Research article

Biological Sciences, Plant Biology

Redox-mediated regulation of ABSCISIC ACID-INSENSITIVE 5 affects seed germination and seedling development in Arabidopsis

Inmaculada Sánchez-Vicente[1]*, Fátima Pollo-Rodríguez, Isabel Mateos, Laura Huebra-Montero, Virginia Corral-Barrio, Pablo Albertos, Oscar Lorenzo & Capilla Mata-Pérez[1]*

Dept. of Botany and Plant Physiology, Institute for Agribiotechnology Research (CIALE), Faculty of Biology, University of Salamanca. C/ Río Duero 12, 37185 Salamanca, Spain

[1]These authors contributed equally to this work

*Corresponding author. E-mail capilla.mata@usal.es; elfik@usal.es; Tel. 0034 923 294500-Ext.5117

HIGHLIGHT

Post-translational modifications and redoxins fine-tune the regulation of ABI5, influencing its redox state and subcellular localization ultimately determining seed germination and seedling establishment.

ABSTRACT

Post-translational modifications (PTMs) of key transcription factors constitute important switches that shape protein function and, consequently, signal transduction and cellular responses. Seed germination and seedling establishment are complex traits regulated by PTMs, which converge on key molecular devices such as the bZIP transcription factor ABI5. The latter represents a molecular hub in the abscisic acid (ABA) signaling pathway, which represses seed germination and seedling establishment. ABI5 is post-translationally modified by nitric oxide (NO) through Cys153-specific *S*-nitrosylation (SNO), leading to its degradation. Despite the physiological effects of redox-sensitive proteins, the specificity and molecular mechanisms underlying this type of regulation during seed germination and post-germination developmental checkpoints remain unknown. Here we show the effect of the redox environment on the formation of ABI5 complexes, emphasizing the relevance of Cys153. In addition, the mutation of this key residue and the phosphorylation status influence the subcellular localization of ABI5. Recent research points to the reversibility of redox-mediated modifications through the action of redoxins. We establish an enzymatic system underlying the reversibility of SNO mediated by thioredoxin *h*5 (TRX*h*5). Furthermore, seeds overexpressing the redoxins *ROXY10* and *ROXY21* show a dysregulation in germination and in the accumulation of the ABI5 protein. These results provide a physiological link between redox regulation and the ABA signaling pathway through the control of ABI5, which is crucial for a successful seedling establishment.

KEYWORDS

ABI5, redox regulation, redoxins, PTMs, nitric oxide, abscisic acid, germination, seed

INTRODUCTION

Seed germination represents a key physiological stage that is essential for the proper establishment of seedlings. This process integrates environmental and internal cues to control

the shift between growth arrest and the progression of seedling development. Precise and specific regulation is essential for ecological success, as it enables plant survival. Abscisic acid (ABA), gibberellins (GAs), and nitric oxide (NO) integrate the central molecular crosstalk that antagonistically controls this process. NO counteracts ABA-mediated inhibition of germination at multiple levels that converge on the bZIP transcription factor (TF) ABSCISIC ACID INSENSITIVE (ABI5) (reviewed in Sánchez-Vicente et al., 2019). ABI5 plays an essential role in regulating seed germination and early seedling development in the presence of ABA and abiotic stress (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001). NO triggers ABI5 degradation through *S*-nitrosylation of Cys153, which facilitates interaction with CULLIN4 and KEEP ON GOING-based E3 ligases (Albertos et al., 2015).

ABI5 constitutes an important regulatory node that controls key developmental decisions by orchestrating the expression of numerous downstream genes. In addition to essential structural changes, which involve protein turnover or irreversible activation/inactivation, other levels of modulation are needed to regulate them precisely and respond efficiently to a range of internal and external conditions. In this regard, post-translational modifications (PTMs) constitute key points for regulating protein properties, but further studies on their reversibility are needed to decipher the complete molecular mechanism.

Redox changes have become fundamental in the regulation of the biological properties of proteins, and the state of functional Cys residues influences the structure, stability, activity, binding capacity or subcellular localization of proteins. Cys residues act as redox sensors due to their ability to adjust their state based on cellular gradients (reviewed in Spadaro et al., 2010; Couturier et al., 2013; Del Río, 2015). Under non-stressful conditions, reduced –SH groups predominate, but stress-related events promote an oxidative burst in the cytoplasm. This redox imbalance modulates the formation of complexes that ultimately redirect gene expression (Comelli et al., 2007; Serpa et al., 2007; Shaikhali et al., 2008; Shaikhali, 2015; Ströher et al., 2009; Giesguth et al., 2015).

Although the redox influence has been analyzed, further research is needed to clarify the molecular mechanism underlying this process. Enzymatic reversibility appears to be a central cue, remodeling this network to improve plant responses to stress situations. One of the best-characterized systems comprises the *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (NPR1), a key transcriptional coactivator during plant immunity, Thioredoxin-*h*5 (TRX*h*5) and NADPH-dependent TRX reductase NTRA (Kneeshaw et al., 2014). The *S*-nitrosylation of NPR1 involves the formation of oligomers in the cytoplasm (Tada et al., 2008;

Lindermayr et al; 2010), which can be reversed by the action of TRX*h*5 as a selective SNO reductase (Kneeshaw et al., 2014). To close the cycle, oxidized TRX*h*5 can be rescued by NTRA. NPR1-SNO oligomers are localized in the cytoplasm, as reduced monomers are translocated to the nucleus, where they modulate gene expression.

As mentioned, the stability of ABI5 is modulated by NO through specific *S*-nitrosylation (Albertos et al., 2015). We recently described the modification of its homologue bZIP67 by NO, involved in the regulation of fatty acid storage in seeds (Mendes et al., 2013), and how this PTM could be reversed by the action of a redoxin, which ultimately affects the lipid profile of the seed (Sánchez-Vicente et al., 2024).

Here we show that ABI5 is a redox-sensitive TF, since under oxidizing conditions, ABI5 predominantly exhibits high molecular weight forms, while treatments with reducing agents promote the accumulation of the monomeric form. Analyses of the ABI5C153S mutant version displayed variations in oligomeric complex formation and subcellular localization, highlighting the relevance of this Cys in sensing and responding to the cellular redox environment and regulating seed germination. Moreover, phosphorylation also appears to be an important PTM for regulating the subcellular localization of ABI5, revealing the contribution of different PTMs that coordinate the same interaction points. To expand our knowledge of the entire molecular mechanism, we analyzed the reversibility of redox regulation by redoxins. Bioinformatics analysis showed that several redoxins are specific to seeds or highly abundant at this developmental stage. Here we define a molecular redox network that controls the state of ABI5 and involves the specific action of seed redoxins. Overexpression of the glutaredoxins *ROXY10* and *ROXY21* affects seed germination and displays differences in the accumulation pattern of ABI5. Additionally, the ROXY10 interacts directly with ABI5 *in vivo*, supporting a possible direct mechanism of ABI5 regulation. Finally, thioredoxin *h*5 (TRX*h*5) may act as a denitrosylase, decreasing the level of ABI5-SNO. Our data provided genetic, biochemical, and physiological evidence of the impact of precise regulation of ABI5 PTMs during the crosstalk between ABA and NO to modulate the switch between seed germination arrest and seedling establishment.

METHODS

Plant materials and treatments

Arabidopsis thaliana accessions Columbia-0 (Col-0) and Landsberg *erecta* (L*er*) were the genetic backgrounds for the wild-type (WT) plants used in this work. Seed stocks of *abi5* mutants were obtained from Arabidopsis Biological Resource Center (ABRC). The single *trxh5* and *trxh3* knockout mutants were kindly provided by Dr. Julio Salinas (CIB-CSIC, Madrid) and double *trxh3trxh5* knockout mutant was kindly provided by Dr. Steven Spoel (University of Edinburgh, United Kingdom). Arabidopsis plants were grown in a growth chamber or greenhouse with a 16-h light/8-h dark photoperiod in pots containing a 1:3 vermiculite/soil mixture. For *in vitro* culture, Arabidopsis seeds were previously sterilized in 75% (v/v) commercial bleach (sodium hypochlorite 3,7%) and 0.01% (v/v) Triton X-100 for 5 min and washed 3 times with sterile water. Seeds were stratified for 3 days at 4°C under dark conditions for dormancy removal and then sowed on MES or Murashige and Skoog (MS) solid media. For the analysis of seed germination, seeds were not stratified and sowed straight on MES solid media to keep the same dormancy status. 5 mM MES was supplemented with 0.8% (w/v) agar and adjusted pH to 5.8 with KOH before autoclaving, and MS was supplemented with 0.6% (w/v) agar, 2% (w/v) sucrose and adjusted to 5.7 with NaOH before autoclaving. Treatments were added to the medium and plates were sealed and incubated in controlled growing chambers.

The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) was purchased from Fisher Scientific. The proteasome inhibitor (MG132), ABA, dithiothreitol (DTT), *S*-Nitroso-N-acetyl-DL-penicillamine (SNAP) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich. Commercial GSNO was purchased from Calbiochem and homemade GSNO was synthesized using equimolar concentrations of $NaNO_2$ and GSH. Reaction was stopped after 5 min by adding 100 mM NaOH and concentration was calculated by measuring the absorbance at 335 nm (ε = 0.92 $mM^{-1} \cdot cm^{-1}$).

Germination assays

Lots of seeds were grown and harvested at the same time. After-ripened seeds were plated on solid medium supplemented with ABA (0.5 µM), cPTIO (100 µM) and GSNO (500 µM). Seeds were shown on MES media (Sigma-Aldrich) adjusted at pH 5.8 while MS media (Duchefa Biochemie) was used for seedling growth and stock multiplication. Plates were incubated in controlled environment growth chamber at 21°C under long day conditions (16 hours of light and 8 hours of dark). The values represent germination or seedling establishment percentages from 50-100 seeds in each experiment, which consists in three technical replicates (from the same

seed batch). This assay was repeated at least two times with different seed batches (except for the preliminary screening).. The germination ratio corresponds to seeds with an emerged radicle and seedling establishment is considered when developing green fully expanded cotyledon.

Generation of transgenic *Arabidopsis* plants

Overexpression *35S:cMyc-ABI5WT* and *35S:cMyc-ABIC153S* lines were obtained as described previously in Albertos et al., (2015). *ROXY10* (AT5G18600), *ROXY21* (AT4G33040), *TRXh3* (AT5G42980), *TRXh5* (AT1G45145) and *NRX1* (AT1G60420) CDS were cloned into pEarleyGate 201 (Earley et al., 2006) using GATEWAY technology using LR clonase (Fisher) to generate the *35S:HA-ROXY10*, *35S:HA-ROXY21*, *35S:HA-TRXh3*, *35S:HA-TRXh5*, *35S:HA-NRX1* transgenes. The *pABI5:GFP-ABI5* construct was kindly provided by Dr. Sandra Bensmihen (CNRS-LIPM, Castanet-Tolosan, France) (Bensmihen et al., 2005).

The constructs generated were used to transform the C58C1 (pGV2260) *Agrobacterium* strain (Deblaere et al., 1985), and to generate Arabidopsis transgenic lines by the floral dip method (Clough and Bent, 1998) as previously described (Albertos et al., 2015). Seeds were plated on the medium supplemented with the corresponding selection marker to identify T1 transgenic plants. Approximately 100 T2 transgenic seeds were plate again on selection medium, and those with 3:1 (resistant/sensitive) segregation ratio were selected, which correspond to one insertion lines. Finally, T3 homozygous seeds were selected by 100% resistance and used for further studies.

Production of recombinant proteins and polyclonal antibodies

Primary antibody against ABI5 was produced as described in Albertos et al., (2015) and purification was carried out by IMMUNOSTEP S.L. (Salamanca, Spain). Primary antibody against the full-length, recombinant NRX1 was kindly provided by Dr. Steven Spoel (University of Edinburgh, United Kingdom). Recombinant TRX*h*5 and ABI5 recombinant proteins were expressed in the *Escherichia coli* BL21 B834 (DE 3) strain and purified using the B-PER Complete Bacterial Protein Extraction Reagent (ThermoFisher) by and Ni-NTA His Bind Resin (Novagen) according to the manufacturer´s protocol. The induction conditions were selected depending on the best obtained results related to the quantity and purification protein values.

Western blotting

Total protein for western blot analysis was extracted from seeds or seedlings of wild type, knockout mutants or transgenic lines, under treatments specified in each assay. Seed tissue was powdered using mortar and pestle and protein from seedlings was obtained by using grinding glass balls. Final powdered tissue was homogenized in 1 volume of extraction buffer (100mM Tris-HCl, 150mM NaCl, 0.25% NP-40) containing 1mM PMSF and 1X cOmplete® EDTA-free proteases inhibitors (Sigma) followed by centrifugation for 10min at 15,800g at 4°C. Final protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad) based on the Bradford method (Bradford, 1976). Total protein was loaded per well in SDS-acrylamide/bisacrylamide gel electrophoresis using Tris-glycine-SDS buffer. Proteins were electrophoretically transferred to an Inmobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) using the Trans-Blot Turbo (Bio-Rad).

Membranes were blocked in Tris buffered saline-0.1% Tween 20 containing 5% blocking agent and probed with antibodies diluted in blocking buffer with 1% blocking agent. Anti-ABI5 purified rabbit immunoglobulin (Biomedal, 1:10,000), anti-NRX1 (ProteinTech, 1:15,000), anti-Actin (Sigma, 1: 10,000), anti-Biotin (Sigma, 1:4,000), anti-His (Qiagen, 1:2,000), anti-HA-HRP (Roche, 1:2,000), and ECL-Peroxidase-labelled anti-rabbit (Amersham, 1:10,000) and anti-mouse (Amersham, 1:10,000) antibodies were used in the western blot analyses. Detection was performed using ECL Advance Western Blotting Detection Kit (Amersham) and the chemiluminescence was detected using an Intelligent Dark-Box II, LAS-1000 scanning system (Fujifilm), and a ChemiDoc Imaging System (Bio-Rad).

In vitro redox assays

Recombinant protein (Induced with 1mM IPTG at 25ºC for 4 hours, and purified by using Ni-NTA His Bind Resin from Novagen) was pretreated with the reducing agent DTT (10mM, Sigma) to obtain the same redox status in all the samples (-SH). To determine *in vitro* ABI5 redox status, DTT was removed by acetone precipitation, and proteins were subjected to treatment with 1mM GSNO, 1 mM GSH or 20 mM of H_2O_2 in the dark at room temperature for 1 hour with regular mixing. Total protein was analyzed in 7% nonreducing SDS-PAGE and 10% reducing SDS-PAGE gels.

S-nitrosylation and denitrosylation assays

For the *in vitro S*-nitrosylation analysis, ABI5 recombinant protein (BIOMEDAL) was pre-treated with the reducing agent 20 mM DTT for 1 hour at room temperature in the dark to obtain the same redox status of cysteine residues in all the samples (-SH). Next, DTT was removed by acetone precipitation and ABI5 was treated with 1 mM GSNO for 30 min at room temperature in the dark with regular mixing. After *S*-nitrosylation, GSNO excess was removed by adding two volumes of -20ºC acetone and centrifugation at 15,000 *xg* for 5 min at 4°C followed by two rinse-steps with ice-cold 70% acetone. After desalting, molar excess of TRX*h*5 (15-20 µM) was incorporated into the reaction and kept for 1 h at 25ºC with gentle mixing. For –SH blocking, SNO-treated protein was incubated with 20 mM N-ethylmaleimide (NEM) and 2.5% SDS at 50°C for 30 min with frequent mixing. Finally, 1 mM biotin HPDP (Pierce, Rockford, IL) and 20 mM sodium ascorbate were added to the reaction and incubated for 1 h at room temperature in darkness and regular mixing. After each step, reagents were removed by precipitation with two volumes of ice-cold acetone and centrifuged at 15,000 *xg* for 5 min at 4°C followed by two rinse-steps with ice-cold 70% acetone. After biotin-substitution, proteins were dissolved in 110 µL of HEN buffer (10 mM Hepes, 1 mM EDTA and 0.1 mM neocuproine, pH 7.7), being 10 µL kept as "input fraction" and the rest incubated with streptavidin agarose beads (Sigma) overnight (o/n) at 4ºC with gentle shaking. Beads were washed three times with washing buffer (10 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine, 600 mM NaCl and 0.5% Triton X-100, pH 7.7) and eluted using 1 mM of the reducing agent β-mercaptoethanol in HEN buffer for 30 min at room temperature with vigorous mixing. Finally, proteins were loaded in SDS-acrylamide/bisacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane, to detect protein-SNO with anti-ABI5 (BIOMEDAL, 1:10000).

Co-immunoprecipitation and pull-down assays

For cMyc pull-down assays, the proteins were extracted from Arabidopsis 7-day-old seedlings with lysis buffer (100mM Tris–HCl, pH 7.5, 150mM NaCl, 0.1% Tween-20) supplemented with 1 mM PMSF and 1X cOmplete® EDTA-free proteases inhibitors (Sigma). Extracts were cleared by centrifugation and protein concentration was determined by Bradford assay. 300 µg of soluble proteins from Col-0; *35S:cMyc-ABI5WT* and 600 µg from Col-0; *35S:FLAG-NRX1*, or Col-0; *35S:cMyc-ABI5WT* and Col-0; *35S:HA-ROXY21*, were treated with or without 1mM GSNO in darkness at room temperature during 30 min. For Fig.4B and Supplementary Fig.4B assay, cMyc-

ABI5 (the bait) was immobilized on the beads (anti-c-Myc Affinity beads Matrix), and it is shown as "pull-down" in the Fig. RbcL also bounds non-specifically to the beads, so this protein may appear in the pull-down. To detect the immobilization on the beads an anti-ABI5 antibody was used. CoIP shows proteins bound to the cMyc-ABI5 protein immobilized in the beads and were detected with the specified antibodies (anti-NRX1 or anti-HA).

After treatment, extracts were immunoprecipitated using anti-c-Myc Affinity Matrix (Sigma) for ABI5/NRX1 and ABI5/ROXY21 interactions, respectively. Extracts and beads were incubated during 2 hours at 4°C at 4rpm. After incubation, beads were washed three times in the lysis buffer and proteins were eluted from the beads in the sample buffer.

Bimolecular fluorescence complementation assay.

The coding regions of *ABI5* and *ROXY21* were cloned into pYFN43 and pYFC43 vectors using GATEWAY technology. The constructs were transiently expressed into *Nicotiana benthamiana* epidermal cells by agroinfiltration of transformed *Agrobacterium tumefaciens* C58C1 strain with pGV2260. Additionally, p19 was added to avoid silencing. Leaves were observed by confocal microscopy after 72-96 hours. For life imaging of YFP, fluorescence was examined with a Leica TCS SP2 confocal microscope. For fluorescence detection, the excitation source was an argon ion laser at 488 nm and detection filters between 515-530 nm.

Subcellular localization in *Arabidopsis thaliana* and *Nicotiana benthamiana*

The subcellular localization of ABI5 was observed using Arabidopsis transgenic lines carrying the *pABI5:GFP-ABI5* construct. Treatments were performed in Arabidopsis seeds imbibed for 24 hours in 5 mM MES buffer pH 5,8 with 100 µM of the proteasome inhibitor Z-leu-leu-leu (MG132, Sigma) and 1 mM of cPTIO (Fisher), to accumulate ABI5 protein. Then, buffer was removed to eliminate them, and seeds were transferred to new MES plates supplemented with NO donors, 1 mM GSNO (Sigma) and SNAP, 1 mM cPTIO or 100 µM MG132, for 6 hours.

To visualize the localization of TRXh3, ROXY10 and ROXY21, the coding sequences were cloned into pGWB6 or pMDC43 using GATEWAY technology. The constructs were transiently expressed in *Nicotiana benthamiana* by agroinfiltration of transformed *Agrobacterium tumefaciens* C58C1

strain. Additionally, p19 was added to avoid silencing. Leaves from these plants were observed by confocal microscopy 72-96 hours after infiltration using a Leica TCS SP2 confocal microscope. GFP was excited using the 488 nm laser and detection filters between 500-550 nm. Chlorophyll was differentiated by using a different channel with a 633 nm laser and detection filters between 610-670 nm. Plant discs (from tobacco) or Arabidopsis germinating seeds were visualized with distilled water and between a glass slide and a coverslip.

Subcellular fractionation and western blot analysis

Subcellular fractionation and western blot analysis Arabidopsis 7-day-old-seedling from Col-0, *abi5-7*, *snrk2.2snrk2.3*, *35S:cMyc-ABI5* and *35S:cMyc-ABI5C153S* with or without the treatments specified in each assay, were harvested and frozen in liquid nitrogen. 1-3 g of tissue was ground by using a mortar and pestle and resuspended in 3 mL of 1X NIB buffer (CelLyticTMPN, Sigma) supplemented with 1 mM DTT freshly prepared. The suspension was then filtered and centrifuged at 1,260 *xg* for 10 min at 4ºC. Pellets were resuspended in 0.5 mL of NIBA (1X NIB, 1X cOmplete® EDTA-free proteases inhibitor, 0.3% Triton X-100) and centrifuged at 1,200 *xg* for 10 min at 4 ºC. The supernatants were collected as cytosolic fractions (C) whereas pellets were washed five times in 1 mL of NIBA for crude nuclei preparation (N). Final pellet was resuspended in 150 µL of working solution ® (CelLyticTMPN) with 5 mM DTT, 1X cOmplete® EDTA-free proteases inhibitor and PMSF. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad) based on the Bradford method. 5 µg of total protein was loaded per well in SDS-acrylamide/bisacrylamide gel electrophoresis using Tris-glycine-SDS buffer. To monitor the cleanness of subcellular fractions, membranes were tested against nuclear H3 histone (Agrisera). In all cases, detection was performed using ECL Advance Western Blotting Detection Kit (Amersham) and the chemiluminescence was detected using a ChemiDoc Imaging System (Bio-Rad).

RESULTS

The promotion of the oligomeric state of ABI5 by oxidative conditions involves Cys153

ABI5 was previously characterized as a prototypical bZIP (Supplementary Fig. S1A) with functional and regulatory relevance in ABA-triggered processes (Finkelstein and Lynch, 2000; Lopez-Molina et al.,2001) and susceptible to redox modification by *S*-nitrosylation (Albertos et al., 2015). To characterize the redox sensitivity of ABI5, we analyzed the protein pattern under different conditions. Incubation of the ABI5 recombinant protein (Supplementary Fig. S1B) in the presence of the NO donor GSNO or hydrogen peroxide (H_2O_2) induced high molecular weight forms, while reduced glutathione (GSH) led to the accumulation of ABI5 monomers (Fig. 1A, B, Supplementary Fig. S1C-E). This observation was also confirmed in Arabidopsis seed extracts, where the application of the reductant agent dithiothreitol (DTT) abolished the formation of protein complexes (Fig. 1C, D). Western blot analyses indicated the specificity of the high molecular weight protein complexes, as these bands were not detected in the *abi5* knockout mutant background. To confirm that the high molecular weight bands appear because of redox treatments, the same recombinant protein or seed extracts were analyzed under reducing conditions by adding β-mercaptoethanol (Fig. 1B, D, F).

We previously reported that Cys153 is a key residue that controls NO-based stability of ABI5. Therefore, Cys153 participates in NO-related destabilization in a proteasome-dependent manner, but the DNA-binding ability of ABI5 is not affected (Albertos et al., 2015). To assess the impact of this residue on protein complex formation, we subjected the mutated version ABI5C153S (Supplementary Fig. S1B) to redox assays. The recombinant protein exhibited migration differences under non-reducing conditions in comparison to the wild-type (WT) version of the protein (Fig. 1A, Supplementary Fig. S1D). Next, we examined protein aggregates by comparing Arabidopsis transgenic lines overexpressing both the WT and C153S versions of ABI5. The results from *in vivo* extracts of seeds and seedlings pointed to monomeric detection only in the *ABI5C153S* overexpression line, suggesting that this Cys represents an essential residue for protein complex formation (Fig. 1E). These protein arrangements are redox-based, as the same extracts were analyzed under reducing conditions, showing bands corresponding to the monomeric form in all studied lines but the *abi5-7* mutant (Fig. 1F). All these results may indicate precise regulation of ABI5 by the redox environment, linking the oligomeric state to oxidizing conditions. The absence of Cys153 favors the accumulation of monomers, revealing the key role of this specific residue during the sensing of the cellular state.

ABA and redox treatments have a low impact on ABI5 nuclear localization

The redox state influences the stability, activity and localization of proteins, and these changes occur in a very specific manner, giving rise to various cellular responses and adaptations (Chi et al., 2013). In a previous study, we observed the impact of NO treatments, both with donors and scavengers, on ANAC089 TF (Albertos et al., 2021). In this regard, and to evaluate the possibility that NO influences the subcellular localization of ABI5, Arabidopsis seeds were treated with the proteosome inhibitor MG132 and the NO scavenger cPTIO to accumulate the protein and then subjected to treatment with NO donors (GSNO or SNAP), cPTIO or MG132 (Fig. 2A). Regardless of the treatment, the highest accumulation was observed in the nucleus, but there was a slight delocalization in the GSNO treatment. To validate these results and analyze whether Cys153 plays a role in this process, we performed subcellular fractioning using transgenic lines that overexpress *ABI5* and *ABI5C153S* versions. Seven-day-old seedlings were grown in MS medium supplemented with ABA and cPTIO (Fig. 2B) or GSNO and MG132 (Fig. 2C) for 3 hours. Under all conditions, and after quantification of the cytoplasm:nuclei ratio ABI5 was mainly detected in the nuclear fraction (Fig. 2D, E), but an increase was observed in the cytoplasm in the ABA and cPTIO treatment, and even in the mutated version (Fig. 2B, D), highlighting that ABA and this residue could also be involved in the regulation of the subcellular localization of ABI5. To test the specific effect of ABA treatment, the *ABI5* overexpression line and the WT, Col-0, were grown in MS media supplemented with 0.5 µM ABA and, under these conditions, in the overexpression line cytoplasmic ABI5 slightly increased compared to the control, (Supplementary Fig. S2A), but not in the wild type, where the amount of ABI5 protein is lower. Given the ABA role in ABI5 accumulation and regulation, and that the phosphorylation of ABI5 by SnRKs was previously described (Nakashima et al., 2009), a double mutant of SnRKs, *snrk2.2/2.3*, has been used to test whether the phosphorylation status influences ABI5 localization. Regardless of the presence of NO, quantification of cytoplasm:nuclei ratio alsoshowed that ABI5 is mostly absent from the cytoplasm of *snrk2.2/2.3* (Supplementary Fig. S2, C-F). Only under the simultaneous treatment with ABA and cPTIO, an increase in the ratio of cytoplasmic ABI5 is detected, especially in ABI5C153S version, suggesting that, individually, these factors do not have a significant effect on its localization. Taken together, these results suggest that ABI5 is mostly localized in the nucleus, and that Cys153 and ABA treatments have a minimal impact individually on the balance between nucleus and cytoplasm during seedling establishment.

Identification and localization of putative redoxins involved in the regulation of germination

Redoxins have been described as playing a key role in the seeds of different plant species, since redox state of the proteins change during germination (Alkhalfioui et al., 2007), but also during the response to stress conditions (Ortiz-Espín et al., 2017) or linked to hormonal crosstalk (Née et al., 2021), highlighting the importance of redox control at this developmental stage. Even, cellular compartments must be specifically controlled by redox system depending on the subcellular localization (Pulido et al., 2009). To identify whether any of them participate in the regulation of the redox state of ABI5, we first verified their expression pattern, selecting those that are expressed during germination (Supplementary Fig. S3). Based on the data in the Arabidopsis Information Resource (TAIR) (http://arabidopsis.org), UniProtKB, and Arabidopsis eFP Browser (Winter et al., 2007) databases, we gathered information on the subcellular localization and physiological processes of redoxins expressed during the germination window as a preliminary *in silico* characterization (Supplementary Fig. S3). Protein targets and the possible redoxins associated with germination regulation must have a coinciding spatiotemporal pattern. As mentioned, the initial selection was based on the temporal pattern, followed by a subcellular analysis that was performed to verify the different redoxins under our experimental conditions. For this purpose, glutaredoxins *ROXY10* and *ROXY21* were transiently expressed in *Nicotiana benthamiana* under the CaMV 35S constitutive promoter (Supplementary Fig. S4). Glutaredoxin ROXY21 was present in both the nucleus and the cytoplasm (Figure 3A, 4A, Supplementary Fig. S4A) and in a manner similar to the glutaredoxin ROXY10 (Supplementary Fig. S4A). Furthermore, NUCLEOREDOXIN 1 (NRX1) was previously characterized as a redoxin involved in protection against oxidative stress, showing oligomeric patterns depending on redox conditions (Kneeshaw et al., 2017), and exhibiting both nuclear and cytoplasmic localization (Kang et al., 2020). Under our control conditions, we also found it in both locations (Fig. 3A), where ABI5 is also present (Fig. 2). Moreover, Thioredoxin *h*3 (TRX*h*3) undergoes a redox-dependent structural and functional shift during heat-shock responses, acting as a chaperone when it forms oligomeric complexes and as a disulphide reductase when low molecular weight forms are present (Park et al., 2009). In this regard, a transgenic construct of TRX*h*3 under CaMV 35S constitutive promoter exhibited cytoplasmic location; however, when we checked the same genetic construct under our experimental conditions, we observed that TRX*h*3 is present in both nucleus and cytoplasm (Fig. 3B, C). In agreement with this, Thioredoxin *h*5 (TRX*h*5), which is very similar to TRX*h*3, has also been described in both compartments, where it is able to control the *S*-nitrosylation level of the TIRK receptor during the response to *Tetranychus urticae* (Arnaiz et al., 2023). Similarly, TRX*h*5

was previously characterized as a selective denitrosylase capable of modulating plant immunity (Kneeshaw et al., 2014). These diverse localizations could be related to the rapid adaptation of plant responses to a variety of stresses. Overall, the redoxins analyzed under our experimental conditions show both nuclear and cytoplasmic localization, suggesting that they may be involved in the redox regulation of ABI5.

ABI5 interacts directly with ROXY10 and NRX1

As mentioned above, ABI5 is a TF that can be modified by redox-dependent PTMs (Albertos et al., 2015), and the latter influences the formation of protein complexes (Fig. 1). It is known that some redox reactions are reversed by the direct action of redoxins (Kneeshaw et al., 2014; Sánchez-Vicente et al., 2024). In this context, redox modulation involves a transient interaction between TFs and redoxins, allowing rapid regulation through the oxidation/reduction of Cys thiols. To this end, we tested the possible interactions between redoxins described above and ABI5. Without ruling out other possible interactions, even between redoxins themselves in other situations such as in response to stress, under our experimental conditions, we detected that ABI5 interacts with ROXY10 *in planta* by BiFC (Fig. 4A) and NRX1 by mixing Arabidopsis extracts (Fig. 4B). Although ROXY10 and ROXY21 belong to the same group of glutaredoxins (de la Fuente et al., 2024), and display a similar expression pattern during the germination process (Supplementary Fig. S3) as well as a similar subcellular localization (Supplementary Fig. S4A), the interaction appears to be very specific, as no interaction between ABI5 and ROXY21 was detected in our system (Supplementary Fig. S4B). Therefore, the direct interaction between ABI5 and NRX1 or ABI5 and ROXY10 could be involved in the redox effects on ABI5.

Overexpression of *ROXY10* and *ROXY21* influence seed germination

As it is previously mentioned, ABI5 accumulation inhibits germination and the *S*-nitrosylation in a specific Cys residue promotes its degradation (Albertos et al., 2015), highlighting that redox state influences seed germination. To analyze the possible implication of redoxins in ABI5 regulation, we focus on the germination phenotype and perform a preliminary screening (data not showed) by using mutants and lines that overexpress the redoxins listed in Supplementary Fig. S3 (except ROXY10 and ROXY21, for which only the overexpression lines were analyzed). For this purpose, seeds were sown in different treatments, such as GSNO, cPTIO and ABA. Under our experimental

conditions and compared to WT Col-0, only the *ROXY10* and *ROXY21* overexpression lines showed a noticeable phenotype (Fig. 5). *ROXY10* overexpression inhibited germination in all treatments, but the effect of *ROXY21* was just the opposite, although only significant in cPTIO and ABA conditions in which ABI5 accumulates (Albertos et al., 2015). Since *ROXY10* overexpression showed inhibition of germination, we studied if ABI5 accumulation was the responsible of the phenotype in this line (Supplementary Fig. S5). We observed a slight increase of ABI5 protein content under control conditions compared to Col-0, which could be related to a decrease in germination. The increase observed in the ABA treatment corroborates that *ROXY10* overexpression does not interfere with the response to ABA. No notable differences were detected between GSNO and cPTIO, suggesting the possible involvement of this redoxin in the redox state of ABI5, since in WT seeds was previously described that GSNO and cPTIO promote ABI5 degradation and accumulation, respectively (Albertos et al., 2015). To corroborate if these effects are directly due to ABI5, we obtained overexpression lines in *abi5-7* mutant background (Supplementary Fig. S5). *ROXY10* overexpression lines in *abi5-7* mutant background presented the opposite phenotype compared to the ones in Col-0 background, with higher germination percentages in control, GSNO and cPTIO treatments, suggesting that the inhibitory effect during germination is related to the presence of an active form of this TF (i.e. presence of ABI5 in a reduced state). Nevertheless, *ROXY21* overexpression lines in the mutant background exhibited a highly different and variable response during germination, and based on this, we cannot conclude a direct relationship between ABI5 and ROXY21.

Thioredoxin*h*5 can control the *S*-nitrosylation level of ABI5

The level of *S*-nitrosylation regulates the localization, activity or stability of specific targets and is considered an important PTM that controls plant growth and development (Liu et al., 2024). Previous results showed the ability of certain redoxins to act as denitrosylates/trans-denitrosylases (Kneeshaw et al., 2014, Sánchez-Vicente et al., 2024), tailoring selective changes that ultimately redirect gene expression. To analyze whether the *S*-nitrosylation of ABI5 (Albertos et al., 2015) could be reversed by redoxin activity, we checked the *S*-nitrosylation level of this TF together with the denitrosylation activity of TRX*h*5 using *in vitro* assays (Fig. 6A). TRX*h*5 has two Cys residues in the active redox domain (ARD) (Fig. 6B), which confers it with denitrosylation activity and the formation of the TRX*h*5-SNO intermediate (Kneeshaw et al., 2014), and its expression is also induced under ABA treatment (Laloi et al., 2004). Recombinant TRX*h*5 was purified (Fig. 6C), reduced with DTT, and then mixed with SNO-ABI5. A decrease in the level of

SNO-ABI5 was observed when the TRX*h*5 was added, and SNO-TRX*h*5 was also detected after 1h of incubation (Fig. 6A), corroborating the trans-denitrosylation mechanism previously described in Kneeshaw et al., (2014) in response to biotic stress. Due to the detection of SNO-TRX*h*5, we performed an *in silico S*-nitrosylation analysis, and one of the three Cys of TRX*h*5 appears to be susceptible to modification by NO (Fig. 6D) with the highest score value, suggesting that this residue could also be involved in the formation of SNO-TRX*h*5. These results point to the possible role of TRX*h*5 in seed germination through the control of ABI5 redox state.

Discussion

Plants have evolved to integrate a large amount of physiological information, from cellular signals to environmental conditions, to respond and adapt efficiently to these changing situations. This capacity must be flexible and finely regulated, coordinating interactions between hormonal signaling pathways, redox status and TFs. Some of these are considered master regulators, as they control key developmental signals or stress response decisions by coordinating the regulation of the expression level of numerous downstream genes. Given that these TFs act as important hubs at the top of gene regulatory networks, their function must be tightly modulated by different strategies involving localization, activity, and stability. Posttranslational modifications (PTMs) have profound effects on the control of protein fate, allowing for dynamic and strictly specific regulation that leads to cellular readjustments throughout the life cycle and in response to various types of stress (Zhenxiang et al., 2025). *S*-nitrosylation regulates different aspects of targets during development and the modulation of stress responses (Sánchez-Vicente et al., 2019; Mata-Pérez et al., 2023). Redox species play a crucial role in germination, from seed maturation to the release of dormancy and viability.

In this regard, it has been previously described that ABI5 is *S*-nitrosylated at Cys153, which promotes interaction with CUL4 and KEG-based E3 ligases, leading to its degradation and, consequently, seed germination (Albertos et al., 2015). Redox-sensitive proteins can exhibit dynamics between oligomeric and monomeric forms. Previous research has shown that *S*-nitrosylation of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) facilitates the formation of oligomeric complexes (Tada et al., 2008). Based on this, the influence of redox treatments on the ABI5 protein was tested *in vitro* and *in vivo*, observing that, similar to NPR1, oxidizing conditions, including treatment with NO, promote oligomeric complexes; while reducing conditions favor the presence of monomers (Fig. 1). In addition, it was observed that the redox-

related pattern is different when comparing the WT version of ABI5 with the mutated version without Cys153, highlighting the relevance of this specific Cys residue in the redox sensitivity of this TF (Fig. 1). In support of these results, ABI5 degradation events involved structural changes related to the formation of high molecular weight complexes, probably related to interaction with other members (Lopez-Molina et al., 2003) and high and low forms in *keg* mutants (Stone et al., 2006), which are also observed in Fig. 1. Oligomeric complexes are not only related to mechanisms associated with degradation, but also to other regulatory functions. In 2015, Kovacs et al. demonstrated that GSNO treatment, which is related to the formation of oligomeric complexes (Tada et al., 2008), is involved in the translocation of SNO-NPR1 from the cytoplasm to the nucleus. ABI5 has previously been described as a bZIP TF located mainly in the nucleus, where it is capable of regulating late embryonic genes during seed formation and germination of ABA signal transduction pathways (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001). Nevertheless, some studies have shown that this TF could be located in the cytoplasm, when its degradation is mediated by the RING-type E3 ligase KEEP ON GOING (Liu and Stone, 2013), or under specific temperature and seed dormancy state, as occurs with ABA accumulation (Xia et al., 2019).

Nucleocytoplasmic movement involves different mechanisms but often requires a nuclear localization signal (NLS) located in the sequence. In this context, ABI5 has three NLS, suggesting that changes in its localization play an important role in its function (Liu and Stone, 2013), perhaps linked to specific signals or eventual responses to stress and specific redox conditions (Igarashi et al., 2001; Srivastava et al., 2013; Albertos et al., 2021). Based on this, we analyzed whether treatments with NO donors or scavengers could be related to ABI5 subcellular localization. Confocal microscopy and subcellular fractioning assays revealed that NO is not the main factor controlling it, at least under the conditions studied (Fig. 2). The phosphorylation state has been described as a molecular switch for protein activity, stability and localization (Zhang et al., 2023). SnRKs2.2 and 2.3 kinases are present in both the nucleus and cytoplasm (Fujita et al., 2009) and has been suggested that ABI5 phosphorylation could induce a conformational change that potentially exposes the domain to interaction partners (Nakamura et al., 2001), which can be related to the formation of high molecular weight protein complexes (Lopez-Molina et al., 2003; Stone et al., 2006). In addition, translocation mechanisms involving proteins capable of regulating ABI5 have been proposed (Xu et al., 2019; Sánchez-Vicente et al., 2019). However, we observed a change in localization under combined treatments with ABA and the NO scavenger, more pronounced in the version without the C153, but not only in ABA or in the double mutant of SnKRs kinases (Fujii et al., 2007) (Fig. 2, Supplementary Fig. S2), suggesting that multiple factors could

influence the subcellular localization during seedling establishment. Cytoplasmic accumulation could also be implied in the temporary maintenance of a pool related to a rapid response to abiotic stress (i.e. salinity, osmotic, or drought), leading to a more dynamic readjustment linked to adaptation in the early stages after germination (Lopez-Molina et al., 2001). In relation to this, it has been reported that some TF are retained in the cytoplasm and relocated to the nucleus in response to stress (Igarashi et al., 2001).

As previously mentioned, ABI5 is a redox-sensitive TF (Fig. 1). Recently, the *S*-nitrosylation of the ABI5 homologue, bZIP67, and the reversibility of this modification by PEROXIREDOXIN IIE (PrxIIE) have been described (Sánchez-Vicente et al., 2024). Based on this, we searched for redoxin candidates that could be involved in the redox regulation of ABI5. To do this, we selected redoxins expressed during seed germination from public databases (EFP Browser, TAIR and UniProtKB) (Supplementary Fig. S3) and analyzed the subcellular localization of those that have not been previously described, or at least not under our experimental conditions, to ensure that they colocalize with ABI5 (Fig. 3). When we overexpress *ROXY10* and *ROXY21*, both are present in the nucleus and cytoplasm (Fig. 3, Supplementary Fig. S4A), as are NRX1 and TRX*h*3 (Fig. 3), corroborating their colocalization with ABI5 (Fig. 2). Once the location of the different redoxins had been analyzed, we studied the possible interaction with ABI5 (Figures 4 and S4). The interaction of ROXY10 and NRX1 with ABI5 was confirmed by BiFC and co-immunoprecipitation assays, respectively, but not that of ROXY21, although both ROXYs have a similar subcellular localization (Supplementary Fig. S4), highlighting that this mechanism occurs specifically depending on the target or conditions analyzed.

To stablish the physiological effects of these interactions and further investigate the possible functions of redoxins during seed germination, we performed a screening (data not shown) in which we analyzed the germination of mutants and transgenic lines of the selected redoxins (Supplementary Fig. S3) (except ROXY10 and 21, for which only overexpression lines were analyzed). Only the latter showed a phenotype related to the modulation of seed germination, promoting it (ROXY21) or inhibiting it (ROXY10) (Fig. 5), but only overexpression of *ROXY10* in *abi5-7* mutant background reverse the observed phenotype (Supplementary Fig. S5). This result together with the interaction between ROXY10, but not ROXY21, with ABI5 (Fig. 4, Supplementary Fig. S4) suggest that only ROXY10 could be directly involved in ABI5 regulation.

Inhibition of germination is related to either increased activity or accumulation of ABI5 (Albertos et al., 2015), and to test whether this occurred in the *ROXY10* overexpression lines, we performed a western blot assay in different treatments with NO donor/scavenger and ABA. Under control

conditions, the accumulation of ABI5 protein is slightly higher compared to Col-0 (Supplementary Fig. S5), which could be related to the inhibition of germination. Our data support that ABI5 activity (i.e. presence of ABI5 in a reduced state) rather than protein levels may influenced the seed germination phenotype. Nevertheless, we cannot exclude that ROXY10 could affect other molecular targets distinct from ABI5, that influence seed viability and germination. ABA promotes accumulation of ABI5, as expected (Finkelstein and Lynch, 2000), but no differences were detected between GSNO and cPTIO treatments, although the phenotypic differences were significant (Fig. 5). This result could be related to the redox regulation of ABI5, which affects its activity and accumulation.

Considering the importance of controlling the redox status of ABI5 in seed germination, we examined whether TRX*h*5 exhibits protein denitrosylation activity on ABI5. Results showed that TRX*h*5 was able to denitrosylate ABI5 *in vitro* (Fig. 6), this data indicating that *S*-nitrosylation of ABI5 is reversible and that protein-SNO reductase ability may modulate SNO-ABI5 levels to control early stages of plant development.

Taken together, these results led us to propose a model that delves deeper into the regulation of ABI5 based on PTMs. ABI5 is a redox-sensitive TF that can exist in different monomeric and oligomeric forms, depending, at least in part, on the presence of the Cys153 residue. Although ABI5 is mainly localized in the nucleus, where it can bind to the promoter of specific genes, redox and phosphorylation states influence its subcellular localization. Finally, redoxins can also modulate ABI5 redox state through direct binding (i.e. ROXY10) or through denitrosylation activity (TRX*h*5), showing additional layers capable of fine-tune the germination process through specific targets (Fig. 7).

Acknowledgements

We thank the Spanish networks RED2022-134072-T and RED2022-134917-T for critical comments and stimulating discussions on the manuscript.

Author contributions

I.S.-V, C.M.-P., F.P.-R, I.M., L.H.-M. and V.C.-B. performed the research. I.S.-V, C.M.-P, P.A. and O.L. analyzed the data. I.S.-V and C.M.-P. wrote the manuscript; and I.S.-V. C.M.-P, P.A. and O.L. designed the research, supervised the work and provided financial support. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Funding

This work was financed by grants PID2023-149447OB-I00 from the Spanish Ministry of Science, Innovation and Universities MCIU/AEI/10.13039/501100011033 (to P.A. and O.L), SA142P23 from the Regional Government of Castile and Leon (to O.L. and C.M-P) and “Escalera de Excelencia” CLU-2O25-2-04 co-funded by the FEDER Operative Program of the Regional Government of Castile and Leon 2021–2027 Spain (to O.L.), grant funded by Fundación Memoria de D. Samuel Solórzano Barruso-Universidad de Salamanca Ref FS/8-2024 (to I. S-V.) and funding from the C2 call of Research Projects of the University of Salamanca (PIC2-2023-08) (to C.M-P).

Data availability

The primary data supporting this study were not made publicly available at the time of publication. For correspondence related to the distribution of materials integral to the findings presented in this article, please email OL (oslo@usal.es).

References

Albertos P, Romero-Puertas MC, Tatematsu K, Mateos I, Sánchez-Vicente I, Nambara E, Lorenzo O. 2015. *S*-nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth. Nat Communications 6,8669.

Albertos P, Tatematsu K, Mateos I, Sánchez-Vicente I, Fernández-Arbaizar A, Nakabayashi K, Nambara E, Godoy M, Franco JM, Solano R, Gerna D, Roach T, Stöggl W, Kranner I, Perea-Resa C, Salinas J, Lorenzo O. 2021. Redox feedback regulation of ANAC089 signaling alters seed germination and stress response. Cell Reports 35,109263.

Alkhalfioui F, Renard M, Vensel WH, Wong J, Tanaka CK, Hurkman WJ, Buchanan BB, Montrichard F. 2007. Thioredoxin-linked proteins are reduced during germination of Medicago truncatula seeds. Plant Physiology 144,1559-79.

Arnaiz A, Romero-Puertas MC, Santamaria ME, Rosa-Diaz I, Arbona V, Muñoz A, Grbic V, González-Melendi P, Mar Castellano M, Sandalio LM, Martinez M, Diaz I. 2023. The Arabidopsis thioredoxin TRXh5 regulates the *S*-nitrosylation pattern of the TIRK receptor being both proteins essential in the modulation of defences to *Tetranychus urticae.* Redox Biology 67,102902.

Bensmihen, S. Giraudat, J. Parcy, F. 2005. Characterization of three homologous basic leuzine zipper transcription factors (bZIP) of the ABI5 family during Arabidopsis thaliana embryo maturation. Journal of Experimental Botany 56,597-603.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Chi YH, Paeng SK, Kim MJ, Hwang GY, Melencion SM, Oh HT, Lee SY. 2013.Redox-dependent functional switching of plant proteins accompanying with their structural changes. Frontiers in Plant Science 4,277.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant Journal 16, 735-43.

Comelli RN, Gonzalez DH. 2007. Conserved homeodomain cysteines confer redox sensitivity and influence the DNA binding properties of plant class III HD-Zip proteins. Archives of Biochemistry and Biophysics 467,41-7.

Couturier J, Chibani K, Jacquot JP, Rouhier N. 2014. Cysteine-based redox regulation and signaling in plants. Frontiers in Plant Science 4,105.

Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, Van Montagu M, Leemans J. 1985. Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. Nucleic Acids Research 13, 4777-88.

de la Fuente C, Grondin A, Sine B, Debieu M, Belin C, Hajjarpoor A, Atkinson JA, Passot S, Salson M, Orjuela J, Tranchant-Dubreuil C, Brossier JR, Steffen M, Morgado C, Dinh HN, Pandey BK, Darmau J, Champion A, Petitot AS, Barrachina C, Pratlong M, Mounier T, Nakombo-Gbassault P, Gantet P, Gangashetty P, Guedon Y, Vadez V, Reichheld JP, Bennett MJ, Kane NA, Guyomarc'h S, Wells DM, Vigouroux Y, Laplaze L. 2024. Glutaredoxin regulation of primary root growth is associated with early drought stress tolerance in pearl millet. Elife 12,RP86169.

Del Río LA. 2015 ROS and RNS in plant physiology: an overview. Journal of Experimental Botany 66,2827-37.

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS. 2006. Gateway-compatible vectors for plant functional genomics and proteomics. Plant Journal 45, 616-29.

Finkelstein RR, Lynch TJ. 2000. The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell 12,599-609.

Fujii H, Verslues PE, Zhu JK. 2007. Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. Plant Cell 1,485-94

Fujita Y, Nakashima K, Yoshida T, Katagiri T, Kidokoro S, Kanamori N, Umezawa T, Fujita M, Maruyama K, Ishiyama K, Kobayashi M, Nakasone S, Yamada K, Ito T, Shinozaki K, Yamaguchi-Shinozaki K. 2009. Three SnRK2 protein kinases are the main positive regulators

of abscisic acid signaling in response to water stress in Arabidopsis. Plant Cell Physiology 50,2123-32.

Giesguth M, Sahm A, Simon S, Dietz KJ. 2015. Redox-dependent translocation of the heat shock transcription factor AtHSFA8 from the cytosol to the nucleus in *Arabidopsis thaliana*. FEBS Letters 589, 718–725

Igarashi D, Ishida S, Fukazawa J, Takahashi Y. 2001. 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. Plant Cell 13,2483-97.

Kang CH, Park JH, Lee ES, Paeng SK, Chae HB, Hong JC, Lee SY. 2020. Redox-Dependent Structural Modification of Nucleoredoxin Triggers Defense Responses against *Alternaria brassicicola* in Arabidopsis. International Journal of Molecular Science 21:9196.

Kneeshaw S, Gelineau S, Tada Y, Loake GJ, Spoel SH. 2024. Selective protein denitrosylation activity of Thioredoxin-h5 modulates plant Immunity. Molecular Cell 56,153-62.

Kneeshaw S, Keyani R, Delorme-Hinoux V, Imrie L, Loake GJ, Le Bihan T, Reichheld JP, Spoel SH. 2017. Nucleoredoxin guards against oxidative stress by protecting antioxidant enzymes. Proceedings of the National Academy of Sciences, USA 114,8414-8419.

Kovacs I, Durner J, Lindermayr C. 2015. Crosstalk between nitric oxide and glutathione is required for NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1)-dependent defense signaling in *Arabidopsis thaliana*. New Phytologist 208, 860-72.

Laloi C, Mestres-Ortega D, Marco Y, Meyer Y, Reichheld JP. 2004. The Arabidopsis cytosolic thioredoxin h5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. Plant Physiology 134, 1006-16.

Liu Y, Liu Z, Wu X, Fang H, Huang D, Pan X, Liao W. 2024. Role of protein S-nitrosylation in plant growth and development. Plant Cell Reports 43,204.

Liu H, Stone SL. 2013. Cytoplasmic degradation of the Arabidopsis transcription factor abscisic acid insensitive 5 is mediated by the RING-type E3 ligase KEEP ON GOING. Journal of Biological Chemistry 201288, 20267-79.

Lopez-Molina L, Mongrand S, Chua NH. 2001. A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proceedings of the National Academy of Sciences, USA 98,4782-7.

Lopez-Molina L, Mongrand S, Kinoshita N, Chua NH. 2003. AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. Genes & Development 173,410-8.

Mata-Pérez C, Sánchez-Vicente I, Arteaga N, Gómez-Jiménez S, Fuentes-Terrón A, Oulebsir CS, Calvo-Polanco M, Oliver C, Lorenzo Ó. 2023. Functions of nitric oxide-mediated post-translational modifications under abiotic stress. Frontiers in Plant Science 14,1158184.

Mendes A, Kelly AA, van Erp H, Shaw E, Powers SJ, Kurup S, Eastmond PJ. 2013. bZIP67 regulates the omega-3 fatty acid content of Arabidopsis seed oil by activating fatty acid desaturase3. Plant Cell 25, 3104-16.

Nakamura S, Lynch TJ, Finkelstein RR. 2001. Physical interactions between ABA response loci of Arabidopsis. Plant Journal 26, 627-35.

Nakashima K, Fujita Y, Kanamori N, Katagiri T, Umezawa T, Kidokoro S, Maruyama K, Yoshida T, Ishiyama K, Kobayashi M, Shinozaki K, Yamaguchi-Shinozaki K. 2009. Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. Plant Cell Physiology 50, 1345-63.

Née G, Châtel-Innocenti G, Meimoun P, Leymarie J, Montrichard F, Satour P, Bailly C, Issakidis-Bourguet E. 2021. A New Role for Plastid Thioredoxins in Seed Physiology in Relation to Hormone Regulation. International Journal of Molecular Sciences 22,10395.

Ortiz-Espín A, Iglesias-Fernández R, Calderón A, Carbonero P, Sevilla F, Jiménez A. 2017. Mitochondrial AtTrxo1 is transcriptionally regulated by AtbZIP9 and AtAZF2 and affects seed germination under saline conditions. Journal of Experimental Botany 68, 1025-1038.

Park SK, Jung YJ, Lee JR, Lee YM, Jang HH, Lee SS, Park JH, Kim SY, Moon JC, Lee SY, Chae HB, Shin MR, Jung JH, Kim MG, Kim WY, Yun DJ, Lee KO, Lee SY. 2009. Heat-shock and redox-dependent functional switching of an h-type Arabidopsis thioredoxin from a disulfide reductase to a molecular chaperone. Plant Physiology 150, 552-61.

Pulido P, Cazalis R, Cejudo FJ. 2009. An antioxidant redox system in the nucleus of wheat seed cells suffering oxidative stress. Plant Journal 57,132-145.

Sánchez-Vicente I, Fernández-Espinosa MG, Lorenzo O. 2019. Nitric oxide molecular targets: reprogramming plant development upon stress. Journal of Experimental Botany 70, 4441-4460.

Sánchez-Vicente I, Albertos P, Lorenzo O. 2019. Protein Shuttle between Nucleus and Cytoplasm: New Paradigms in the ABI5-dependent ABA Responses. Molecular Plant 12,1425-1427.

Sánchez-Vicente I, Albertos P, Sanz C, Wybouw B, De Rybel B, Begara-Morales JC, Chaki M, Mata-Pérez C, Barroso JB, Lorenzo O. 2024. Reversible *S*-nitrosylation of bZIP67 by peroxiredoxin IIE activity and nitro-fatty acids regulates the plant lipid profile. Cell Reports 43, 114091.

Serpa V, Vernal J, Lamattina L, Grotewold E, Cassia R, Terenzi H. 2007. Inhibition of AtMYB2 DNA-binding by nitric oxide involves cysteine S-nitrosylation. Biochemical and Biophysical Research Communications 361, 1048–1053.

Shaikhali J, Heiber I, Seidel T, Ströher E, Hiltscher H, Birkmann S, Dietz KJ and Baier M. 2008. The redox-sensitive transcription factor Rap2.4a controls nuclear expression of 2-Cys peroxiredoxin A and other chloroplast antioxidant enzymes. BMC Plant Biol 8, 48.

Shaikhali J. 2015.GIP1 protein is a novel cofactor that regulates DNA-binding affinity of redox-regulated members of bZIP transcription factors involved in the early stages of Arabidopsis development. Protoplasma 252, 867-83.

Spadaro D, Yun BW, Spoel SH, Chu C, Wang YQ, Loake GJ. 2010. The redox switch: dynamic regulation of protein function by cysteine modifications. Physiologia Plantarum 138, 360-71.

Stone SL, Williams LA, Farmer LM, Vierstra RD, Callis J. 2006. KEEP ON GOING, a RING E3 ligase essential for Arabidopsis growth and development, is involved in abscisic acid signaling. Plant Cell 18, 3415-28.

Srivastava R, Deng Y, Shah S, Rao AG, Howell SH. 2013. BINDING PROTEIN is a master regulator of the endoplasmic reticulum stress sensor/transducer bZIP28 in Arabidopsis. Plant Cell 25, 1416-29.

Ströher E, Wang XJ, Roloff N, Klein P, Husemann A, Dietz KJ. 2009. Redox-dependent regulation of the stress-induced zinc-finger protein SAP12 in Arabidopsis thaliana. Molecular Plant 2:357-67.

Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X. 2008. Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. Science 321, 952-6

The UniProt Consortium, UniProt: the universal protein knowledgebase. Nucleic Acids Research 45, D158–D169.

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ. 2007. An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One**,** :e718.

Xia Q, Ponnaiah M, Thanikathansubramanian K, Corbineau F, Bailly C, Nambara E, Meimoun P, El-Maarouf-Bouteau H. 2019. Re-localization of hormone effectors is associated with dormancy alleviation by temperature and after-ripening in sunflower seeds. Scientific Reports 9, 4861.

Xu X, Wan W, Jiang G, Xi Y, Huang H, Cai J, Chang Y, Duan CG, Mangrauthia SK, Peng X, Zhu JK, Zhu G. 2019. Nucleocytoplasmic Trafficking of the Arabidopsis WD40 Repeat Protein XIW1 Regulates ABI5 Stability and Abscisic Acid Responses. Molecular Plant 12,1598-1611.

Zhang WJ, Zhou Y, Zhang Y, Su YH, Xu T. 2023. Protein phosphorylation: A molecular switch in plant signaling. Cell Reports 42,112729.

Zhenxiang Li, Liqian Qin, Dongjiao Wang, Tianzhen Cui, Yanlan Liang, Shoujian Zang, Tingting Sun, Wanying Zhao, Qibin Wu, Youxiong Que. 2025. Protein post-translational modification in plants: Regulation and beyond. Horticultural Plant Journal.

Figure legends

Fig. 1. Redox-dependent structural forms of ABI5 and ABI5C153S.

(A-B) Recombinant ABI5 and ABI5C153S proteins were analyzed under 7% non-reducing SDS-PAGE (A) and 10% reducing SDS-PAGE (B) gels. Proteins were treated for 1 hour with GSNO (1 mM), GSH (1 mM) and H_2O_2 (20 mM) after removal of DTT (10 mM). The position of monomeric low mobility (LM), monomeric high mobility (HM), oligomeric (O) and monomeric (M) ABI5 protein is highlighted with black arrows. NT means non-treated. The quantification of ABI5 monomer levels relative to the total amount is shown in Supplemental Fig. S1C.

(C-D) Protein seed extracts of Col-0 and *abi5*-7 knockout mutant were analyzed under 7% non-reducing SDS-PAGE (C) and 10% reducing SDS-PAGE (D) gels. Extracts were treated for 1 hour with GSNO (1 mM), H_2O_2 (20 mM) and DTT (20 mM). The position of monomeric (M) and oligomeric (O) ABI5 protein is highlighted with black arrows. NT means non-treated.

(E-F) Arabidopsis after-ripened seeds were stratified for 3 days in water at 4ºC under dark conditions. Protein seed extracts of Col-0 and the different transgenic lines were also analyzed under 7% non-reducing SDS-PAGE (E) and 10% reducing SDS-PAGE (F) gels. Monomeric (M) and oligomeric (O) forms are highlighted with black bars. Reducing SDS-PAGE gels were used as loading control. All transgenic lines are in Col-0 background.Antibody against ABI5 (αABI5) was used in all immunoblots.

Fig. 2. Subcellular localization of ABI5 analyzed by confocal microscopy and subcellular fractioning.

(A) Localization of ABI5 in the root tip of an Arabidopsis embryo using a transgenic line with the *pABI5:GFP-ABI5* construct in the *abi5-1* knockout mutant background. Seeds were imbibed for 24 hours in MES buffer supplemented with 1 mM cPTIO and 100 µM MG132 for ABI5 accumulation, after being treated for 6h with different compounds as indicated. Bar scale: 50µm.

(B, C) Subcellular localization of ABI5 protein by a subcellular fractioning assay in 7-day-old Arabidopsis seedlings grown in MS medium (non-treated, NT) or MS medium supplemented with 0.5 µM ABA plus 100 µM cPTIO at 0 hours (B); and with 1 mM GSNO combined with 100 µM

MG132 for 3 hours (C). Histone H3 and RbCL were used as loading controls from nucleus and cytoplasm, respectively. N: nuclear fraction; C: cytoplasm. All transgenic lines are in Col-0 background. Antibodies against ABI5 (αABI5) and anti-H3 (αH3) were used for the immunoblots.

(D, E) Quantification of the ratio of cytoplamic:nuclear ABI5 protein relative to the nuclear and cytoplasmic loading controls. Bars represent the media of three measurements and error bars correspond to the standard error. For normal and homoscedastic data, one-way ANOVA tests were carried out.

Fig. 3. Subcellular localization of TRX*h*3, ROXY21 and NRX1.

(A) Subcellular localization of ROXY21 and NRX1 proteins by a subcellular fractioning assay in 7-day-old Arabidopsis seedlings grown in MS medium. Histone H3 and RbCL were used as loading controls from nucleus and cytoplasm, respectively. N: nuclear fraction; C: cytoplasm. Antibodies against NRX1 (αNRX1), anti-HA (αHA) and anti-H3 (αH3) were used for the immunoblots.

(B) TRX*h*3 subcellular localization by transient expression of *GFP-TRXh3* in *N. benthamiana.* Bar scale: 100µm.

(C) Subcellular localization of TRX*h*3 protein by a subcellular fractioning assay in 7-day-old Arabidopsis seedlings grown in MS medium. Histone H3 and RbCL were used as loading controls from nucleus and cytoplasm, respectively. N: nuclear fraction; C: cytoplasm. Antibodies against anti-HA (αHA) and anti-H3 (αH3) were used for the immunoblots.

Fig. 4. Interaction between ABI5 and the different redoxins.

(A) *In planta* interaction between ABI5 and ROXY10 using Bimolecular Fluorescence Complementation assays (BiFC). The interaction was localized specifically in the nucleus of the tobacco epidermal cells. Bar scale: 100µm.

(B) *In vivo* co-immunoprecipitation (CoIP) assay to test the interaction between ABI5 and NRX1 using *35S:cMyc-ABI5WT* and *35S:Flag-NRX1* transgenic lines pretreated or not with 1mM GSNO for 30min. Pull-down and input protein levels regarding ABI5 protein were analyzed using anti-ABI5, and CoIP and input protein levels related to NRX1 were observed by using anti-NRX1 antibody.

Fig. 5. Germination percentage of ROXY overexpression lines.

Seed stocks were generated and maintained in the same conditions. Col-0, *35S:HA-ROXY10* and *35S:HA-ROXY21* overexpression lines, and *abi5-7* mutant were sown in MES media not supplemented or supplemented with 100 µM cPTIO, 500 µM GSNO or 0.5 µM ABA. Germination was followed for 7 days. Graphs show germination 48 hours after sowing. Statistical analysis was performed using GraphPad Prism 9.0. For normal and homoscedastic data, one-way ANOVA

tests with Bonferroni post-hoc tests were carried out, while for non-parametric data, a Kruskal Wallis test with Bonferroni correction was chosen. Each assay was repeated three times with 50-60 seeds.

Figure 6. *In vitro* trans-denitrosylation of ABI5 by TRX*h*5 and analyses of relevant residues for its activity.

(A) *In vitro* denitrosylation assay between ABI5-SNO and TRXh5. DTT-reduced ABI5 recombinant protein was first treated with GSNO and then incubated with reduced TRXh5. Antibodies against ABI5 (αABI5) and anti-His (αHis) were used for the immunoblots. Treatment without ascorbate was used as a negative control.

(B) Scheme of TRX*h*5 protein structure displaying the active redox domain (ARD) and the position of the cysteine residues within the structure.

(C) Purification of 6xHIS-TRX*h*5 by using a Ni-NTA His Bind Resin. Different fractions were collected and shown, UB: unbound, E1-3: elutions 1-3.

(D) Computational prediction of TRX*h*5 *S*-nitrosylation sites. GPS-SNO (Xue et al., 2010) was used to calculate Cys targets of *S*-nitrosylation using the High Threshold option.

Fig. 7. Impact of redox state, Cys153 and ABA signaling pathway during ABI5 posttranslational regulation.

ABI5 is predominantly located in the nucleus, where it controls the expression of target genes in the ABA signaling pathway (1). Nitric oxide (NO) promotes *S*-nitrosylation of ABI5, impacting on protein degradation depending on proteasome system (E3 ligase KEG) (2), which can be regulated by redoxins, such as ROXY10 and TRX*h*5. Redox state in the cell influences ABI5 oligomerization status, in which the Cys153 residue has a prominent role. Together, these molecular mechanisms, including ABI5 subcellular localization, influenced also by the presence of NO (donor GSNO or scavenger cPTIO) and ABA, fine-tune the redox-dependent PTMs of ABI5 and, consequently, the germination and the seedling establishment processes.

Created in BioRender. Sánchez-Vicente, I (2025) https://BioRender.com

(1) Nakashima et al., 2009; (2) Albertos et al., 2015; (3) Liu and Stone, 2013

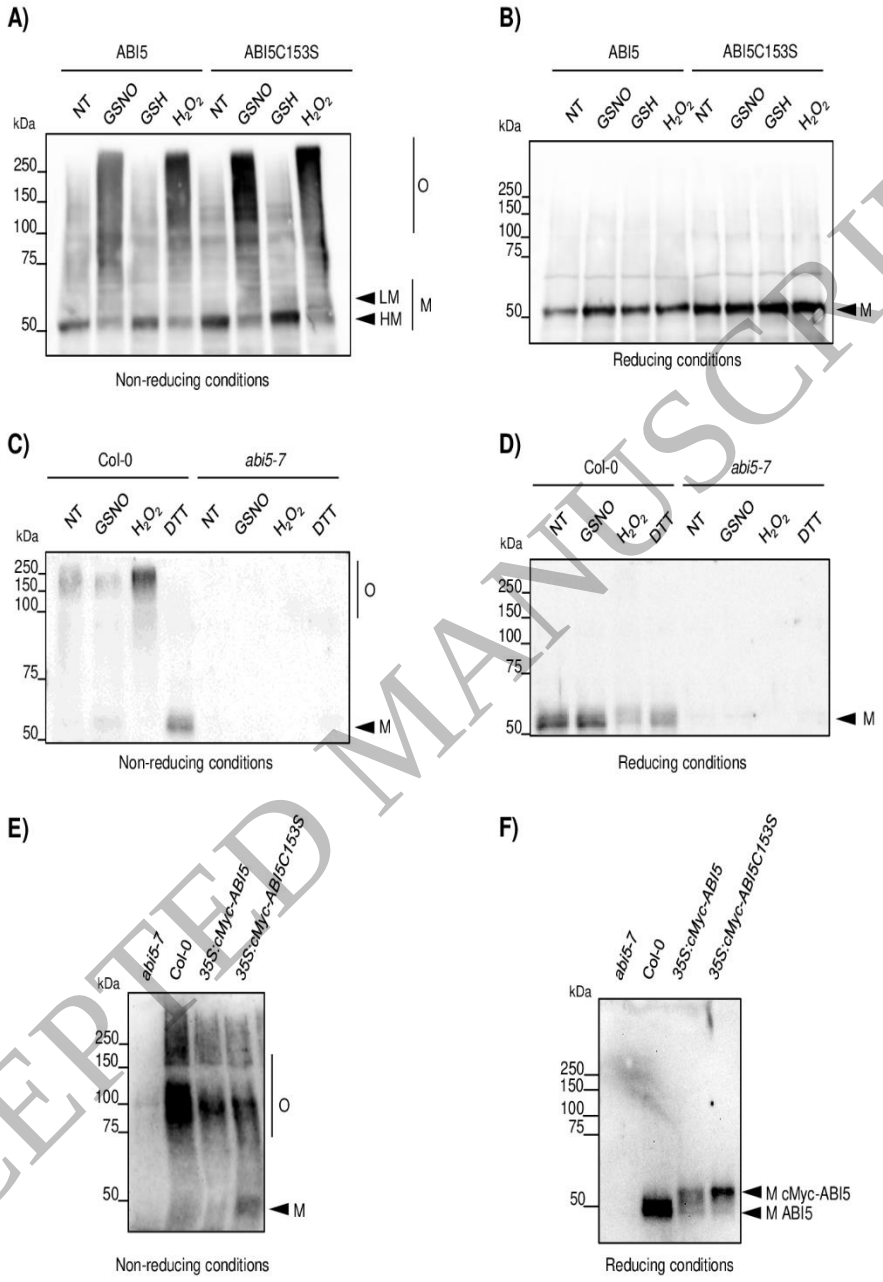

Figure 1
190x254 mm (x DPI)

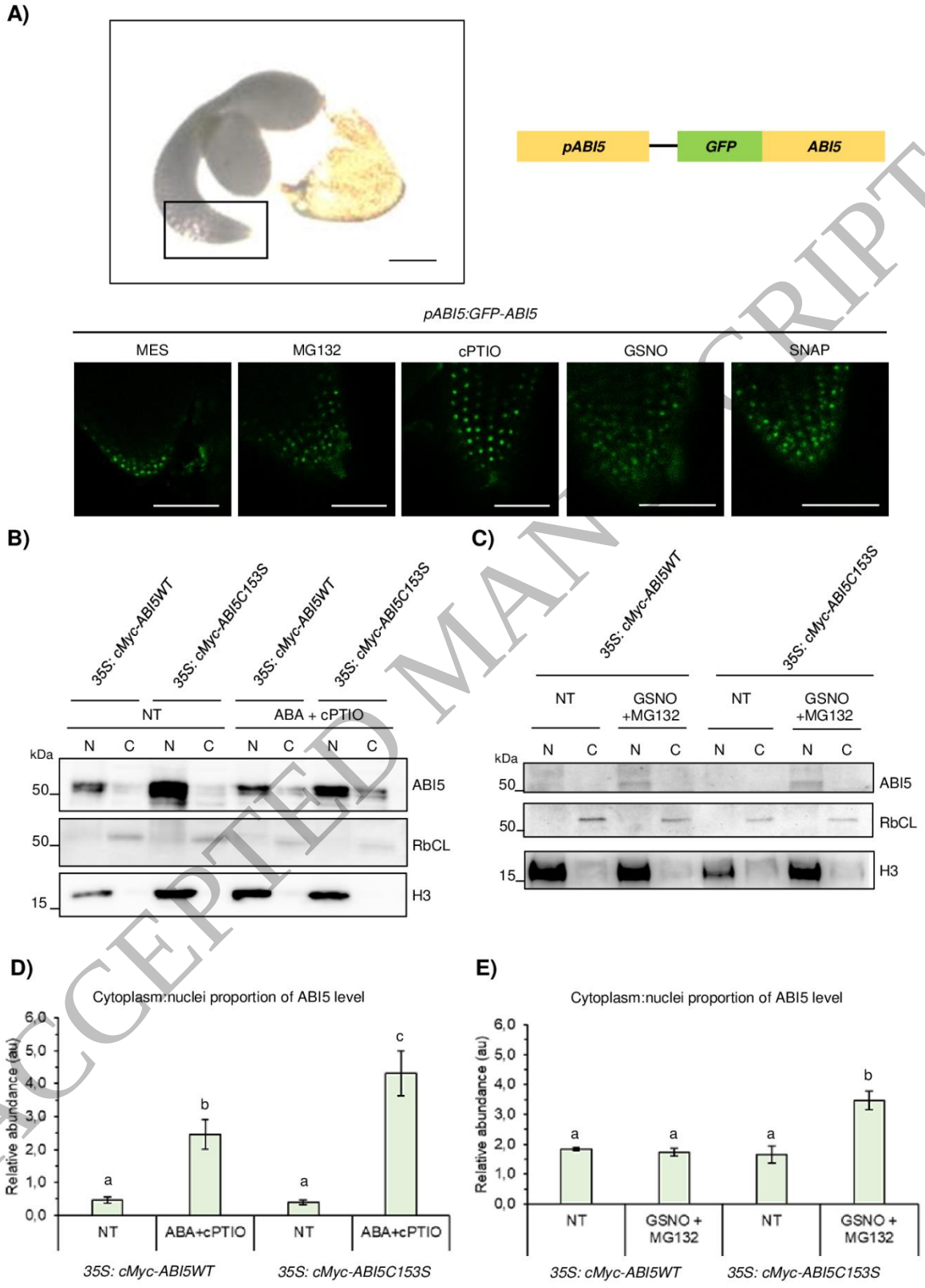

Figure 2
190x254 mm (x DPI)

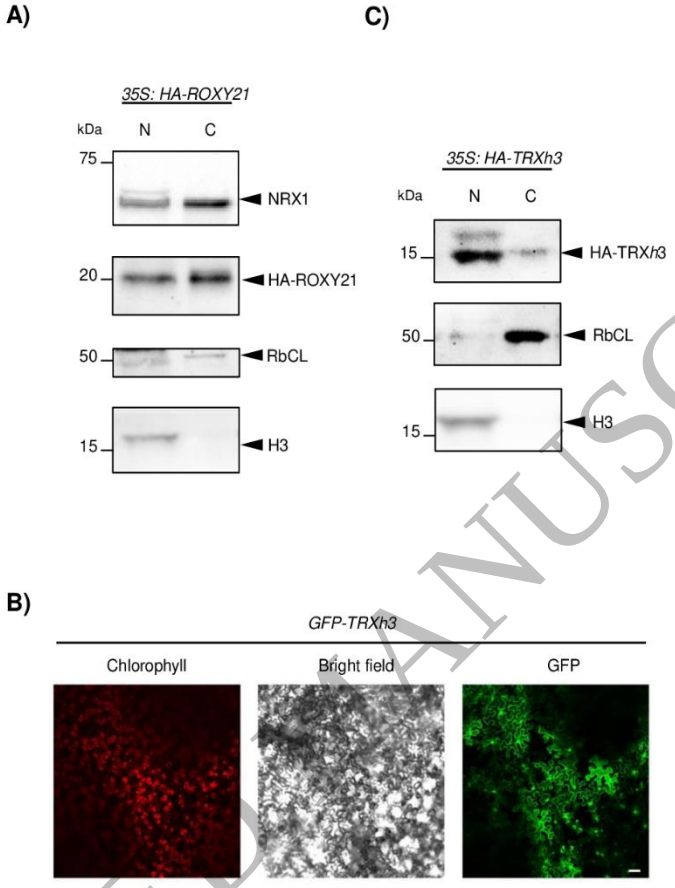

Figure 3
190x254 mm (x DPI)

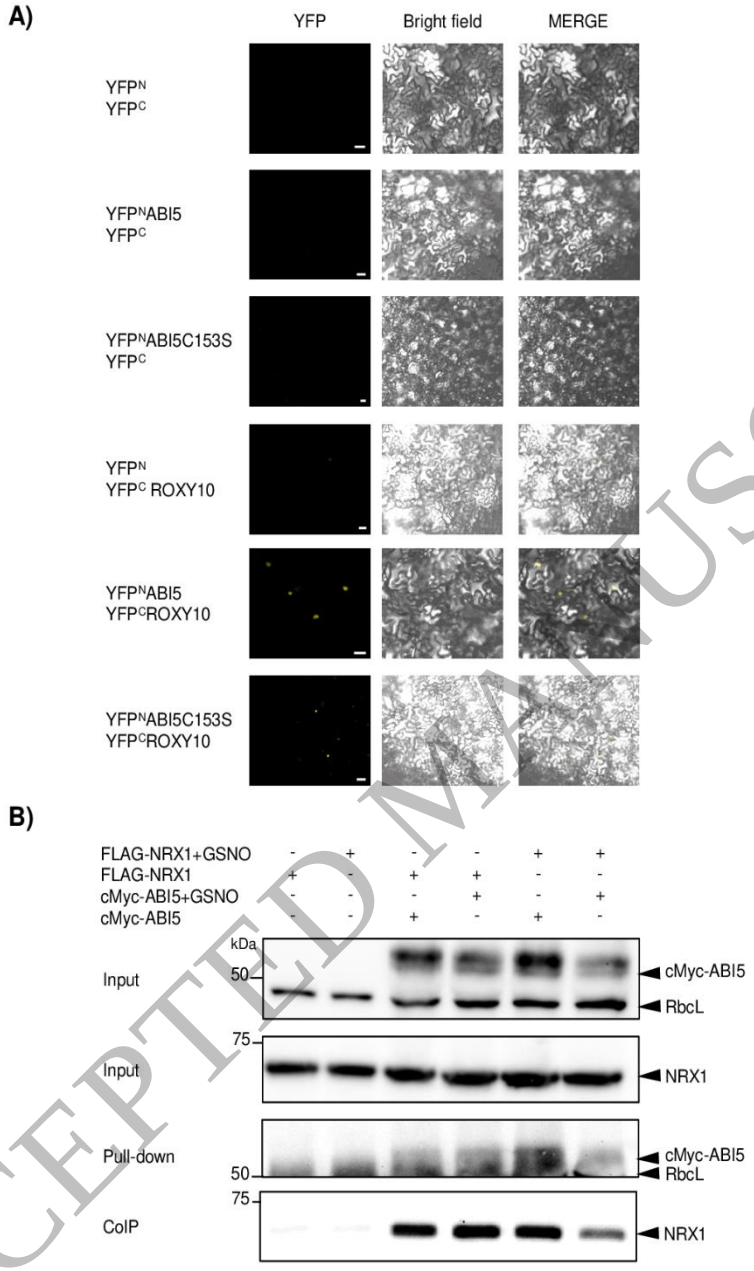

Figure 4
190x254 mm (x DPI)

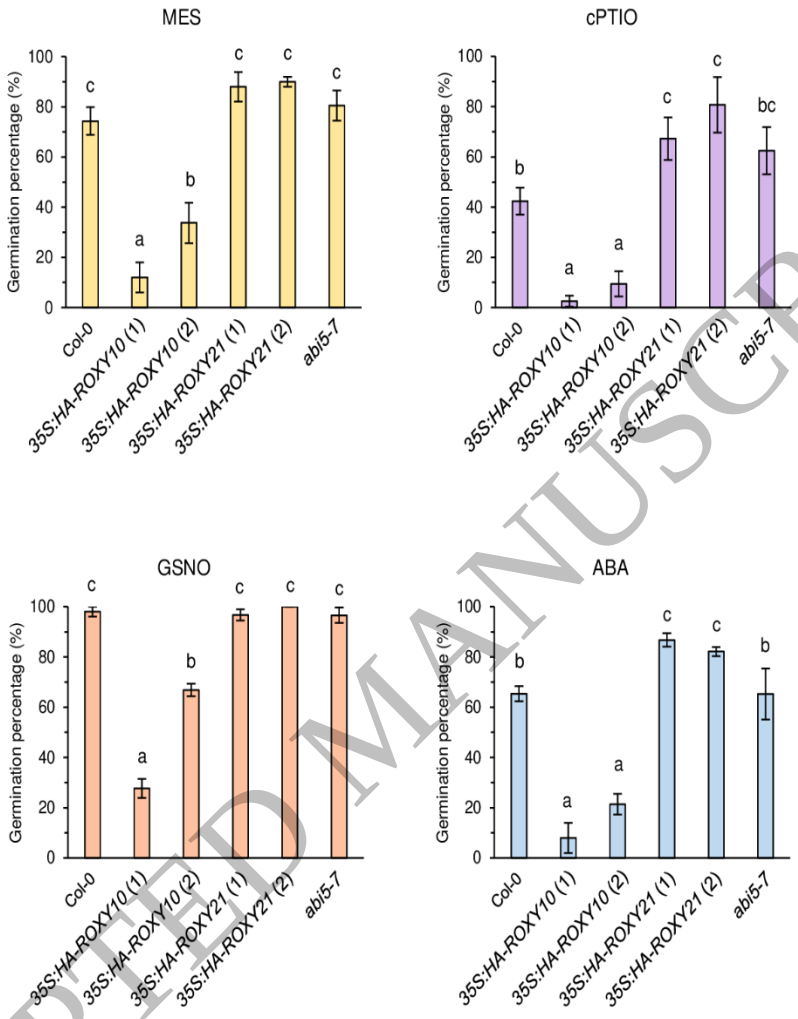

Figure 5
190x254 mm (x DPI)

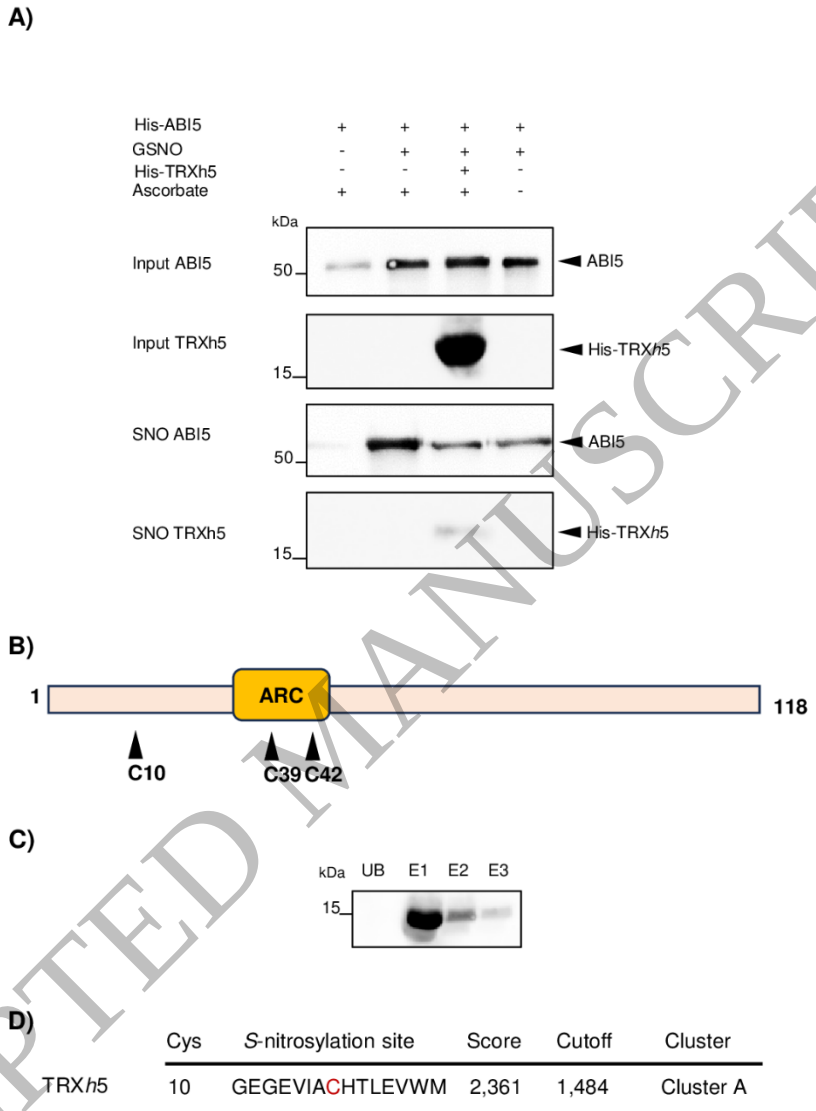

Figure 6
190x254 mm (x DPI)

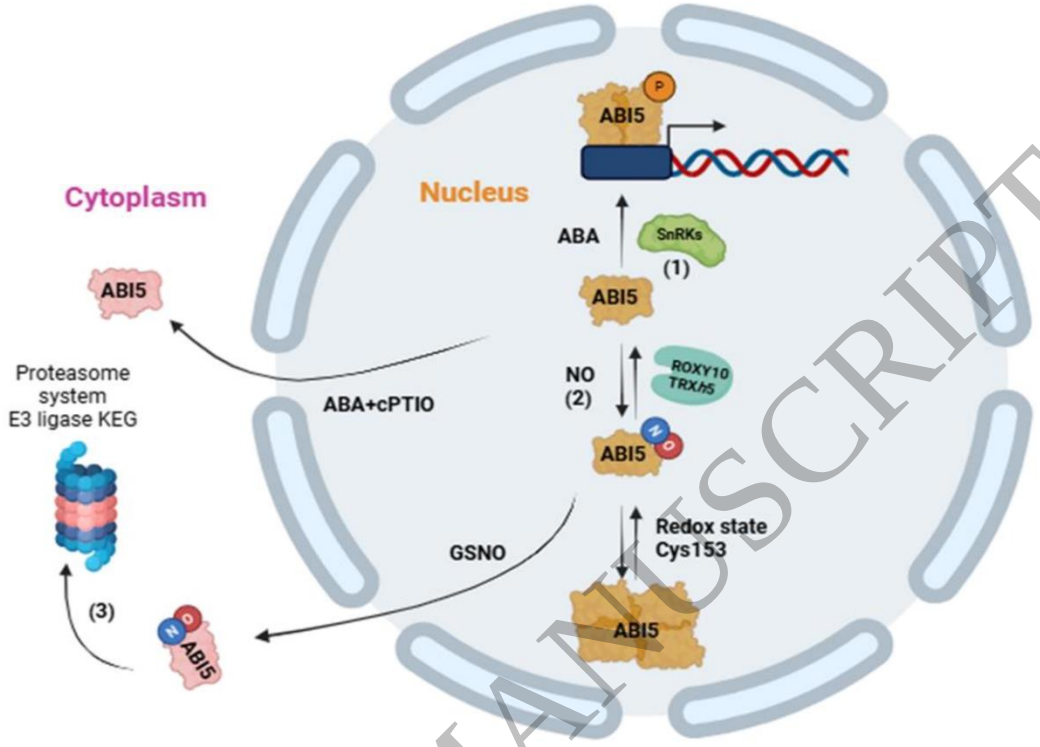

Figure 7
190x254 mm (x DPI)