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NITRIC OXIDE INFLUENCES AUXIN SIGNALING THROUGH S-NITROSYLATION OF THE *ARABIDOPSIS* TRANSPORT INHIBITOR RESPONSE1 AUXIN RECEPTOR

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Summary

Previous studies demonstrated that auxin and nitric oxide (NO) are plant growth regulators that coordinate several plant physiological responses determining root architecture. Nonetheless, the way in which these factors interact to affect these growth and developmental processes is not well understood. The *Arabidopsis thaliana* F-box proteins TIR1/AFBs are auxin receptors that mediate degradation of Aux/IAA repressors to induce auxin-regulated responses. A broad spectrum of NO-mediated protein modifications are known in eukaryotic cells. Here, we provide evidence that NO donors increase auxin-dependent gene expression while NO depletion blocks Aux/IAA protein degradation. NO also enhances TIR1-Aux/IAA interaction as evidenced by pull-down and two hybrid assays. In addition, we provide evidence for NO-mediated modulation of auxin signaling through S-nitrosylation of the TIR1 auxin receptor. S-nitrosylation of cysteine is a redox-based post-translational modification that contributes to the complexity of the cellular proteome. We show that TIR1 C140 is a critical residue for TIR1-AuxIAA interaction and TIR1 function. These results suggest that TIR1 S-nitrosylation enhances TIR1-Aux/IAA interaction facilitating Aux/IAA degradation, and subsequently promoting activation of gene expression. Our findings underline the importance of NO in phytohormone signaling pathways.

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Short Legend for Supporting Information

The following materials are available in the on-line version of this article.

Figure S1. NO requirement for auxin-dependent gene expression.

Figure S2. NO enhances TIR1/AFB2-Aux/IAA interaction.

Figure S3. TIR1 and tir1 mutant protein levels in wild-type and *tir1-1* transgenic lines.

Keywords

Arabidopsis thaliana; auxin; nitric oxide; TIR1 receptor; S-nitrosylation; root growth

Introduction

Auxin (indole-3-acetic acid) coordinates many plant growth processes by modulating gene expression, which leads to changes in cell division, elongation and differentiation. It activates transcription by stimulating the degradation of transcriptional repressors called AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins through the action of the E3-ubiquitin ligase complex, $SCF^{TIR1/AFB}$ (Ruegger *et al.*, 1998; Gray *et al.*, 2001). The substrate receptor subunits of the complex, the TRANSPORT INHIBITOR RESPONSE1/ AUXIN SIGNALING F-BOX (TIR1/AFB) proteins, function also as auxin receptors. Auxin acts as a "molecular glue" to stabilize the interaction between TIR1/AFBs and Aux/IAA proteins, thus promoting their ubiquitination and degradation (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005; Tan e*t al.*, 2007). TIR1 is a member of a small gene family that contains 5 additional AFB proteins (AFB1 to AFB5), that collectively mediate auxin response and are essential for *Arabidopsis thaliana* growth and development (Dharmasiri *et al.*, 2005b; Parry *et al.*, 2009).

Nitric oxide (NO) is a hydrophobic, highly diffusible gaseous molecule with a broad spectrum of regulatory functions involved in controlling growth, developmental, and patho-physiological processes (Wendehenne *et al.*, 2004; Delledonne, 2005). Frequently under the control of hormonal stimuli, NO acts as a second messenger implicated in many plant cell signaling events. In particular, NO is an important molecule in the auxin-regulated signaling cascade determining root morphology during growth and development (Correa-Aragunde *et al.*, 2007). NO accumulation was reported in response to auxin treatment during adventitious root (AR), lateral root (LR) and root hair formation (RH), as well as asymmetric NO accumulation in the root tip during the gravitropic response (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2004; Hu *et al.*, 2005; Lombardo *et al.*, 2006). Auxin-induced AR, LR, and RH formation as well as root gravitropic response were prevented by application of the specific NO scavenger cPTIO, suggesting a key role for endogenous NO in the control of those processes (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2004; Hu *et al.*, 2005; Lombardo *et al.*, 2006).

In general, different ways by which NO interacts with proteins have been described. Besides its capacity to bind metal ions of heme groups, NO participates in important post-translational modifications through S-nitrosylation and nitration. These events are currently under study in plants (Lindermayr and Durner, 2009).

S-nitrosylation is the reversible binding of a NO moiety to a reactive cysteine residue of a protein to form an S-nitrosothiol (Hess *et al.*, 2005). Even though NO redox-based modification of target proteins by S-nitrosylation is emerging as an efficient regulatory mechanism in plant and mammalian signal transduction, few S-nitrosylated proteins are known in plants (Lindermayr and Durner, 2009; Lindermayr *et al.*, 2010; Palmieri *et al.*, 2010).

In this study, we demonstrate that NO modulates auxin-dependent gene expression through the stimulation of TIR1/AFB-Aux/IAA interaction. The results presented here provide evidence for a link between the auxin signaling pathway and NO through S-nitrosylation of TIR1. Our findings emphasize the physiological impact of two key cysteine residues in TIR1 that are responsible at least in part, for NO action in auxin signaling.

Results

NO Activates Auxin Signaling

To explore the role of NO on auxin signaling, we examined auxin-induced NO production in *Arabidopsis* roots. Seedlings were pre-loaded with the permeable NO-sensitive fluorophore diaminofluorescein-FM diacetate (DAF-FM DA) and then exposed to 1 μM IAA for 1.5 h. We observed increased NO-associated fluorescence in IAA-treated roots (Figure 1). Further, we analyzed auxin response after NO donor application in *BA3:GUS* transgenic seedlings (Oono *et al.*, 1998). This line carries auxin response elements fused to the β-glucuronidase encoding gene (GUS). Seedlings were treated with a low dose of IAA (10 nM). While GUS was expressed poorly in IAA-treated seedlings, simultaneous application of IAA and the NO-donor sodium nitroprusside (SNP) caused a substantial increase in GUS reporter expression (Figure 2a). Treatment of *BA3:GUS* seedlings with SNP alone did not induce significant GUS activity (Figure 2a and Figure S1a). Furthermore, we used the specific NO scavenger carboxy-PTIO (cPTIO) to study the NO requirement for IAA-dependent gene expression. *BA3:GUS* seedlings treated with 50 nM IAA showed GUS activity but the application of cPTIO prior to IAA treatment prevented such induction (Figure 2b). Hemoglobin, another NO scavenger, and cPTIO inhibited auxin-dependent BA3:GUS induction in a dose-dependent manner (Figure S1c). Moreover, the auxin-regulated reporter *DR5:GUS* (Ulmasov *et al.*, 1997) displayed a similar response as *BA3:GUS* to NO-donor and NO-scavenger treatments (Figures S1b and S1d). We also directly analyzed the expression of auxin-responsive genes in response to NO donor treatment. The expression of both *IAA1* and *IAA5* genes seems to be potentiated by SNP in IAA-treated wild-type seedlings (Figure 2c) suggesting that NO might be indeed required for auxin-dependent gene expression in *Arabidopsis*.

Considering that auxin-mediated gene expression is regulated via degradation of Aux/IAA repressors, we hypothesized that NO action may result in Aux/IAA destabilization. To address this possibility, we tested the stability of the reporter protein AXR3NT-GUS. This reporter is a fusion between the amino terminus of the Aux/IAA protein AXR3/IAA17 (AXR3NT) and GUS under control of a heat-shock inducible promoter (HS) (Gray *et al.*, 2001). After heat shock treatment, *Arabidopsis* seedlings were treated with IAA in the presence of NO donor, S-nitrosoglutathione (GSNO) and NO scavengers, cPTIO and hemoglobin, and subsequently stained for GUS activity. It is important to note that we used low- or fast-release NO donors according to treatment times required for each type of experiments (Floryszak-Wieczorek *et al.*, 2006; Noble and William, 2000). IAA treatment (50 nM) caused a decrease in AXR3NT-GUS stability that is substantially enhanced with 10 μM GSNO (Figure 2c). Moreover, seedlings treated with cPTIO and hemoglobin and in combination with high IAA concentration (1 μM) exhibited much stronger GUS staining (Figure 2d). Taken together all these findings show that auxin-dependent gene expression and Aux/IAA degradation might rely on NO availability.

TIR1 Protein Undergoes S-Nitrosylation

Since NO enhances auxin signaling by increasing Aux/IAA degradation, a possible mechanism that could explain this modulation is via the S-nitrosylation of cysteine residues in target proteins. In this scenario, members of Aux/IAA and TIR1/AFB families are attractive candidates for S-nitrosylation. We therefore searched for cysteine residues proposed to constitute the acid-basic S-nitrosylation motif described by Stamler *et al.* (2001) in Aux/IAA and TIR1-AFBs. None of Aux/IAA protein members contain an obvious putative cysteine that matched the consensus. However, two cysteine residues within TIR1, C140 and C480, are putative candidates to undergo S-nitrosylation. Interestingly, both cysteine residues are highly conserved within the TIR1/AFB receptor family (Figure 3a).

C140 residue is located at the LRR 4 loop and C480 is placed at the end of the α-helix of LRR16 in TIR1/AFBs (Figure 3b).

To determine if TIR1 could be S-nitrosylated, baculovirus-expressed and -purified TIR1 protein was incubated with the physiological NO donor, GSNO and subjected to the biotin-switch assay (Jaffrey and Snyder, 2001). In this method, nitrosothiol groups in nitrosylated proteins are substituted for a more stable biotin moiety via chemical reduction by ascorbate, and then identified by western blot. As shown in Figure 4, biotinylated TIR1 was detected upon the biotin-switch and the immunoreactivity was GSNO-dependent. Moreover, TIR1 signal was drastically diminished when DTT was added after GSNO treatment. This result strongly supports TIR1 S-nitrosylation.

S-nitrosylation of TIR1 Affects TIR1-Aux/IAA Interaction

To analyze if S-nitrosylation of TIR1 affects auxin-dependent TIR1-Aux/IAA interaction, we performed GST pull-down experiments. c-Myc-tagged TIR1 was *in vitro* –TNT synthesized in wheat germ extracts and incubated with GST-IAA3 in the presence of an NO source. The proteins were incubated with the NO donor, S-nitrosocysteine (CysNO) and IAA simultaneously. At low IAA concentration (5 μM), CysNO addition resulted in a significant increase in recovery of TIR1-Myc protein, as compared to the control (Figure 5a and Figure S2a). On the other hand, incubation with cPTIO completely abolished TIR1 recovery. cPTIO exerts its effect by scavenging endogenous NO that could be present in the TNT-wheat germ extract. The fact that IAA, as a nitrate compound may produce NO in aqueous solution or cell-free system must not be excluded (Feelisch and Noack, 1987; Pataricza *et al.*, 1998). The NO-dependent interaction was also observed for the AFB2-IAA3 pair (Figure 5b and Figure S2b). Next, we investigated the effect of NO on *in vivo* TIR1/AFB2-Aux/IAA interactions using the LexA yeast two-hybrid system (Prigge *et al.*, 2010). In yeast cells, the NO donor, SNP enhanced the interaction of TIR1 and AFB2 with IAA7 in a dose-dependent fashion (Figure 5c). Altogether, *in vitro* and *in vivo* experiments indicate that NO is required and enhances both TIR1/AFB2-Aux/IAA interactions.

Two TIR1 Cysteines Are Critical for Auxin-Dependent TIR1-Aux/IAA Interaction

Since C140 and C480 may act as putative targets for S-nitrosylation in TIR1, we expected that mutation of these residues may interfere with TIR1-Aux/IAA interaction. To address this, we generated single TIR1 mutant versions, tir1 C140A and tir1 C480A, in which each cysteine was independently changed to an alanine residue by site-directed mutagenesis. TIR1 and the mutated versions were *in vitro* –TNT synthesized in wheat germ extracts and tested in pull-down assays with GST-tagged IAA3 protein. As Figure 6a shows, the C140A mutation abolished the auxin-induced recovery of the tir1-IAA3 complex, whereas C480A severely reduced it. Moreover, yeast two-hybrid assays with C140A and C480A mutated version of TIR1 validated our *in vitro* assays. Even with the addition of IAA, tir1 C140A-IAA7 interaction was completely abolished and tir1 C480A-IAA7 interaction was strongly impaired compared to the control (Figure 6b). These results indicated that the TIR1-Aux/IAA interaction is dependent on C140 and, to a lesser extent, on C480. Interestingly, mutations on other single amino acids surrounding C140, such as S138A, S139A and E141A had no effect on their binding to GST-IAA3 (Figure 6c). This result supports specificity of S-nitrosylation in C140 and C480 TIR1 residues and ruled out the possibility that these mutations simply disrupted TIR1 overall structure independently of any effect of NO.

C140 Residue Has a Pivotal Role for *In Vivo* Auxin-Mediated TIR1 Activity

To assess the functional relevance of C140 and C480 of TIR1 *in vivo*, we introduced the single mutants, tir1 C140A and tir1 C480A, under the control of the constitutive cauliflower mosaic virus 35S, into both *Arabidopsis* wild-type and *tir1-1* backgrounds (Figure S3a–b

and Figure 7). Auxin-dependent induction of LR and inhibition of primary root elongation is affected in the *tir1-1* mutant plants, due to the inability of the receptor to sense the hormone. Overexpression of the wild-type TIR1 protein in the *tir1-1* background rescued the normal root sensitivity to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (Figures 7a–c). We assayed two different overexpressing transgenic lines of both tir1 C140A (L1 and L3) and tir1 C480A (L2 and L4) for auxin-resistance. Although the introduction of tir1 C480A in the *tir1-1* background partially restores auxin sensitivity, tir1 C140A did not recover TIR1 functionality and seedlings remained resistant to 2,4-D treatment. These data provide further evidence about the relevance of C140 as putative S-nitrosylation site in TIR1 as well as NO-mediated modification for proper *in vivo* TIR1 function.

Discussion

In this study, we present evidence that NO acts as a positive modulator of the TIR1/AFB auxin signaling pathway. As previously reported in other plant species, IAA induces NO accumulation in *Arabidopsis* roots (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2004; Hu *et al.*, 2005). In addition, NO-dependence of auxin-responsive gene expression together with NO-mediated Aux/IAA degradation and NO-enhancement of TIR1-Aux/IAA interaction provide further support for this idea. We were able to show the putative S-nitrosylation of the TIR1 protein by biotin-switch assay indicating an important role for a redox-based mechanism in the control of TIR1 action by NO. In support of our data, Kepinski and Leyser (2004) reported that NEM and juglone, which form cysteine adducts, blocked TIR1-Aux/IAA interaction. However, treatments with oxidative stressors or with skewed ratios of GSH/oxidized GSH had only a modest effect. More recently, Yan *et al.* (2009) demonstrated that CORONATINE INSENSITIVE1 (COI1), which is an F-box protein essential for all the jasmonate responses, depends to a great extent on a reducing environment for its function. Similarly, S-nitrosylation of TIR1 may occur in a very particular cell-redox environment. For example, Jiang *et al*. (2003) reported that the formation of the quiescent center in maize is correlated with an auxin oxidizing environment. In this sense, auxin modulates NO production and because of its reported capacity to influence redox status of tissues, it probably provides an environment suitable for S-nitrosylation.

The mechanism of auxin perception by the E3-ubiquitin ligase, $SCF^{TIR1/AFB}$ has been recently elucidated. Since our results reveal a redox-based post-translational modification of a plant E3-ubiquitin-ligase by NO, one important further issue could be to get insights into the action of S-nitrosylation on the binding between TIR1 and its ligand, auxin. Interestingly, the discovery that inositol hexakiphosphate (IP6) is associated with the TIR1 protein (Tan *et al.*, 2007) suggests that TIR1 activity might be regulated by additional cofactors and that an ubiquitin ligase might integrate diverse signals. Moreover, our experiments also showed that AFB2-IAA3 interaction is also modulated by NO. Even though a weak TIR1/AFBs-Aux/IAA interaction occurs in the absence of exogenous auxin it is possible that NO just enhances the interaction even under this former condition (Greenham *et al*., 2011). S-nitrosylation could also modify the ability of TIR1 to bind auxin. However, this issue is worth addressing in a future work. Consequently, S-nitrosylation of the TIR1/AFB proteins could be a versatile point of control of auxin signaling. Nevertheless, an additional mechanism of activation of auxin signaling by NO could not be discarded. Since other proteins involved in auxin signaling may also be subjected to a redox-based modification by NO, a complete understanding of its action requires the direct identification of S-nitrosylated targets. Remarkably, it was recently demonstrated that salicylic acid signaling requires regulation of NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) and TGACG MOTIF BINDING FACTOR1 (TGA1) proteins by NO for a proper systemic acquired resistance in plants (Lindermayr *et al.*, 2010). Much more recently, S-nitrosylation of *Arabidopsis* NADPH oxidase and its consequence on activity have been

also demonstrated (Yun et al, 2011). It is likely that these S-nitrosylation events are important in plant defense mechanisms. Nevertheless, S-nitrosylated-TIR1 could exert its action in plant growth and developmental responses. Thus, S-nitrosylation in plants appeared to be a general mechanism implicated in broad spectrum of plants processes.

In TIR1 protein, the mutation of two putative S-nitrosylated residues, C140 and C480, resulted in reduced interaction with Aux/IAA proteins *in vitro* and *in vivo*. In contrast, substitution of residues adjacent to C140 had no effect on TIR1 activity. The physiological relevance of TIR1 S-nitrosylation is supported by the analysis of tir1 C140A and tir1 C480A function in *tir1-1* receptor mutant plants. When primary root growth and LR formation were analyzed, *tir1 C140A* transgenic seedlings displayed an auxin-resistant phenotype. However, mutations in C140 and C480 of TIR1 have different effects on root growth. This fact allows us to suggest that each cysteine residue could be important for the specific redox-based post-translational modification by NO, depending on the intracellular oxidation/reduction status in the cell. As mention before, C140 is located at the LRR 4 loop in TIR1 protein and it is not close to other cysteine residue discarding the formation of disulphide bonds. Notably, the attenuation of TIR1-IAA3 interaction by the C140A mutation was comparable in magnitude with that shown in the absence of NO (e.g. NO-scavenger treatments) suggesting that the effect of the C140A mutation could be ascribed most likely to S-nitrosylation of C140 than to a structural disruption. Experimental determination for mapping S-nitrosylated residues in TIR1 will consolidate our hypothesis. Likewise, different cysteines may be selective for diverse modifications and this fact may affect protein function in distinct ways. In *Arabidopsis*, C260 and C266 of the TGA1 protein were found to be S-nitrosylated and also S-glutathionylated by GSNO whereas C172 was only S-glutathionylated under low GSNO concentrations (Lindermayr *et al.*, 2010). Moreover, the mammalian ryanodine receptor channel, RyR1, contains different reactive thiols with selectivity for S-nitrosylation or S-glutathionylation each leading to specific functional consequences (Martinez-Ruiz and Lamas, 2007).

It has been well documented that one of the effects of S-nitrosylation is to influence protein stability via modulation of ubiquitination and proteasome-dependent degradation (Hess *et al.*, 2005). In human cells, ubiquitin ligases themselves have also been identified as targets for S-nitrosylation. For instance, S-nitrosylation of Parkin, a ubiquitin E3-ligase important for the survival of dopamine neurons in Parkinson's disease, controls its E3-ubiquitin ligase activity and thereby affects its neuroprotective function (Chung *et al.*, 2004; Yao *et al.*, 2004). Taking into account all these findings and our data, we propose that NO might operate in multiple ways, including the negative but also positive regulation of ubiquitin-dependent protein degradation.

In summary, our results reveal an important new aspect of TIR1/AFB-mediated auxin signaling. We present evidence that S-nitrosylation of TIR1 promotes the interaction between TIR1 and Aux/IAA proteins facilitating Aux/IAA degradation. Certainly, the characterization of GSNO reductase (GSNOR) mutants, *atgsnor1-1* and *atsgnor1-3*, with altered levels of S-nitrosothiols (Feechan *et al.*, 2005) is the most suitable genetic tool that could strongly complement our chemical approach. However, in support of our hypothesis, previous studies at our laboratory using *Arabidopsis* mutants which display perturbed NO levels evidenced changes in different auxin-mediated physiological responses. Flores *et al.* (2008) for instance, reported that the arginase-negative mutants *argah1-1* and *argah2-1*, with high NO levels, show enhanced formation of lateral and adventitious roots. The *nia1 nia2* double mutant on the other hand, which has low NO levels, has been shown to be affected in root hair elongation, which is a well-known auxin-mediated response (Lombardo *et al.*, 2006).

Finally, our findings pave the way for studies on the potential of F-Box ubiquitin ligases to sense and integrate signals in a transduction network and to explore the role of NO on other phytohormone signaling pathways.

Experimental procedures

Plant Material, Growth Conditions and Treatments

Arabidopsis transgenic lines *BA3:GUS, DR5:GUS* and *HS:AXR3NT-GUS* lines have been previously described (Ulmasov *et al.*, 1997; Oono *et al.*, 1998; Gray *et al.*, 2001). Seeds were surface-sterilized and stratified at 4 °C for 2–4 days in the dark and plated on ATS medium (Wilson *et al.*, 1990) containing 1% sucrose with 0.8% agar, and vertically grown at 23 °C under 120 μmol photons m^{-2} s^{-1} with 16: 8 h light: dark cycles. Where necessary, 8- to 10-day-old seedlings were transferred to soil and grown at 23°C under the same photoperiod.

For auxin growth assays, 5-day-old seedlings growing on minimal medium on vertical agar plates were transferred onto fresh media ± 2,4-D for an additional 2 days after which the length of roots upon transfer was measured. Lateral root assays were performed in a similar manner except that the number of emerged lateral roots was measured after additional 4 days. Emerged lateral roots were counted using a dissecting microscope.

Generation of Transgenic Lines

Arabidopsis Pro35S:tir1-Myc C140A and Pro35S:tir1-Myc C480A transgenic lines were created by introducing the point mutation in TIR1 sequence using QuikChange Site-Directed Mutagenesis kit (Stratagene, www.stratagene.com) and pENtr-TIR1 (Invitrogen, www.invitrogen.com) vector as template. After LR Clonase reaction the sequences were cloned into the destination vector pGWB17 (Nakagawa *et al.*, 2007). The primer sequences used for the mutagenesis reactions were as follows (altered residues underlined):

tir1 C140A fw: 5′-GTTCTTGTGCTTTCTTCCGCCGAAGGCTTCTCCACC-3′

tir1 C140A rv: 5′-GGTGGAGAAGCCTTCGGCGGAAGAAAGCACAAGAAC-3′

tir1 C480A fw: 5′-
CATCATGTTTTGTCCGGGGCCGATAGCTTGAGGAAACTAG-3′

tir1 C480A rv: 5′-
CTAGTTTCCTCAAGCTATCGGCCCCGGACAAAACATGATG-3′

We transformed each destination binary vector into electrocompetent *Agrobacterium tumefaciens* strain GV3101 and then transformed wild-type (*Col-0*) and *tir1-1* plants using the floral dip method (Clough and Bent, 1998). Selection of transgenic seedlings was performed by growth on hygromycin-containing plates (35 μg/ml).

To analyze mutated tir1-Myc C140A and tir1-Myc C180A protein levels, 10-day-old seedlings were collected from wild-type and *tir1-1* tir1-Myc C140A and tir1-Myc C480A transgenic lines, homogenized in ice-cold buffer (50 mM Tris pH 7.5, 200 mM NaCl, 10% glycerol, 0.1% Tween-20, containing 1 mM phenylmethylsulphonyl fluoride [PMSF] and complete protease inhibitor cocktail [Roche, www.roche.com]), and centrifuged twice at 10,000 × g for 15 min at 4°C. Equal amounts of protein were loaded onto SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were incubated with α-Myc antibody (Sigma, www.sigmaaldrich.com) and visualized using the ECL kit (Amersham, www.amersham.com).

Measurement of NO Production

For determination of NO production, 5-day-old seedlings were loaded with 5 mM of the cell-permeable fluorescent probe 3-amino, 4-aminomethyl-2,7-difluorofluorescein diacetate (DAF-FM DA) (Calbiochem, www.merck-chemicals.com) in 20 mM HEPES–NaOH pH 7.5 for 30 min in darkness. Then, seedlings were washed with fresh buffer and incubated with 1 μM IAA for additional 1.5 h in the same conditions. After three washes, seedlings were examined by epi-fluorescence (DAF-FMDA excitation 490 nm, emission 525nm) in an Eclipse E200 microscope (Nikon, www.nikon.com) connected with a high resolution digital camera (Nikon, www.nikon.com).

GUS Staining and Analysis

Five-day-old seedlings from *BA3:GUS* and *DR5:GUS* transgenic lines were transferred into liquid ATS medium containing the different compounds and incubated for 6 h at room temperature. For NO-scavenger treatments, seedlings were pretreated with these compounds 45 min before IAA addition. After treatment, seedlings were fixed in 90% acetone at −20°C for 1 h, washed twice in 50 mM sodium phosphate buffer pH 7 and incubated in staining buffer (50 mM sodium phosphate buffer pH 7, 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (GBT) and stained for GUS activity from 2 h to overnight at 37 °C. Seedlings were cleared by ethanol series (70%, 50%, 30%, and 10%). Bright-field images were taken using Nikon SMZ800 magnifier.

Six-day-old *HS:AXR3NT-GUS* seedlings were heat shocked at 37°C for 2 h in liquid ATS medium. The seedlings were transferred into new ATS medium containing the indicated compounds, incubated for 1 h at room temperature and stained for GUS activity as indicated above.

RNA Gel Blot Analysis

Ten-day-old seedlings were transferred from vertical agar plates growing on minimal medium into fresh ATS medium with the indicated compounds. The seedlings were incubated with mild shaking for 3 h and ground in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen, www.invitrogen.com). RNA gel blots were undertaken using standard techniques. ^{32}P-labeled specific DNA probes were produced using Megaprime™ DNA labeling system (Amersham Biosciences, www.gelifesciences.com).

Protein Expression and Pull-down Reactions

tir1-Myc S138A, tir1-Myc S139A, tir1-Myc C140A, tir1-Myc E141A and tir1-Myc C480A proteins were obtained by introducing the point mutation in TIR1 sequence using QuickChange Site-Directed Mutagenesis kit (Stratagene, www.stratagene.com) and pTNT-TIR1-Myc vector as template. The primers used for the mutagenesis reactions were as followed (altered residues underlined):

tir1 S138A fw: 5′-GGTTCTTGTGCTTGCTTCCTGCGAAGGCTTC-3′

tir1 S138A rv: 5′-GAAGCCTTCGCAGGAAGCAAGCACAAGAACC-3′

tir1 S139A fw: 5′-GTTCTTGTGCTTTCTGCCTGCGAAGGCTTCTCCACC-3′

tir1 S1390A rv: 5′-GGTGGAGAAGCCTTCGCAGGCAGAAAGCACAAGAAC-3′

tir1 E141A fw: 5′-GTGCTTTCTTCCTGCGCAGGCTTCTCCAC-3′

tir1 E141A rv: 5′-GGTGGAGAAGCCTGCGCAGGAAGAAAGCAC-3′

For C140A and C480A point mutations, the set of primers used were the same as indicated above. TIR1, AFB2 and mutated versions of the proteins were obtained by *in vitro*

translation using TNT-coupled wheat germ extract system (Promega, www.promega.com) in the presence of [^{35}S] translabeled-methionine where indicated. GST-IAA3 protein was expressed in *Escherichia coli* and purified using GSH-sepharose according to the manufacturer's instructions.

For pull-down assays, 20 μl of TIR1-Myc, tir1-Myc S138A, tir1-Myc S139A, tir1-Myc C140A, tir1-Myc E141A, tir1-Myc C480A and AFB2-Myc proteins were incubated for 2 h at 4°C with >10 μg of GSH-agarose immobilized GST-IAA3 protein in 200 μl of lysis buffer (20 mM Tris pH 8.0, 200 mM NaCl, 5 mM DTT) in the presence of indicated compounds. After washing samples with 10 bed volumes of lysis buffer, beads were resuspended in one bed volume of sample buffer, denaturated, and separated on 12% SDS-PAGE. Products were detected by autoradiography or by immunoblotting with α-Myc antibody coupled to peroxidase and visualized using the ECL kit (Amersham Biosciences, www.gelifesciences.com).

Yeast Two-hybrid Analysis

The cDNAs of TIR1 and AFB2 were cloned into pGILDA (Clontech, www.clontech.com) and the cDNA of IAA7 was cloned into pB42AD (Clontech, www.clontech.com). To obtain mutated versions of TIR1 protein, site directed mutagenesis was performed using pGILDA-TIR1 vector as template and the primers indicated above. Yeast two-hybrid vectors pGILDA and pB42AD containing the different cDNAs were transformed into the yeast strain EGY48 [pSH18-34] (Clontech, www.clontech.com) by the lithium acetate method (Gietz *et al.*, 1992). Handling of yeast cultures, plate growth assays and β-galactosidase assays were performed as described in the Clontech Yeast Protocols Handbook™.

Biotin-Switch Assay

Recombinant TIR1 was co-expressed with ASK1 as a glutathione S-transferase (GST)-fusion protein, in High Five™ (Invitrogen, www.invitrogen.com) suspension insect cells as previously published (Tan *et al.*, 2007). The TIR1–ASK1 complex was isolated from the soluble cell lysate by glutathione affinity chromatography. After cleavage by TEV, the complex was further purified by anion exchange and gel filtration chromatography. TIR1 was S-nitrosylated with the stated concentrations of GSNO, NO donor, for 30 min in darkness and then subjected to the biotin-switch assay (Jaffrey and Snyder, 2001) including controls according to Sell *et al.* (2008) and Forrester *et al.* (2009). Subsequently, proteins were subjected to western blot analysis using an anti-biotin antibody (Sigma, www.sigmaaldrich.com).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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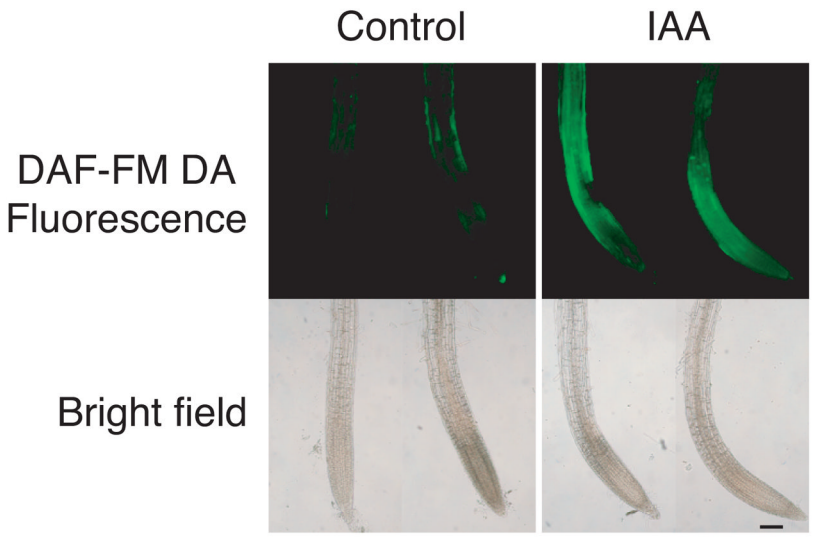

Figure 1. IAA increases endogenous NO in *Arabidopsis* roots

Wild-type seedlings grown on ATS media were pre-loaded with the probe DAF-FM DA, and exposed to 1 μM IAA for 1.5 h at room temperature in darkness. NO accumulation is shown as green fluorescence in representative roots. Bar = 100 μM

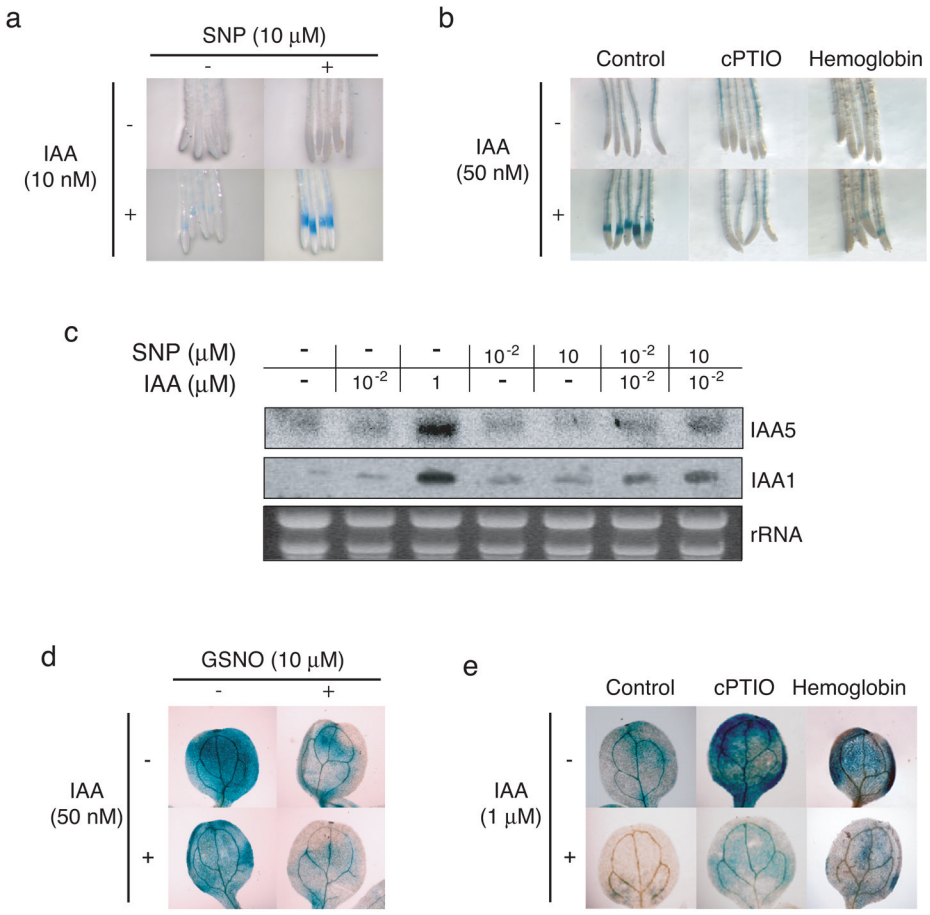

Figure 2. NO is required for IAA-dependent gene expression and Aux/IAA protein degradation

(a) *Arabidopsis BA3:GUS* seedlings were treated with SNP with or without 10 nM IAA, and **(b)** pretreated for 45 min with cPTIO (0.5 mM) or hemoglobin (50 μM) prior 50 nM IAA treatment and them subjected to GUS staining **(c)** Wild-type seedlings were treated with different concentrations of IAA with or without increasing concentrations of SNP. Total RNA was isolated and transcript levels of *IAA1* and *IAA5* were analyzed using specific ^{32}P labeled probes. rRNA is shown as loading control. **(d)** *HS:AXR3NT-GUS* seedlings were treated with GSNO with or without 50 nM IAA or **(e)** with 1 μM IAA in combination with cPTIO (1 mM) or hemoglobin (50 μM) and stained for GUS activity.

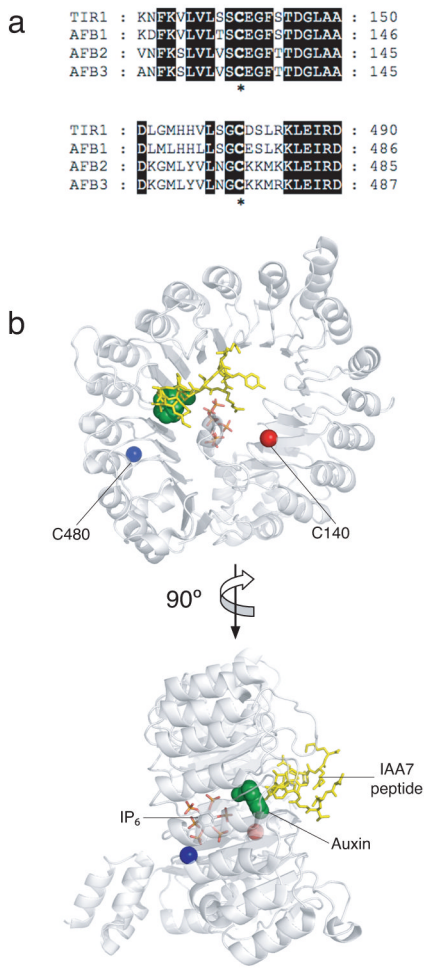

Figure 3. Conservation of C140 and C480 in TIR1/AFB auxin receptor family and location in TIR1 crystal structure

(a) Alignment of amino acid sequences surrounding C140 and C480 in TIR1/AFB receptor family using ClustalX program. Asterisks stand for the cysteine residues.

(b) Two views of the TIR1 structure shown as a ribbon diagram. Carbon alphas of C140 (red) and C480 (blue) are represented by spheres. IP6 (colored by element) and the IAA7 substrate peptide (yellow) molecules are shown as stick models. IAA is represented by a space filling model (green).

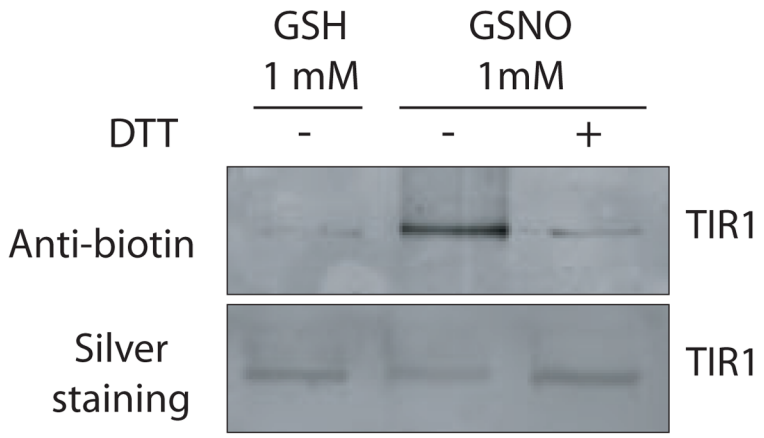

Figure 4. S-nitrosylation of TIR1 recombinant protein

Purified TIR1 protein was incubated with GSNO or GSH for 30 min and subjected to biotin-switch assay. S-nitrosylated TIR1 was detected with an anti-biotin antibody (upper panel). As a negative control GSNO-treated TIR1 was incubated with 20 mM DTT before the biotin-switch. Silver-stained TIR1 is shown as loading control.

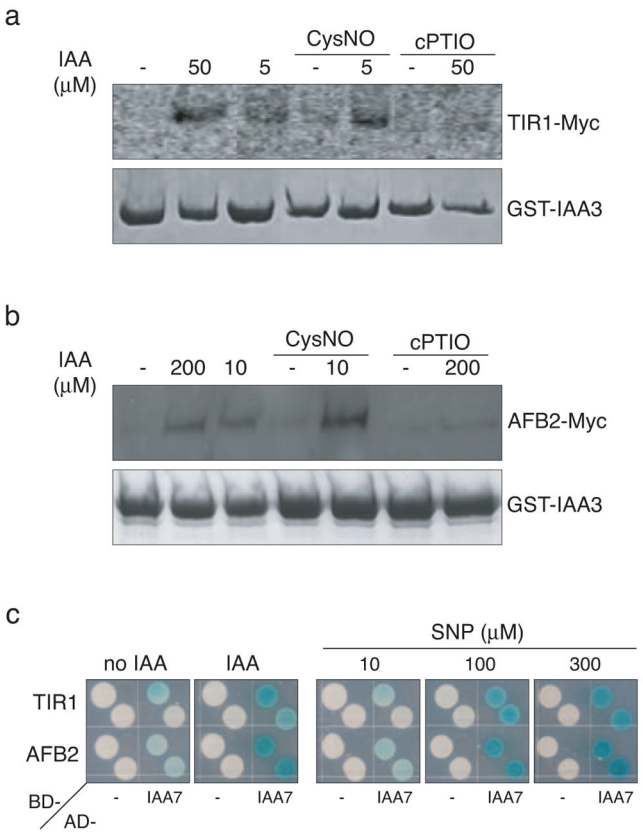

Figure 5. NO enhances TIR1/AFB2-Aux/IAA interaction

Pull-down reactions were performed using ^{35}S-methionine *in vitro* synthesized **(a)** TIR1-Myc or **(b)** AFB2-Myc and recombinant GST-IAA3 proteins. Reactions were carried out in the presence of the indicated IAA concentrations and the addition of 1mM CysNO or 1 mM cPTIO. TIR1/AFB2 proteins were detected using Phosphoimager. Coomassie blue-stained GST-IAA3 was used as loading control. **(c)** Yeast two-hybrid assays were carried out with cells co-transformed with the indicated constructs and grown on SD-U-H-T selective media plus the addition of 50 μM IAA or increasing concentrations of SNP and X-Gal to develop β-galactosydase activity.

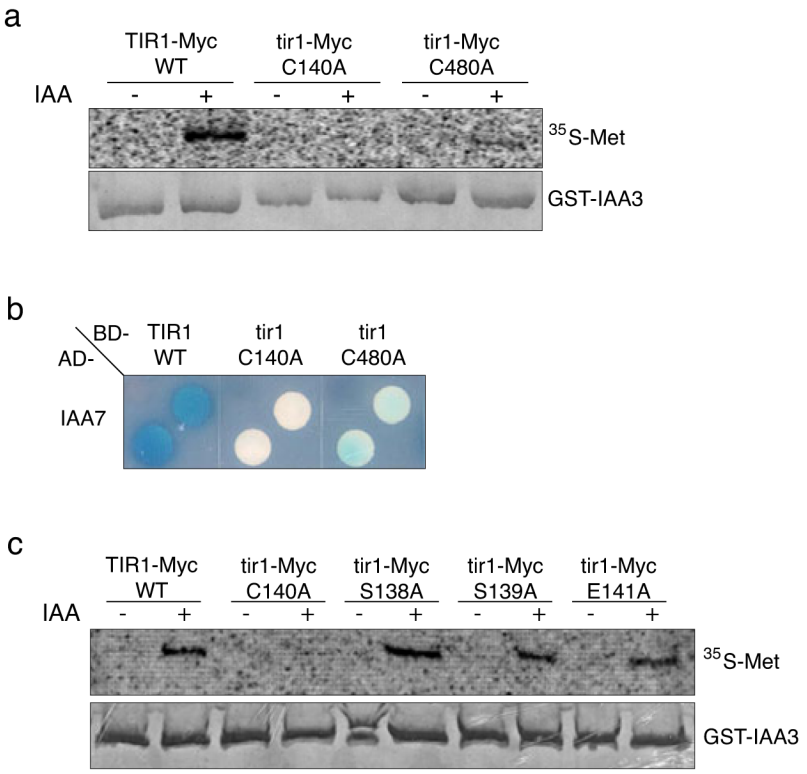

Figure 6. TIR1-Aux/IAA interaction is dependent on C140 and C480 of TIR1

(a) tir1-Myc C140A and tir1-Myc C480A mutated proteins were ^{35}S-methionine *in vitro* synthesized and used in pull-down reactions along recombinant GST-IAA3 proteins. Reactions were carried out in the presence or absence of 50 μM IAA and TIR1-Myc and mutant proteins were detected using Phosphoimager. Coomassie blue-stained GST-IAA3 was used as loading control. **(b)** Yeast two-hybrid assays were carried out with cells co-transformed with the indicated constructs and grown on SD-U-H-T selective media plus the addition of 50 μM IAA. **(c)** Mutations in amino acids surrounding C140 do not affect TIR1-Aux/IAA interaction. TIR1-Myc mutated versions on the S-nitrosylation consensus motif around C140 were used to carry out pull-down reactions. Experiments were performed as **(a)**.

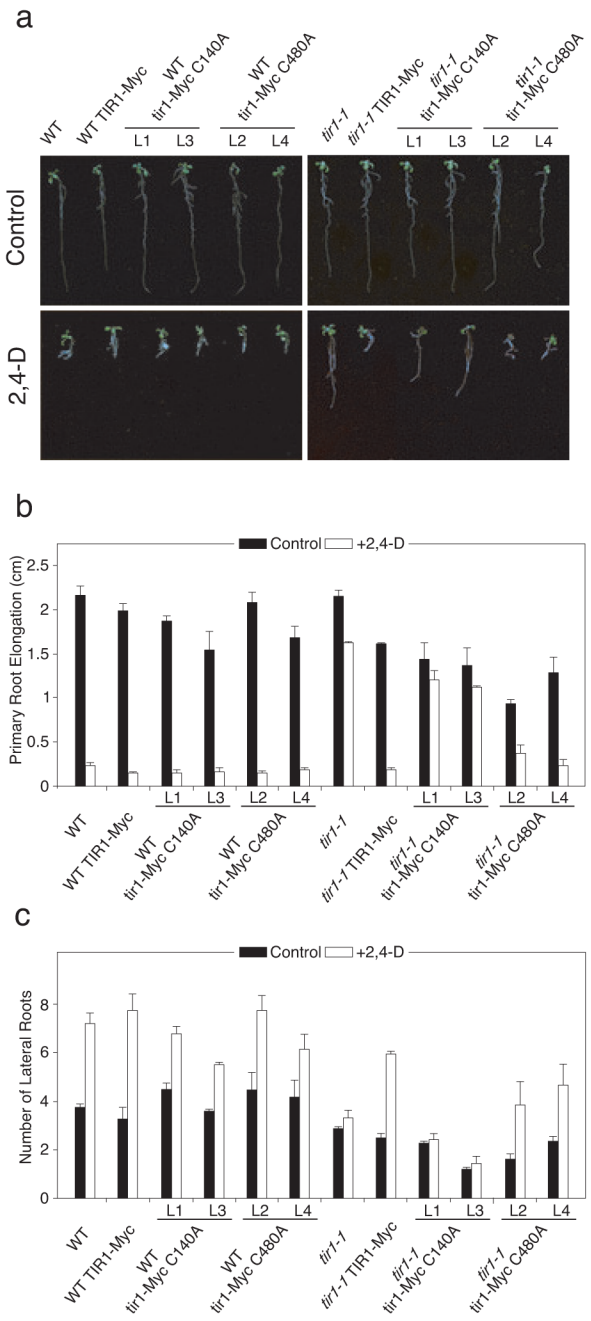

Figure 7.

tir1 C140A overexpression does not rescue the *tir1-1* auxin resistant phenotype. Four-day-old seedlings grown on ATS media were transferred to media containing 2,4-D. **(a)** Representative seedlings after 6 days under 2,4-D treatment. **(b)** Root elongation was measured after 2 days in 2,4-D treatment. **(c)** Lateral root formation was measured after 4 days in 2,4-D treatment. Values represent the mean of at least three independent experiments ± SE.