S-nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth

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Plant survival depends on seed germination and progression through post-germinative developmental checkpoints. These processes are controlled by the stress phytohormone abscisic acid (ABA). ABA regulates the basic leucine zipper transcriptional factor ABI5, a central hub of growth repression, while the reactive nitrogen molecule nitric oxide (NO) counteracts ABA during seed germination. However, the molecular mechanisms by which seeds sense more favourable conditions and start germinating have remained elusive. Here, we show that ABI5 promotes growth via NO, and that ABI5 accumulation is altered in genetic backgrounds with impaired NO homeostasis. S-nitrosylation of ABI5 at cysteine-153 facilitates its degradation through CULLIN4-based and KEEP ON GOING E3 ligases, and promotes seed germination. Conversely, mutation of ABI5 at cysteine-153 deregulates protein stability and inhibition of seed germination by NO depletion. These findings suggest an inverse molecular link between NO and ABA hormone signalling through distinct posttranslational modifications of ABI5 during early seedling development.

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In the plant life cycle, the development of a new seedling depends on both appropriate timing of seed germination and the perception of environmental conditions. Consequently, germination and early seedling development must be tightly regulated by exogenous and endogenous signal molecules. Among them, abscisic acid (ABA) plays an important role in the inhibition of seed germination and in post-germinative seedling arrest under unfavourable environmental conditions. ABA-dependent growth arrest after germination relies on the basic leucine zipper-type transcription factor ABI5 (refs 3–5). In many plant developmental processes and stress responses, the precise changes in cellular status are regulated not solely by ABA signalling but by a complex network of ABA and other signalling pathways. Several examples of cross-talk between ABA and the gaseous signalling molecule nitric oxide (NO) have been recently reported. These interactions include the link of SnRK2.6/OST1 S-nitrosylation to the negative regulation of ABA signalling in the stomata and the NO regulation of ABI5 transcription through control of group VII ethylene response factors (ERFs) stability 

NO is a signalling molecule involved in a variety of physiological processes during plant growth and development. Extensive research has shown that NO affects seed dormancy, seed germination and ABA sensitivity, as evidenced by exogenous application of NO donors, or by genetic analysis of mutants with altered endogenous NO levels in Arabidopsis. However, despite the abundant involvement of NO in different plant cell signalling pathways, the actual knowledge about its direct targets is poorly understood. A key feature of NO biology is the posttranslational modification of cysteine thiols to form nitrosothiols (S-nitrosylation) in target proteins. In animals, this posttranslational modification has also been related to protein degradation via the ubiquitin-dependent proteasome pathway. Here we establish a molecular mechanism for NO and ABA antagonism in the regulation of seed germination and post-germinative development through ABI5 protein stability. Genetic analysis identified that abi5 mutants are insensitive to NO scavenging during seed germination. ABI5 protein levels are high in NO-deficient mutant backgrounds and low in NO-overaccumulating plants. S-nitrosylation of ABI5 at Cys 153 facilitates its degradation and promotes seed germination. Conversely, mutation of ABI5 Cys 153 reduces protein degradation through CULLIN4 (CUL4)-based and KEEP ON GOING E3 (KEG) ligases, and deregulates the inhibition of seed germination by NO depletion. Thus, ABI5 is regulated through the antagonistic action of ABA and NO, as evidenced by the synergistic effect of S-nitrosoglutathione (GSNO) on ABI5 destabilization. These findings suggest an inverse molecular link between NO and ABA hormone signalling through distinct posttranslational modifications of ABI5 during gene regulation of early seedling development.

**Results**

**Identification and genetic characterization of gap mutants.** NO affects seed dormancy, seed germination and ABA sensitivity, as shown by exogenous application of NO donors or by genetic analysis of mutants with altered endogenous NO levels in Arabidopsis. By exploiting the cross-talk between ABA and NO in the transition from dormancy to germination, we performed a genetic screen using (+)-S-ABA coupled to the effect of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). We isolated 7 (+)-S-ABA-insensitive mutants that were able to germinate on 3 μM (+)-S-ABA and also display a cPTIO-insensitive phenotype (Supplementary Fig. 1a). These mutants were named gap (germination in ABA and cPTIO). Allelism tests indicated that these fell into two different loci, five of these mutants corresponding to new abi5 alleles verified by candidate gene sequencing (Supplementary Fig. 1b). Germination of Col-0 wild-type seeds was delayed by 100 μM cPTIO (Fig. 1a,b), in agreement with previous reports; however, germination of the abi5 mutant alleles was less affected by NO depletion than the wild type. Hence, these results showed that abi5 mutants were insensitive not only to ABA but also to NO scavenging by cPTIO during seed germination.

**NO induces degradation of the ABI5 protein.** To extend these findings, we determined the localization of the ABI5 protein (Fig. 1c) and ABI5 transcript (Fig. 1d) in seeds after treatment with ABA, the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP) and the NO scavenger cPTIO. ABI5 accumulated to high levels after treatment with ABA for 48 or 72 h and even earlier after NO was depleted (Fig. 1d). In contrast, application of the NO donor SNAP quickly reduced ABI5-GUS levels (Fig. 1c and Supplementary Fig. 2).

To further investigate the role of NO in the regulation of seed germination in Arabidopsis, we examined endogenous NO levels in 2-day-old wild-type seeds by using the fluorescence indicator 4,5-diaminofluorescein diacetate (DAF-2DA). We observed NO-dependent fluorescence in the seed endosperm and rapid elongation zone after radicle protrusion (Fig. 1c), as previously described. Application of the NO scavenger cPTIO reduced the DAF-2DA fluorescence pattern in treated seeds, signal being limited to the autoflorescence of the testa tissues (Supplementary Fig. 1c–e). Co-localization of NO burst and ABI5 tissue-specific reduction of ABI5-GUS protein during radicle protrusion (seed imbibition/germination) was confirmed after GUS histochemical analysis using pABI5:ABI5-GUS lines as highlighted in Fig. 1c.

Recently, the N-end rule pathway of targeted proteolysis has been shown to regulate ABI5 expression through group VII ERF transcription factors. To this end, application of MG132 did not transcriptionally induce ABI5 gene expression, as a putative consequence of enhanced group VII ERF stabilization (Fig. 2a).

The effect of SNAP and the physiological NO donor GSNO in the promotion of seed germination was correlated by the disappearance of ABI5 during germination and post-germinative growth (Fig. 2b–d and Supplementary Fig. 3a–c). Conversely, endogenous NO depletion by the cPTIO scavenger inhibited seed germination and maintained high ABI5 protein levels, similar to ABA (Fig. 2b,d). It is noteworthy that the proteasome inhibitor MG132 or the proteasome inhibitor cocktail (including MG115, MG132 and epoxomicin) restored ABI5 accumulation, even in the presence of NO donors, and prevented seed germination (Fig. 2b,c and Supplementary Fig. 3c). In addition, cycloheximide alongside the MG132 and NO donor/scavenger treatments proved that ABI5 protein stability was being affected (Fig. 2a,c). Collectively, these findings implied that NO function during seed germination was through ABI5 degradation. After this developmental checkpoint and at high concentrations, NO dramatically affected post-germinative seedling growth, inhibiting root growth and development.

**ABI5 level is altered when NO homeostasis is impaired.** We corroborated the above pharmacological findings by the use of mutants and transgenic lines impaired in NO homeostasis. Thus, NO-deficient atn ao1-2nia1;nia2 triple mutant impaired in NIA/NR- and AtNOA1-dependent NO biosynthesis in Arabidopsis was hypersensitive to ABA, underscoring its effect on germination inhibition (Supplementary Fig. 3d). Non-symbiotic...
after 24, 48 and 72 h and in the absence of (Control) or the presence of 100 μM cPTIO after 2 days. Error bars represent ± s.e. (n = 3). Asterisk indicates significant differences compared with Col-0 (Control) (t-test, P < 0.05). (c) Co-localization of ABI5 expression, protein localization and NO production. pABI5:ABI5-GUS seeds were stratified for 3 days at 4°C and grown for 1 to 2 days at 21°C on MS agar plates and then subjected to DAF-2DA incubation or GUS staining after treatment with NO scavenger (cPTIO) and donor (SNAP). Arrows indicate high NO accumulation (left), and ABI5 expression and protein localization (middle). Scale bars, 100 μm. (d) qRT-PCR analysis of ABI5 relative transcript abundance in Col-0 seeds untreated (Control) and after treatments with ABA, cPTIO, SNAP and GSNO after 24, 48 and 72 h and in the abi5-1 background. Error bars represent ± s.e. (n = 3).

Figure 1 | NO depletion phenotypes of ABI5 loss-of-function mutants during seed germination. (a) Insensitivity of abi5 mutants to NO scavenging by cPTIO during seed germination. Photographs of 2-day-old germinated seeds after imbibition of wild type (Col-0) and the ABA-insensitive abi5-1 and abi5-7 mutants, in the absence of (Control) or the presence of 100 μM cPTIO. Scale bar, 1 mm. (b) Germination of wild-type (Col-0), abi5-1 and abi5-7 seeds in media containing 0 and 100 μM cPTIO after 2 days. Error bars represent ± s.e. (n = 3). Asterisk indicates significant differences compared with Col-0 (Control) (t-test, P < 0.05). (c) Co-localization of ABI5 expression, protein localization and NO production. pABI5:ABI5-GUS seeds were stratified for 3 days at 4°C and grown for 1 to 2 days at 21°C on MS agar plates and then subjected to DAF-2DA incubation or GUS staining after treatment with NO scavenger (cPTIO) and donor (SNAP). Arrows indicate high NO accumulation (left), and ABI5 expression and protein localization (middle). Scale bars, 100 μm. (d) qRT-PCR analysis of ABI5 relative transcript abundance in Col-0 seeds untreated (Control) and after treatments with ABA, cPTIO, SNAP and GSNO after 24, 48 and 72 h and in the abi5-1 background. Error bars represent ± s.e. (n = 3).

haemoglobin 1 (Ahb1) is an endogenous scavenger of NO, and thus Ahb1-overexpressing and -silenced lines contain lower and higher NO levels, respectively. As expected, the ABA response phenotype of these Ahb1 lines during seed germination differed from the hypersensitivity to ABA of the Ahb1-overexpressing lines to the wild-type germination of the Ahb1-silenced lines (Supplementary Fig. 3e,f).

We investigated ABI5 protein accumulation in the Arabidopsis atnoa1, nia1;nia2 and atnoa1;nia1;nia2 mutant seeds, which exhibit decreased levels of cellular NO (Fig. 2e). NO-deficient mutants, defective in either the oxidative or reductive NO synthesis pathways, respectively, or in both pathways, accumulated higher ABI5 protein levels. In agreement with the enhanced ABA sensitivity of NO-deficient mutant backgrounds, ABI5 protein levels were increased with respect to those observed in the wild type (Col-0). To corroborate these findings, Ahb1-overexpressing and -silenced lines were also analyzed for their ABI5 accumulation pattern (Fig. 2f). Collectively, these findings implied that genetic backgrounds where the endogenous NO levels were enhanced (Ahb1-silencing lines) or diminished (atnoa1, nia1;nia2, atnoa1;nia1;nia2 and Ahb1-overexpressing lines) displayed altered ABI5 levels, which were mirrored by changes in their ABA response. These pharmacological and genetic approaches suggest that NO promoted ABI5 protein degradation via a proteasome-dependent pathway.

ABI5 is S-nitrosylated in vivo and in vitro. One possible mechanism of NO action in plant tissues is the redox-based posttranslational modification of target proteins through S-nitrosylation. NO is able to reversibly modify thiol groups of specific cysteine residues in target proteins, hence altering protein function. To determine whether ABI5 was S-nitrosylated by NO, the recombinant protein was exposed to either GSNO or SNAP, which are typically used to evaluate S-nitrosylation (that is, SNO formation) in vitro. The formation of SNO-ABI5 was monitored by the biotin switch method. As shown in Fig. 3a–c, ABI5 was S-nitrosylated by either GSNO or SNAP. Furthermore, the addition of dithiothreitol (DTT) strongly reduced the formation of SNO-ABI5, consistent with the presence of a reversible thiol modification. The biotin switch was assayed without ascorbate, to assure that the presence of the protein was nitrosylated by either GSNO or SNAP, which are typically used to evaluate S-nitrosylation. This Cys 153 is only S-nitrosylated in vitro. In-silico prediction suggested that Cys 153 was potentially S-nitrosylated (Supplementary Fig. 4b). Mass spectrometry analysis confirmed S-nitrosothiol formation at only Cys 153 of ABI5 (Fig. 3a,b and Supplementary Figs 5 and 6). This residue was therefore mutated individually and the resulting protein was expressed and treated with GSNO before analysis with the biotin switch method. The Cys153 to Ser mutation abolished S-nitrosylation of ABI5 (Fig. 3c). Collectively, these findings indicated that Cys 153 of ABI5 was specifically S-nitrosylated in vitro. This Cys 153 is only present in the closest ABI5 homologue AthZIP67 (Supplementary Fig. 4c), sharing a seed expression pattern with ABI5 (Supplementary Fig. 7). Together, these data suggest that redox modification by S-nitrosylation may regulate the activity of these proteins.
b-ZIP transcription factors in plants to govern seed germination and seedling establishment.

To understand whether S-nitrosylation of Cys 153 could modulate the function of ABI5, we assessed the effect of this NO-mediated posttranslational modification on the previously reported homodimerization of the ABI5 protein by using a yeast two-hybrid assay. Interestingly, neither the ability of ABI5 to interact (Supplementary Fig. 8) nor the DNA-binding capacity to the ABRE cis-consensus motif (Supplementary Fig. 9) were disturbed by the Cys→Ser mutation or in the presence of NO-related compounds.

To determine whether ABI5 was S-nitrosylated in vitro during seed germination, protein extracts from wild-type and 35S:ABI5 transgenic plants were treated with GSNO and MG132, and assayed by the biotin switch method, then S-nitrosylated proteins were immunopurified (Fig. 3d,e). Protein gel blot analysis of purified proteins probed with an antibody specifically recognizing the ABI5 protein showed the corresponding S-nitrosylation in vitro. In addition, in vivo studies with transgenic ABI5 lines expressing either Myc-tagged wild-type ABI5 or mutant derivatives were analysed during seed germination and treatment with MG132. To this end, endogenous proteins were subjected to biotin-switch analysis, immunoprecipitated and detected with an anti-ABI5 antibody. Wild-type ABI5 was S-nitrosylated during seed germination, but the Cys153Ser mutant was not (Fig. 3f).

Therefore, these results suggest that NO may regulate ABI5 redox state by S-nitrosylation at Cys 153 during seed imbibition.

**ABI5 is a NO sensor during seed germination.** To assess a physiological role for S-nitrosylation of the ABI5 protein during seed germination, AB15- and AB15Cys153Ser-overexpressing lines were generated in Arabidopsis by expressing this protein under control of the 35S promoter, in the wild-type and abi5-1 mutant background (Supplementary Fig. 10). The phenotype of the transgenic plants was evaluated in response to NO and ABA, and was compared with that of abi5 mutant and 35S:ABI5 transgenic plants. Intact AB15- and mutated AB15-Cys153Ser-overexpressing lines exhibited ABA-hypersensitive phenotypes during seed germination and seedling establishment (Supplementary Fig. 10d), demonstrating that AB15Cys153Ser was able to largely restore the abi5-1 mutation (Supplementary Fig. 10e). To further explore the possible biological consequence of ABI5-SNO formation at Cys 153, ABI5 protein accumulation was monitored in the presence of the protein synthesis inhibitor cycloheximide with or without GSNO (Fig. 4a). The data...
of S seed extracts 24 h after proteasome inhibitor MG132 (100 Actin protein levels are shown as a loading control. (abi5–1 rate of germination. In addition, there was a significant increase overexpressors and also in the establishment of a new plant Ser

Samples were initially immunopurified with anti-biotin before immunoblot analysis of ABI5 protein levels in seed extracts of Col-0 (abi5–1). The C153 from the tryptic fragment QGSLTLPAPLCR (peptide MS/MS spectra shown with Cys modified by biotin-HPDP). (Figure 3 | S-nitrosylation of ABI5 in vivo and in vitro. (a) Mass spectrometric analyses identify C153 as the S-nitrosylation site. MS/MS spectra of C153 from the tryptic fragment QGSLTLPAPLCR (peptide MS/MS spectra shown with Cys modified by biotin-HPDP). (b) The LC-MS spectra of the corresponding peaks (‘+562 m/z (+) and 842.49 m/z (+2)) of this peptide fragment is shown in the inset. (c) The C153 mutation blocks S-nitrosylation of ABI5. In vitro S-nitrosylation of wild-type ABI5 and mutant ABI5C153S recombinant proteins by the NO donors GSNO (200 μM) and SNAP (200 μM). This modification is reversed by treatment with DTT (20 mM). No signal was observed with glutathione (200 μM) treatment showing specificity of the biotin-switch assay. ABI5 protein loading was detected by anti-His antibody. (d,e) S-nitrosylation of ABI5 induced by GSNO in after-ripened seed extracts. Samples were initially immunopurified with anti-biotin before immunoblot analysis of ABI5 protein levels in seed extracts of Col-0 (d), 35S:ABI5 (e) and abi5–1 untreated (C) or treated with the indicated compounds. No signal was observed in the absence of biotin (– Biotin) or after DTT (20 mM) treatment. Actin protein levels are shown as a loading control. (f) In-vivo S-nitrosylation of ABI5 in abi5–1;35S:αMyc-ABI5 and abi5–1;35S:αMyc-ABI5C153S after-ripened seed extracts 24 h after proteasome inhibitor MG132 (100 μM) incubation. Immunoblot analysis of in vivo ABI5 protein levels after immunopurification of 5-nitrosylated proteins. No signal was observed in the absence of sodium ascorbate (– Asc) or after cPTIO (1 mM) treatment. Input protein levels were also determined using anti-ABI5 anti-serum.

obtained implied that the mutation of ABI5 at Cys 153, considering similar seed germination stages, impaired NO-promoted ABI5 degradation, resulting in elevated levels of ABI5 protein. CUL4 and KEG are well-known factors that directly bind and regulate ABI5 ubiquitin-mediated proteolysis20–24. Accordingly, mutations in either CUL4 or KEG abolished NO-promoted ABI5 protein degradation (Fig. 4b and Supplementary Fig. 11). Intact ABI5 protein interacted with both CUL4 and KEG in the presence of GSNO, whereas ABI5Cys153Ser mutation failed to interact (Fig. 4c,d), supporting that S-nitrosylation triggered ABI5 destabilization through CUL4-based and KEG E3 ligases.

To determine the possible impact of the ABI5Cys153Ser mutation on NO sensing during seed germination and seedling establishment, we treated intact ABI5- and mutated ABI5Cys153Ser-overexpressing lines with NO scavengers (cPTIO) and donors (SNAP) (Fig. 4e,f and Supplementary Fig. 10f). As depicted in Fig. 4e,f, inhibition of seed germination by NO depletion during radicle protrusion was enhanced in ABI5 but not in ABI5Cys153Ser overexpressors and also in the establishment of a new plant (Supplementary Fig. 10f), resulting in a prominent decrease in the rate of germination. In addition, there was a significant increase in seedling establishment after NO treatment in the ABI5-overexpressing lines relative to the overexpression of the ABI5Cys153Ser mutant version that instead was able to maintain growth arrest (Supplementary Fig. 10f). Germination of the transgenic plants under unfavourable conditions such as high salinity and hyperosmotic stress showed NaCl- and mannitol-hypersensitive inhibition of post-germinative growth in 35S:ABI5 and 35S:ABI5C153S lines as compared with wild-type plants (Fig. 4g). Indeed, 35S:ABI5C153S lines were more deeply hypersensitive than the ABI5-overexpressing lines. Together, this information implies that one way in which seeds sense NO was by the S-nitrosylation of ABI5 at Cys 153, and that disruption of this mechanism was able to arrest seedling growth under adverse environmental conditions.

The involvement of group VII ERF transcription factors in NO sensing and NO downregulation of ABI5 transcription has been previously reported7. Consistently, ABI5 protein levels accumulated to a greater extent after ABA treatment in prt6–1 mutant seeds and seedlings, which are impaired in the degradation of group VII ERFs by the N-end rule pathway25,26 (Fig. 5a–c). Thus, Rubisco large subunit was detected in those
Figure 4 | The ABI5 C153S mutant shows decreased proteasomal degradation by CUL4 and KEG, and confers NO insensitivity during seed germination. (a) Immunoblot analysis of ABI5 protein levels in 8-day-old seedling extracts of similar germination stages abi5-1;35S:cMYC-ABI5 and abi5-1;35S:cMYC-ABI5C153S, in the presence of cycloheximide (1 mM) and cycloheximide (1 mM) plus GSNO (500 μM) from 0 to 9 h. Actin protein levels are shown as a loading control. (b) ABI5 protein levels in wild type (Col-0), c4lcs, keg4 and abi5-1 mutant backgrounds. Stratified seeds were incubated with 5 μM ABA for 48 h and treated with GSNO (1 mM) for 6 h after ABA removal. Immunoblot analysis of ABI5 protein levels in seed extracts of wild type and mutants. Actin protein levels are shown as a loading control. (c) Co-immunoprecipitation assays between CUL4 and transgenic ABI5/ABI5C153S proteins in the presence of GSNO. Input protein levels were also determined using anti-HA and anti-MYC antisera, respectively. (d) Co-immunoprecipitation assays between KEG and ABI5/ABI5C153S proteins in the presence of GSNO. Input protein levels were also determined using anti-FLAG and anti-MYC antisera, respectively. (e) NO-insensitive inhibition of seed germination by NO scavenging in 35S:ABI5C153S lines as compared with 35S:ABI5 plants. Total seed germination of wild type (Col-0), abi5-1, abi5-7 and two (1, 2) 35S:ABI5- and 35S:ABI5C153S-independent lines grown for 2 days on MS agar plates untreated (Control) or supplemented with 50 and 100 μM of the NO-scavenger cPTIO. Values represent the mean ± s.e. (n = 3). Asterisks indicate significant differences compared with 0 μM cPTIO (t-test, *P < 0.05, **P < 0.01). (f) ABI5 levels in 35S:ABI5 and 35S:ABI5C153S transgenic lines used for the germination assay. Immunoblot analysis of ABI5 protein levels in seed extracts. Actin protein levels are shown as a loading control. (g) NaCl- and mannitol-hypersensitive inhibition of post-germinative growth in two 35S:ABI5 and 35S:ABI5C153S lines as compared with wild-type plants. Seedling growth of wild type (Col-0), abi5-1, 35S:ABI5 and 35S:ABI5C153S lines grown for 9 days on MS agar plates untreated (Control) or supplemented with 100 mM of NaCl and 250 mM of mannitol. Values represent the mean ± s.e. (n = 3). Letters indicate significant differences compared with wild-type (Col-0) (a), 35S:ABI5-1 (b), 35S:ABI5-2 (c), abi5-1;35S:ABI5-1 (e), abi5-1;35S:ABI5-2 (d), (t-test, P < 0.05).
ABIs1−1 Col-0 prt6−1
- - -
50 -
37 -
αABI5
α-Actin

Col-0
- + -
50 -
37 -
αABI5

Col-0
- + +
ABA
αABI5

Col-0
- + +
αABI5

Col-0
- + +
ABA (μM)

Col-0
- + +
αABI5

Col-0
- + +
RbcL

Col-0
- + +
αActin

Col-0
- + +
αABI5

Col-0
- + +
α-Actin

Dormant/dry seed
Imbibed seed
Germinated seed

Group VII ERF
N-end rule (PRT6)
Degradation

ABIs
NO

NO

Cys153-SH

Cys153-SNO

CUL4/KEG

Figure 5 | ABIs accumulation and degradation in prt6−1 mutant background during seed germination and post germination. (a,b) ABIs1 treatment promotes ABIs1 accumulation in germinating seeds. Immunoblot analysis of ABIs1 protein levels in seed extracts of Col-0 and prt6−1 before (a) and after 48 h treatment with (+) or without (−) 0.25 μM ABA (b). Actin protein levels are shown as a loading control. (c) Post-germinative ABIs1 accumulation in seedlings. Immunoblot analysis of ABIs1 protein levels in 10-day-old extracts of Col-0, prt6−1 and abi5−1, treated with or without (−) 0.25, 0.5 and 1 μM ABA. Actin protein levels are shown as a loading control and Rubisco large subunit (RbcL) detection is indicated. (d) ABIs1 protein levels in wild-type (Col-0), prt6−1 and abi5−1 mutant backgrounds. Stratified seeds were incubated with 5 μM ABA for 48 h (T0) and treated after ABA removal with H2O, ABA, SNAP (1 mM) and GSNO (1 mM) for 12 h. Immunoblot analysis of ABIs1 protein levels in seed extracts of wild type and mutants. Actin protein levels are shown as a loading control.

Discussion
In conclusion, our data establish a molecular mechanism for NO and ABA antagonism in the regulation of seed germination and post-germinative growth (Fig. 6). We identify two new loci involved in ABA and NO signalling (GERMINATION IN ABA AND cPTIO, GAP) and characterize one of these loci that correspond to the basic leucine zipper transcription activator ABIs1. S-nitrosylation of ABIs1 targets proteasomal degradation through CUL4-based and KEG ligases and acts as a regulatory switch for seed germination in Arabidopsis. Thus, S-nitrosylation offers new insights into the regulation of protein stability through CULLIN-related degradation pathways in plants.

The findings by Gibbs et al.7 and those reported here demonstrate that NO targets ABIs1 at both the transcriptional level (through NO-mediated degradation of ERFVIIIs) and the posttranslational level (through NO-mediated degradation of ABIs1), respectively, highlighting the fact that ABIs1 is depleted via a dual NO-responsive mechanism. These two independent mechanisms converging on ABIs1 may have evolved to ensure that NO irrevocably removes ABIs1 from the seed to promote germination. Several previous reports emphasize that ABIs1 protein stability is regulated tightly by multiple different mechanisms converging on ABIs1 may have evolved to ensure that NO irreversibly removes ABIs1 from the seed to promote germination. Several previous reports emphasize that ABIs1 protein stability is regulated tightly by multiple different mechanisms21–24. Thus, it is reasonable to speculate that S-nitrosylation-mediated ABIs1 protein degradation plays an important role in the NO function to regulate seed germination, rather than only through transcriptional regulation7. In addition, it is likely to be that other mechanisms of NO-induced ABIs1 degradation independently of C153 S-nitrosylation may be involved, probably nitrosylating different regulators for ABIs1 degradation.

The identification of ABIs1 as a direct NO target involved in NO-mediated effects in plant growth and development contributes to our understanding of NO role in plant signal transduction networks and establishes a molecular framework for the NO function during seed germination.

Methods
Plant materials and treatments. Arabidopsis thaliana ecotype Columbia-0 (Col-0) was the genetic background for all wild-type plants used in this work. Seed stocks of abi5, atroa1;nia1;nia2 and key mutants were obtained from Arabidopsis Biological Resource Center. The atroa1;nia1;nia2 mutants11 and 35S:AHb1 (H3, H7) and 35S:SantiAHb1 (L1, L3) lines27 were kind gifts from Dr José León.
Arabidopsis plants were grown in a growth chamber or greenhouse under 50–60% humidity, a temperature of 22 °C and with a 16 h light/8 h dark photo-period at 80–100 μl m⁻² s⁻¹ in pots containing a 1:3 vermiculite/sand mixture. For in vitro culture, Arabidopsis seeds were surface sterilized in 75% (v/v) sodium hypochlorite and 0.1% (v/v) Triton X-100 for 5 min, and washed three times in sterile water before sowing. Seeds were stratified for 3 days at 4 °C and then sown on Murashige and Skog (MS) solid medium with 2% (w/v) Suc and 0.5% agar (pH 5.8). After incubation at 22 °C and 70% humidity, cotyledons were determined and developed green, fully expanded cotyledons was determined every day during 10 days after sowing.

Western blotting. Total protein for western blot analysis was extracted from dormant seeds of Col-0, abi5-1 and after-ripened seeds of Col-0, abi5-1 and transgenic lines untreated or treated during 72 h with ABA, cPTIO, GSNO, SNAP, GSNO and SNAP plus MG132 or the protease inhibitor cocktail including MG115 (1 μM), MG132 (100 μM) and epoxomicin (0.75 μM). For cycloheximide treatments, transgenic plants were germinated and grown on MS liquid medium during 7 days and cycloheximide (1 mM) alone or in combination with GSNO (500 μM) was added to the medium, and samples were taken at indicated intervals. Tissue was powdered using mortar and pestle, and incubated for 10 min on ice with extraction buffer (50 mM Tris–HCl, pH 7.5, 75 mM NaCl, 15 mM EDTA, 1% SDS, 0.2 M NaV, 0.1% Tween 20, 1 mM Na-pyrophosphate, 60 mM β-glycerolphosphate and 1 × proteases inhibitor mix, Roche) followed by centrifugation for 10 min at 15,800 g at 4 °C. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad) based on the Bradford method. Sixty micrograms of total protein was loaded per well in SDS-acrylamide/bisacrylamide gel electrophoresis using Tris–glycine–SDS buffer. Proteins were electrophoretically transferred to an Immobilon-P polyvinylidine difluoride 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. Anti-ABI5 Purified Rabbit IgG Antibod (Bioss, 1:5,000), anti-ABI5 purified Mouse IgG (Sigma A0480, 1:20,000), monoclonal anti-polyHistidine – Peroxidase produced in mouse (Sigma A0758, 1:2,000) and ECL-Peroxidase-labelled anti-rabbit (Amer sham NA934, 1:2,000) and anti-mouse (Amer sham NA931, 1:2,000) antibodies were used in the western blot analyses. Detection was performed using ECL Advance Western Blotting Detection Kit (Amer sham) and the chemiluminescence was detected using an Intelligent Dark-Box II, LAS-1000 scanning system (Fujifilm). Quantification of band intensity was performed with ImageJ software. Full-sized uncropped immunoblots of cropped blottings used in figures are included in Supplementary Fig. 12.

Site-directed mutagenesis. Site-directed mutagenesis of ABI5 was performed using the QuickChange II Site-Directed Mutagenesis Kits (Stratagene Corporate). Plasmid pET28a-ABI5 was used as template and primers were designed using the tools from Stratagene and synthesized by Isogen. The primers were as follows: forward primer 5'-CACCTTACACGTGCCTTAGGTACGCTTGTG-3' and reverse primer 5'-ATACAAACAGTCCTCCTACTAACGAGGCACTTGAGTAGTGG-3'. Mutations were confirmed by sequencing.
In vivo and in vitro S-nitrosylation assays. We used the biotin switch method\(^{18}\) that converts –SNO into biotinylated groups, to detect S-nitrosylated proteins in Arabidopsis seed extracts and recombinant purified ABI5 and ABI5C153S, with slight modification.

For in vitro S-nitrosylation, purified ABI5 recombinant protein was pre-treated with NO donors SNAP and GSNO (200 μM, Calbiochem), and the glutathionylating agent glutathione (100 μM, Sigma) in the dark at room temperature for 30 min with regular vortexing with the reducing agent (DTT, 20 mM; Sigma) after GSNO incubation was carried out for 1 h under the same conditions, to check reversibility of the modification. Reagents were removed by precipitation with two volumes of cold acetone and proteins were assayed by the biotin switch method.

In vivo S-nitrosylation of ABI5 was carried out with Arabidopsis dry seeds homogenized in extraction buffer (50 mM Tris–HCl, pH 7.5, 75 mM NaCl, 15 mM EGTA, 15 mM MgCl\(_2\), 0.1% Tween 20, 1 mM EDTA, 0.2% Mn Na\(_2\), 2 mM Na pyrophosphate, 60 mM β-glycerophosphate) containing Complete Protease Inhibitor Cocktail (Roche). Seeds extracts (1 mg) were incubated with GSNO (1 mM) and proteinase inhibitors (Sigma) at 4°C for 20 min in the dark for 30 min with repeated vortexing. Samples treated with DTT (20 mM) were also kept for 1 h under the same conditions and were used as a negative control. Proteins were recovered by precipitation with two volumes of acetone for 20 min at –20°C, to remove excess of GSNO/DTT, and assayed by the biotin switch method.

For the biotin switch, extracts or recombinant proteins were incubated with 20 mM S-methyl-methanethiosulfonate and 2.5% SDS at 50°C for 30 min with frequent vortexing, to block free Cys. S-methyl-methanethiosulfonate was removed by protein precipitation with two volumes of cold acetone and proteins were dissolved in 0.1 ml of RB buffer (25 mM Hepes, 1 mM EDTA and 1% SDS, pH 7.7) per mg of protein. After addition of 100 mM HEPES, 50 mM NaCl, 20% acetic acid, the mixture was incubated for 1 h at room temperature in the dark with intermittent vortexing. In vivo biotinylated proteins were purified by immunoprecipitation with neutravidin or an IPA (protein A/G UltraLink Resin, Pierce) anti-biotin antibody, overnight at 4°C. Proteins were eluted with 10 mM DTT in SDS-PAGE solubilization buffer, loaded in 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane, to detect ABI5 with anti-ABI5 Purified Rabbit Immunoglobulin (Biomedal, 1:5,000). Mass spectrometry (MALDI–TOF/TOF). Alternatively, purified proteins were analysed directly by nano-ESI-MS/MS in the pEarleyGate 203 vector 38 using the GATEWAY technology and the following primers (ABRE-binding site motif underlined)\(^{36}\) was incubated with 1.5 μg wild-type ABI5 or mutant ABI5C153S recombinant protein in the presence and absence of GSNO (1 mM) and DTT (1 mM) as described previously\(^{37}\). Based on these data, we concluded that ABI5C153S is more susceptible to GSNO-induced nitrosylation than ABI5.

**Electrophoretic mobility shift assay.** For the electrophoretic mobility shift assay, 10 ng of a double-stranded biotinylated probe 5’-GATGCTTCTCGGTACAA-TAAAAGTCAAGAGACGGGCACATGACCTAAGATGATGCTGTA-3’ (ABRE-**G** motif underline)\(^{36}\) was incubated with 1.5 μg wild-type ABI5 or mutant ABI5C153S recombinant protein in the presence and absence of GSNO (1 mM) and DTT (1 mM) as described previously\(^{37}\). Based on these data, we concluded that ABI5C153S is more susceptible to GSNO-induced nitrosylation than ABI5.

The remaining 80% of the samples were analysed by LC coupled to electrospray ion-trap mass spectrometry MS/MS using Ultimate 3000 nano LC ( Dionex, Amsterdam, The Netherlands) and a 75-mm I.D., 100 mm reverse-phase column, at 300 nl min \(^{-1}\) flow, coupled to a Bruker HCT Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) working in dynamic exclusion mode. For protein identification, LC coupled to electrospray ion-trap mass spectrometry MS/MS experiments were transferred to the Bruker Biflex T.20 interface (Bruker Daltonics). Data were searched in the NCBI database using a licensed version of Mascot v.2.2.04 search engine (www.matrixscience.com; Matrix Science). Search parameters were set as follows: in reduced samples, carbamidomethyl cystein was set as fixed modification by the treatment with iodoacetamide, oxidized methionines as variable modification, peptide mass tolerance of 0.5 Da for the parental mass and fragment masses, and one missed cleavage site. In the case of non-reduced samples, biotin-HPDP cysteine modification was set as variable modification.

**Yeast two-hybrid assay.** ABI5 and ABI5C153S were cloned in the pDEST22 and pDEST32 vectors using the GATEWAY technology. Prey and bait clones were grown for 3 days on DOB-W and DOB-L (MP Biomedicals) plates from their corresponding frozen stocks. YPAD medium was inoculated in parallel with bait and prey cells, and incubated overnight at 28°C with shaking (200 r.p.m.). After overnight incubation, the bait culture was added to prey and mating was allowed 48 h by incubating at 28°C without shaking. Settled cells were resuspended and used to inoculate plates containing diploid selection media (DOB-L-W). After 1 day of growth at 28°C and vigorous shaking, diploid cells were resuspended and spotted onto diploid selection and screening (DOB-L-W-Histidine ± 3-Amino-1,2,4-triazole; 3-AT, Sigma) plates. Positive colonies were visible after 2–5 days of growth at 28°C.

**Co-immunoprecipitation and pull-down assays.** For haemagglutinin (HA) and FLAG pull-down assays, the proteins were extracted with lysis buffer (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Roche). Extractions were cleared by centrifugation and protein concentration was determined by Bradford assay. One milligram of soluble proteins was treated with GSNO (1 mM) in darkness at room temperature during 1 h. After treatment, extracts were immunoprecipitated using anti-HA Affinity Matrix (Roche) and anti-FLAG M2 Matrix (Sigma) for ABI5/KEG and ABI5/CUL4 interactions, respectively. Extracts and beads were incubated during 2 h at 4°C and beads were washed twice in lysis buffer. Next, the beads were incubated at 4°C during 2 h with 1 μg of ABI5 and ABI5C153S soluble proteins previously treated with GSNO (1 mM) in darkness at room temperature during 1 h. After incubation, beads were washed twice and proteins were eluted from beads in SDS sample buffer. The proteins were visualized using anti-MYCY antibodies (Abcam ab62928, 1:10,000), anti-HA antibodies (Roche 12013891001, 1:2000) and anti-FLAG antibodies (Sigma F1804, 1:1,000).

**Generation of transgenic Arabidopsis plants.** ABI5 and ABI5C153S were cloned in the pEarleyGate 203 vector using the GATEWAY technology and the following primers (ABI5-F 5’-ATGGTAACTAGAGAAACGAAGTTGACG-3’; ABI5-R 5’-TTAGCCTGACGTAGGAC-3’). Similarly, ABI5C153S was cloned in the binary pGW3137 vector fusing to GUS. The constructs generated (Supplementary Fig. 11a) were used to transform the C58C1 (pGVT2260) Agrobacterium strain\(^{40}\). For plant transformation, Arabidopsis plants (Col-0, abi5-1 or abi5-7) were transformed by the floral dip method\(^{41}\) as described previously\(^{42}\). Seeds were harvested and grown on medium to identify T1 transgenic plants. Approximately 100 of the T2 seeds were plated on selection medium MS agar plates and transgenic lines with a 3:1 (resistant/sensitive) segregation ratio were selected. T3 progenies homozygous for selection medium resistance were used for further studies.

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