



## Research paper

# Regulation of SCF<sup>TIR1/AFBs</sup> E3 ligase assembly by S-nitrosylation of Arabidopsis SKP1-like1 impacts on auxin signaling



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## ABSTRACT

The F-box proteins (FBPs) TIR1/AFBs are the substrate recognition subunits of SKP1–cullin–F-box (SCF) ubiquitin ligase complexes and together with Aux/IAAs form the auxin co-receptor. Although tremendous knowledge on auxin perception and signaling has been gained in the last years, SCF<sup>TIR1/AFBs</sup> complex assembly and stabilization are emerging as new layers of regulation. Here, we investigated how nitric oxide (NO), through S-nitrosylation of ASK1 is involved in SCF<sup>TIR1/AFBs</sup> assembly. We demonstrate that ASK1 is S-nitrosylated and S-glutathionylated in cysteine (Cys) 37 and Cys118 residues *in vitro*. Both, *in vitro* and *in vivo* protein-protein interaction assays show that NO enhances ASK1 binding to CUL1 and TIR1/AFB2, required for SCF<sup>TIR1/AFB2</sup> assembly. In addition, we demonstrate that Cys37 and Cys118 are essential residues for proper activation of auxin signaling pathway *in planta*. Phylogenetic analysis revealed that Cys37 residue is only conserved in SKP proteins in Angiosperms, suggesting that S-nitrosylation on Cys37 could represent an evolutionary adaption for SKP1 function in flowering plants. Collectively, these findings indicate that multiple events of redox modifications might be part of a fine-tuning regulation of SCF<sup>TIR1/AFBs</sup> for proper auxin signal transduction.

## 1. Introduction

Auxin is an omnipotent regulator of growth and development throughout the entire lifespan of the plants. Within the multifaceted function in different processes, auxin exerts a pivotal role in the establishment of root architecture by inducing adventitious root, lateral root, root hair formation, and also in the regulation of the gravitropic response [12]. The modulation of endogenous auxin level and its redistribution along the root is responsible for the dynamic growth to the highly changeable environmental conditions, including the formation

of new roots [56,57]. In addition to auxin, nitric oxide (NO) is considered a ubiquitous signal in plants which contributes to determining the morphology and developmental pattern of roots, in part by the modulation of auxin response. Auxin-NO crosstalk involves indole-3-acetic acid (IAA)-mediated NO production, where peroxisomes are a major cellular site of NO production during the IBA to IAA conversion by  $\beta$ -oxidation [12,74,76]. Moreover, NO also modulates auxin metabolism, transport and signaling including the post-translational regulation of the auxin efflux protein PIN-FORMED 1 (PIN1) and Transport Inhibitor Resistant 1 (TIR1) auxin receptor [21,25,80]. Besides the

Abbreviations: ASK1, Arabidopsis SKP1-like1; FBP, F-box protein; IAA, indole-3-acetic acid; SCF, SKP1–cullin–F-box; SKP1, S-phase kinase-associated protein 1

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peroxisome-derived NO origin, many sources of NO production have been described in different plant cell compartments [9]. Enzymatic and non-enzymatic activities have been characterized as generating NO through the reduction of nitrites, or through an oxidative pathway involving an NO-synthase like activity and arginine (revised by [23]). All of those pathways could potentially co-exist in the same cell, depicting a complex scenario for its analysis.

Multimeric cullin-RING ligases (CRLs) are the largest group of E3 ubiquitin ligases in eukaryotic organism. CRLs that contain a Cullin1 (CUL1) subunit, also called SCF-type complex are pivotal for hormone sensing and transduction in plants. SCF E3 ligase complex is involved in the last step of protein ubiquitination to be degraded through the 26S proteasome and is composed by four primary subunits: the CUL1 scaffold protein, S-phase kinase-associated protein 1 (SKP1), Ring Box 1 (RBX1), and a substrate receptor F-box protein (FBP) [6,72,8]. CUL1 and RBX1 subunits recruit the E2-ubiquitin conjugating enzyme, while SKP1 serves as a bridge between CUL1, and one of the interchangeable and highly variable FBPs. Among the 21 Arabidopsis SKP1-like genes (ASKs), Arabidopsis SKP1-like1 (ASK1) protein plays important roles in multiple cellular processes in plants. The *ask1* mutation causes male sterility since ASK1 is essential for early nuclear reorganization during male meiosis [95,96]. A proteomic analysis revealed that several proteins involved in growth processes, photomorphogenesis, circadian clock oscillation and defense response against stress are altered in the *ask1-1* mutant [86]. In the nuclear auxin signaling pathway, ASK1 acts as a bridge between CUL1 and the TIR1/Auxin Signaling F-Box (AFBs) receptors which are the FBPs subunits of the SCF<sup>TIR1/AFBs</sup> ubiquitin ligase E3 complex. Auxin signaling activation initiates when the hormone physically interacts with TIR1/AFBs and the auxin co-receptor Auxin/Indole-3-Acetic Acid (Aux/IAA) proteins [14,15,36,78]. This interaction promotes the polyubiquitination and degradation of Aux/IAA repressors through the 26S proteasome leading to the consequent induction of three families of early auxin-response genes: *Aux/IAAs*, *Small Auxin Up Rna (SAURs)* and *Gretchen Hagen 3 (GH3)* [8,45,88].

Since the Arabidopsis genome encodes hundreds of FBPs, and ASK1 is able to associate with FBPs in an interchangeable manner [66], the challenge of regulating SCF assembly is particularly relevant. Several FBPs show a tissue-specific preference interaction with particular ASKs and more than two hundred FBPs do not interact with any of 19 different assayed ASK proteins, implying that additional regulations for their *in vivo* interactions within the SCF complex could be necessary [39]. The role of SCF<sup>TIR1/AFBs</sup> complex during auxin signaling activation has been extensively studied and several proteins including the COP9 signalosome (CSN) complex, RUB/NEDD8, CAND1 and ALF4 have been associated to the exchange of substrate adapters and the regulation of SCF<sup>TIR1/AFBs</sup> activity [16,18,3,60,65,89]. In addition, HSP90 and the co-chaperone SGT1 have been related to the stabilization of TIR1 [85], which may also involve an autocatalytic mechanism [97]. However, recent reports suggest that additional post-translational modifications including thiol redox regulation are emerging as new regulatory mechanisms in the modulation of E3 ligases [71].

The reactive thiol groups of cysteine (Cys) residues can sense fluctuations in redox status leading to protein post-translational modifications including S-sulfonation, S-glutathionylation and S-nitrosylation [13,47,50,73]. Redox post-translational modifications are central for multiple cellular signaling in plants, of which NO-based S-nitrosylation has been consolidated as a ubiquitous signal in physiological processes requiring a rapid and versatile regulation [2,41]. S-nitrosylation consists in the incorporation of a NO moiety to a reactive thiol group in a Cys residue to form an S-nitrosothiol (SNO) regulating the stability, subcellular localization, conformational changes, and biochemical activities of a target protein [28,46,48]. S-nitrosylation has been associated to the regulation of plant growth and development processes, as well as stress responses [1,29,42,77,83,84,94,98]. In contrast to other post-translational protein modifications, S-nitrosylation is generally considered to be a non-enzymatic process. However, a recent report

described the hybrid cluster protein Hcp involving activities of three types of enzymes that coordinately generate NO, convert NO to SNO, and propagate SNO-based signaling in *Escherichia coli* [68]. The dynamics of cellular S-nitrosylated proteins are also influenced by different enzymes with de-nitrosylation activities such S-nitrosoglutathione reductase (GSNOR) and thioredoxin [20,43,5,66,77]. GSNOR was reported to be a key enzyme in the regulation of the cellular level of SNOs under different physiological and stress processes in plants [40,90]. Impairment in GSNOR1 function causes defects in growth and development due to deregulation of auxin signaling and transport, suggesting that multiple events of S-nitrosylation could be affecting the auxin response [69].

Auxin signaling activation by modulating TIR1-Aux/IAA interaction and facilitating Aux/IAA degradation is affected by S-nitrosylation of TIR1 protein [80]. In order to fully understand the regulation of auxin signaling by NO, we evaluated whether in addition to the FBP TIR1/AFBs, the adaptor protein ASK1 could be also a target for S-nitrosylation. We demonstrate that ASK1 is sensitive to S-nitrosylation on Cys37 and Cys118 residues. Our results show that S-nitrosylation enhanced protein-protein interactions between ASK1 and its partners, TIR1, AFB2 and CUL1, modulating thereby the SCF<sup>TIR1/AFBs</sup> complex assembly and auxin signaling response in plants. Collectively, our results shed light on the biochemistry of NO and its multiple roles in the regulation of SCF<sup>TIR1/AFBs</sup> complex. These findings underline even more the impact of NO signaling and S-nitrosylation in auxin transduction pathway in plants.

## 2. Material and methods

### 2.1. Plant material

*Nicotiana benthamiana* L. seeds were grown in soil mixed with vermiculite at a 3:1 ratio in a greenhouse with a 16-h photoperiod (150  $\mu\text{E m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation) at 25 °C and 60% relative humidity.

### 2.2. Plasmid constructs

ASK1 ORF was amplified *via* PCR using the primers described in Table S1 and first strained cDNA from 2-week-old Arabidopsis plants as template. The resulting amplicon was cloned into a Gateway pENTR/TOPO vector by BP reaction (Gateway; Life Technologies, USA). The resultant plasmid pENTR-ASK1 was subjected to site directed mutagenesis using QuickChange Site-Directed Mutagenesis kit (Stratagene, USA) and the primers listed in Table S1 (altered residues underlined) to generate the ask1Q27K, ask1C37A, ask1C59A and ask1C118A mutations. Then, plasmids were subjected to the LR reaction using gateway technology with the following destination vectors: pGEX-4T-2 (GE Healthcare Life Sciences, USA) for recombinant protein expression in *E. coli* as GST-ASK1 fusion proteins; pB42AD (Clontech, USA) for Yeast two-hybrid system (Y2H); and pEarleyGate203 [17] for transient expression in *N. benthamiana*.

### 2.3. Transformation assay and auxin treatment

*N. benthamiana* leaves from 4-week-old plants were infiltrated with *Agrobacterium tumefaciens* strain GV3101 carrying Pro35S-ASK1 pEarleyGate203 expression vector (or alternatively, Pro35S-ask1C37A, Pro35S-ask1C59A, Pro35S-ask1C118A) together with p19 (a gene silencing suppressor) for transient expression of ASK1 protein or with infiltration solution (10 mM MgCl<sub>2</sub>, 10 mM MES pH 5.6, 100  $\mu\text{M}$  acetoxyringone) as control. Twenty four h post-infiltration, plants were sprayed with 10  $\mu\text{M}$  IAA or mock solution (supplemented with an equal amount of ethanol used to prepare the IAA solution) as control. Leaves were harvested 1 h later and stored at -80 °C.

#### 2.4. RNA isolation and quantitative real-time RT-qPCR

Total RNA from *N. benthamiana* leaves treated as described in 2.3 was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's recommendations, and samples were treated with RQ1 RNase-free DNase (Promega, USA) for DNA contamination removal. For cDNA synthesis, 1 µg of total RNA was reverse transcribed by IMPROM II (Thermo Fisher Scientific, USA) using random primers (Biodynamics SRL, Argentine). The expression of a subset of early auxin response genes (*NbGH3.6* -Niben101Scf13270g03004.1-; *NbGH3.2* -Niben101Scf12751g00003.1-; *NbGH3.1* -Niben101Scf12751g00003.1-; *NbIAA3*-Niben101Scf12159g-09021.1-; *NbIAA19* -Niben101Scf02572g04006.1-; *NbEF-1α* -Niben101-Scf12941g01003.1-) was analyzed by qPCR. The primers used are listed in Table S1. qPCR reactions were conducted in triplicates (40 cycles at 95 °C for 10 min and 1 min at 60 °C) in a Step One real-time PCR system (Applied Biosystems, USA) using SYBR green PCR master mix (Applied Biosystems, USA) following manufacturer's instructions. Primer pairs were tested for specificity and for amplification efficiency with a standard cDNA dilution curve. Data presented are normalized to the expression level of the control gene *EF-1α* [63] of three independent experiments involving 2 plants per treatment with similar ASK1 overexpression.

#### 2.5. Recombinant ASK1 protein expression

The GST-tagged ASK1 proteins were expressed in *E. coli* BL21 (DE3) cells. Harvested cells were resuspended and sonicated in lysis buffer (50 mM Tris pH 8.0, 200 mM NaCl, and EDTA-free protease inhibitor -Roche, USA-). Lysate supernatant was used for purification using GSH-sepharose according to the manufacturer's instructions (GE Healthcare Life Sciences).

#### 2.6. Immunoblotting

Proteins were run on SDS-PAGE and electrotransferred to nitrocellulose membranes, probed with primary antibody overnight, and then incubated with secondary antibody coupled to peroxidase (Invitrogen, USA). The visualization was performed using the ECL kit (Amersham Biosciences, USA) in a FUJI ImageQuant LAS-4000 system (Fujifilm, Japan). All immunoblottings were repeated at least three times. Ponceau or Sypro Ruby (Molecular Probes, USA) staining was used to visually check the loading uniformity.

#### 2.7. Biotin switch assay

ASK1, wild type (WT) and mutant purified proteins were S-nitrosylated with the stated concentrations of NO-Cys (0, 10, 50 and 100 µM) or diethylamine NONOate (DEANO; 0, 10, 50 and 100 µM) for 15 min in the dark with frequent vortexing. Then, proteins were subjected to biotin switch assay [33,49] including control treatments according to Forrester et al [24]. Briefly, proteins were incubated with 30 mM methyl-methanethiosulfate (MMTS; Sigma-Aldrich, USA) and 3.3% SDS in HEN buffer (25 mM HEPES pH 7.7, 1 mM EDTA, 0.1 mM neocuproine -Sigma-Aldrich, USA-) at 50 °C for 20 min to block free Cys residues. Proteins were precipitated with 2 volumes of cold acetone and resuspended in HEN buffer with 1% SDS. After the addition of 20 mM ascorbic acid and 1 mM biotin-HPDP (Pierce Chemical, USA), the mixture was incubated for 1 h at room temperature in the dark. Subsequently, proteins were subjected to protein blotting analysis using anti-biotin antibody (Sigma-Aldrich, USA).

#### 2.8. Fluorescence switch assay

Proteins were incubated with different oxidant agents: S-nitroglutathione (10 and 100 µM GSNO), hydrogen peroxide (100 µM

H<sub>2</sub>O<sub>2</sub>) or glutathione disulfide (100 µM GSSG) for 15 min in the dark and then subjected either to the fluorescence switch assay to detect S-nitrosylation [79] or to the redox fluorescence switch to detect reversibly oxidized Cys [31]. Proteins were blocked with 4 volumes of blocking buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 2.5% SDS and 30 mM NEM) at 37 °C for 30 min. Then, proteins were precipitated with 2 volumes of cold acetone and resuspended in 250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS. After the addition of 100 mM ascorbic acid and 40 µM Bodipy-FL -(2-aminoethyl) maleimide (Life Technologies, USA), the mixture was incubated for 1 h at room temperature. In the case of redox fluorescence switch, after acetone precipitation proteins were resuspended in 250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS and 2.5 mM DTT and incubated for 10 min at room temperature. Samples were again precipitated with acetone, resuspended in 250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine and 1% SDS with 40 µM Bodipy-FL -(2-aminoethyl) maleimide and incubated for 30 min at 37 °C. Finally, proteins were acetone-precipitated before separation by 15% SDS-PAGE and visualized by Fuji ImageQuant LAS-4000 system (Fujifilm, Japan).

#### 2.9. Pull-down assays

Five µg of GST-ASK1 protein or its mutated versions were immobilized in GSH-sepharose beads (GE Healthcare, USA), and incubated with 100 µM NO-Cys in 200 µl of 1X PBS buffer during 1 h in the dark. DTT was added for 10 min as control. After washing samples with 10 bed volumes of 1X PBS, beads were incubated with TIR1-myc during 30 min. TIR1-myc was obtained by *in vitro* translation using TNT coupled wheat germ extract system (Promega, USA) according to Terrile et al. [80]. Finally, proteins were eluted in 50 mM Tris-HCl pH 8.0 containing 200 mM NaCl and 10 mM GSH, denatured and separated on 15% SDS-PAGE. TIR1-myc was detected by immunoblotting with anti-myc antibody (Sigma-Aldrich, USA).

#### 2.10. Yeast two-hybrid system

pGILDA-TIR1, pGILDA-AFB2 [7] and pB42AD-ASK1 or the corresponding ASK1 mutant constructs were used to transform *Saccharomyces cerevisiae* strain EGY48 [pSH18-34] (Clontech, USA; [27]). Yeast cells co-expressing DBD-TIR1/AFB2 and AD-ASK1 (or AD-mutated ask1) were grown on SD-U-H-T selective media containing or not different concentrations (100 or 300 µM) of sodium nitroprusside (SNP) and 5-bromo-4-chloro-indolyl-*b*-D-galactopyranoside (X-Gal) to develop β-galactosidase activity under ambient light. Handling of yeast cultures and β-galactosidase assays were performed according to Clontech Yeast Protocols Handbook (Protocol PT3024-1, Version PR973283, 2009).

#### 2.11. In-gel digestion for mass spectrometry (MS) analysis

After drying, gel bands or spots were washed in acetonitrile: water (ACN:H<sub>2</sub>O, 1:1) and digested *in situ* in non-reducing conditions with sequencing grade trypsin (Promega, USA) as described by Shevchenko et al [68] with minor modifications [53]. The gel pieces were shrunk by removing all liquid using sufficient ACN. ACN was pipetted out and the gel pieces were dried in a speedvac. The dried gel pieces were re-swollen in 50 mM ammonium bicarbonate pH 8.8 with 12.5 ng/µl trypsin for 1 h in an ice bath. The digestion buffer was removed and gel pieces were covered again with 50 mM NH<sub>4</sub>CO<sub>3</sub> and incubated at 37 °C for 12 h. Digestion was stopped by the addition of 1% trifluoroacetic acid. Whole supernatants were dried down and then desalted onto ZipTip C18 Pipette tips (Millipore, USA) before the MS analysis.

## 2.12. Reverse phase-liquid chromatography MS (RP-LC-MS/MS) analysis in SMIM mode

Protein identification by LC-MS/MS and identification of post-translational modifications were carried out in the Centro de Biología Molecular Severo Ochoa (CBMSO, España) protein chemistry facility, a member of ProteoRed network.

The desalted protein digest was dried, resuspended in 10  $\mu$ l of 0.1% formic acid and analyzed by RP-LC-MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro mass spectrometer (Thermo Scientific, USA). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 mm  $\times$  20 mm precolumn Acclaim PepMap C18, 5  $\mu$ m, 100 Å (Thermo Scientific, USA), and then separated using a 0.075 mm  $\times$  100 mm column Acclaim PepMap C18, 3  $\mu$ m, 100 Å (Thermo Scientific, USA) operating at 0.3  $\mu$ l/min. Peptides were eluted using a 90-min gradient from 5% to 40% solvent B (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid, 80% ACN in water). Electrospray ionization (ESI) was done using a Nano-bore emitters stainless steel ID 30  $\mu$ m interface. The Orbitrap resolution was set at 30,000. The mass spectrometer was operated in the selected MS/MS ion monitoring mode (SMIM mode; [34]). In this mode, the LTQ-Orbitrap-Velos-Pro detector was programmed to perform, along the same entire gradient, a continuous sequential operation in the MS/MS mode on the doubly or triply charged ions corresponding to the peptide/s selected previously from the theoretical prediction. The MS/MS spectra from the peptide were analyzed by assigning the fragments to the candidate sequence, after calculation the series of theoretical fragmentations, according to the nomenclature of the series as previously described [62].

## 2.13. Circular dichroism (CD)

CD measurements were performed on a Jasco Model J-715 Spectropolarimeter (Japan Spectroscopic Co., Japan). Measurements were carried out at 25 °C with a thermostated cell holder and a thermostatic Neslab RTE-110 circulating water bath, at 0.2 nm s<sup>-1</sup> scanning speed. The far-UV spectra were recorded from 190 to 250 nm at a protein concentration of 40  $\mu$ M, as an average of 5 scans after being corrected by subtraction of a buffer blank [51]. Mean residue weight ellipticities were expressed in terms of residue molar ellipticity in deg cm<sup>2</sup> dmol<sup>-1</sup>. For GSH and GSNO treatments, proteins were incubated during 30 min at room temperature in buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1 mM neocuproine) with the addition of 0.5 mM GSNO or 0.5 mM GSH in the dark.

## 2.14. Bioinformatic and phylogenetic analysis

Protein sequences alignments were performed using MEGA7 version 7.0.14 [38]. Phylogenetic trees were constructed using the neighbor-joining method and the default settings of MEGA7 version 7.0.14 [38]. Optimal trees are shown in Fig. 1D (the sum of branch length = 2.29501151), Supplementary Fig. S1 (sum of branch length = 4.39274666) and Supplementary Fig. S2 (sum of branch length = 8.11938003). The evolutionary distances were computed using the Poisson correction method [99] and are in the units of the number of amino acid substitutions per site. Analysis involved 15 amino acid sequences and a total of 213 positions in the final dataset (Fig. 1D), 21 amino acid sequences and a total of 419 positions (Supplementary Fig. S1) and 22 amino acid sequences and a total of 328 positions in the final dataset (Supplementary Fig. S2). Graphic display of identities was visualized using Geneious (9.1.4 version, <http://www.geneious.com>) based on an identity matrix [35].

Molecular modeling was built via PyMOL package (<https://pymol.org>). Crystallographic data from Protein Data Bank were used to build the model, ASK1 (3ogl), TIR1 (2p1q) and CUL1 (1ldk). 1ldk was used to model the cartoon representation of AtCUL1 amino acid sequence (At4g02570) with SWISS-MODEL (<https://swissmodel.expasy.org/>)

## 2.15. Densitometry analysis

The densitometry analyses were performed with the ImageJ software (<http://rsb.info.nih.gov/ij/>).

## 2.16. Statistical Analysis

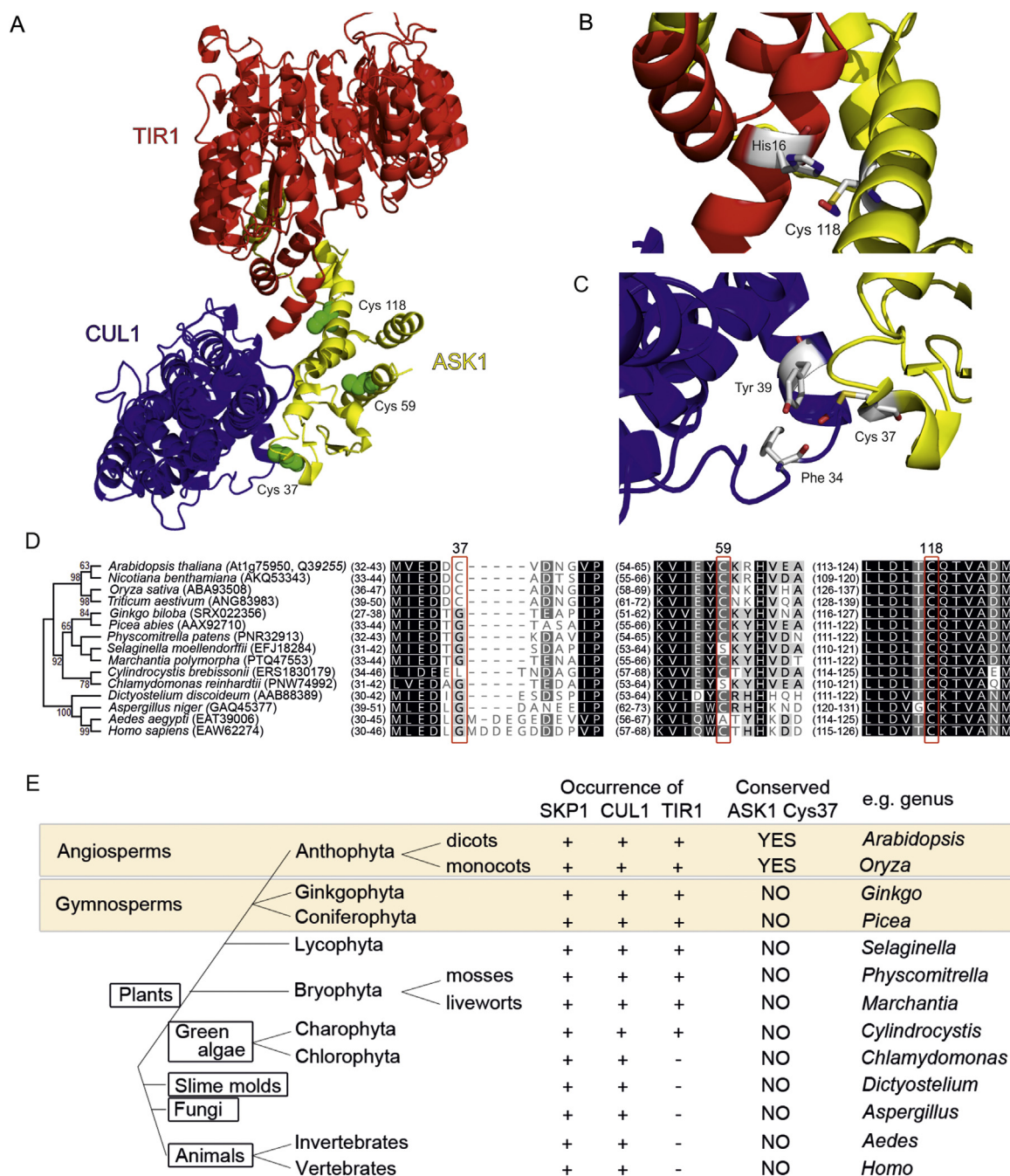
The values shown in each figure are mean values  $\pm$  SE. The data were subjected to *t*-test or analysis of variance (one-way or two-way ANOVA) and post hoc comparisons with Tukey's multiple range test (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001) using Graphpad Prism version 5.01 software.

## 3. Results

### 3.1. ASK1 protein structure exhibits two conserved Cys residues in the interaction interface with CUL1 and TIR1

We firstly explored the potential functional implications of redox post-translational modification on the three Cys residues in ASK1 (Cys37, Cys59 and Cys118) by analyzing localization and conservation through a bioinformatic approach. We modeled SCF<sup>TIR1</sup> complex from crystallographic data and determined the location of putative nitrosylated Cys residues of ASK1 in the respective interfaces: ASK1-TIR1 and ASK1-CUL1 (Fig. 1A). According to crystal structure, Cys37 is in the flexible loop of the N-terminal region of ASK1 in the interface involved in the interaction with CUL1 (Fig. 1C). SNO-Cys118 would reside in the H6 helix of ASK1 in the interaction interface with the H1 helix of TIR1 (Fig. 1B). Contrary, SNO-Cys59 would localize in H3 helix of ASK1, in a region with no interaction with SCF protein partners. Modeling predictions indicate that all Cys residues are exposed to the solvent and therefore, would be accessible to redox-mediated modifications. To address whether specific Cys residues work as cis-acting regulators of ASK1 protein function we reasoned that they should exhibit a high degree of conservation within SKP1 family in eukaryotic organisms. An exhaustive Blast search of SKP1 sequences along all life Kingdoms using ASK1 as a query was performed. A protein sequence alignment of SKP1s from representative members of different eukaryotic Kingdoms showed a high degree of evolutionary conservation of Cys59 and Cys118 residues (Fig. 1D). However, the Cys37 residue is only present in SKPs of Angiosperms including monocots and dicots, but not in sequences neither of the rest of Viridiplantae group nor in fungi or animals (Fig. 1D and E). Cys37 is conserved in 19 of 21 members of the SKP1 family in Arabidopsis, and 14 of the 22 members in rice (Supplementary Figs. S1, S2, respectively). This suggests that S-nitrosylation of Cys37 in ASK1 could probably emerged as an adaptation in the most recent common ancestor of both dicots and monocots before the multiple duplication events that expand SKPs in Angiosperms [37]. Although Cys118 is highly conserved among Kingdoms, 9 members of ASKs and 8 of OsSKPs have lost this residue, allowing differential regulatory responses inside each family (Supplementary Figs. S1, S2, respectively).





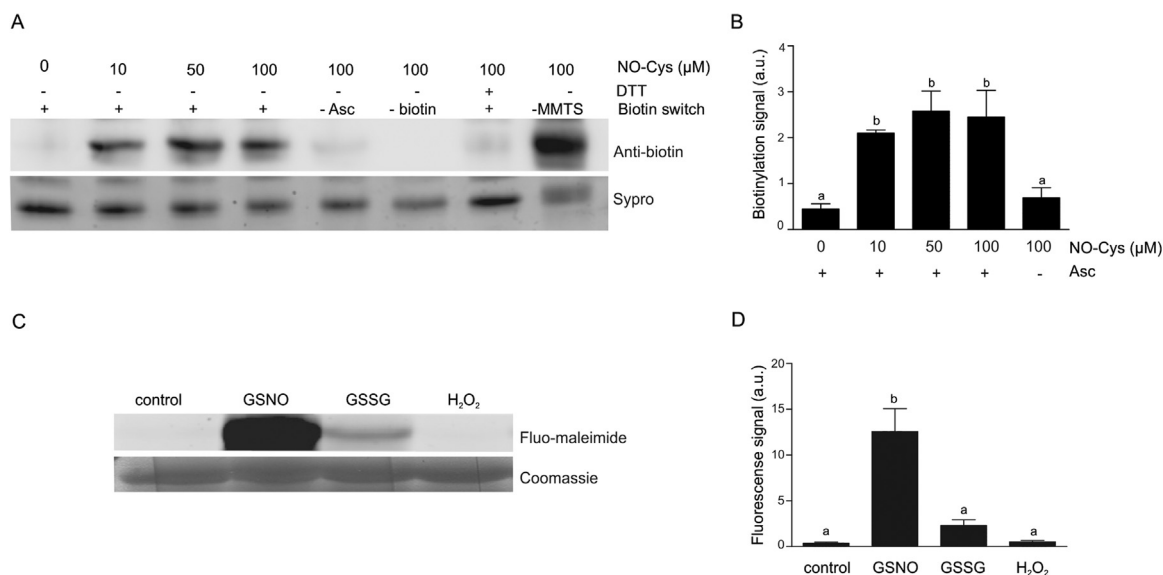
**Fig. 1.** *In silico* analysis of predicted localization and conservation of ASK1 Cys. (A) Cartoon representation of the N-term CUL1-ASK1-TIR1 interaction where Cys residues in ASK1 are represented by green spheres. Detailed views of the interfaces between (B) ASK1-TIR1 and (C) ASK1-CUL1 are shown. ASK1, CUL1 and TIR1 are represented in yellow, blue and red, respectively. S-nitrosylation of Cys residues are modeled and proximal residues are shown as stick models colored by element (yellow: sulphur; cyan: nitrogen; red: oxygen). Crystallographic data from Protein Data Bank were used to build the model, ASK1 (3og1), TIR1 (2p1q), CUL1 (1ldk). (D) Sequence conservation among SKP family members across phylogeny. Partial sequence alignments of representative SKP1 sequences showing regions including conserved Cys. Residues aligned with ASK1 Cys37, Cys59 and Cys118 are boxed. The phylogenetic tree was constructed using the complete protein sequences. Bootstrap values higher than 60% (1000 replicates over) are shown. Shaded residues correspond to conservation higher than 50%; more intense shading represent higher degree of conservation. (E) Tree of life representing the occurrence of SKP1, CUL1, TIR1 and the conservation of the ASK1 Cys37 residue in the SKP1 homologs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

### 3.2. Purified ASK1 protein undergoes redox regulation

To study if the thiol groups of Cys residues in ASK1 are sensitive to redox regulation, recombinant ASK1 protein was purified from bacteria extracts and incubated with increasing concentrations of NO-Cys. Then, ASK1 was subjected to the biotin switch assay [32,49] by which S-nitrosylation is specifically reduced by ascorbate and a biotin moiety is attached to the Cys residues that were previously S-nitrosylated. After

15 min of treatment, 10  $\mu$ M NO-Cys triggers ASK1 biotinylation, and the signal correlated with the dosage of added NO-Cys (Fig. 2A and B). As expected, the biotin signal was abolished when the reducing agent DTT was added after NO-Cys treatment. No signal was obtained in the absence of reduction by ascorbate, indicating the specificity of the biotin switch method. S-nitrosylation of ASK1 was also validated by the addition of the NO donors, GSNO and DEANO (Supplementary Fig. S3).

Since Cys residues are postulated as redox sensors susceptible to

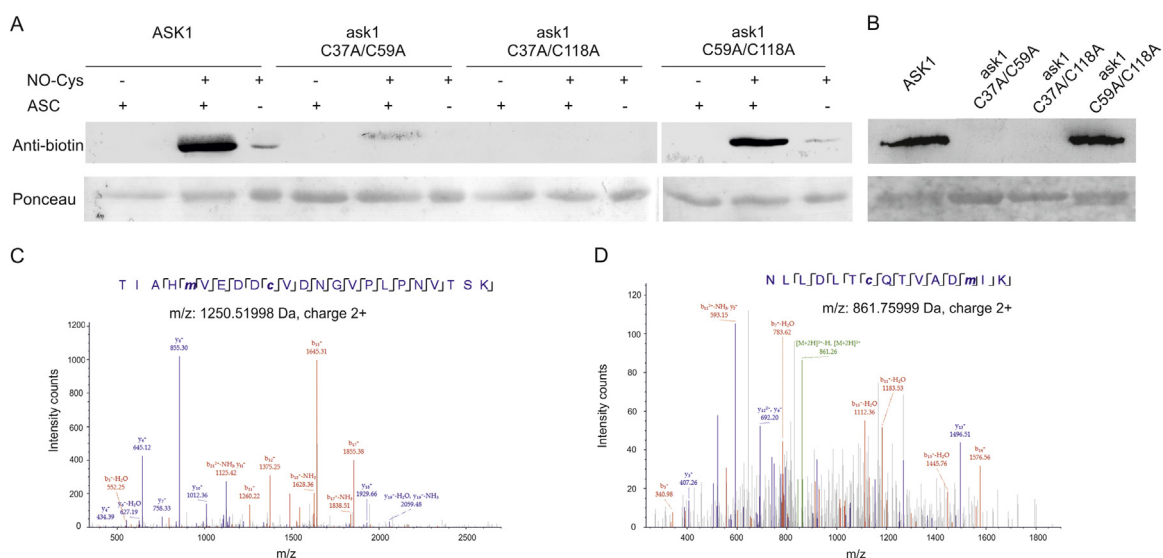


**Fig. 2. S-nitrosylation of recombinant ASK1 recombinant.** ASK1 recombinant protein was incubated with increasing concentrations of NO-Cys for 15 min and subjected to biotin-switch assay. S-nitrosylated proteins were detected by immunoblot using an anti-biotin antibody (upper panels). Sypro ruby staining is shown as a loading control (lower panels). Controls of the biotin switch assay minus ascorbate (Asc), minus biotin, minus methyl methanethiosulfonate (MMTS) and with DTT reduction before the biotin switch are shown. (A) Representative experiment and (B) quantification of the biotin signal of three independent experiments are shown. (C) ASK1 recombinant protein was incubated with 100 μM GSNO, 100 μM GSSH and 100 μM H<sub>2</sub>O<sub>2</sub> for 15 min and subjected to redox fluorescence switch assay to detect reversibly oxidized Cys. Modified proteins were detected by their fluorescent maleimide signal (upper panel). Coomassie staining is shown as a loading control (lower panel). (D) Quantification of fluorescence signal of three independent experiments. Different letters indicate a significant difference at P ≤ 0.05 (one way ANOVA, post hoc Tukey).

several covalent post-translational modifications, ASK1 oxidations by H<sub>2</sub>O<sub>2</sub>, GSSG and the physiological nitrosative agent GSNO were also evaluated. A modified version of the fluorescence switch, called redox fluorescence switch where oxidized Cys are labeled with fluorescent maleimide was carried out. In this assay, DTT as reducing agent was employed, which reduced S-nitrosylation but also all the putative reversible oxidative modifications triggered by the other oxidants [31]. Fig. 2C and D show weak oxidations with H<sub>2</sub>O<sub>2</sub> and GSSG in comparison with the strong modification of ASK1 by GSNO. Although S-glutathionylation could not be discarded, this result suggests a major susceptibility of ASK1 Cys residues to S-nitrosylation.

### 3.3. Cys37 and Cys118 residues are S-nitrosylated in vitro

In order to identify the S-nitrosylated residues in ASK1, recombinant double mutant ask1C37A/C59A, ask1C59A/C118A and ask1C37A/C118A proteins were generated by replacing two Cys by Ala residues, leaving a single Cys in each mutant protein. WT and all mutated proteins were analyzed by biotin switch assays. Cys residues in ASK1 were differentially modified by the NO donor, NO-Cys (Fig. 3). While Cys37 residue (C59A/C118A mutant) clearly undergoes S-nitrosylation at 10 and 100 μM NO-Cys treatments (Fig. 3B and A, respectively), Cys118 (C37A/C59A mutant) was only modified at 100 μM NO-Cys and to a



**Fig. 3. S-nitrosylation of recombinant ASK1 and its mutants in Cys37, Cys59 and Cys118.** (A, B) Double Cys mutants of ASK1 recombinant protein were incubated with (A) 100 μM NO-Cys or (B) 10 μM NO-Cys for 15 min and subjected to biotin switch assay. S-nitrosylated proteins were detected by immunoblot using an anti-biotin antibody (upper panel). Ponceau staining is shown as a loading control (lower panel). The experiment was repeated three times with similar results. Treatment without ascorbate (Asc) reveals the specificity of the biotin switch assay. (C, D) MS/MS spectra showing fragmentation patterns that correspond with ions of the y (blue) and the b (red) series of (C) S-nitrosylated ask1Q27K Cys37 peptide and (D) S-biotinylated ask1Q27K Cys118 peptide. m, methionine sulfoxide.

very limited extent. Cys59 (C37A/C118A mutant) was not detected S-nitrosylated under our assayed conditions (Fig. 3A and B). Next, to confirm redox modifications of Cys residues, MS analyses were performed. Since the trypsin-digested peptide containing the Cys37 residue was not clearly detected by MS, an ask1Q27K mutant protein including a new site for trypsin digestion was generated. S-nitrosylation of this mutant using 100  $\mu$ M NO-Cys is shown in Supplementary Fig. S4. Purified ask1Q27K protein was treated with 10 and 100  $\mu$ M NO-Cys or GSNO for 15 min, digested with trypsin and analyzed by MS. In addition, the mutant protein was subjected to biotin switch prior to MS analyses. At 10  $\mu$ M NO-Cys, Cys37 was found to be S-nitrosylated as shown in the MS/MS spectrum of 28TIAHmVEDDc<sup>NO</sup>VDNGVPLPNV-TSK50 (Fig. 3C), while Cys118 was detected biotinylated upon 100  $\mu$ M NO-Cys and 10–100  $\mu$ M GSNO treatment followed by biotin switch assay in the peptide 112NLLDLTc<sup>biotin</sup>QTVADmIK126 (Fig. 3D and Supplementary Table S2). At 10  $\mu$ M GSNO, S-glutathionylation was also observed in Cys37 and Cys118 (Supplementary Fig. S5A and B). Supplementary Table S2 summarizes the observed S-nitrosylated and S-glutathionylated Cys residues and the tryptic peptides in which they have been found.

In order to evaluate whether S-nitrosylation of Cys37 and Cys118 residues or even point mutations could affect ASK1 protein conformation, CD analysis was performed. The CD spectrum in the far UV revealed a typical  $\alpha$ -helix secondary structure profile with minimal valleys at 208 nm and 222 nm (Supplementary Fig. S6A). ask1C37A and ask1C118A mutants have the same CD spectra than ASK1 suggesting that at least the secondary structure is not affected by these mutations. Treatment with 100  $\mu$ M GSNO produced a slight alteration of the CD spectrum (Supplementary Fig. S6B); however, this cannot be attributed to S-nitrosylation, as treatment with reduced GSH produces the same variation of the spectrum, probably due to the optical effect of the thiol group added at relatively high concentration.

### 3.4. NO modulates ASK1-TIR1/AFB2 and ASK1-CUL1 interactions through Cys118 and Cys37

Based on the function of ASK1 as a bridge between TIR1/AFBs and CUL1 in the SCF<sup>TIR1/AFBs</sup>, we investigated the effect of ASK1 S-nitrosylation on the interaction with its partners. First, we studied the interaction of ASK1 with TIR1, as Cys118 lies in the interface between both proteins. We performed pull-down assays, where GST-ASK1 immobilized in GSH-sepharose beads was S-nitrosylated in the presence of 100  $\mu$ M NO-Cys and incubated with *in vitro*-translated TIR1-myc. ASK1 S-nitrosylation resulted in a significant increase of TIR1-myc protein recovery (Fig. 4A). However, ASK1-TIR1 interaction was diminished when ASK1 was treated with NO-Cys followed by DTT reducing agent which abolished S-nitrosylation (Fig. 4A). In order to confirm NO-mediated regulation *in vivo*, TIR1-ASK1 and AFB2-ASK1 interactions were tested by yeast two-hybrid system. Yeast co-expressing DBD-TIR1 or DBD-AFB2 and AD-ASK1 were treated with the slow-release NO donor, SNP [22].  $\beta$ -Galactosidase reporter expression was analyzed two days after spotting, observing that SNP treatment enhanced both ASK1-TIR1 and ASK1-AFB2 interactions (Fig. 4B and Supplementary Fig. S7A). We next investigated the *in vivo* role of putatively S-nitrosylated Cys residues. In comparison to AD-ASK1, AD-ask1C118A mutant showed weak interaction with DBD-TIR1 and DBD-AFB2 proteins, while ask1C37A and ask1C59A showed similar interactions with ASK1 (Fig. 4C and F), strongly suggesting the role of Cys118 S-nitrosylation in favoring the interaction. In support of these findings, a reduced interaction between ask1C118A and TIR1 was also detected by GST pull-down assay (Supplementary Fig. S8A).

Since Cys37 residue is located in the interface involved in the interaction with CUL1, we further investigated whether NO could also modulate ASK1-CUL1 interaction. Yeast cells co-expressing AD-ASK1 and DBD-CUL1 were grown in the presence of increasing concentrations of SNP. A positive increase of  $\beta$ -galactosidase activity revealed the *in*

*vivo* and NO-dependent ASK1-CUL1 interaction (Fig. 4D and Supplementary Fig. S7B). While ask1C37A-CUL1 was highly diminished, mutations on Cys59 and Cys118 residues of ASK1 had no effect on their binding to CUL1 protein (Fig. 4E and F, Supplementary Fig. S8B).

### 3.5. Cys37 and Cys118 have a pivotal role in auxin signaling activation in *planta*

To assess the functional relevance of S-nitrosylation of Cys37 and Cys118 in ASK1 *in planta*, each single mutant was transiently expressed under the control of the constitutive cauliflower mosaic virus 35S promoter in *N. benthamiana* leaves. Then, the ability to activate auxin signaling was tested. Leaves were sprayed with 10  $\mu$ M IAA and *Aux/IAAs* and *GH3s* gene expression was analyzed 1 h later. The median and dispersion of the expression of five early auxin response genes with and without IAA treatment for each ASK1 mutant are shown in Fig. 5A. While overexpressing ASK1 and ask1C59 showed an increased auxin response gene expression upon IAA treatment, the overexpression of ask1C37 and ask1C118 mutants failed to activate the auxin signaling pathway. All these findings provide further evidence about the relevance of ASK1 Cys37 and Cys118 residues on SCF<sup>TIR1/AFBs</sup> action *in planta*.

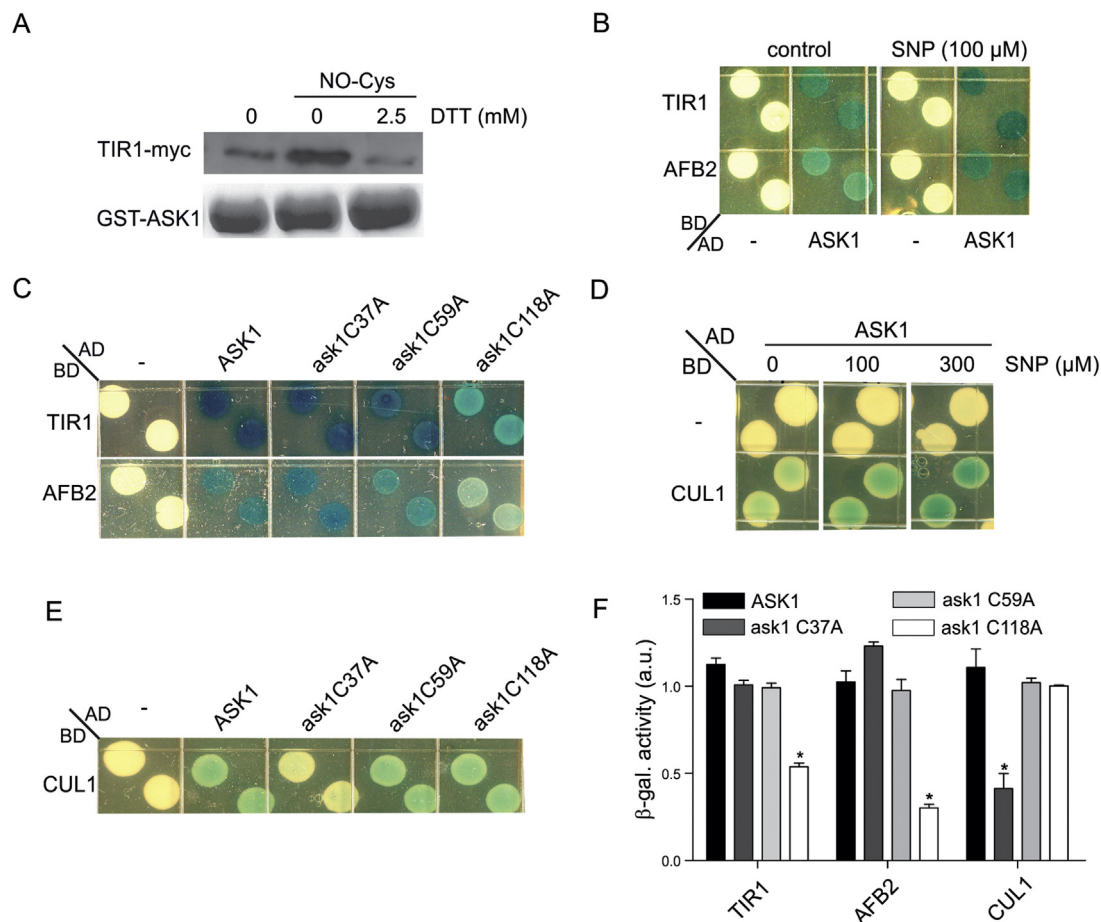
## 4. Discussion

Our data suggest a novel mechanism by which NO directly regulates SCF<sup>TIR1/AFBs</sup> E3 ubiquitin ligase complex assembly through S-nitrosylation of ASK1 impacting on auxin signaling activation (Fig. 5B). Since TIR1 F-box protein is also redox-regulated by S-nitrosylation [80], collectively our results substantiate the interplay between S-nitrosylation and ubiquitination regulation which may constitute a robust control mechanism to fine-tune auxin responses during plant growth and development. Reciprocal regulation of multiple post-translational modifications constitutes a common strategy in plant signaling regulation. Recent evidence described S-nitrosylation of histone deacetylases modulating histone acetylation [52]. In addition, S-nitrosylation of the arginine methyltransferase PRMT5 leads to methylation control of pre-mRNA splicing in response to environmental changes [29].

Interestingly, *in vitro* ASK1 undergoes NO-induced redox modifications, including S-nitrosylation on Cys37 and Cys118 (Figs. 2 and 3), as well as S-glutathionylation on these same residues (Supplementary Fig. S5). Here, it is necessary to highlight the yet unsolved problem derived from unknowing the real concentration of NO in specific subcellular microdomains. Thus, it is not recommendable to establish conclusions from comparisons between concentrations of NO required for modifying Cys residues *in vitro* and physiological NO concentrations. In other words, the *in vitro* experiments hardly can reconstruct in a precise way and with the same efficiency, the cellular environments where the S-nitrosylation and S-glutathionylation of proteins takes place. Several methods have been assessed to approach the measuring of NO concentrations in different plant organs *in vivo*. Even if some discrepancies appear, it is conceivable that the range of NO concentrations under normal growth conditions varies between 10 nM and 1  $\mu$ M [82], very close to NO concentrations found in animal tissues [30].

NO-mediated modification of Cys118 and Cys 37 regulates ASK1 interaction with TIR1/AFB2 and CUL1 scaffold protein, respectively (Fig. 4). Consequently, mutations in those residues significantly reduce these interactions impairing auxin signaling activation in plant tissues (Fig. 5). ASK1 together with TGA, NPR1 and APX1 constitute valuable *Arabidopsis* examples where the diversity of putative thiol redox states offers the possibility to use Cys residues of a single protein for a wide range of molecular switches [59,71,10]. S-glutathionylation was initially considered to protect proteins from over-oxidation of Cys during oxidative stress but actually, it is also emerging in association with S-nitrosylation as a regulatory modification in mammals [26,50]. In





**Fig. 4. S-nitrosylation on Cys37 and Cys118 modulates assembly of ASK1 in an SCF E3 ubiquitin ligase complex.** (A) Pull-down reactions were performed using *in vitro* synthesized TIR1-myc and recombinant GST-ASK1 proteins. Reactions were carried out without any addition or in the presence of 100 μM NO-Cys in combination or not with DTT. TIR1 protein was detected using anti-myc antibody. Coomassie blue-stained GST-ASK1 was used as a loading control (lower panel). (B, D) 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 100 and 300 μM SNP and X-Gal to develop β-galactosidase activity. (C, E) Yeast two-hybrid interaction experiments between the different ASK1 Cys mutants, and TIR1-AFB2 or CUL1, respectively. The experiment was repeated at least three times with similar results. (F) Densitometry of yeast two-hybrid activity (n = 3; \* p < 0.01, t-test).

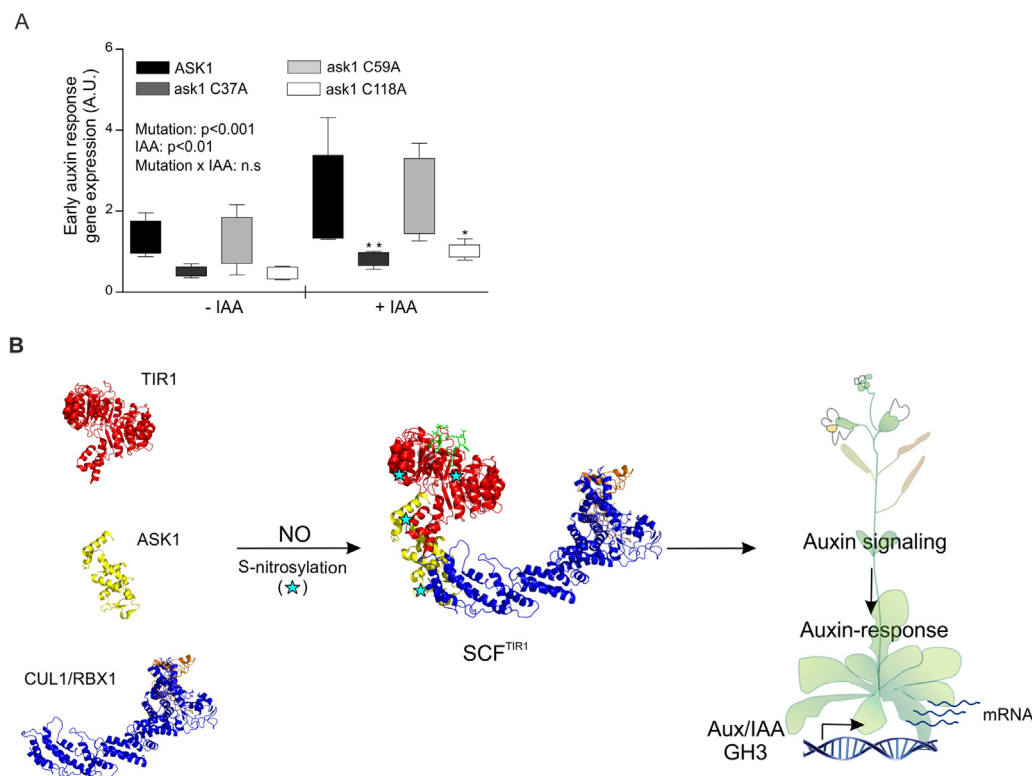
plants, evidences of regulation by S-glutathionylation comes from *in vitro* and proteomic studies, but *in vivo* function of S-glutathionylation is in its infancy [4]. GSNO was reported to mediate β-amylase3 activity inhibition by S-glutathionylation under cold stress in Arabidopsis, although S-nitrosylation was not analyzed [75]. It will be of interest in future studies to explore events that distinctively can be mediated by S-glutathionylation or S-nitrosylation of ASK1.

Highlighting the role of proteasome degradation on the regulation of multiple signaling transduction pathways during plant life, the subunits of ubiquitin ligase complexes represents the 6% of Arabidopsis proteome with approximately 700 F-box proteins comparing to 69 identified in humans [81]. In addition, whereas protists, algae, fungi, and vertebrates have a single SKP1 gene, vascular plants possess multiple SKP1 homologs [19,37,55,93]. The presence of a multi-gene SKP1 family is probably a general feature of plants where more dynamic regulation of protein level is required to modulate their responses to environmental stimuli. In addition, diversification of this gene family could allow acquisition of new regulation points through inclusion of amino acids susceptible to versatile and reversible redox control. Phylogenetic analysis of Cys residues conservation in SKP1s reveals that Cys37 is conserved only in Angiosperms (Fig. 1), where SKP gene family suffered a large expansion mainly through repeated tandem duplication [37]. Cys37 S-nitrosylation in ASK1 could represent an evolutionary leap for the assembly dynamics of SCF complexes with multiple impacts

in flowering plants. The relatively rapid evolution of F-box proteins and SKPs partners suggests that SKP1 modifications could constitute an outsized role in environmental regulation of unique lineage with species-specific functions. Coincidentally, a particular post-translational modification which includes hydroxylation and subsequent glycosylation in Pro143/154 that serves as O<sub>2</sub>-sensing mechanism controlling development was reported in SKP1 from two unrelated protists, the amoeba *Dictyostelium* and the parasite *Toxoplasma*, respectively [61,87,91,92]. Validation of this mechanism in diverged unrelated protists suggests that SKP1 hydroxylation and glycosylation occurred in ancestral eukaryotes and was lost in fungi, higher plants and animals.

SCF E3 ubiquitin ligases have been shown to be essential for sensing and signaling in response to various hormones in plants, where NO acts as a versatile and extensive second messenger [70]. It is well reported that auxin promotes the induction of NO in different plant species [11,44,58,80]. It appeared that NO-dependent post-translational modification of different components of SCF ubiquitin ligase complex may function as a key strategy to determinate precise SCF<sup>TIR1/AFBs</sup> assembly. This regulation might involve a time-dependent and tissue-specific proper activation of auxin signal transduction pathway. Although S-nitrosylation of TIR1 and ASK1 is currently the only evidence of NO directly regulating the ubiquitin machinery in plants that we know, the regulation of ubiquitin-proteasome system by S-nitrosylation is a conserved mechanism associated to neurodegenerative diseases in animals





**Fig. 5. ASK1 Cys37 and Cys118 impact on auxin signaling activation.** (A) ASK1 WT or its mutants in Cys37, Cys59 and Cys118 were transiently expressed in leaves of 4-week-old *N. benthamiana* plants. Leaves were sprayed with 10  $\mu$ M IAA or mock solution as control 24 h post-infiltration. The expression of a subset of early auxin response genes (NbGH3.6 -Niben101Scf13270g03004.1; NbGH3.2 -Niben101Scf12751g00003.1; NbGH3.1 -Niben101Scf12751g00003.1; NbIAA3 -Niben101Scf12159g09021.1; NbIAA19 -Niben101Scf02572g04006.1; NbEF-1 $\alpha$  -Niben101Scf12941g01003.1) was analyzed by qPCR 1 h later in three independent experiments. Box-plot (median, 1–3 interquartile range and 95% confidence interval) of normalized expression of the subset of early auxin response genes relative to EF1. The significance of the effect of auxin treatment and Cys mutation in two-way ANOVA is indicated (post-hoc Dunnett compare to ASK1 \* $p < 0.05$ ; \*\* $p < 0.01$ ). (B) A model for redox regulation of SCF<sup>TIR1-AFBs</sup> complex assembly. S-nitrosylation of Cys37 and Cys118 residues in ASK1 impacts on its binding with CUL1 and the F-box proteins, TIR1 and AFB2, leading to enhanced SCF<sup>TIR1</sup> and SCF<sup>AFB2</sup> assembly.

In addition, S-nitrosylation of TIR1 increases its interaction with Aux/IAA [80]. This redox-mediated complex stabilization augments the ubiquitination/degradation of Aux/IAA proteins which, in turn, directs the activation of auxin-response genes. ASK1, TIR1, CUL1, RBX1 and IAA7 peptide are colored in yellow, red, blue, orange and green, respectively. IAA is shown in grey.

[54]. Our results establish a molecular framework for NO modulation of SCF complexes in plants. The universality of both S-nitrosylation and SCF complexes associated to the regulation of ubiquitin-proteasome system provides a valuable platform to extend this study to other eukaryotic organisms.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.redox.2018.07.003](https://doi.org/10.1016/j.redox.2018.07.003).

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