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Loss of GSNOR1 Function Leads to Compromised Auxin Signaling and Polar Auxin Transport

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ABSTRACT

Cross talk between phytohormones, nitric oxide (NO), and auxin has been implicated in the control of plant growth and development. Two recent reports indicate that NO promoted auxin signaling but inhibited auxin transport probably through S-nitrosylation. However, genetic evidence for the effect of S-nitrosylation on auxin physiology has been lacking. In this study, we used a genetic approach to understand the broader role of S-nitrosylation in auxin physiology in *Arabidopsis*. We compared auxin signaling and transport in Col-0 and *gsnor1-3*, a loss-of-function GSNOR1 mutant defective in protein de-nitrosylation. Our results showed that auxin signaling was impaired in the *gsnor1-3* mutant as revealed by significantly reduced DR5-GUS/DR5-GFP accumulation and compromised degradation of AXR3NT-GUS, a useful reporter in interrogating auxin-mediated degradation of Aux/IAA by auxin receptors. In addition, polar auxin transport was compromised in *gsnor1-3*, which was correlated with universally reduced levels of PIN or GFP-PIN proteins in the roots of the mutant in a manner independent of transcription and 26S proteasome degradation. Our results suggest that S-nitrosylation and GSNOR1-mediated de-nitrosylation contribute to auxin physiology, and impaired auxin signaling and compromised auxin transport are responsible for the auxin-related morphological phenotypes displayed by the *gsnor1-3* mutant.

Key words: phytohormone cross talk, *S*-nitrosoglutathione reductase (GSNOR), S-nitrosylation, auxin signaling, auxin transport, *Arabidopsis*

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INTRODUCTION

Nitric oxide (NO) is a small gaseous hormone that plays a wide range of physiological roles across kingdoms. In plants, NO is involved in diverse functions such as stomatal closure (Neill et al., 2002), cell death, disease resistance (Durner et al., 1998; Klessig et al., 2000; Wendehenne et al., 2004; Zeidler et al., 2004; Lin et al., 2012; Ye et al., 2012), abiotic stress (Xuan et al., 2010; Fan and Liu, 2012; He et al., 2012), flowering (He et al., 2004), as well as many other processes (Lamattina et al., 2003).

S-Nitrosylation is the addition of an NO moiety to thiol groups, including those in cysteine residues in proteins, to form *S*-nitrosothiols (SNOs). S-Nitrosylation is an enzyme-independent post-translational and labile modification that can function as an on/off switch for protein activity (Stamler, 1994; Grennan, 2007; Hess and Stamler, 2012; Astier et al., 2012). In mammals, NO is synthesized from arginine and is catalyzed by three nitric oxide synthases (NOS) (reviewed in Crawford and Guo, 2005). There is also evidence that NOS-like activity is detectable in plants (Durner et al., 1998; Foissner et al., 2000). However, identification of NOS in plants has remained an enigma (Moreau et al., 2010; Fröhlich and Durner, 2011). Other routes for NO biosynthesis in plants exist and can proceed via reduction of nitrate/nitrite by nitrate reductase and the polyamine pathway, respectively (reviewed in Crawford and Guo, 2005). Plant tissues also form NO via the non-enzymatic reduction of

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apoplastic nitrite (Bethke et al., 2004). In addition, microorganisms in the soil produce NO, which may also play a role in signaling in plants (reviewed in Crawford and Guo, 2005). In cells, the extent of S-nitrosylation of proteins and other molecules is dynamic and determined by NO levels (S-Nitrosylation) and de-nitrosylation catalyzed by *S*-nitrosoglutathione reductase (GSNOR) (Liu et al., 2001; Feechan et al., 2005) and thioredoxin (Tada et al., 2008; Benhar et al., 2009; Sengupta and Holmgren, 2012).

GSNOR is a conserved protein in prokaryotes and eukaryotes and it catalyzes GSNO reduction and maintenance of cellular SNO homeostasis (Liu et al., 2001; Feechan et al., 2005). Loss of GSNOR leads to increased cellular levels of S-nitrosylated proteins (Liu et al., 2001; Feechan et al., 2005). Mouse *gsnor1* mutants show increased susceptibility to nitrosative challenge, displaying severe tissue damage, and mortality when exposed to endotoxic or bacterial challenge (Liu et al., 2004). *R* gene-mediated resistance, basal defense, and non-host resistance are all compromised in the *gsnor1-3* mutant of *Arabidopsis* (Feechan et al., 2005). In addition, *Arabidopsis* GSNOR1 also participates in cell death (Chen et al., 2009), heat stress response (Lee et al., 2008) as well as growth and development (Lee et al., 2008; Chen et al. 2009; Holzmeister et al., 2011; Kwon et al., 2012).

An increasing number of proteins have been identified as targets of S-nitrosylation; their functions are regulated by this post-translational modification (Lindermayr et al., 2005; Forrester et al., 2009; Hess and Stamler, 2012; Hu et al., 2015). In plants, the S-nitrosylated cysteine residues of some target proteins have been identified and reveal how this post-translational modification modulates protein functions (Lindermayr et al., 2006; Serpa et al., 2007; Belenghi et al., 2007; Romero-Puertas et al., 2007; Tada et al., 2008; Wang et al., 2009; Lindermayr and Durner, 2009; Lindermayr et al., 2010; Yun et al., 2011; Astier et al., 2012; Feng et al., 2013; Yang et al., 2015).

Auxin is an extensively studied plant hormone that plays a battery of roles in development such as regulation of apical dominance, lateral root formation, gravitropic responses, meristem maintenance, cell division, and cell elongation (Teale et al., 2006). Endogenous auxin levels are determined by the dynamics of auxin biosynthesis, primarily by the TAA family of amino transferases and the YUCCA (YUC) family of flavin monooxygenases (Won et al., 2011; Mashiguchi et al., 2011; Tivendale et al., 2014), auxin degradation, and auxin conjugation/de-conjugation with sugars and amino acids (Woodward and Bartel, 2005; Korasick et al., 2013).

In the auxin signaling pathway, two independent studies have demonstrated that TIR1, a nuclear F-box protein is an auxin receptor (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Upon binding of auxin, TIR1 mediates the degradation of the Aux/IAA repressors by the 26S proteasome. As a result, auxin response factor (ARF)-mediated transcription is de-repressed (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). A previously postulated auxin receptor, Auxin-binding protein 1 (ABP1), was proposed to function at both the plasma membrane and in the cytoplasm (Robert et al., 2010; Xu et al., 2010; Shi and Yang, 2011) and ABP1-mediated auxin responses were proposed as faster than TIR1-mediated auxin responses and independent of gene expression (Dahlke et al., 2010). However, a recent detailed report brings those previous studies regarding ABP1 as an essential receptor into question (Gao et al., 2015; Liu, 2015) and suggests the need for an increased focus on the TIR1 receptor.

Auxin gradients, which are established and maintained by a network of plasma membrane-localized transporters that facilitate directed auxin influx and efflux from individual cells, are also essential to auxin functions in the regulation of stem cell differentiation, gravitropic responses, and the initiation of lateral organs (Woodward and Bartel, 2005; Leyser, 2006; Petrásek and Friml, 2009). These transporters include the AUX/LAX family of auxin influx proteins (Marchant et al., 1999), the PIN-FORMED (PIN) family of auxin efflux proteins (Chen et al., 1998; Gälweiler et al., 1998; Müller et al., 1998; Geldner et al., 2001; Friml et al., 2002; Blilou et al., 2005; Wisniewska et al., 2006; Pan et al., 2009), and the P-glycoprotein/multidrug resistance B family of ATP-binding cassette transporter B-type (ABCB) proteins (Noh et al., 2001; Lewis et al., 2007; Wu et al., 2007). Mutations in the genes encoding these transporters result in dramatic defects in shoot and root architecture and gravitropism.

Cross talk between NO and auxin in controlling plant growth and development has been studied previously (Pagnussat et al., 2002, 2003; Correa-Aragunde et al., 2004; Pagnussat et al., 2004; Lanteri et al., 2006; Lombardo et al., 2006). However, the molecular mechanism of cross talk between NO and auxin in controlling plant growth and development is not well understood. Two recent reports indicate that cross talk between NO and auxin controls plant development probably through protein S-nitrosylation (Fernández-Marcos et al., 2011; Terrile et al., 2012). One study used NO inhibitors and an *Arabidopsis* NO-overproducing mutant, *nox1*, to show that NO causes root apical meristem defects and growth inhibition through compromising PIN1-dependent acropetal auxin transport (Fernández-Marcos et al., 2011). However, a second study showed that application of GSNO increased S-nitrosylation of TIR1 and resulted in enhanced TIR1-Aux/IAA interaction and Aux/IAA degradation leading to increased auxin signaling (Terrile et al., 2012). Although these studies indicate a role for S-nitrosylation in NO-auxin cross talk, additional genetic evidence is required to understand a broader role for S-nitrosylation in auxin transport and signaling in controlling plant growth and development. In this study, we confirm that loss of GSNOR1-mediated de-nitrosylation in *Arabidopsis* leads to defective growth and development and we provide genetic evidence that these defects result from compromised auxin signaling and polar transport. Our results suggest that GSNOR1-mediated de-nitrosylation is required for auxin signaling and polar auxin transport.

RESULTS

Auxin-Related Phenotypes in *gsnor1-3*

We confirmed earlier reports that loss of GSNOR1 function causes developmental defects by investigating the morphological phenotypes of *gsnor1-3* mutant under our growth conditions. In agreement with previous reports (Lee et al., 2008; Chen et al.,

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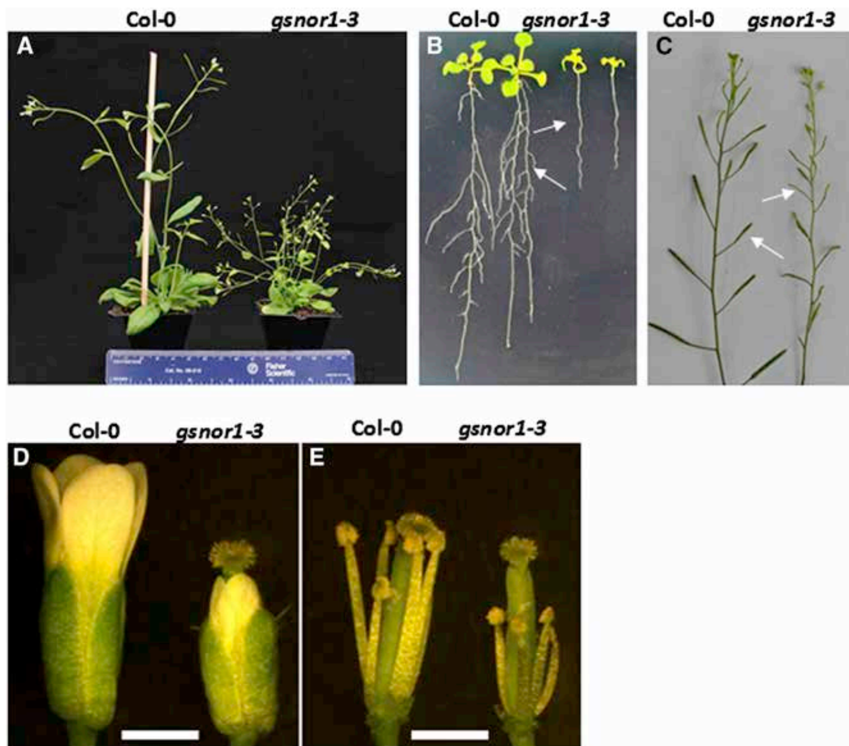

Figure 1. Morphological Defects Displayed by the *gsnor1-3* Mutant.

(A) Loss of apical dominance.

(B) Short primary roots and lack of the lateral roots (see the differences pointed by the white arrows).

(C) Reduced fertility reflected by seedless and/or short siliques (see the differences pointed by the white arrows).

(D and E) Floral phenotypes in Col-0 **(D)** and the *gsnor1-3* mutant displaying protrusion of stigma and shortness of stamen filaments **(E)**. Bar represents 1 mm.

2009; Holzmeister et al., 2011; Kwon et al., 2012), *gsnor1-3* plants displayed a wide range of developmental defects including severe bushiness and reduced stature or loss of apical dominance (Figure 1A and Figure 2A and 2B), shorter primary roots (Figure 1B and Figure 2C), fewer lateral roots (Figure 1B and Figure 2D), shorter hypocotyls (Figure 2E), and fewer rosette leaves (Figure 2F), all of which are reminiscent of auxin-related phenotypes. In addition, as reported previously (Lee et al., 2008), fertility of the *gsnor1-3* mutant was severely reduced and its siliques were significantly shorter compared with those of Col-0 (Figure 1C). Flowers of the *gsnor1-3* mutant had elongated gynoecia and shorter anther filaments compared with Col-0, leading to a physical separation of stamina from stigma (compare Figure 1D and 1E). This separation is likely the major cause of the reduced fertility of this mutant (Figure 1C and 1E; Lee et al., 2008). Some of the developmental defects observed in *gsnor1-3* plants, such as short and branched plant stature and reduced fertility, were also reported for the *Arabidopsis hot5-2* and *par2-1* mutant lines that carry two different *gsnor1* alleles (Lee et al., 2008; Chen et al., 2009; Kwon et al., 2012).

Similar Levels of Indole Acetic Acid in *gsnor1-3* and Col-0 Wild-Type

Because the *gsnor1-3* mutant phenotypes are similar to those of auxin mutants (Noh et al., 2001; Dai et al., 2006), we speculated that some of the defects might be caused by aberrant auxin biosynthesis/degradation in different tissues of different development stages. To explore the possibility, we used gas chromatography–mass spectrometry to quantify free indole acetic acid (IAA) levels in the leaves of 30-day-old plants and 12-day-old seedlings of both Col-0 and *gsnor1-3* (see Materials and Methods). As shown in Figure 3A and 3B, free IAA levels in the leaves and seedlings of *gsnor1-3* were similar to those of Col-0. As many assays were performed in the roots of seedlings (Figures 4–8), we also subdivided whole 12-day-old seedlings below the cotyledonary node and at the root-shoot junction to yield three fractions (leaves and epicotyl [labeled leaves], hypocotyl, and root) and used a sensitive isotope dilution assay based on selective reaction monitoring using gas chromatography–tandem mass spectrometry (GC–MS/MS) to determine the free IAA levels in each tissue fraction. No significant differences in IAA levels were observed between Col-0 and *gsnor1-3* mutants in any of the tissues (Figure 3C), indicating that the auxin-related developmental phenotypes observed for *gsnor1-3* plants do not result from changes in free IAA levels.

Expression of DR5-GUS or DR5-GFP Is Reduced in *gsnor1-3* Mutant

To investigate the role of GSNOR1 in auxin signaling, we monitored the expression of the *35S:DR5-GUS* and *35S:DR5-GFP* reporters, in which multiple tandem repeats of a highly active synthetic auxin response element (AuxRE) are fused upstream of a minimal 35S promoter-GUS or GFP reporter gene (Ulmasov et al., 1997; Sabatini et al., 1999). Such constructs have been widely used to indicate a local auxin maximum (Benková et al., 2003; Geldner et al., 2004). The *35S:DR5-GUS* and *35S:DR5-GFP* transgenes were independently introgressed into the *gsnor1-3* line and GUS staining and GFP florescence were visually inspected to monitor DR5-GUS and DR5-GFP accumulation in the roots of 7-day-old seedlings. As shown in Figure 4A, GFP fluorescence was clearly reduced in the roots of the *gsnor1-3* mutant relative to Col-0 in the absence of exogenous IAA (compare regions indicated by the white arrows in Figure 4A). In addition, we detected increased GUS staining in the presence of IAA in both Col-0 and *gsnor1-3* in a concentration-dependent manner (compare the regions indicated by the red and black arrows in the upper and lower panels in Figure 4B). However, the degree of GUS staining in *gsnor1-3* roots was consistently lower than in roots of Col-0 in either the absence or presence of IAA (Figure 4B). Similar analyses have been used previously for investigating the role of LEAFY in controlling auxin response (Li et al., 2013) in the absence of changing auxin levels.

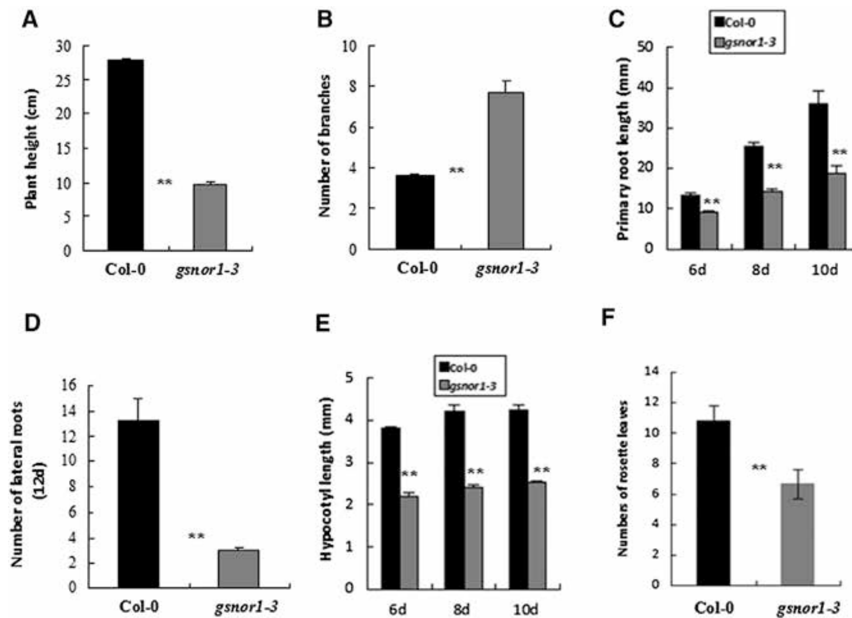

Figure 2. Growth Phenotypes of the WT Col-0 and the *gsnor1-3* Mutant Plants.

Shown are comparisons of:
(A) the height of 40-day-old plants (cm).
(B) the number of branches of 40-day-old plants.
(C) the length of primary roots (mm) of seedlings on different days as indicated on the X axis.
(D) the number of lateral roots on 12-day-old seedlings.
(E) the length of hypocotyls (mm) between Col-0 (black) and the *gsnor1-3* seedlings (gray) at different days as shown on the X axis.
(F) the number of rosette leaves.
**Significant differences between Col-0 and *gsnor1-3* at the 0.01 level by Student's *t*-test.

Auxin-Dependent Lateral Root Formation Is Impaired in *gsnor1-3* Mutant

Lateral root induction in the presence of exogenous auxin is often used as an assay for measuring auxin responses. To test whether auxin signaling was altered in the *gsnor1-3* plants, 7-day-old seedlings of both Col-0 and *gsnor1-3* grown on 1/2 Murashige and Skoog (MS) agar plates were transferred to 1/2 MS agar plates containing various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and grown for an additional 5 days. Under our growth conditions, lateral root formation was induced in Col-0 seedlings by 2,4-D in a concentration-dependent manner (Figure 5). In the absence of 2,4-D, the average number of lateral roots of Col-0 was only 2.9 ± 1.4. However, in the presence of 0.02 and 0.06 μM 2,4-D, the average numbers of lateral roots increased to 7.3 ± 1.4 and 10 ± 1.3, respectively. However, no lateral root formation was induced in *gsnor1-3* seedlings at these 2,4-D concentrations and the lateral root formation was induced only when 2,4-D was increased to 0.5 μM (Figure 5), suggesting that auxin signaling is impaired but not completely abolished in the *gsnor1-3* mutant.

Exogenous GSNO Treatment Reduces GUS Accumulation in Wild-Type *DR5*:GUS Transgenic Plants

Terrile et al. (2012) reported that in the presence of IAA, the NO donor sodium nitroprusside (SNP) substantially increased GUS reporter expression in *DR5*-GUS and *BA3*-GUS transgenic plants, which carried an auxin response element fused to GUS. However, we observed a significantly reduced accumulation of *DR5*-GUS and *DR5*-GFP in the roots of the *gsnor1-3* mutant compared with wild-type (WT) Col-0 (Figure 4), suggesting that loss of GSNOR1 function and exogenous NO donor might have different effects on DR5-GUS expression. To corroborate the difference, we treated *DR5*-GUS/Col-0 transgenic plants with different concentrations of GSNO in combination with different concentrations of IAA. A substantial increase in GUS expression was not observed in the *DR5*-GUS/Col-0 plants that received simultaneous application of IAA and GSNO (Figure 6). Instead, GUS expression was clearly reduced at root elongation zones of *DR5*-GUS/Col-0 plants treated by 10 μM and 100 μM GSNO in the presence of 0.1 and 1 μM IAA, respectively (Figure 6, compare the areas indicated by black arrows with those indicated by red arrows). These observations are consistent with our genetic results that showed *DR5*-GUS expression was reduced in the *gsnor1-3* mutant (Figure 4) and with a previous report showing that *DR5*-GUS expression was attenuated both by NO donor treatments and in a NO-overproducing mutant *nox1*/*cue1* (Fernández-Marcos et al., 2011).

IAA-Induced Degradation of Heat Shock-Induced AXR3NT-GUS Is Compromised in *gsnor1-3* Plants

Transgenic *Arabidopsis* plants carrying *HS*:*AXR3NT-GUS* (HS, heat shock promoter) provide a useful reporter for investigating auxin-mediated degradation of Aux/IAA by the auxin receptor, AFB F-box proteins (Gray et al., 2001). AXR3, a member of the Aux/IAA family, is a target for auxin receptor-mediated degradation. After heat shock, degradation of AXR3NT-GUS occurs in the presence of auxin (Gray et al., 2001). To further investigate the effect of loss-of-function of *GSNOR1* on auxin signaling, we introduced *HS:AXR3NT-GUS* into the *gsnor1-3* background and compared GUS staining in WT and *gsnor1-3* roots following heat shock (2 h) in the presence and absence of 5 μM IAA. As shown in Figure 7A, after heat shock for 2 h, the IAA-induced degradation of AXR3NT-GUS was delayed in the *gsnor1-3* mutant compared with Col-0 even though the initial GUS staining was lower in *gsnor1-3* plants than in Col-0 (Figure 7A, see arrow). The GUS staining result was further confirmed by a parallel GUS activity assay (Figure 7B), further suggesting that TIR1-mediated auxin signaling is compromised in the *gsnor1-3* mutant.

The Increased Accumulation Level of AXR3NT-GUS in *gsnor1-3* Relative to Col-0 Is Not Correlated with its Transcript Level

To examine whether the delayed degradation of AXR3NT-GUS in *gsnor1-3* versus Col-0 plants as shown in Figure 7A and 7B, is controlled at the transcription level, qRT–PCR was performed for the same set of samples using a pair of GUS primers. The qRT–PCR results indicated that the higher accumulation level of

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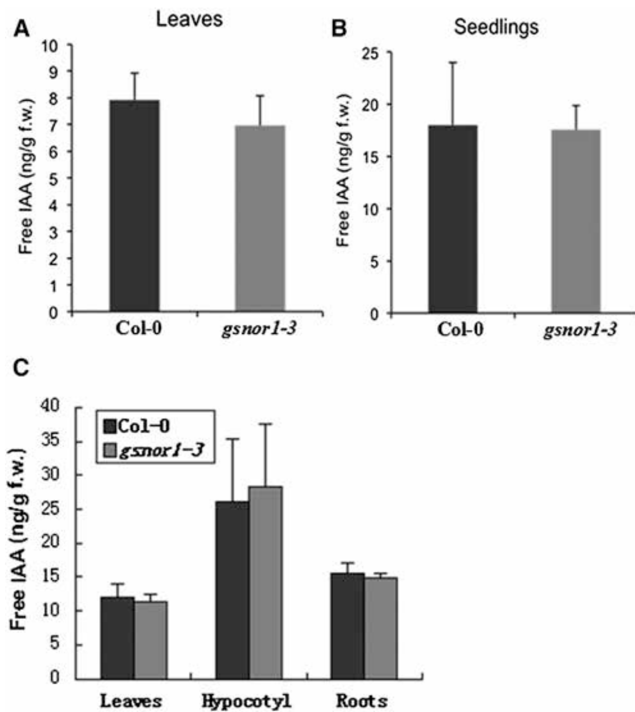

Figure 3. The *gsnor1-3* Mutant Contains Normal Levels of Free IAA.

(A) Free IAA levels were measured in 30-day-old Col-0 and *gsnor1-3* plants.

(B) Free IAA levels were measured in 12-day-old Col-0 and *gsnor1-3* seedlings.

(C) Free IAA levels were measured in 12-day-old seedlings subdivided below the cotyledonary node and at the root-shoot junction to yield three fractions (leaves and epicotyl [labeled 'leaves'], hypocotyl, and root). Errors bars represent the SE for four to six independent samples. The differences in **(C)** are not significant ($p > 0.1$ for Student 's *t*-tests).

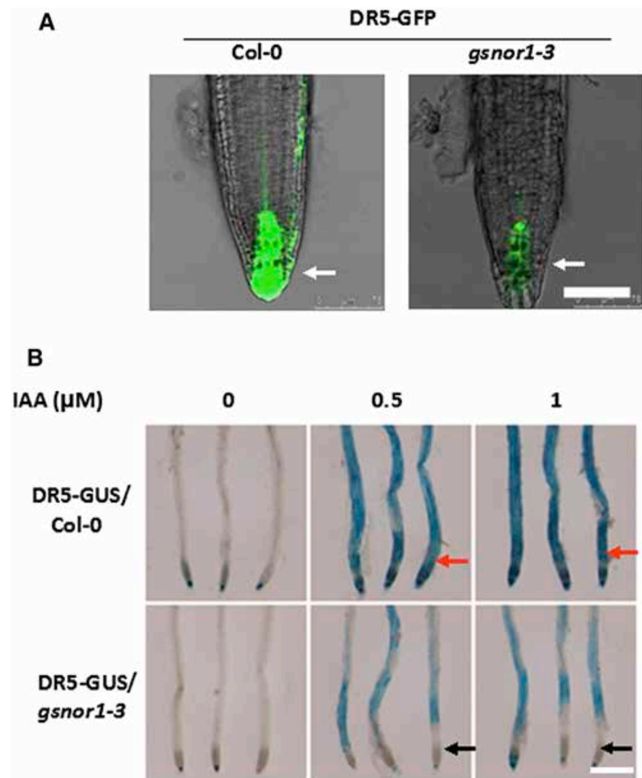

Figure 4. Auxin Sensitivity Is Reduced in the Roots of *gsnor1-3* Mutant Seedlings.

(A) Comparison of DR5-GFP expression between Col-0 and *gsnor1-3* (compare the white arrow-pointed regions). Bar represents 75 μm.

(B) DR5-GUS induction by different concentrations of IAA between Col-0 and *gsnor1-3* mutant (compare the regions pointed by the red arrows in Col-0 with those pointed by the black arrows in *gsnor1-3*). Upper panel, DR5-GUS/Col-0; lower panel, DR5-GUS/*gsnor1-3*. Seven-day-old seedlings were used in these experiments. The experiments were repeated three times with similar results. Bar represents 0.5 mm.

AXR3NT-GUS in *gsnor1-3* relative to Col-0 plants was not correlated with its transcription level as the transcript of *AXR3NT-GUS* was reduced in *gsnor1-3* plants relative to Col-0 after a 40-min induction by 5 μM IAA (Figure 7C).

GSNO Inhibits *HS*:AXR3NT-GUS Degradation

Terrile et al. (2012) reported that degradation of AXR3NT-GUS induced by auxin treatment was substantially promoted by 10 μM GSNO treatment. To confirm this result, we applied different concentrations of GSNO to *HS*:AXR3NT-GUS/Col-0 plants treated with different concentrations of IAA after heat shock. To our surprise, we found that, instead of promoting the IAA-induced AXR3NT-GUS degradation, treatments of 1 and 10 μM GSNO significantly delayed the degradation of AXR3NT-GUS induced by different concentrations of IAA (Figure 8). Together, these results clearly indicated that GSNO had a negative effect on auxin signaling in the roots.

Polar Auxin Transport Is Compromised in the *gsnor1-3* Mutant

Auxin signaling and polar auxin transport are closely intertwined processes. To test whether auxin transport is compromised in the *gsnor1-3* mutant, we measured basipetal [^{3}H]IAA transport from the shoot apex to the root of 7-day light-grown seedlings of Col-0 and *gsnor1-3*. Our results showed that basipetal [^{3}H] IAA transport in *gsnor1-3* seedlings was reduced by 1.9-fold compared with that in WT (Figure 9A), indicating that auxin transport is also compromised in the *gsnor1-3* seedlings. A similar result was also previously observed in the NO-overproducing mutant, *cue1/nox1* (Fernández-Marcos et al., 2011), suggesting that the mutants were either defective in de-nitrosylation or overproducing NO leading to compromised polar auxin transport.

If IAA transport capacity in the *gsnor1-3* stem is reduced, auxin levels in the basal segment of the stems should be reduced (Bennett et al., 2006). To test this, we examined *DR5*-GUS activity in the basal stem for both *DR5-GUS*/Col-0 and *DR5-GUS/gsnor1-3* plants. GUS activity was clearly visible in the basal stem and basal stem vasculature of *DR5-GUS*/Col-0 (Figure 9B and 9C, left), but barely detectable in the same tissues of *DR5-GUS/gsnor1-3* plants (Figure 9B and 9C, right), suggesting that the flow of auxin transported from the sites of auxin biosynthesis was impaired in *gsnor1-3* plants. Taken together, these results indicate that polar auxin transport and polar auxin transport-dependent auxin distribution are impaired in *gsnor1-3* plants.

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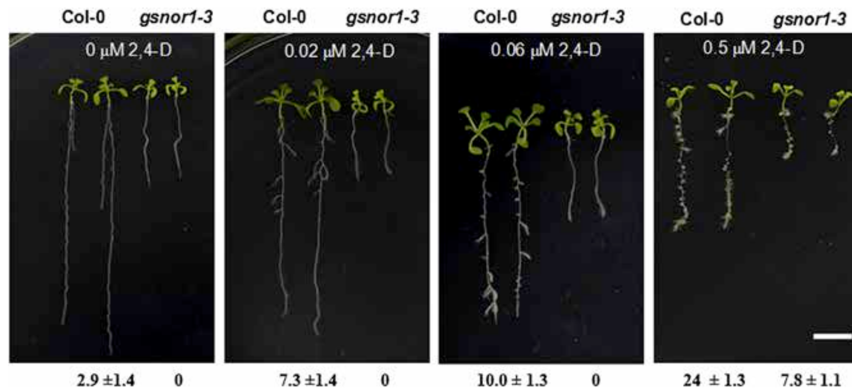

Figure 5. Comparison of Lateral Root Induction by 2,4-D between Col-0 and *gsnor1-3* Seedlings.

Seven-day-old seedlings of Col-0 and *gsnor1-3* were grown on 1/2 MS medium and then transferred to 1/2 MS agar plates containing different concentrations of 2,4-D as indicated, and then they remained on these plates for an additional 5 days before photographs were taken. The average numbers of lateral roots of Col-0 and *gsnor1-3* seedlings are shown below the figure. Bar represents 0.5 cm.

The Accumulation Levels of Both Endogenous PIN Proteins and Transgenic Expressed GFP-PIN Proteins Are Significantly Reduced in the Roots of *gsnor1-3* Mutant

PIN proteins are major players in auxin transport. It has been reported that both exogenous NO donor treatment and mutation in *NOX1* (NO over expresser), which leads to over accumulation of cellular NO in *Arabidopsis* (He et al., 2004), caused decreased PIN1-GFP fluorescence (Fernández-Marcos et al., 2011). However, NO donor treatments did not significantly reduce the level of GFP-PIN2 in the $PIN2_{pro}$:*GFP-PIN2* line (Fernández-Marcos et al., 2011). To test whether *gsnor1-3* mutation has a similar effect, as noted in the *nox1* mutant (loss of denitrosylation versus NO overproduction) on PIN accumulations, we compared the endogenous levels of PIN1 and PIN2 in both the WT and *gsnor1-3* mutant backgrounds using immunofluorescence microscopy. As shown in Figure 10A, the levels of endogenous PIN1 and PIN2 in the *gsnor1-3* roots were significantly reduced relative to WT even though the polarities of both PIN1 and PIN2 were not altered in the *gsnor1-3* mutant.

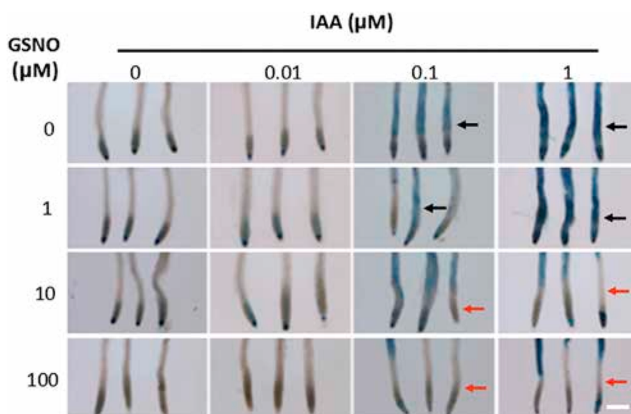

Figure 6. GSNO Inhibits DR5-GUS Expression Induced by IAA in Col-0 in a Concentration-Dependent Manner.

DR5-GUS expression was induced by IAA in a concentration-dependent manner (upper panel). The induction of DR5-GUS was strongly inhibited by 10 and 100 μM GSNO treatments (3rd and 4th panels). Red arrows point to the regions with reduced accumulation of DR5-GUS in comparison with the regions indicated by black arrows. Seven-day-old DR5-GUS/Col-0 seedlings were used for this experiments. Bar represents 0.4 mm.

To test whether the levels of other members of the PIN family of proteins are similarly reduced in *gsnor1-3* plants, the constructs of *GFP-PIN1, GFP-PIN2*, *GFP-PIN3*, *GFP-PIN4*, and *GFP-PIN7* driven by their native promoters were introgressed into the *gsnor1-3* background. Similar to the immunofluorescence results (Figure 10A), GFP-PIN1 and GFP-PIN2 fluorescence was reduced in *gsnor1-3* plants compared with Col-0 (Figure 10B), indicating that the levels of both endogenous PIN1 and PIN2 as well as transgenically expressed GFP-PIN1 and GFP-PIN2 were all reduced in the *gsnor1-3* mutant. Similarly, the levels of GFP-PIN3, GFP-PIN4, and GFP-PIN7 were also reduced in *gsnor1-3* plants relative to WT Col-0 (Figure 10B). Together, our results suggest that the compromised polar auxin transport seen in *gsnor1-3* plants (Figure 9A) is most likely due to reduced accumulation of various PIN proteins (Figure 10A and 10B) in addition to PIN1 (Fernández-Marcos et al., 2011).

The Accumulation Levels of Different Members of GFP-PIN Proteins in the Roots Are Not Correlated with the Expression of *GFP-PINs*

To gain insight into the molecular mechanism of the reduced fluorescence of various GFP-PINs, qRT–PCRs were performed using a pair of *GFP* primers and total RNAs isolated from roots of WT and *gsnor1-3* seedlings expressing different *GFP-PINs*, respectively. As shown in Figure 10C, the transcript level was reduced in *gsnor1-3* plants relative to Col-0 for *GFP-PIN1* and *GFP-PIN7.* However, the reduction of transcript of *PIN1* was not proportional to the reduction of intensity of GFP fluorescence (compare Figure 10B and 10C). Moreover, while the expression of *GFP-PIN2* and *GFP-PIN3* was significantly increased in *gsnor1-3* compared with Col-0 (Figure 10C), the fluorescence intensities of GFP-PIN2 and GFP-PIN3 were significantly reduced in *gsnor1-3* (Figure 10B), indicating that the regulation of PINs in *gsnor1-3* is not at the transcriptional level.

Assembly of the SCF^{TIR1} protein Degradation Complex Is Not Disrupted by GSNO Treatment

To test whether the assembly of the SCF^{TIR1} complex is affected by GSNO, we used IAA7 tagged with glutathione *S*-transferase (GST-IAA7) to pull down the components in SCF^{TIR1} complex *in vitro* from transgenic plants carrying dexamethasone (Dex)-inducible *TIR1-Myc* in the presence or absence of GSNO. The SCF^{TIR1} complex consists of F-box protein TIR1, ASK1, Culin, and RXB (Dharmasiri et al., 2005).

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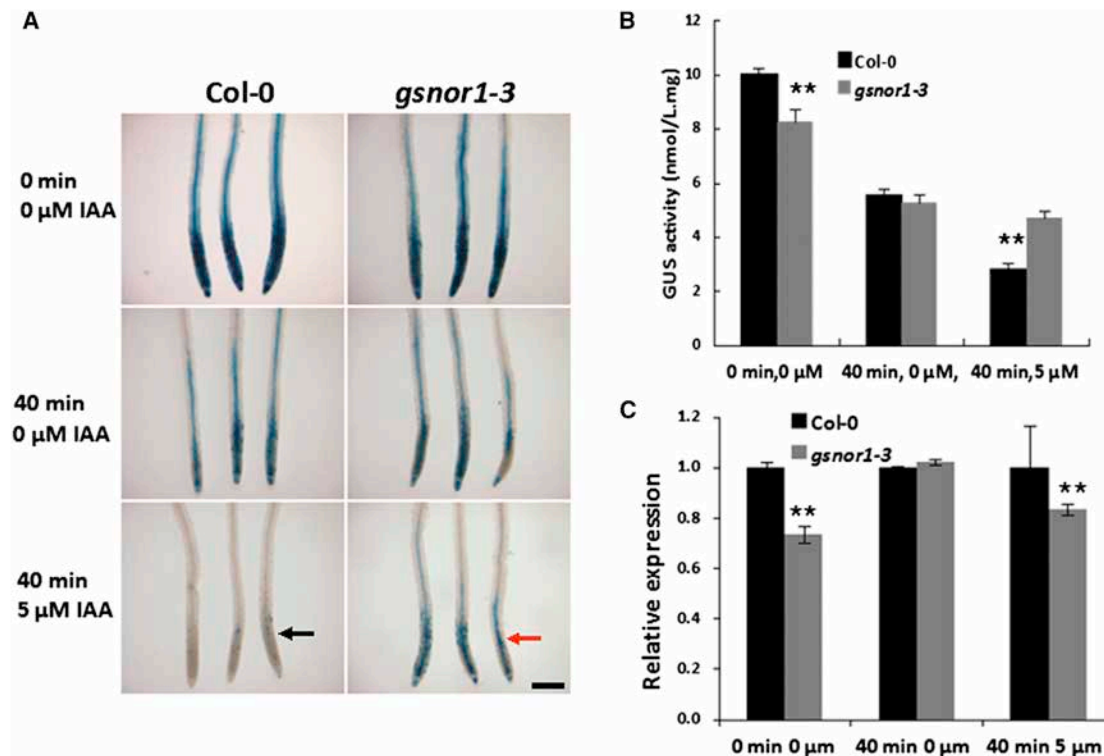

Figure 7. Auxin-mediated Degradation of AXR3NT-GUS Is Compromised in the Roots of *gsnor1-3* Mutant Seedlings.

(A) Degradation of AXR3NT-GUS was delayed in the *gsnor1-3* mutant compared with Col-0 seedlings. Seven-day-old seedlings of both HT:AXR3NT-GUS/Col-0 and HT:AXR3NT-GUS/*gsnor1-3* were heat shocked at 37°C for 2 h and then transferred to room temperature in growth medium containing 0 or 5 μM IAA for 40 min. DR5-GUS expression was visualized by GUS staining. A compromised degradation of AXR3NT-GUS in *gsnor1-3* seedlings compared with Col-0 seedlings was observed (compare the regions indicated by the red arrow and the black arrow). Bar represents 0.4 mm.

(B) The GUS activity was measured in a parallel experiment as in **(A)**.

(C) qRT–PCR analysis of AXR3NT-GUS expression in both Col-0 and *gsnor1-3* seedlings using a pair of GUS primers. *Actin2* was used a reference gene. **Significant differences between Col-0 and *gsnor1-3* seedlings at the 0.01 level as determined by Student 's *t*-test.

Antibodies raised against Myc, Culin, and RXB were used to perform western blot analyses on the GST-IAA7 pull-down extracts. It appeared that GSNO treatment did not disrupt the assembly of the SCF^{TIR1} complex as the presence of TIR1-Myc, Culin, and RXB were detected in the pull-down extracts (Figure 11A), indicating that they are present in the same complexes. Interestingly, the results indicate that GSNO treatment enhanced the amount of TIR1-Myc in the pull-down extracts, consistent with the enhanced Aux/IAA degradation observed by Terrile et al. (2012).

The Overall Level of Protein S-Nitrosylation Is Increased in *gsnor1-3* Mutant

We postulated that the impaired auxin signaling and polar auxin transport in *gsnor1-3* mutant could be partially, if not fully, due to S-nitrosylation of key components participating in these two processes. To provide evidence of such changes, we performed a biotin switch assay to compare the level of protein S-nitrosylation between Col-0 and *gsnor1-3* lines. Consistent with the results obtained by Tada et al. (2008), the overall level of S-nitrosylated proteins was significantly increased in the *gsnor1-3* mutant compared with Col-0 (Supplemental Figure 1, see arrows), suggesting that one or more components in auxin signaling or transport might be regulated by S-nitrosylation.

The Accumulation of GFP-PINs Is Independent of 26S Proteasome-Mediated Degradation

It has been reported previously that the NO-dependent reduction in PIN1 protein levels does not require 26S proteasome activity (Fernández-Marcos et al., 2011). Consistent with this prior report, our inhibitor results showed that the fluorescence intensities of GFP-PIN1, GFP-PIN2, GFP-PIN3, and GFP-PIN7 were not increased in the presence of MG132, a potent inhibitor of 26S proteasome machinery (Supplemental Figure 2). This result indicates that the degradation of various GFP-PINs is independent of 26S proteasome machinery.

Gravitropic Response Is Compromised in *gsnor1-3* Mutant

Compromised polar auxin transport usually results in delayed gravitropic response (Rashotte et al., 2000) and PIN2 plays a critical role in polar auxin transport in the root (Chen et al., 1998). To test whether the gravitropic response was altered in the *gsnor1-3* mutant, vertically grown 7-day-old seedlings of both Col-0 and the *gsnor1-3* mutant on 1/2 MS agar plates were turned 90° and placed horizontally to induce a gravitropic response. The curvatures of the roots were measured during the course of the gravitropic induction. Consistent with decreased polar auxin transport (Figure 9A), the gravitropic

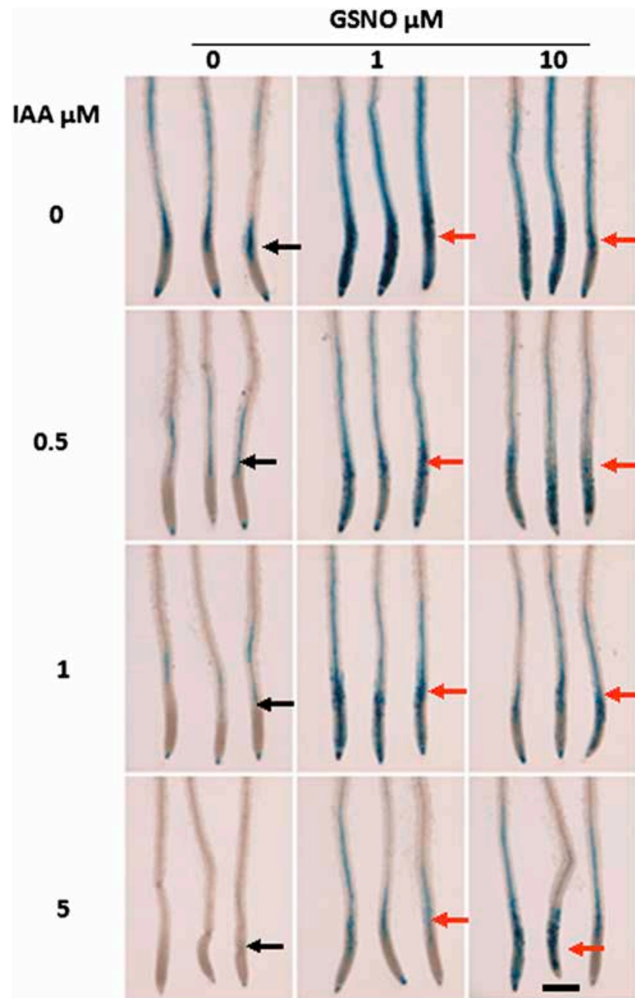

Figure 8. GSNO Treatments Inhibited Degradation of AXR3NT-GUS.

Seven-day-old seedlings of HT:AXR3NT-GUS/Col-0 were heat shocked at 37°C for 2 h and then transferred to medium at room temperature containing 0, 0.5, 1, and 5 μM IAA in combination with 0, 1, and 10 μM GSNO for 40 min. AXR3NT-GUS expression was visualized by GUS staining. A compromised degradation of AXR3NT-GUS was observed in the presence of 1 and 10 μM GSNO regardless of IAA concentration (compare the regions indicated by the red and the black arrows). Bar represents 0.4 mm.

response was clearly delayed in the *gsnor1-3* seedlings compared with Col-0 (Figure 11B).

DISCUSSION

Loss of *GSNOR1* results in pleotropic developmental phenotypes (Figure 1 and Figure 2; Feechan et al., 2005; Lee et al., 2008; Chen et al., 2009; Holzmeister et al., 2011; Kwon et al., 2012), many of which are reminiscent of phenotypes displayed by the mutants defective in auxin physiology. Here, we further investigated the potential link between auxin and S-nitrosylation. We observed a reduced expression of DR5-GUS or DR5-GFP in the roots in the absence of exogenous IAA in *gsnor1-3* relative to Col-0 seedlings (Figure 4), suggesting that auxin physiology is impaired in this mutant. The fact that IAA levels are similar in *gsnor1-3* and Col-0 WT plants (Figure 3) indicates that the auxin-related phenotype of the *gsnor1-3* mutant is not due to an altered auxin level. The reduced responsiveness of DR5-GUS to exogenous IAA clearly showed, however, that auxin signaling is severely impaired in *gsnor1-3* mutant plants (Figure 4B). This reduced responsiveness of the DR5-GUS phenotype is correlated with the impaired lateral root induction by exogenous IAA seen with this mutant (Figure 5).

In the presence of exogenous auxin, the degradation of AXR3NT-GUS depends on an intact auxin signaling pathway. As the degradation of AXR3NT-GUS was delayed in *gsnor1-3* plants compared with that seen in Col-0 (Figure 7), it is most likely that the TIR1-mediated auxin signaling pathway was compromised in the *gsnor1-3* mutant, which is inconsistent with the data showing that NO donor treatments enhances auxin sensitivity through promoting TIR1-Aux/IAA interaction and thus Aux/IAA degradation (Terrile et al., 2012). These results raise the possibility that the high level of protein S-nitrosylation resulting from an artificially applied NO donor may not recapitulate the high S-nitrosylation situation *in planta*. We observed an increased amount of TIR-myc in our pull-down assay using IAA7-GST from protein extracts treated with GSNO (Figure 11A), consistent with the conclusion that GSNO treatment can enhance the TIR1–AUX/IAA interaction (Terrile et al., 2012). However, instead of an increased auxin response (Terrile et al., 2012), a reduced auxin response was observed both in the roots of *gsnor1-3* mutant and GSNO-treated Col-0 seedlings (Figure 7 and Figure 8), suggesting that either auxin signaling is impaired downstream of TIR1-AUX/IAA interaction or the enhanced TIR1-IAA7 interaction has a negative impact on auxin signaling. Both RUB and CUL1 were present in the pull-down assay, indicating that the assembly of SCF^{TIR1} protein degradation complex was not disrupted by GSNO (Figure 11A) and the impairment of AXR3NT-GUS degradation may occur after assembly of the SCF^{TIR1} complex. We currently do not know the exact reason for the differences between our results and the results reported by Terrile et al. (2012). Given the promiscuous nature of S-nitrosylation, TIR1 may not be the only protein that is targeted for S-nitrosylation in the auxin signaling pathway. Multiple proteins could determine the final outcome of AXR3NT-GUS degradation in the presence of higher levels of GSNO. It is possible that the functions of other components in addition to TIR1 in the SCF^{TIR1} complex and the components in the 26S proteosome pathway are targeted and thus inhibited by S-nitrosylation. This idea is supported by a report that ubiquitin-conjugating enzyme E2 and 26S proteasome non-ATPase regulatory subunit 9 are targets of S-nitrosylation in human cells (Liu et al., 2010). The alternative explanation for reduced accumulation of DR5-GUS (Figure 4) and delayed degradation of AXR3NT-GUS in the *gsnor1-3* mutant (Figure 7) is that GSNOR1 may play an additional role other than S-nitrosylation, which is critical to auxin signaling or transport or both.

In addition to auxin signaling, polar auxin transport was also compromised in *gsnor1-3* plants (Figure 9). The reduced polar auxin transport observed in *gsnor1-3* plants was most likely a result of globally reduced levels of different members of the PIN protein family (Figure 10A and 10B). A recently published study observed a similarly reduced accumulation of *PIN1pro*:GFP-PIN1 in the *nox1/cue1* mutant that correlated

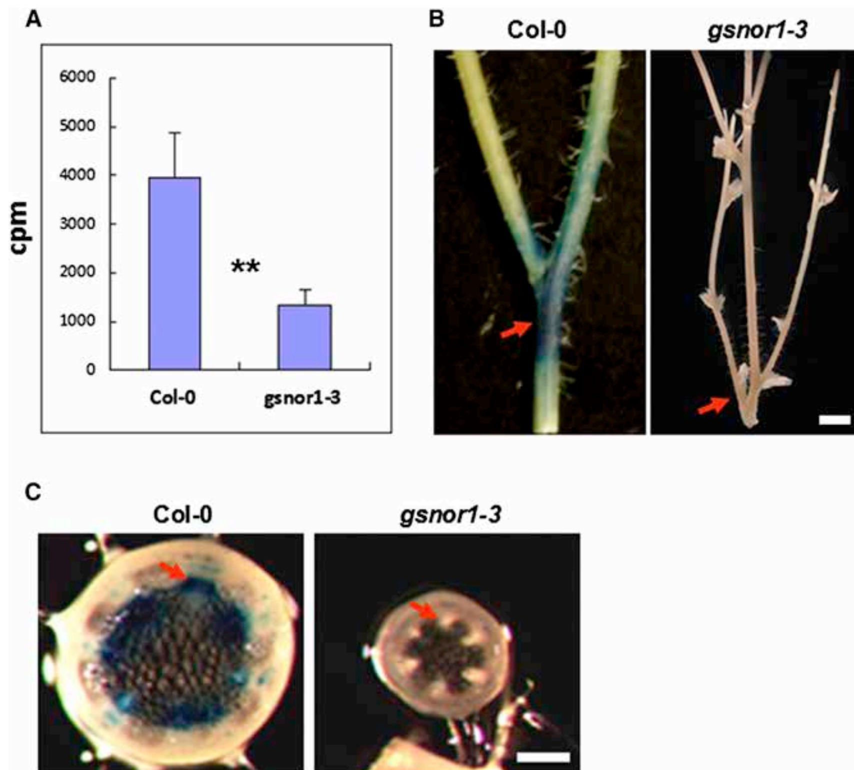

Figure 9. Polar Auxin Transport Is Compromised in the *gsnor1-3* Mutant Seedlings and the DR5-GUS Expression Is Reduced in the Basal Stems of Mature *gsnor1-3* Mutant Plants.

(A) Basipetal transport of [^{3}H]IAA from the shoot apex to the root was measured in 7-day light-grown seedlings of Col-0 and *gsnor1-3* lines. Each assay used 10 seedlings. **p < 0.01 (Student's t-test). CPM, counts per minute.

(B) Comparison of DR5-GUS expression in the basal stems of Col-0 and *gsnor1-3* plants (30-day-old mature plants). Bar represents 1 mm.

(C) DR5-GUS expression in the vasculatures of the basal stems of Col-0 (left) and *gsnor1-3* (right) plants. Bar represents 400 μm.

See the differences pointed by the red arrows in **(B)** and **(C)**.

with reduced polar auxin transport (Fernández-Marcos et al., 2011), suggesting that either defects in cellular de-nitrosylation or excessive accumulation of NO has a similar effect on PIN1 accumulation (Figure 10A and 10B; Fernández-Marcos et al., 2011). However, the effect of the *nox1/cue1* mutation on *PIN2pro*:GFP-PIN2 accumulation was not shown in that prior report and the NO donor treatments did not result in a significant reduction of *PIN2pro*:GFP-PIN2 fluorescence in Col-0 plants (Fernández-Marcos et al., 2011). Contrary to the results obtained using the NO donor in Col-0, both endogenous PIN2 and ectopic expressed *PIN2pro*:GFP-PIN2 levels were significantly reduced in the roots of *gsnor1-3* plants (Figure 10A and 10B), suggesting that treatment with exogenous NO donors and loss of *GSNOR1* have differential effects on PIN2 accumulation in the roots. The alternative explanation is that exogenous NO donors may not recapitulate the physiological situation of nitrosative stress in the *gsnor1-3* mutant.

The phenotype of the *gsnor1-3* mutant differs from the *nox1/cue1* mutant in branching properties and in lateral root/root hair formation (Figure 1; Fernández-Marcos et al., 2011). The *gsnor1-3* plants were highly branched and lacked lateral roots (Figure 1B), whereas the *nox1* mutant had normal branches and root hairs (Fernández-Marcos et al., 2011). These different phenotypes indicate that NO overproduction and lack of de-nitrosylation (loss of GSNOR1), both of which result in increased level of protein S-nitrosylation, lead to different effects on plant development. This may in part be due to the subcellular location of NO actions. GSNOR1 has both nuclear and cytoplasmic localizations (data not shown), but NOX1/CUE1 is exclusively chloroplast localized (Streatfield et al., 1999). As a result, the subset of proteins being S-nitrosylated in *gsnor1-3* and *nox1/cue1* mutants are likely different, or the extent of S-nitrosylation of shared target proteins could be different and thus lead to the different phenotypes.

Since the reduced PIN protein levels are not regulated at the transcriptional level (Figure 10) and are independent of the 26S proteasome-degradation machinery (Supplemental Figure 1), it is possible that their regulation is at the level of protein synthesis. This is supported by the fact that 15 proteins involved in protein synthesis have been identified as targets of S-nitrosylation in humans (Liu et al., 2010) and this is also consistent with our results that the accumulation levels GFP-PIN2 and GFP-PIN3 (Figure 10B) were significantly lower in *gsnor1-3* plants than in Col-0 even though their transcripts were significantly higher in *gsnor1-3* (Figure 10C). Intriguingly, the degradation of AXR3NT-GUS was also delayed in the *gsnor1-3* mutant relative to Col-0 (Figure 7A and 7B) even though the transcript level was reduced (Figure 7C), raising a possibility that protein synthesis and auxin-mediated degradation are both compromised in *gsnor1-3* plants.

The activities of many ion channels in animals, including Na^+ and Ca^{2+} channels, are regulated by S-nitrosylation (Xu et al., 1998; Renganathan et al., 2002) and S-nitrosylation modifies an outward-rectifying K^+ channel in *Vicia faba* (Sokolovski and Blatt, 2004). The regulation of ion transport proteins by S-nitrosylation suggests the possibility that other transporter proteins, including auxin carriers (including efflux and influx carriers), could be similarly regulated by S-nitrosylation. Given the non-specific nature of S-nitrosylation, it is also possible that the physiology regulated by other hormones might also be affected. The overall phenotype of the *gsnor1-3* mutant might result from impairment of multiple hormone pathways. The stigma protruding phenotype of the *gsnor1-3* mutant is also displayed in the *coi1* and *jaz1* mutants (Xie et al., 1998; Chini et al., 2007; Thines et al., 2007), both of which are key players in the JA signaling pathway. Since COI1 is a F-box protein similar to TIR1, it is likely that COI1 is also targeted by S-nitrosylation. Recently, it has been shown that *S*-nitrosylation of AHP1 at Cys 115 leads to repression of cytokinin signaling (Feng et al., 2013). This

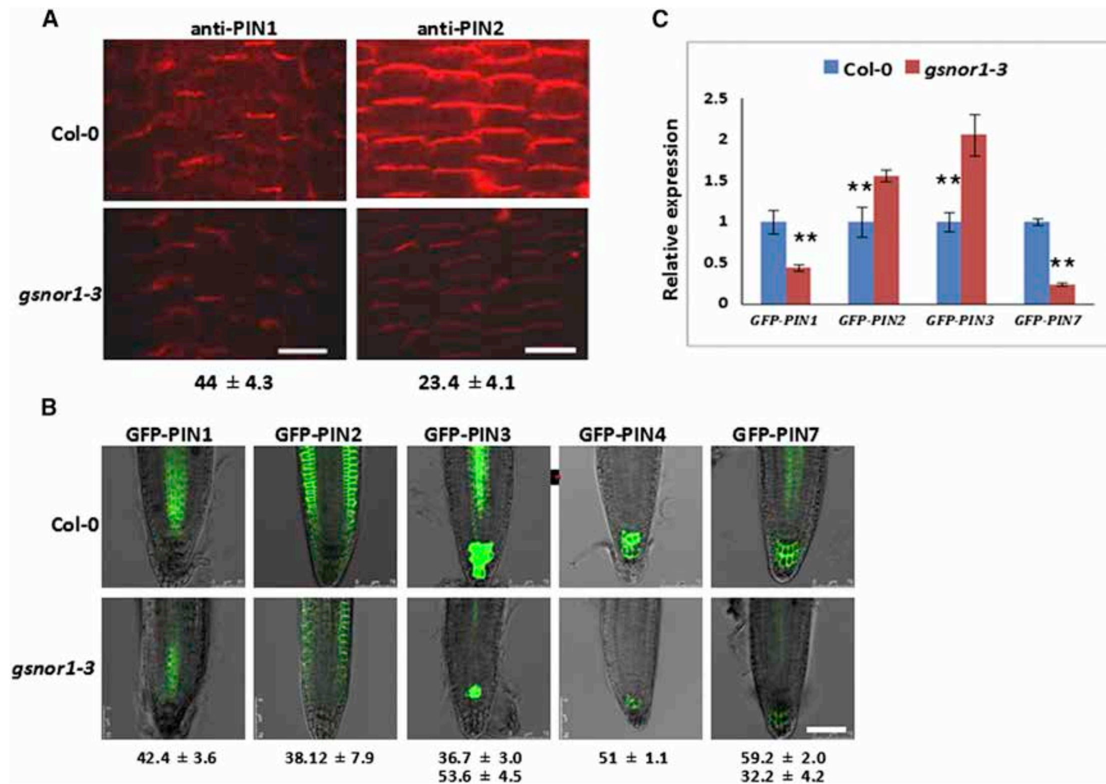

Figure 10. The Levels of Multiple PIN Proteins Are Universally Reduced in the Roots of *gsnor1-3* Mutant Seedlings in a Manner Independent of Transcription.

(A) Both PIN1 and PIN2 levels were significantly reduced in *gsnor1-3* mutant. Immunolocalizations of PIN1 and PIN2 were performed using anti-PIN1 and anti-PIN2 at 1:50 dilution and anti-rabbit-CY3 was used as secondary antibody (1:100) to visualize PIN1 and PIN2. Images were captured using Leica confocal laser scanning microscopy and the intensities of fluorescent signals were measured using ImageJ. The relative fluorescence intensities are presented as percentages of WT controls and shown below the figures. Bars represent 10 μm.

(B) The levels of multiple members of PIN proteins fused with GFP are universally reduced in the roots of *gsnor1-3* seedlings. GFP-PIN2, GFP-PIN3, GFP-PIN4, and GFP-PIN7 constructs driven by their native promoters were introgressed into *gsnor1-3* mutant by crossing with transgenic Col-0 lines that were expressing these constructs. Images were captured using Leica confocal laser scanning microscopy and shown are the merged images of the GFP channel and the bright light channels. The intensities of fluorescent signals were measured using ImageJ. The relative fluorescence intensities of *gsnor1-3* are presented as percentages of WT controls and shown below the figures. The numbers in the upper layer for GFP-PIN3 and GFP-PIN7 are relative GFP intensities in stelle cells and those in the lower row are relative intensities in columella cells. Bar represents 75 μm.

(C) qRT–PCR analysis of the expression of GFP-PIN1, GFP-PIN2, GFP-PIN3, and GFP-PIN7 in both Col-0 and *gsnor1-3* seedlings using a pair of GFP primers. Actin2 was used as a reference gene. **Significant differences for expression between Col-0 and *gsnor1-3* seedlings at the 0.01 level by Student's *t*-test.

observation seems to conflict with the highly branched (loss of apical dominance) (Figure 1A) and significantly delayed senescence phenotypes displayed by the loss of *GSNOR1* mutants (Lee et al., 2008; Chen et al., 2009; Kwon et al., 2012) as cytokinin promotes axillary bud growth and delays the senescence (Campbell et al., 2008). It is highly possible that the phenotype displayed by the *gsnor1-3* mutant might result from an altered ratio of auxin and cytokinin responses. The ability of NO to mediate opposite processes should likely not be viewed as conflicting data but rather as an illustration of the complexity of NO signaling (Wendehenne et al., 2014).

Several studies suggest that redox homeostasis plays an important role in auxin physiology (Joo et al., 2001; Wang et al., 2007; Bashandy et al., 2010; Cheng et al., 2011). Joo et al. (2001) reported that the generation of ROS plays a role in root gravitropism and ROS may function as a downstream component in auxin-mediated signal transduction. Thioredoxin (TRX) and glutaredoxin (GRXS) are key regulators of redox homeostasis. A TRX triple loss-of-function mutant displays a phenotype reminiscent of several mutants affected in auxin transport or biosynthesis, and auxin transport capacities and auxin levels are perturbed in the mutant (Bashandy et al., 2010). Both cytosolic and mitochondrial thioredoxins play key roles in protein denitrosylation (Tada et al., 2008; Benhar et al., 2008). Glutaredoxin loss-of-function mutants, such as *atgrxs17*, also display auxin-related phenotypes and have impaired auxin sensitivity and transport (Cheng et al., 2011). Salicylic acid inhibits auxin responses through global repression of auxin-related genes (Wang et al., 2007). Altered ROS homeostasis has been shown for the *gsnor1-3* mutant (Lee et al., 2008) and the high levels of endogenous NO and its derivatives could lead to oxidative stress. This might, in turn, negatively regulate auxin transport and response. Therefore, we cannot exclude the possibility that the additional effects of altered redox homeostasis other than increased S-nitrosylation in the *gsnor1-3* mutant is partially

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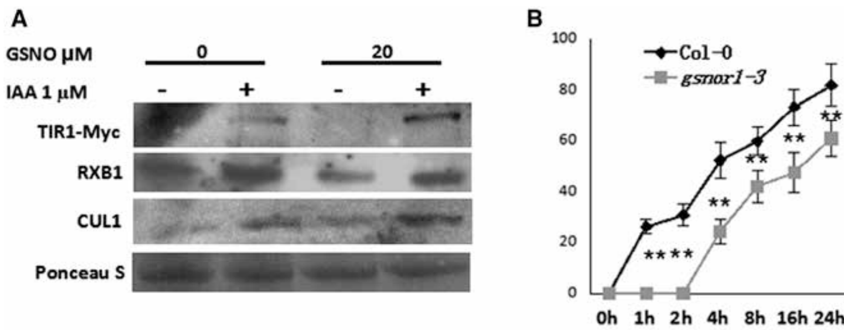

Figure 11. GSNO Does Not Inhibit the Assembly of SCF^{TIR1} Complex Formation and Gravitropic Response Was Compromised in *gsnor1-3* Mutant.

(A) Formation of SCF^{TIR1} complex was not disrupted by the GSNO treatment. Bacterium-expressed and purified GST-IAA7 was used to pull down the components of the SCF^{TIR1} complex from extracts of 7-day-old seedlings of transgenic plants expressing an inducible TIR1-myc in the absence or presence of 1 μM IAA and 20 μM GSNO. The presence of TIR1-myc, Culin1, and RXB were detected by immunoblotting with anti-myc, anti-CUL1, and anti-RXB antibodies, respectively. Ponceau S was used to stain the membrane used for western blot analysis as a loading and transfer control.

(B) Gravitropic response was compromised in *gsnor1-3* mutant. Root curvatures of 5-day-old seedlings were measured at the time points indicated after 90° re-orientation. Shown are means ± SD. $^{**}p < 0.01$ (Student's *t*-test).

responsible for the impaired auxin signaling and compromised polar auxin transport. It is likely that the perturbed auxin signaling and transport observed in *gsnor1-3* plants could be caused by the combined effects of both increased S-nitrosylation of key components required for auxin signaling/transport and impaired cellular ROS homeostasis.

Conditions associated with oxidative or nitrosative stress increase protein glutathiolation via changes in the glutathione redox status (GSH/GSSG) or through the formation of nitrosylated (SNO) cysteine intermediates (Klatt and Lamas, 2000; Hill and Bhatnagar, 2012). These activated thiols promote reversible S-glutathiolation of key proteins involved in cell signaling, energy production, ion transport, and cell death (Martinez-Ruiz and Lamas, 2007; Hill and Bhatnagar, 2012). For example, it has been reported that S-glutathiolation of the ubiquitin- and ATP-independent free 20S proteasome (20SPT) in yeast triggers gate opening and thereby activates the proteolytic activities of free 20SPT and in this way promotes the removal of oxidized or unstructured proteins in stressful situations (Silva et al., 2012). Using the biotin switch method in conjunction with nano-liquid chromatography and mass spectrometry, Palmieri et al. (2010) identified 11 candidate proteins that were S-nitrosylated and/or glutathionylated in mitochondria of *Arabidopsis* leaves. These included the glycine decarboxylase complex (GDC), a key enzyme of the photorespiratory C2 cycle in C3 plants. GDC activity was inhibited by S-nitrosoglutathione due to S-nitrosylation/S-glutathionylation of several cysteine residues. Lindermayr et al. (2010) revealed that Cys residues 260 and 266 of TGA1 are S-nitrosylated and S-glutathionylated even at GSNO concentrations in the low micromolar range and S-nitrosoglutathione protects TGA1 from oxygen-mediated modifications and enhances the DNA binding activity of TGA1 to the *as-1* element in the presence of NPR1. It is possible that S-glutathiolation may also contribute to the impaired auxin signaling and transport in *gsnor1-3* mutant.

Based on our results and the data from others, a working model for the roles of GSNOR1 in auxin signaling and transport can now be proposed (Figure 12). Loss of GSNOR1 results in the increased cellular level of GSNO. The increased GSNO leads to the universally reduced accumulation of various PINs independent of transcriptional regulation and proteasome degradation, and as a result, a compromised polar auxin transport system; it is also possible that a direct S-nitrosylation of PINs could lead to the reduced PIN activities and thus reduced polar auxin transport. On the other hand, increased levels of GSNO could inhibit auxin signaling through S-nitrosylation of components like TIR1 and E2 in the auxin signaling pathway. Collectively, both the compromised polar auxin transport and auxin signaling would thus contribute to the auxin-related phenotypes displayed by the *gsnor1-3* mutant.

In conclusion, our results indicate that the loss-of-function *GSNOR1* mutant, *gsnor1-3*, is a powerful tool to investigate the cross talk between NO and auxin in controlling plant growth and development and the pleiotropic morphological defects observed in *gsnor1-3* mutant result from impaired auxin signaling and compromised auxin transport.

MATERIALS AND METHODS

Arabidopsis Lines and Growth Conditions

In addition to *Arabidopsis* Col-0 and *gsnor1-3* (Feechan et al., 2005), the following transgenic lines were used in this study: *DR5*:*GFP* (Benková et al., 2003), *DR5*:*GUS* (Benková et al., 2003), *HS:AXR3NT-GUS* (Gray et al., 2001), *ProPIN1*:*PIN1-GFP* (Heisler et al., 2005), *ProPIN2*:*PIN2-GFP* (Xu and Scheres, 2005), *ProPIN3*:*PIN3-GFP* (Blilou et al., 2005), *ProPIN7*:*PIN4-GFP* (Vieten et al., 2005), and *ProPIN7*:*PIN7-GFP* (Blilou et al., 2005). These transgenes were subsequently introgressed into the *gsnor1-3* background by crossing. These plants were grown under 16 h of light at 22°C and 8 h of darkness at 18°C. Seedlings were grown on 0.5 × MS medium containing 3.0% sucrose (pH 5.7).

RNA Extraction, RT–PCR, and qRT–PCR

Total RNA was extracted from *Arabidopsis* seedlings using a RNeasy Plant Mini Kit followed by DNase I treatment according to the manufacturer's manual (Qiagen). cDNA was synthesized using a Rever Tra Ace qPCR RT Kit (Toyobo) as described (Liu et al., 2005). qRT–PCR was performed using a StepOnePlus™ real-time PCR system (Applied Biosystems) and Thunderbird SYBR qPCR Mix (Toyobo). Actin2 was used as a control for normalization. The primers used for qRT–PCR were as follows:

GFP-F: TCCATGGCCAACACTTGTCA
GFP-R: CCTTCAGGCATGGCACTCTT
GUS-F: TACCGACGAAAACGGCAAGA
GUS-R: CGGTGATATCGTCCACCCAG
At-Actin2-F: CTCAGCACATTCCAGCAGATG
AtActin2-R: AACCCCAGCTTTTTAAGCCTTTG

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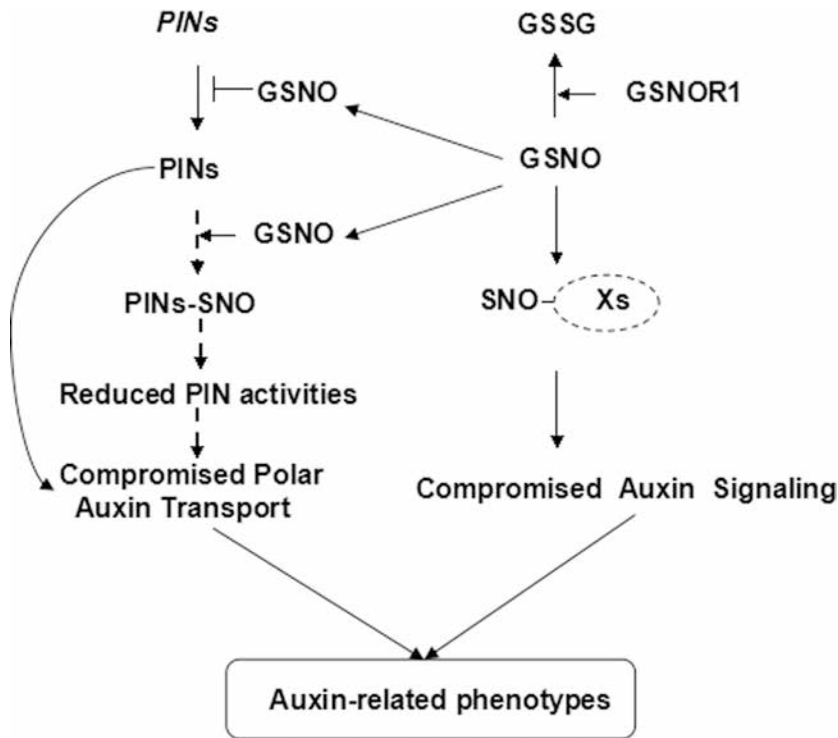

Figure 12. A Working Model for the Roles of GSNOR1 in Auxin Signaling and Transport Based on Our Results and the Data from Others.

Loss of GSNOR1 results in an increased cellular level of GSNO. The increased GSNO leads to the reduced accumulation of PIN proteins independent of transcriptional regulation and proteosome degradation and as a result, compromised polar auxin transport; it is also possible that S-nitrosylation of PINs could lead to the reduced PIN activities and, thus polar auxin transport. On the other hand, increased level of GSNO could inhibit auxin signaling through S-nitrosylation of components such as TIR1 and E2 (indicated as Xs) in the auxin signaling pathway. Together, compromised polar auxin transport and impaired auxin signaling result in the auxin-related phenotypes displayed by the *gsnor1-3* mutant. Solid lines in the model are supported by experimental evidence and dashed lines indicate that no direct evidence has been presented.

β-Glucuronidase Histochemical Staining and Activity Assay

HS::AXR3NT-GUS (Gray et al., 2001) and *DR5-GUS* (Ulmasov et al., 1997) were crossed into the *gsnor1-3* mutant background. For DR5-GUS assays, 7-day-old seedlings from DR5:GUS/Col-0 and/or DR5:GUS/*gsnor1-3* transgenic lines were transferred into 1 ml of ddH_2O containing different concentrations of IAA in combination with different concentrations of GSNO and incubated overnight at room temperature. Plants were then stained for GUS activity at 37°C for 3–4 h. For the HS:AXR3NT-GUS assays, 7-day-old *HS::AXR3NT-GUS*/Col-0 and *HS::AXR3NT-GUS/gsnor1-3* seedlings were heat shocked at 37°C for 2 h. After heat shock, the seedlings were transferred to medium at 20°C in the absence or presence of IAA and incubated for 0 or 40 min before staining for GUS activity. Different concentrations of IAA in combination with different concentrations of GSNO were added to the medium at 20°C where indicated. GUS staining and the activity assay were performed as described previously (Jefferson et al., 1987).

Generation of *GST-IAA7* and *GST-TIR1* Fusion Constructs and Protein Expression and Purification

Full-length cDNA of *IAA7* was amplified via RT–PCR using following primers:

IAA7-F: caccATGATCGGCCAACTTATGAAC
IAA7-R: TCAAGATCTGTTCTTGCAGTACTTC

The amplified full-length cDNA was first cloned into the pENTR/D vector and subsequently cloned into the pDEST15 destination vector to generate the GST-IAA7 fusion protein according to the manufacturer's manual (Invitrogen, Carlsbad, CA). GST-IAA7 was expressed in *Escherichia coli* and purified using glutathione beads according to the manufacturer's instructions (GE Healthcare Bio-Sciences, Pittsburgh, PA).

Pull-Down Assays

The *tir1 GVG::TIR1-myc* line was described elsewhere (Gray et al., 1999). The inducible *TIR1-myc* transgenic seedlings were treated with Dex overnight to induce TIR1-Myc production. Crude protein extracts were prepared as described previously (Dharmasiri et al., 2005). Total protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA). TIR1-Myc was pulled down with purified GST-IAA7 in the absence or the presence of 50 μM IAA. GSNO was added during Dex induction and during the pull-down assay. After washing the pull-down reactions with washing buffer three times, proteins were separated on SDS–PAGE. The proteins on the gel were transferred onto polyvinylidene fluoride membrane using a semi-dry transfer system (Bio-Rad, Hercules, CA). TIR1-myc, CUL1, and RXB1 proteins were detected by immunoblotting with a α-myc antibody, a CUL1 antibody, and a RXB1 antibody, respectively (Gray et al., 1999). Proteins were visualized using the ECL kit (Pierce) after secondary antibody incubation.

Free IAA Extraction and Measurement

Free IAA extraction and quantification were performed essentially as described previously (Barkawi et al., 2008, 2010). Levels of endogenous IAA were calculated by monitoring ions at *m/z* 130 and 189 for endogenous IAA, and *m/z* 136 and 195 for the [$^{13}C_6$]IAA added internal standard as their methyl esters. For studies of smaller tissue sections, selected reaction monitoring GC-MS/MS was used following the *m/z* 189 to *m/z* 130 and *m/z* 195 to *m/z* 136 transitions exactly as described (Liu et al., 2012). Quantities were calculated by using standard isotope dilution equations (Cohen et al., 1986).

IAA Transport and Root Gravitropic Response Assays

Auxin transport from the shoot apex into the roots was assayed using intact light-grown seedlings as described (Murphy et al., 2000; Dai et al., 2006). Root gravitropic responses were evaluated using the gravitropic index, which was defined as the ratio between the vertical distance/ordinate from the root tip to the root base and the root length (Grabov et al., 2005).

Immunolocalization Analysis

Immunolocalization analysis of PIN1 and PIN2 in primary roots was performed as previously described by Wang et al. (2013). Dilutions of primary antibodies for anti-PIN2 and anti-PIN2 were 1:50. A secondary antibody, anti-rabbit-Cy3 (Sigma-Aldrich), was diluted 1:100. Images were captured using a confocal laser scanning microscope (Leica TCS SP5 AOBS).

Biotin Switch Assay

The extraction of proteins from leaf tissues for biotin switch assays was performed according to Lindermayr et al. (2005, 2008). Briefly, the proteins were extracted with HEN buffer (25 mM HEPES [pH 7.7], 1 mM EDTA, and 0.1 mM neocuproine). The final concentration of 250 μM GSNO was added to the protein extracts and incubated at room temperature for 20 min. After treating with GSNO, the protein extracts were incubated with 20 mM methyl methanethiosulfenate (MMTS) and 2.5% SDS at 50°C for 20 min with frequent vortexing for blocking non-nitrosylated free Cys residues. Residual MMTS was removed by precipitation with two volumes of acetone at –20°C and the proteins were resuspended in HENS buffer (HEN buffer containing 1% SDS). Biotinylation was achieved by adding 2 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (Thermo Scientific, Rockford, IL) and 1 mM ascorbate and incubating at room temperature for 1 h. After biotinylation, proteins were separated by SDS–PAGE and immunoblotted with an anti-biotin antibody (Sigma-Aldrich, St. Louis, MO). The bands were visualized by incubating the blots with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate after secondary antibody incubation.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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