an anti-biotin antibody. The asterisk indicates a cross-reacting band. (d, e) Mass spectrometry analysis of the tryptic biotin-charged MBP-COP1$^{336-675}$ recombinant protein. The b- and y-type product ions are indicated. C425 (d) and C607 (e) were identified as the S-nitrosylated residues.

Figure 2. The activity of COP1 is reduced in *gsnor1-3* mutant seedlings.

(a, b) *gsnor1-3* mutant accumulated higher HY5 levels than WT both in white light (a) and in darkness (b). Immunoblotting analysis with anti-HY5. Protein extracts were prepared from WT, *hy5hyh* and *gsnor1-3* knock-out mutant grown in darkness (a) and white light (b) for ten days. The asterisk indicates a cross-reacting band. Ponceau staining of Rubisco Large chain serves as a loading control.

Figure 3. COP1 interacts with TRXh3, TRXh5 and CAT3.

(a) COP1 interacts with TRXh3 and TRXh5 in yeast two-hybrid assays. EV indicates empty vector. BD and AD indicate DNA binding domain vector and activation domain vector, respectively. (b,c) COP1 interacts with CAT3 in yeast two-hybrid assays (b) and in Split-Luciferase assays (c). Split-luciferase assays were carried out in *N. benthamiana* leaves. The luminescence images were captured using a CCD imaging system.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Primers used in this study.

Figure S1. The *Arabidopsis* COP1 Cysteine 425 and 607 are evolutionarily highly conserved.

Figure S2. COP1 protein levels are not altered by NO

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