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Research Article

Nitrate attenuates abscisic acid signaling via NIN-LIKE PROTEIN8 in Arabidopsis seed germination

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Abstract

Abscisic acid (ABA) suppresses Arabidopsis (*Arabidopsis thaliana*) seed germination and post-germinative growth. Nitrate stimulates seed germination, but whether it directly regulates ABA signaling and the associated underlying molecular mechanisms remain unknown. Here, we showed that nitrate alleviates the repressive effects of ABA on seed germination independently of the nitric oxide (NO) pathway. Moreover, nitrate attenuates ABA signaling activated by ABSCISIC ACID INSENSITIVE3 (ABI3) and ABI5, two critical transcriptional regulators of the ABA pathway. Mechanistic analyses demonstrated that ABI3 and ABI5 physically interact with the nitrate signaling-related core transcription factor NIN-LIKE PROTEIN 8 (NLP8). After ABA treatment, NLP8 suppresses ABA responses during seed germination without affecting ABA content. Notably, nitrate represses ABA signaling mainly through NLP8. Genetic analyses showed that NLP8 acts upstream of ABI3 and ABI5. Specifically, NLP8 inhibits the transcriptional functions of ABI3 and ABI5, as well as their ABA-induced accumulation. Additionally, *NLP8* overexpression largely suppresses the ABA hypersensitivity of mutant plants exhibiting impaired NO biosynthesis or signaling. Collectively, our study reveals that nitrate counteracts the inhibitory effects of ABA signaling on seed germination and provides mechanistic insights into the NLP8–ABI3/ABI5 interactions and their antagonistic relationships in ABA signaling.

Introduction

Seed germination and post-germinative growth are critical for the propagation and survival of plant populations. Seed germination in a specific physiological context and the subsequent establishment of a young seedling are regulated precisely by multiple signals. Among them, the phytohormone abscisic acid (ABA) is a prominent signal that suppresses seed germination and post-emergence growth in *Arabidopsis thaliana* (Gubler et al. 2005; Finkelstein et al. 2008; Cutler et al. 2010; Golldack et al. 2013; Nakashima and Yamaguchi-Shinozaki 2013; Sajeev et al. 2024; Zhao et al. 2024a). When the ABA concentration is low, type 2C protein phosphatases (PP2Cs) physically associate with and repress the kinase activity of SNF1-RELATED KINASE2 (SnRK2s), thereby inhibiting the downstream signaling events of the ABA pathway (Umezawa et al. 2009; Vlad et al. 2009; Soon et al. 2012). When the ABA concentration increases, however, the PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) proteins perceive ABA and then disrupt the PP2Cs co-receptors, leading to the release of SnRK2s from PP2C–SnRK2 complexes (Ma et al. 2009; Miyazono et al. 2009; Nishimura et al. 2009; Park et al. 2009; Santiago et al. 2009). The activated SnRK2s subsequently phosphorylate and stimulate the downstream targets of the ABA signaling pathway, such as the basic leucine zipper (bZIP) family protein ABSCISIC ACID INSENSITIVE5 (ABI5) and its close homologs, to modulate the expression of ABA-responsive genes (Kobayashi et al. 2005; Furihata et al. 2006; Fujii et al. 2007; Fujii and Zhu 2009; Nakashima et al. 2009).

The transcription factors ABI3 (a B3 domain-containing protein) and ABI5 are master activators of ABA-suppressed seed germination and the subsequent seedling establishment (Giraudat et al. 1992; Finkelstein 1994; Finkelstein and Lynch 2000; Lopez-Molina and Chua 2000; Lopez-Molina et al. 2001, 2002; Nakamura et al. 2001; Brocard et al. 2002; Finkelstein et al. 2005). Both *ABI3* and *ABI5* are highly expressed in mature seeds and are strongly responsive to ABA (Giraudat et al. 1992; Finkelstein and Lynch 2000). The loss-of-function *abi3* and *abi5* mutant seeds are substantially less sensitive to ABA than the wild-type controls (Koornneef et al. 1984; Giraudat et al. 1992; Finkelstein 1994; Finkelstein and Lynch 2000). Mechanistic investigations revealed that ABI3 and ABI5 are modulated by several regulators through post-translational modifications (Kobayashi et al. 2005; Furihata et al. 2006; Fujii et al. 2007; Garcia et al. 2008; Fujii and Zhu 2009; Miura et al. 2009; Nakashima et al. 2009; Hu and Yu 2014; Albertos et al. 2015; Zhou et al. 2015;

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Lynch et al. 2017; Ji et al. 2019; Varshney et al. 2023; Du et al. 2024). For example, the E3 ubiquitin ligases, such as KEEP ON GOING (KEG), ABI3-INTERACTING PROTEIN2 (AIP2), and PLANT U-BOX8 (PUB8), ubiquitinate and destabilize ABI3 and ABI5 in the absence of ABA (Zhang et al. 2005; Stone et al. 2006; Lee et al. 2010; Liu and Stone 2010; Seo et al. 2014; Li et al. 2023). Notably, ABI3 and ABI5 are also crucial integrators of ABA and other interactive signaling pathways (Lim et al. 2013; Guan et al. 2014; Kim et al. 2016; Yang et al. 2016, 2021; Ju et al. 2019; Guo et al. 2022; Li et al. 2022; Peng et al. 2022; He et al. 2023; Mei et al. 2023; Varshney et al. 2023). Although there have recently been considerable advances in our understanding of the ABA signaling network, the precise mechanisms underlying how ABA responses are strictly regulated in specific environmental and physiological contexts remain to be investigated in depth.

Nitrogen is a macronutrient essential for plants and its availability is a major factor influencing crop growth and yield. Plants take up nitrogen from the soil mainly as nitrate (NO_3^-), which is further assimilated to nitrite, ammonium, and amino acids for various biological processes (Crawford 1995; Jia et al. 2022). Additionally, nitrate functions as a critical signaling compound that modulates multiple morphological and physiological responses throughout the plant (Hilhorst et al. 1989; Fitter et al. 2002; Alboresi et al. 2005; Ho et al. 2009; Krouk et al. 2010; Canales et al. 2014; Yan et al. 2016; Landrein et al. 2018; Liu et al. 2020; Li et al. 2021; Chu et al. 2021). In terms of seed germination, nitrate has a key stimulatory effect in Arabidopsis and many other plants (Hilhorst et al. 1989; Fitter et al. 2002; Ali-Rachedi et al. 2004; Alboresi et al. 2005; Finch-Savage et al. 2007; Matakiadis et al. 2009; He et al. 2015; Duermeyer et al. 2018; Ciou et al. 2020; Zhang et al. 2020). Accordingly, NITRATE TRANSPORTER 1.1 [NRT1.1, which is also known as CHLORINA 1 (CHL1)], a nitrate sensor and dual-affinity nitrate transporter, and NIN-LIKE PROTEIN 8 (NLP8), a core transcription factor of the nitrate signaling pathway, are required for nitrate-induced dormancy release (Tsay et al. 1993; Wang et al. 1998; Liu et al. 1999; Alboresi et al. 2005; Remans et al. 2006; Matakiadis et al. 2009; Ho et al. 2009; Sun et al. 2014; Duermeyer et al. 2018; Lin et al. 2020). In the presence of nitrate, NLP8 binds to the promoter of *Cytochrome P450 family 707 subfamily A polypeptide 2* (*CYP707A2*, encoding an ABA catabolic enzyme) and upregulates gene expression, which results in a decrease in ABA levels during seed imbibition (Yan et al. 2016). Furthermore, nitric oxide (NO), the reactive nitrogen molecule produced during the reduction of nitrate to nitrite, also promotes Arabidopsis seed germination (Bethke et al. 2006; Mur et al. 2013; Gibbs et al. 2014; Zhao et al. 2024b). More specifically, NO functions through the PROTEOLYSIS6 (PRT6)-mediated N-end rule pathway to negatively affect *ABI5* transcription and ABA responses (Gibbs et al. 2014). In addition, NO induces the S-nitrosylation of ABI5 protein and triggers its degradation via the proteasome pathway (Albertos et al. 2015). Interestingly, nitrate and NO signaling pathways are distinct, but they likely interact during seed germination; this is supported by the fact NO-insensitive mutant plants (e.g. *prt6*) are still responsive to nitrate (Yan et al. 2016; Duermeyer et al. 2018). Despite recent advances, whether nitrate serves as a crucial cue that directly regulates ABA signaling during seed germination and the molecular basis of the convergence of nitrate and ABA signaling remain unknown.

In this study, we demonstrated that nitrate plays a crucial inhibitory role as a regulatory signal in ABA signaling-induced delayed seed germination. Our findings also unveiled the underlying mechanism of a previously unexplored signaling module in which the NLP8 transcription factor of nitrate signaling directly interacts with ABI3 and ABI5 to counteract their modulatory effects. This integration of the nitrate and ABA signal transduction pathways ensures that ABA signaling is maintained at an appropriate level under nitrate-sufficient conditions.

Results

Nitrate is a critical signal for attenuating ABA signaling independently of the NO pathway during seed germination

Previous studies showed that nitrate stimulates seed germination in Arabidopsis (Hilhorst et al. 1989; Fitter et al. 2002; Ali-Rachedi et al. 2004; Alboresi et al. 2005). To investigate whether nitrate modulates this process by attenuating ABA signaling, we initially analyzed whether nitrate regulates ABA responses during seed germination. We examined wild-type seed germination and cotyledon greening on water agar medium with different concentrations of ABA and 1 mM KNO_3 as the only nitrogen source. As expected, the presence of ABA decreased the wild-type seed germination and cotyledon greening percentages (Fig. 1, A to C; Supplementary Fig. S1, A and B). However, the inclusion of 1 mM KNO_3 in the medium appeared to mitigate some of the adverse effects of ABA on seed germination and cotyledon greening (Fig. 1, A to C). In addition, parallel experiments indicated that 1 mM KCl (as a control) did not influence the effects of 1 μM ABA on the seeds (Fig. 1, A to C). These observations imply that nitrate may counteract the inhibitory effect of ABA on seed germination.

To clarify the role of nitrate during ABA responses, we performed quantitative real-time PCR (RT-qPCR) analyses to examine the expression of several well-characterized ABA-responsive genes in ABA- and nitrate-treated germinating wild-type seeds. These genes included *LATE EMBRYOGENESIS ABUNDANT 1* (*EM1*), *EM6*, and *RESPONSIVE TO ABA 18* (*RAB18*). The ABA treatment strongly induced the expression of *EM1*, *EM6*, and *RAB18* during seed germination (Fig. 1D). Notably, the *EM1*, *EM6*, and *RAB18* transcript levels in the germinating seeds were clearly lower in the presence of both ABA and KNO_3 than in the presence of only ABA (Fig. 1D), suggesting nitrate downregulates the expression of these ABA-induced genes. These results further support the idea that nitrate has an inhibitory effect on ABA signaling during seed germination.

In addition to nitrate, the reactive nitrogen molecule NO generated during the assimilation of nitrate also promotes Arabidopsis seed germination (Bethke et al. 2006; Mur et al. 2013; Gibbs et al. 2014; Duermeyer et al. 2018). To assess whether NO is essential for the nitrate-mediated decrease in ABA signaling in germinating seeds, we analyzed the *nia1 nia2* double mutant (CS2356) with strongly reduced endogenous NO content [lacking NITRATE REDUCTASE1 (NIA1) and NIA2 involved in NO production; Bright et al. 2006; Modolo et al. 2006] and the NO-insensitive *prt6* mutant (Salk_051088) on medium containing different concentrations of ABA and 1 mM KNO_3 or KCl. The *nia1 nia2* and *prt6* seeds displayed significantly higher percentages of germination and expanded green cotyledons on medium with ABA and KNO_3 than on medium supplemented with ABA with or without KCl (Fig. 1, A to C; Supplementary Fig. S1, A and B). Consistent with these observations, the *EM1*, *EM6*, and *RAB18* expression levels in germinating *nia1 nia2* and *prt6* seeds were much lower after the treatment with both ABA and KNO_3 than after the treatment with ABA and KCl (Fig. 1D). Nevertheless, the responses of the *nia1 nia2* and *prt6* seeds to both ABA and KNO_3 differed from those of the

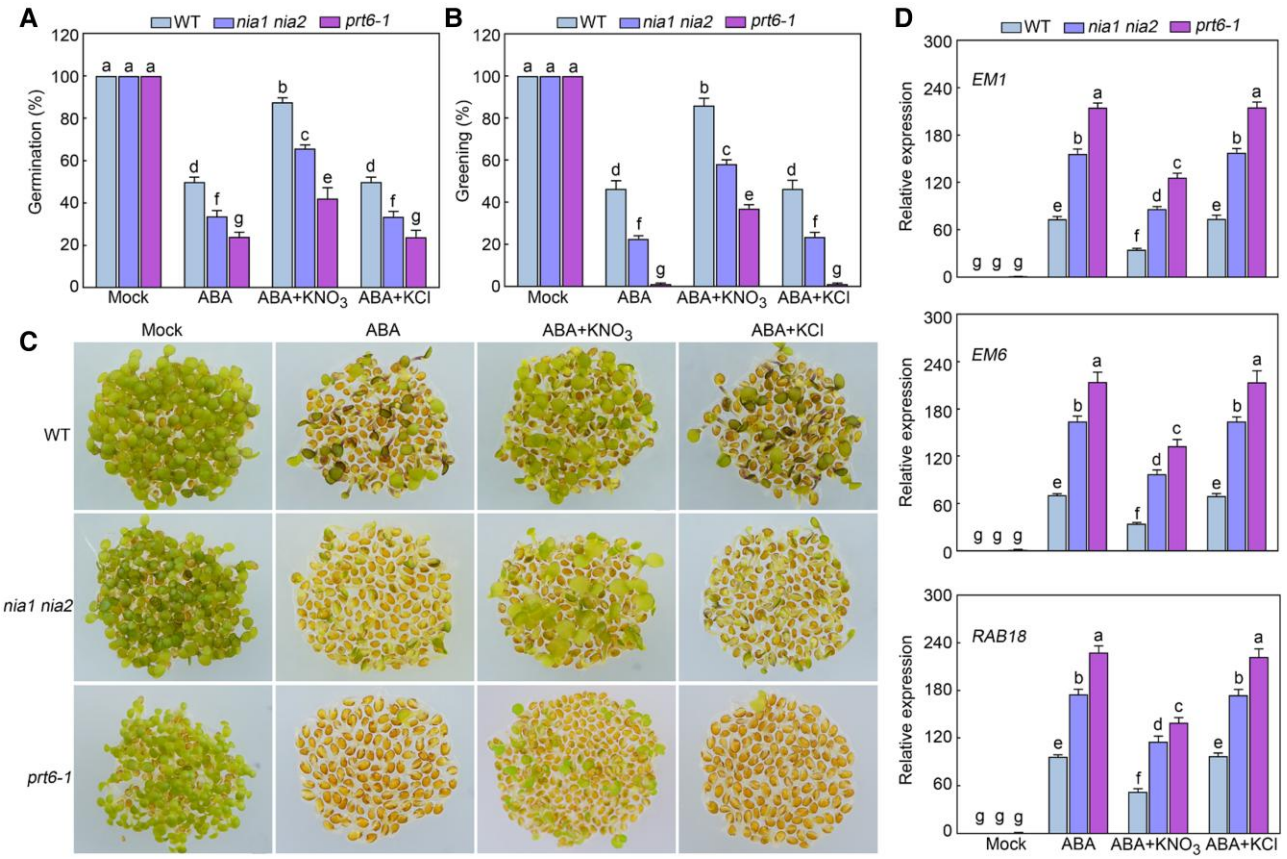

Figure 1. Nitrate attenuates ABA responses during seed germination. **A)** Germination of the wild-type (WT), *nia1 nia2*, and *prt6-1* seeds on water agar medium containing 1 μM ABA with or without 1 mM KNO_3 or KCl. Seed germination was recorded 2 d after stratification. In the mock treatment, an equal volume of 10% (v/v) ethanol was added, and the values (i.e. 100%) indicated that all seeds germinated. **B)** Cotyledon greening of WT, *nia1 nia2*, and *prt6-1* on water agar medium containing 1 μM ABA with or without 1 mM KNO_3 or KCl. Cotyledon greening was scored 5 d after stratification. The experiment was performed 5 times by analyzing different batches of seeds. Each batch of seeds was pooled from >80 individual plants. For each biological replicate, >120 seeds were examined. **C)** Seedlings of WT, *nia1 nia2*, and *prt6-1* 5 d after germination on water agar medium containing 1 μM ABA with or without 1 mM KNO_3 or KCl. **D)** RT-qPCR analysis of the ABA-induced expression of *EM1*, *EM6*, and *RAB18* in germinating seeds of WT, *nia1 nia2*, and *prt6-1*. Total RNA was extracted from 5 different batches of germinating seeds (2 d) treated with 1 μM ABA with or without 1 mM KNO_3 or KCl on water agar medium. The *PP2A* (AT1G13320) gene was used as a control. Values are means ± SD from 5 independent biological replicates using different batches of seeds. Bars with different letters are significantly different from each other ($P < 0.05$). Data were analyzed by a two-way analysis of variance (ANOVA) using Tukey's honest significant difference (HSD) test. The statistical analyses described apply to all statistical analyses presented in this figure. ABA, abscisic acid.

wild-type seeds. These findings show that the ABA-sensitive phenotype of germinating *nia1 nia2* and *prt6* seeds is partially suppressed by nitrate. Moreover, we performed a pharmacological analysis by using 150 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), an NO-specific scavenger (Bethke et al. 2006), to treat the wild-type seeds on medium containing 1 μM ABA and 1 mM KNO_3 or KCl. In the presence of cPTIO, nitrate also attenuated ABA inhibitory effect on germination and cotyledon greening of wild-type seeds (Supplementary Fig. S1, C and D). Taken together, these results suggest that nitrate functions as a regulatory signal independently of the NO pathway to mediate ABA signaling during seed germination.

Nitrate attenuates ABI3/ABI5-mediated ABA signaling during seed germination

The ABI3 and ABI5 transcription factors are two essential regulators of ABA-repressed seed germination and post-germinative growth (Yu et al. 2015; Carbonero et al. 2017). To further clarify the effects of nitrate on ABA responses, we examined whether ABI3/ABI5-mediated ABA signaling is negatively modulated by nitrate. Initially, we analyzed the influence of nitrate on the expression of *ABI3* and *ABI5*. The results showed that the transcript levels of *ABI3* and *ABI5* were decreased in germinating wild-type seeds upon simultaneous treatment with 1 μM ABA and 1 mM KNO_3, compared with those in seeds exposure to ABA with or without 1 mM KCl (Supplementary Fig. S2, A and B). Next, we investigated the performance of wild-type, *ABI3*-overexpressing [*MYC-ABI3*; containing a functional 3MYC-ABI3 construct driven by the Cauliflower mosaic virus 35S promoter (*Pro35S*)], and *ABI5*-overexpressing (*ABI5-MYC*; containing a functional ABI5-4MYC construct under the control of *Pro35S*) plants on water agar medium containing 1 μM ABA and 1 mM KNO_3 or KCl. As expected, compared with the wild-type control treated with ABA alone, the wild-type control simultaneously treated with ABA and KNO_3 had significantly higher seed germination and cotyledon greening percentages (Fig. 2, A to C). Interestingly, the application of KNO_3, but not KCl, also partially suppressed the ABA hypersensitivity of the *MYC-ABI3* and *ABI5-MYC* plants (Fig. 2, A to C). In accordance with this finding, the ABA-induced *EM1*, *EM6*, and *RAB18* expression levels in germinating *MYC-ABI3* and

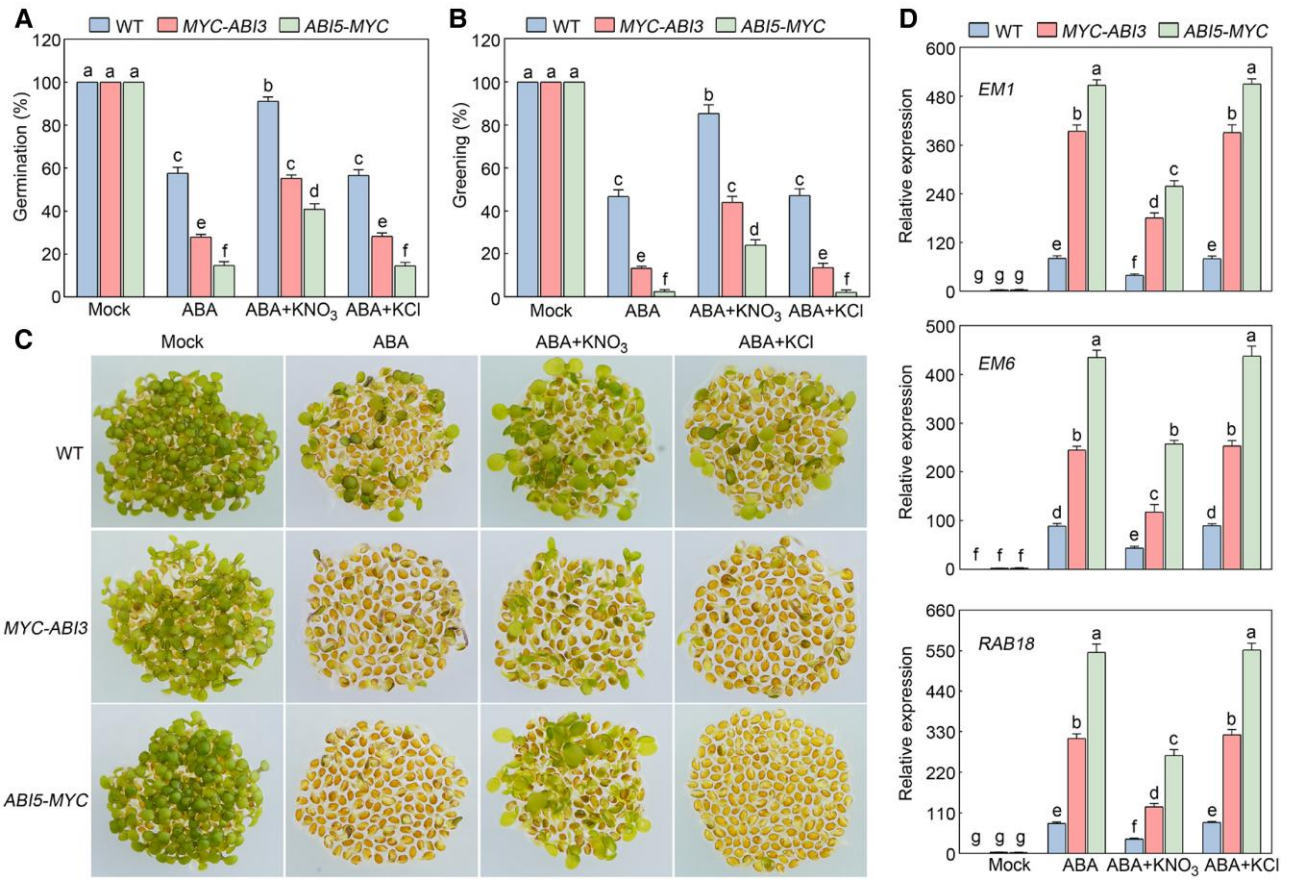

Figure 2. Nitrate represses ABI3- and ABI5-mediated ABA signaling during seed germination. **A)** Germination of the wild-type (WT), *MYC-ABI3*, and *ABI5-MYC* seeds on water agar medium containing 1 μM ABA with or without 1 mM KNO_3 or KCl. Seed germination was recorded 2.5 d after stratification. In the mock treatment, an equal volume of 10% (v/v) ethanol was added, and the values (i.e. 100%) indicated that all seeds germinated. **B)** Cotyledon greening of WT, *MYC-ABI3*, and *ABI5-MYC* on water agar medium containing 1 μM ABA with or without 1 mM KNO_3 or KCl. Cotyledon greening was scored 5 d after stratification. The experiments were performed 5 times by analyzing different batches of seeds. Each batch of seeds was pooled from more than 80 individual plants. For each biological replicate, more than 120 seeds were examined. **C)** Seedlings of WT, *MYC-ABI3*, and *ABI5-MYC* 6 d after germination on water agar medium containing 1 μM ABA with or without 1 mM KNO_3 or KCl. **D)** RT-qPCR analysis of the ABA-induced expression of *EM1*, *EM6*, and *RAB18* in germinating seeds of WT, *MYC-ABI3*, and *ABI5-MYC*. Total RNA was extracted from 5 different batches of germinating seeds (2 d) treated with 1 μM ABA with or without 1 mM KNO_3 or KCl on water agar medium. The *PP2A* (AT1G13320) gene was used as a control. Values are means ± SD from 5 independent biological replicates using different batches of seeds. Bars with different letters are significantly different from each other ($P < 0.05$). Data were analyzed by a two-way analysis of variance (ANOVA) using Tukey's honest significant difference (HSD) test. The statistical analyses described apply to all statistical analyses presented in this figure. ABA, Abscisic acid.

ABI5-MYC seeds were clearly lower after the ABA and KNO_3 treatment than after the ABA treatment (Fig. 2D). Considered together, these results imply that nitrate suppresses ABI3/ABI5-mediated ABA signaling during seed germination.

ABI3 and ABI5 physically interact with NLP8, a critical transcription factor of nitrate signaling in seeds

Considering that ABI3 and ABI5 bind to several transcriptional regulators to integrate the ABA signaling pathway with other signaling pathways (Lim et al. 2013; Pan et al. 2020; Mei et al. 2023), we wondered whether they directly interact with key components of the nitrate signaling pathway and mediate the crosstalk between ABA and nitrate signaling pathways. Hence, we analyzed the possible interactions of ABI3 and ABI5 with several NLP transcription factors that play crucial roles in nitrate-modulated gene expression and physiological processes (Konishi and Yanagisawa 2013). The full-length *ABI3* and *ABI5* sequences were fused to the Gal4 DNA-binding domain of the bait vector to generate BD-ABI3 and BD-ABI5. The full-length *NLP* (such as *NLP1*, *NLP5*, *NLP7*, *NLP8*, and *NLP9*) sequences were cloned and ligated to the Gal4 activation domain of the prey vector (AD-NLP). Both ABI3 and ABI5 interacted with NLP7, NLP8, and NLP9 in yeast (Supplementary Fig. S3A). However, there was no obvious interaction between ABI3 (or ABI5) and NLP1 or NLP5, indicative of the specificity of the associations between ABI3/ABI5 and NLP proteins.

To identify the protein region(s) required for the ABI3/ABI5–NLP interactions, we performed additional directed yeast two-hybrid assays. We selected *NLP8*, which is highly abundant in mature dry and imbibed seeds (Winter et al. 2007; Yan et al. 2016), as the representative NLP transcription factor for further analyses. Four truncated NLP8 fragments were produced and fused to the Gal4 activation domain of the prey vector (Fig. 3A). None of the truncated NLP8 fragments interacted with ABI3 in yeast (Fig. 3A), suggesting that the full-length NLP8 is required for the physical association. Parallel experiments demonstrated that removing the C-terminal of NLP8 (amino acids 671 to 934) completely eliminated the interaction with ABI5 (Fig. 3A). In contrast, deleting the N-terminal residues of NLP8 (amino acids 1 to 670) did not affect the interaction with ABI5 in yeast. These observations suggest that the C-terminal of NLP8 is sufficient for the

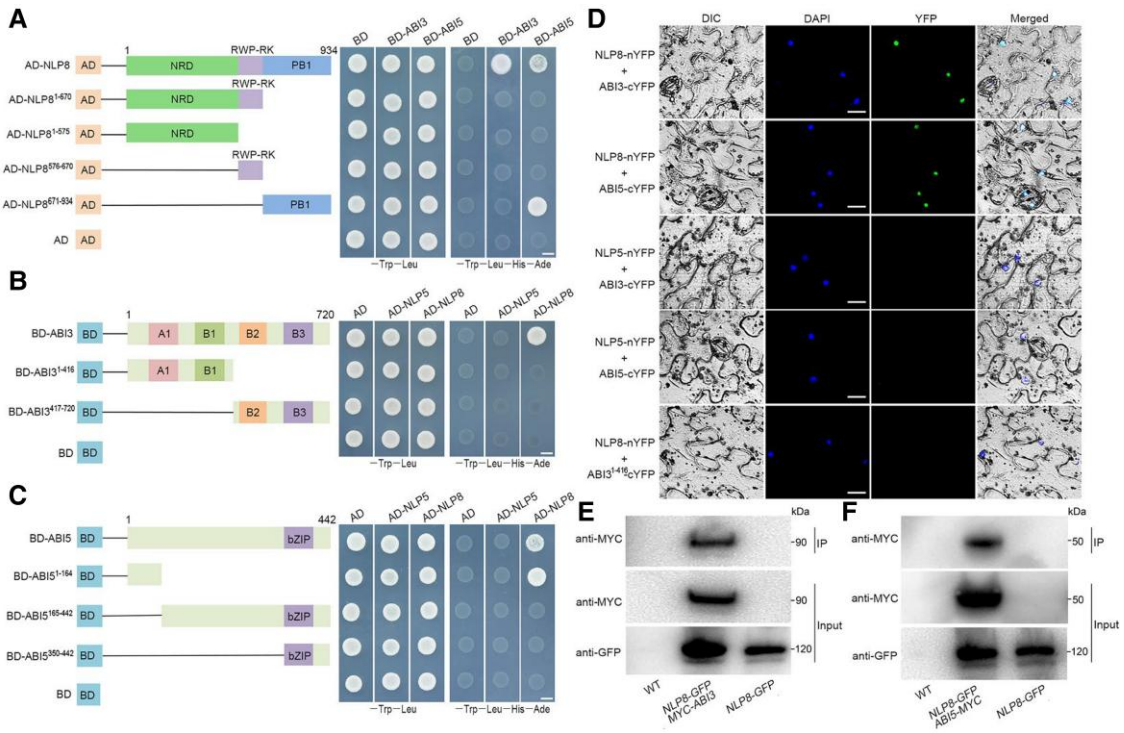

Figure 3. Physical interactions of NLP8 with ABI3 and ABI5. **A)** Mapping the ABI3/ABI5-interacting domain of NLP8 using a yeast two-hybrid assay. Left: Diagram of the full-length and truncated NLP8 constructs with specific deletions. Right: Interactions of NLP8 with ABI3 and ABI5 were indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade and containing 15 mM 3-aminotriazole after a 4-d incubation. pGBKT7 (BD) and pGADT7 (AD) were used as negative controls. Bars = 2.5 mm. **B)** The full-length of ABI3 is required for the NLP8–ABI3 interaction. Left: Diagram of the full-length and truncated ABI3 constructs with specific deletions. Right: Interaction was indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade and containing 15 mM 3-aminotriazole after a 4-d incubation. BD, AD, and AD-NLP5 were used as negative controls. Bars = 2.5 mm. **C)** The N-terminal domain of ABI5 (amino acids 1 to 164) is sufficient for the NLP8–ABI5 interaction. Left: Diagram of the full-length and truncated ABI5 constructs with specific deletions. Right: Interactions were indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade and containing 15 mM 3-aminotriazole after a 4-d incubation. BD, AD, and AD-NLP5 were used as negative controls. Bars = 2.5 mm. **D)** BiFC analyses. Fluorescence was detected in the nuclear compartment of transformed *Nicotiana benthamiana* cells, resulting from the complementation of NLP8-nYFP with ABI3-cYFP or ABI5-cYFP. No signal was observed for the negative controls in which NLP5-nYFP was co-expressed with ABI3-cYFP or ABI5-cYFP, or $ABI3^{1–416}$-cYFP (the sequence encoding the N-terminal part of ABI3 fused with cYFP) was co-expressed with NLP8-nYFP. Nuclei are indicated by DAPI staining. Bars = 15 μm. **E** and **F)** CoIP analyses. Total proteins were extracted from 0.5 μM ABA-treated germinating seeds (2.5 d) of transgenic Arabidopsis plants simultaneously overexpressing *NLP8* and *ABI3* (*NLP8-GFP MYC-ABI3*) or *NLP8* and *ABI5* (*NLP8-GFP ABI5-MYC*) under the control of *Pro35S*. GFP-fused NLP8 was immunoprecipitated using an anti-GFP antibody (1:250) and the co-immunoprecipitated MYC-ABI3 **(E)** or ABI5-MYC **(F)** was detected using an anti-MYC antibody (1:10,000). Protein input for GFP-fused NLP8 in the immunoprecipitated complexes was also detected and is shown. Experiments were performed 3 times with similar results. NRD, nitrate signal-responsive domain; RWP-RK, RWP-RK-type DNA-binding domain; PB1, Phox and Bem1; bZIP, basic leucine zipper; DIC, differential interference contrast; DAPI, 4′,6-diamidino-2-phenylindole; YFP, yellow fluorescence protein; IP, immunoprecipitation.

interaction with ABI5. Moreover, we conducted similar analyses to identify the ABI3 and ABI5 fragments essential for the binding to NLP8. The results indicated that the full-length of ABI3 and the N-terminal of ABI5 (amino acids 1 to 164) are necessary for the interaction with NLP8 in yeast (Fig. 3, B and C).

To further confirm the associations of ABI3 and ABI5 with NLP8 in plant cells, we performed bimolecular fluorescence complementation (BiFC) assays in *Nicotiana benthamiana*. The sequence encoding the C-terminal of the yellow fluorescent protein (cYFP) under the control of *Pro35S* was fused to full-length *ABI3* and *ABI5* sequences to produce ABI3-cYFP and ABI5-cYFP, respectively. The sequence encoding the N-terminal of YFP (nYFP) under the control of *Pro35S* was ligated to the full-length *NLP8* sequence to generate NLP8-nYFP. When ABI3-cYFP or ABI5-cYFP was co-expressed with NLP8-nYFP in leaves of *N. benthamiana*, strong YFP fluorescence was detected in the nucleus of the infiltrated area cells stained with 4′,6-diamidino-2-phenylindole (Fig. 3D; Supplementary Fig. S3B). However, fluorescence was undetectable in the negative controls in which NLP5-nYFP (NLP5 fused to nYFP) was co-expressed with ABI3-cYFP or ABI5-cYFP or NLP8-nYFP was co-expressed with $ABI3^{1–416}$-cYFP (416 amino acids of the ABI3 N-terminal fused to nYFP; Fig. 3D; Supplementary Fig. S3B). In addition to the BiFC assays, co-immunoprecipitation (CoIP) assays involving plant proteins provided further *in vivo* evidence of the interactions between NLP8 and ABI3/ABI5 in transgenic Arabidopsis plants simultaneously overexpressing *NLP8* and *ABI3* (*NLP8-GFP MYC-ABI3*) or *ABI5* (*NLP8-GFP ABI5-MYC*) with or without ABA treatment (Fig. 3, E and F; Supplementary Fig. S3, C and D). These plants were generated by introducing the *NLP8* overexpression construct (i.e. functional NLP8-GFP construct driven by *Pro35S*) into *MYC-ABI3* and *ABI5-MYC* plants, respectively. Collectively, these results suggest that ABI3 and ABI5 can physically associate with seed-expressed NLP8 (transcription factor regulating nitrate signaling), implying that ABI3 and ABI5 may function together with NLP8 to mediate the convergence of ABA and nitrate signaling pathways during seed germination.

Previous studies revealed that ABI5 is phosphorylated and stabilized in response to ABA (Kobayashi et al. 2005; Furihata et al. 2006; Fujii et al. 2007; Fujii and Zhu 2009). To further clarify

whether the phosphorylation status of ABI5 is involved in the interaction with NLP8, we simultaneously mutated several ABI5 phosphorylation sites, i.e. serine (Ser) or threonine (Thr) residues including Thr35, Ser36, Ser41, Ser42, Ser145, Thr201, Ser368, Ser372, and Ser439 (Lopez-Molina et al. 2002; Kobayashi et al. 2005; Hu and Yu 2014; Zhou et al. 2015), to alanine (Ala) to produce a dephosphorylation mutant form of ABI5 (designated as ABI5-A9) or to aspartic acid (Asp) to generate a stable mimic of phosphorylated ABI5 (designated as ABI5-D9). Yeast two-hybrid analyses showed that both ABI5-A9 and ABI5-D9 interacted with NLP8 in yeast (Supplementary Fig. S4A). Interestingly, ABI5-D9 displayed a stronger interaction with NLP8 compared with ABI5-A9 or the wild-type form of ABI5 (Supplementary Fig. S4A). To verify this observation, we expressed ABI5, ABI5-A9, or ABI5-D9 at similar accumulation levels in the wild-type mesophyll protoplasts with NLP8 co-expression. Further CoIP analyses demonstrated that ABI5-D9 had an enhanced interaction with NLP8 (Supplementary Fig. S4B). These results suggest that the NLP8–ABI5 physical association is stimulated by the phosphorylation of ABI5.

Considering the involvement of ABI5 phosphorylation status in the interaction with NLP8, we speculated whether ABA modulates the strength of the interaction. To test this idea, we analyzed the interaction between the wild-type ABI5 and NLP8 in response to ABA. Similar amounts of ABI5 or NLP8 were expressed in the mesophyll protoplasts with or without ABA treatment and used for the CoIP assays. As shown in Supplementary Fig. S4B, the NLP8–ABI5 interaction was clearly promoted by ABA. To further uncover the association of NLP8 and ABI5, we also investigated the possible effect of nitrate on the interaction. The results showed that, in the presence of KNO_3, ABI5 had a strengthened interaction with NLP8 in the mesophyll protoplasts (Supplementary Fig. S4C). Moreover, our parallel experiments demonstrated that ABA and KNO_3 also positively affected the interaction of NLP8 with ABI3 (Supplementary Fig. S4, D and E). Taken together, these findings imply that the NLP8–ABI3/ABI5 interactions are responsive to ABA and nitrate.

NLP8 is a negative regulator of ABA responses during seed germination

Having demonstrated that NLP8 interacts with ABI3 and ABI5, we investigated whether NLP8 contributes to the regulation of ABA signaling during seed germination. We first analyzed the expression of *NLP8* in germinating wild-type seeds with or without an ABA treatment. A putative *NLP8* promoter fragment (*ProNLP8*; 2,321 bp) upstream of the β-glucuronidase (GUS)-encoding reporter gene was cloned to generate a reporter construct (*ProNLP8:GUS*), which we introduced into wild-type plants. The GUS staining results revealed that *NLP8* was highly expressed in dry seeds and was induced by ABA during seed germination (Supplementary Fig. S5, A and B). Further RT-qPCR data reflected the substantial accumulation of *NLP8* transcripts in ABA-treated germinating seeds (Supplementary Fig. S5C), indicative of the stimulation of ABA on *NLP8* transcription during seed germination. Consistent with the results of an earlier study (Yan et al. 2016), the KNO_3 treatment exerted little impact on *NLP8* expression with or without the ABA exposure (Supplementary Fig. S5D). To examine the effects of ABA and KNO_3 on NLP8 more precisely, we generated transgenic plants (further referred to as *NLP8-GFP*; Supplementary Fig. S5E) overexpressing *NLP8* under the control of *Pro35S* and subsequently analyzed the abundance of NLP8-GFP fusion protein in response to ABA and KNO_3. The results showed that the accumulation levels of NLP8-GFP in germinating seeds were not responsive to KNO_3 but enhanced by ABA regardless of the absence or presence of KNO_3 (Supplementary Fig. S5F).

To assess whether NLP8 helps mediate responses to ABA, we analyzed the seed germination of the loss-of-function *nlp8* mutants *nlp8-2* (Salk_140298) and *nlp8-Cas9* (Supplementary Fig. S6) on water agar medium supplemented with different concentrations of ABA. The *nlp8-Cas9* mutant was generated using the CRISPR/Cas9 gene-editing system; the resulting deletion of 26 bases in the *NLP8* coding region was a frameshift mutation (Supplementary Fig. S6). As shown in Fig. 4A, the seeds of the *nlp8-2* and *nlp8-Cas9* mutants had much lower germination percentages than the wild-type seeds following treatments with different ABA concentrations. Moreover, the progeny of *nlp8-2* and *nlp8-Cas9* displayed significantly less greening than the progeny of the wild-type control (Fig. 4, B and C). To verify these observations, we examined the expression of several ABA-responsive genes, including *EM1*, *EM6*, and *RAB18*, in germinating *nlp8-2* and *nlp8-Cas9* seeds treated with 1 μM ABA. These genes were significantly more highly expressed in the germinating *nlp8-2* and *nlp8-Cas9* seeds than in the wild-type seeds after the ABA treatment (Fig. 4D). Hence, NLP8 may negatively modulate ABA responses during seed germination.

To confirm the NLP8 role in ABA signaling, we further investigated seed germination and cotyledon greening of *NLP8*-overexpressing plants (i.e. *NLP8-GFP-3* and *NLP8-GFP-5*; Supplementary Fig. S5E) on water agar medium containing various concentrations of ABA. As shown in Fig. 4, A to C, seeds of *NLP8-GFP-3* and *NLP8-GFP-5* displayed higher percentages of germination and cotyledon greening in response to ABA, compared with the wild-type controls. Correspondingly, the ABA-induced expression of *EM1*, *EM6*, and *RAB18* was lower in the germinating *NLP8*-overexpressing seeds than in the germinating wild-type seeds (Fig. 4D). Accordingly, the overexpression of *NLP8* apparently decreased the sensitivity of the germinating seeds to ABA. Overall, these results further support the idea that NLP8 suppresses ABA signaling and stimulates seed germination and early seedling growth in Arabidopsis.

In addition to NLP8, we considered whether the NLP8 homologs NLP7 and NLP9, which can also interact with ABI3 and ABI5 in yeast (Supplementary Fig. S3A), participate in ABA signaling during seed germination. Specifically, we examined the loss-of-function *nlp7-1* (Salk_026134) and *nlp9-1* (Salk_025839) mutants regarding their germination and cotyledon greening on water agar medium with or without 1 μM ABA. There were no significant differences in the seed germination and cotyledon greening between these mutants and the wild-type control (Supplementary Fig. S7, A and B), implying mutation to NLP7 or NLP9 had little effect on ABA responses during seed germination. Additionally, we analyzed the ABA sensitivity of the *chl1-5* mutant, in which a deletion eliminates the production of the nitrate sensor and transporter NRT1.1 (Wang et al. 1998; Liu et al. 1999; Ho et al. 2009). Compared with the wild-type seeds, the *chl1-5* seeds had lower germination and cotyledon greening percentages after the ABA treatment (Supplementary Fig. S7, A and B), suggesting NRT1.1 negatively affects ABA signaling during seed germination.

Nitrate inhibits ABA signaling mainly through NLP8

Because NLP8 is a transcription factor that negatively regulates ABA responses during seed germination, we speculated whether NLP8 is essential for the nitrate-induced decrease in ABA signaling. Hence, we assessed the possibility that NLP8 modulates the nitrate-repressed ABA-mediated inhibition of seed germination

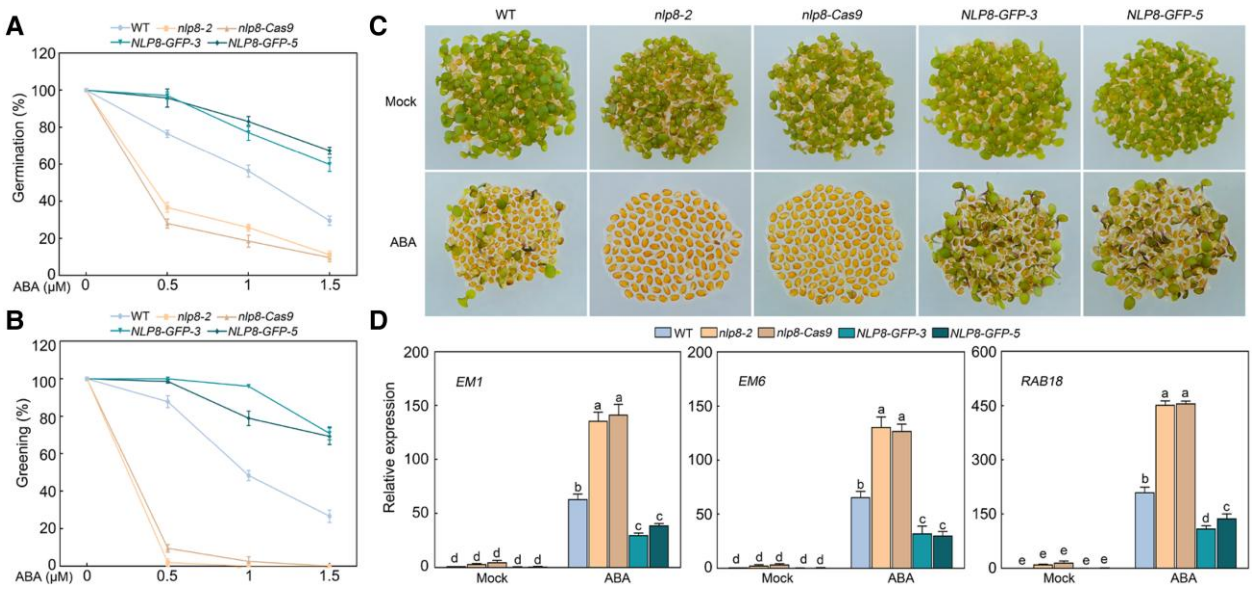

Figure 4. NLP8 negatively modulates ABA responses during seed germination. **A)** Germination of the wild-type (WT), *nlp8-2*, *nlp8-Cas9*, *NLP8-GFP-3*, and *NLP8-GFP-5* seeds on water agar medium supplemented with different concentrations of ABA. Seed germination was recorded 2.5 d after stratification. In the mock treatment, an equal volume of 10% (v/v) ethanol was added, and the values (i.e. 100%) indicated that all seeds germinated. **B)** Cotyledon greening of WT, *nlp8-2*, *nlp8-Cas9*, *NLP8-GFP-3*, and *NLP8-GFP-5* on water agar medium supplemented with different concentrations of ABA. Cotyledon greening was scored 5 d after stratification. The experiments were performed 5 times by analyzing different batches of seeds. Each batch of seeds was pooled from more than 80 individual plants. For each biological replicate, >120 seeds were examined. **C)** Seedlings of WT, *nlp8-2*, *nlp8-Cas9*, *NLP8-GFP-3*, and *NLP8-GFP-5* 5 d after germination on water agar medium containing 1 μM ABA. **D)** RT-qPCR analysis of the ABA-induced expression of *EM1*, *EM6*, and *RAB18* in germinating seeds of WT, *nlp8-2*, *nlp8-Cas9*, *NLP8-GFP-3*, and *NLP8-GFP-5*. Total RNA was extracted from 5 different batches of germinating seeds (2 d) treated with 1 μM ABA on water agar medium. The *PP2A* (AT1G13320) gene was used as a control. Values are means ± SD from 5 independent biological replicates using different batches of seeds. Bars with different letters are significantly different from each other ($P < 0.05$). Data were analyzed by a two-way ANOVA using Tukey's honest significant difference (HSD) test. The statistical analyses described apply to all statistical analyses presented in this figure. ABA, abscisic acid.

and seedling establishment. We germinated the seeds of *nlp8-2* and *NLP8-GFP-3* plants on water agar medium containing different concentrations of ABA and 1 mM KNO_3 or KCl. As expected, compared with the wild-type seeds, the *nlp8-2* seeds had significantly lower germination and cotyledon greening percentages on the medium supplemented with ABA with or without KCl (Fig. 5, A to C; Supplementary Fig. S8, A and B). More importantly, on the medium containing both ABA and KNO_3, the *nlp8-2* seeds exhibited notably delayed germination and cotyledon greening compared with the wild-type controls (Fig. 5, A to C; Supplementary Fig. S8, A and B). In contrast, in response to both ABA and KNO_3, the progeny of the *NLP8-GFP-3* plants had higher seed germination and cotyledon greening percentages than the wild-type controls (Fig. 5, A to C; Supplementary Fig. S8, A and B). Consistent with the phenotypic observations, the relative transcript levels of *EM1*, *EM6*, and *RAB18* were much higher in the germinating *nlp8-2* seeds than in the germinating wild-type seeds following the treatment with both ABA and KNO_3 (Fig. 5D). However, their expression levels were lower in the *NLP8-GFP-3* seeds than in the wild-type seeds (Fig. 5D). These findings demonstrate that NLP8 is crucial for the nitrate-suppressed ABA signaling during seed germination.

An earlier study showed that nitrate induces the catabolism of endogenous ABA in an NLP8-dependent manner in imbibed seeds (Yan et al. 2016). Conversely, environmental nitrate enhances the ABA accumulation in root tips (Ondzighi-Assoume et al. 2016). These observations prompted us to investigate whether the ABA sensitivity of *nlp8-2* and *NLP8-GFP-3* plants during seed germination was due to the altered ABA or nitrate accumulation after the ABA and KNO_3 treatment. We conducted measurements of ABA content in freshly harvested seeds of wild type, *nlp8-2*, and *NLP8-GFP-3*, as well as in germinating seeds subjected to various concentrations of ABA, with or without 1 mM KNO_3 (or KCl). Consistent with the results of Yan et al. (2016), the freshly harvested wild-type, *nlp8-2*, and *NLP8-GFP-3* seeds accumulated similar levels of ABA, but wild-type seeds had lower ABA content after 12 h KNO_3 (not KCl) treatment than *nlp8-2* seeds (Supplementary Table S1). Moreover, the ABA levels were much higher in the wild-type seeds germinated (4 d) on water agar medium containing ABA than in the wild-type seeds germinated on water agar medium lacking ABA (Supplementary Table S1). Notably, the germinating *nlp8-2* and *NLP8-GFP-3* seeds and the germinating wild-type seeds exhibited comparable ABA levels in the presence of exogenous ABA (Supplementary Table S1). Additionally, upon transferring the ABA-treated germinating seeds (at 2 d) to a medium with 1 mM KNO_3 or KCl for an additional 2 d, the levels of ABA present in the *nlp8-2* and *NLP8-GFP-3* seeds were found to be in alignment with those in wild-type seeds (Supplementary Table S1). These findings reflect the limited effect of NLP8 on the ABA content during seed germination following an exposure to ABA. Similarly, we also measured the nitrate content in germinating seeds (2 d) of wild-type, *nlp8-2*, and *NLP8-GFP-3* (as well as other related lines) in response to ABA with or without KNO_3 (or KCl). As shown in Supplementary Table S2, the nitrate levels in the *nlp8-2* and *NLP8-GFP-3* seeds were consistent with those in wild-type seeds, suggesting that NLP8 has a negligible impact on nitrate accumulation in germinating seeds after treatment with ABA and KNO_3.

NLP8 functions upstream of ABI3 and ABI5 to modulate ABA signaling

Having ascertained that NLP8 physically associates with ABI3/ABI5 and modulates nitrate-decreased ABA signaling, we

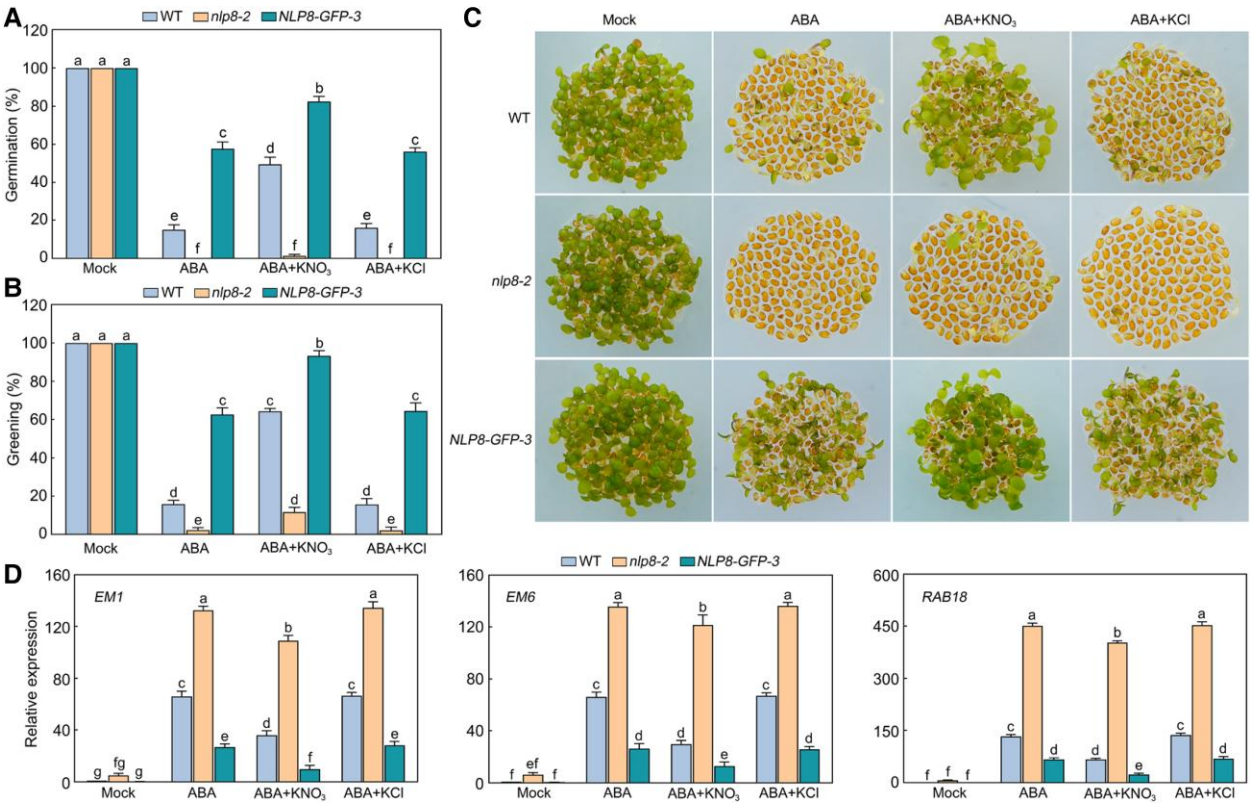

Figure 5. Nitrate suppresses ABA signaling mainly through NLP8. **A)** Germination of the wild-type (WT), *nlp8-2*, and *NLP8-GFP-3* seeds on water agar medium supplemented with 1 μM ABA with or without 1 mM KNO_3 or KCl. Seed germination was recorded 1.5 d after stratification. In the mock treatment, an equal volume of 10% (v/v) ethanol was added, and the values (i.e. 100%) indicated that all seeds germinated. **B)** Cotyledon greening of WT, *nlp8-2*, and *NLP8-GFP-3* on water agar medium supplemented with 1 μM ABA with or without 1 mM KNO_3 or KCl. Cotyledon greening was scored 4 d after stratification. The experiments were performed 5 times by analyzing different batches of seeds. Each batch of seeds was pooled from more than 80 individual plants. For each biological replicate, more than 120 seeds were examined. **C)** Seedlings of WT, *nlp8-2*, and *NLP8-GFP-3* 4 d after germination on water agar medium containing 1 μM ABA with or without 1 mM KNO_3 or KCl. **D)** RT-qPCR analysis of the ABA-induced expression of *EM1*, *EM6*, and *RAB18* in germinating seeds of WT, *nlp8-2*, and *NLP8-GFP-3*. Total RNA was extracted from 5 different batches of germinating seeds (2 d) treated with 1 μM ABA with or without 1 mM KNO_3 or KCl. The *PP2A* (AT1G13320) gene was used as a control. Values are means ± SD from 5 independent biological replicates using different batches of seeds. Bars with different letters are significantly different from each other ($P < 0.05$). Data were analyzed by a two-way analysis of variance (ANOVA) using Tukey's honest significant difference (HSD) test. The statistical analyses described apply to all statistical analyses presented in this figure. ABA, abscisic acid.

investigated whether there are genetic interactions between *NLP8* and *ABI3*/*ABI5* during ABA-induced delayed seed germination. To test this possibility, we generated the *nlp8-2 abi3-8* and *nlp8-2 abi5-8* double mutants by crossing *nlp8-2* with the *abi3-8* (*ABI3* loss-of-function mutant; Nambara et al. 2002) and *abi5-8* (*ABI5* loss-of-function mutant; Zhou et al. 2015) mutants, respectively. Not surprisingly, compared with the wild-type control, the *abi3-8* and *abi5-8* mutants had much higher seed germination and cotyledon greening percentages on water agar medium supplemented with 1 μM ABA (Fig. 6, A to C; Supplementary Fig. S9). Moreover, the progeny of the *nlp8-2 abi3-8* and *nlp8-2 abi5-8* double mutants were hyposensitive to ABA, with much higher seed germination and cotyledon greening percentages than the wild-type control and *nlp8-2* mutant (Fig. 6, A to C; Supplementary Fig. S9). Nevertheless, the ABA responses of *nlp8-2 abi3-8* and *nlp8-2 abi5-8* differed from those of the *abi3-8* and *abi5-8* single mutants. We also crossed *nlp8-2* with the *abi3-8 abi5-8* double mutant to generate the *nlp8-2 abi3-8 abi5-8* triple mutant. Phenotypic analyses showed that the *nlp8-2 abi3-8 abi5-8* mutant had the same seed germination and cotyledon greening percentages as the *abi3-8 abi5-8* double mutant in response to ABA (Fig. 6, A to C; Supplementary Fig. S9). These results demonstrate that the ABA hypersensitivity of *nlp8-2* during seed germination requires functional ABI3 and ABI5, indicating that NLP8 functions upstream of ABI3 and ABI5 to modulate the ABA-mediated suppression of seed germination and seedling establishment.

NLP8 negatively modulates the transcriptional activation and ABA-induced accumulation of ABI3 and ABI5

Because NLP8 physically and genetically interacts with ABI3 and ABI5 in mediating ABA responses during seed germination, we characterized the biochemical mechanisms underlying the regulatory relationships between NLP8 and ABI3/ABI5. Recently, the modulatory effects of several critical proteins on ABI3 and/or ABI5 were revealed by altering transcription factor activities via protein–protein interactions (Lim et al. 2013; Kim et al. 2016; Hu et al. 2019; Ju et al. 2019; Zhao et al. 2019; Pan et al. 2018, 2020; Yang et al. 2021; Mei et al. 2023). Hence, we analyzed the possible regulatory effects of NLP8 on ABI3 and ABI5 functions in Arabidopsis mesophyll protoplasts by performing dual-luciferase (LUC) reporter assays (Yoo et al. 2007). The effectors contained *NLP8*, *ABI3*, *ABI5*, or *GFP* under the control of *Pro35S* (Supplementary Fig. S10A). Because *EM1* and *EM6* are downstream targets of ABI3 and ABI5 (Lopez-Molina and Chua 2000;

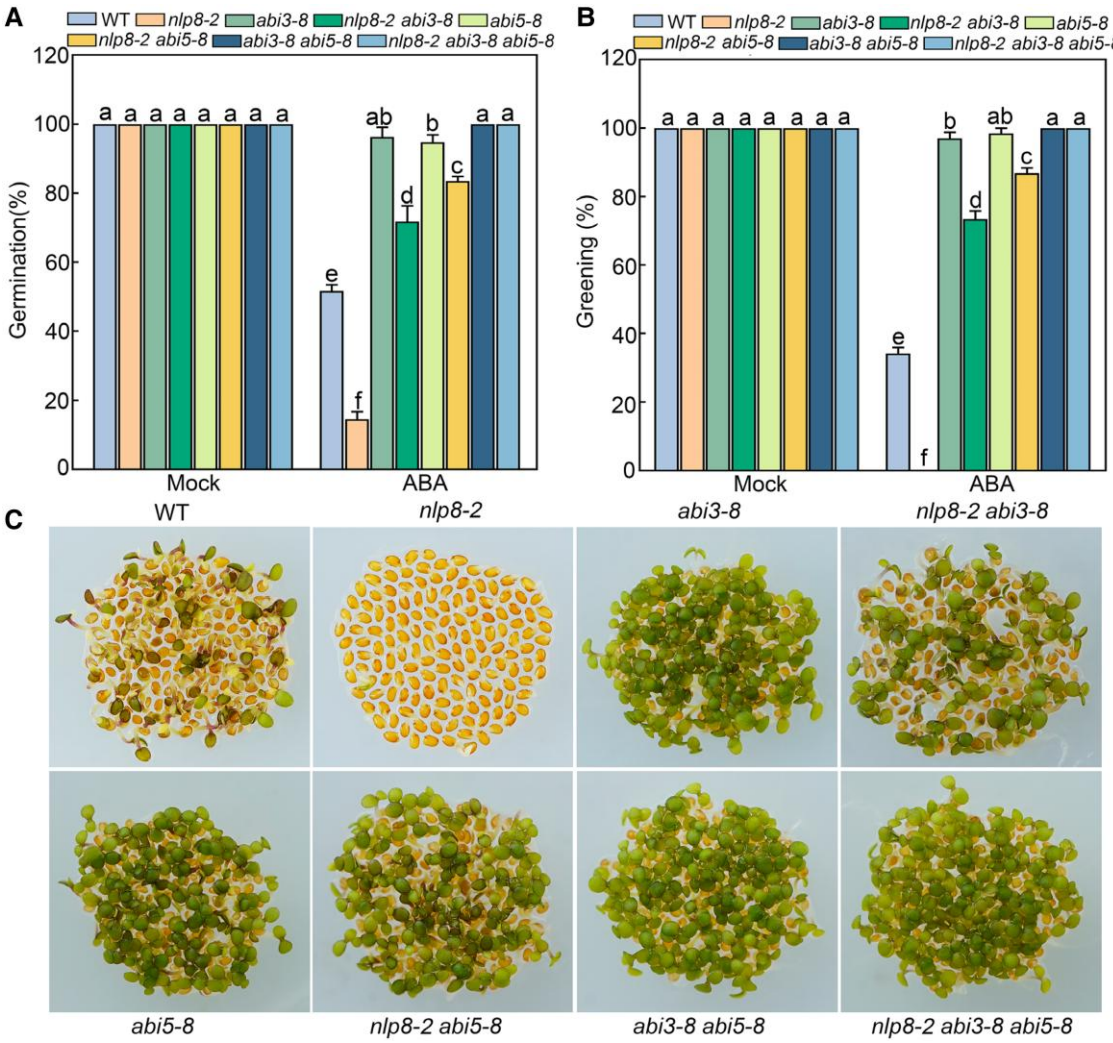

Figure 6. The ABA hypersensitivity of *nlp8-2* during seed germination requires ABI3 and ABI5. **A)** Germination of the wild-type (WT), *nlp8-2*, *abi3-8*, *nlp8-2 abi3-8*, *abi5-8*, *nlp8-2 abi5-8*, *abi3-8 abi5-8*, *nlp8-2 abi3-8 abi5-8* seeds on water agar medium containing 1 μM ABA. Seed germination was recorded 2 d after stratification. In the mock treatment, an equal volume of 10% (v/v) ethanol was added, and the values (i.e. 100%) indicated that all seeds germinated. **B)** Cotyledon greening of WT, *nlp8-2*, *abi3-8*, *nlp8-2 abi3-8*, *abi5-8*, *nlp8-2 abi5-8*, *abi3-8 abi5-8*, *nlp8-2 abi3-8 abi5-8* on water agar medium supplemented with 1 μM ABA. Cotyledon greening was scored 5 d after stratification. The experiments were performed 5 times by analyzing different batches of seeds. Each batch of seeds was pooled from more than 80 individual plants. For each biological replicate, more than 120 seeds were examined. Values are means ± SD from 5 independent biological replicates using different batches of seeds. Bars with different letters are significantly different from each other ($P < 0.05$). Data were analyzed by a two-way analysis of variance (ANOVA) using Tukey's honest significant difference (HSD) test. The statistical analyses described apply to all statistical analyses presented in this figure. **C)** Seedlings of WT, *nlp8-2*, *abi3-8*, *nlp8-2 abi3-8*, *abi5-8*, *nlp8-2 abi5-8*, *abi3-8 abi5-8*, *nlp8-2 abi3-8 abi5-8* 5 d after germination on water agar medium containing 1 μM ABA. ABA, abscisic acid.

Nakamura et al. 2001; Carles et al. 2002; Lopez-Molina et al. 2002), their promoters were ligated to the *LUC* gene to produce reporter constructs (Supplementary Fig. S10A). The expression of *ABI3* or *ABI5* dramatically increased the *LUC* expression levels driven by the *EM1* or *EM6* promoter in wild-type mesophyll protoplasts treated with 5 μM ABA (compared with the expression in the presence of GFP alone) (Fig. 7, A and B; Supplementary Figs. S10, B and C and S11, A and B; Zhou et al. 2015; Hu et al. 2019; Pan et al. 2020; Mei et al. 2023). However, the ABA-induced *LUC* expression was much lower when *NLP8* and *ABI3* or *ABI5* were co-expressed than when *GFP* and *ABI3* or *ABI5* were co-expressed (Fig. 7, A and B; Supplementary Figs. S10, B to E and S11, A and B). These findings suggest that NLP8 adversely affects ABI3 and ABI5 functions, thereby influencing the transcription of the downstream target genes *EM1* and *EM6*.

To confirm the effect of NLP8 on ABI3 and ABI5, we assessed whether a mutation to NLP8 that alters its functions can affect ABI3 and ABI5 activities. The abilities of ABI3 and ABI5 to activate *EM1* or *EM6* transcription were examined in *nlp8-2* protoplasts. We observed that the expression of *LUC* driven by the *EM1* or *EM6* promoter in response to ABA was higher in ABI3- or ABI5-expressing *nlp8-2* protoplasts than in the wild-type protoplasts (Fig. 7, C and D; Supplementary Figs. S10, F and G and S11, C and D). In contrast, *EM1* or *EM6* promoter-driven *LUC* expression was lower in the *NLP8*-overexpressing *NLP8-GFP-3* protoplasts than in the wild-type protoplasts (Fig. 7, C and D; Supplementary Figs. S10, F and G and S11, C and D). To determine whether the NLP8–ABI3/ABI5 protein interaction is necessary for the regulatory effect, we constructed an effector comprising a truncated NLP8 protein (referred to as NLP8-N) which contains amino acids 1 to 670 of the NLP8 N-terminal and does not interact with ABI3 and ABI5 (Fig. 3A; Supplementary Fig. S10A). The *EM1* or *EM6* promoter-driven *LUC* expression level did not differ significantly between the protoplasts co-expressing *NLP8-N* and *ABI3* or *ABI5* and the protoplasts

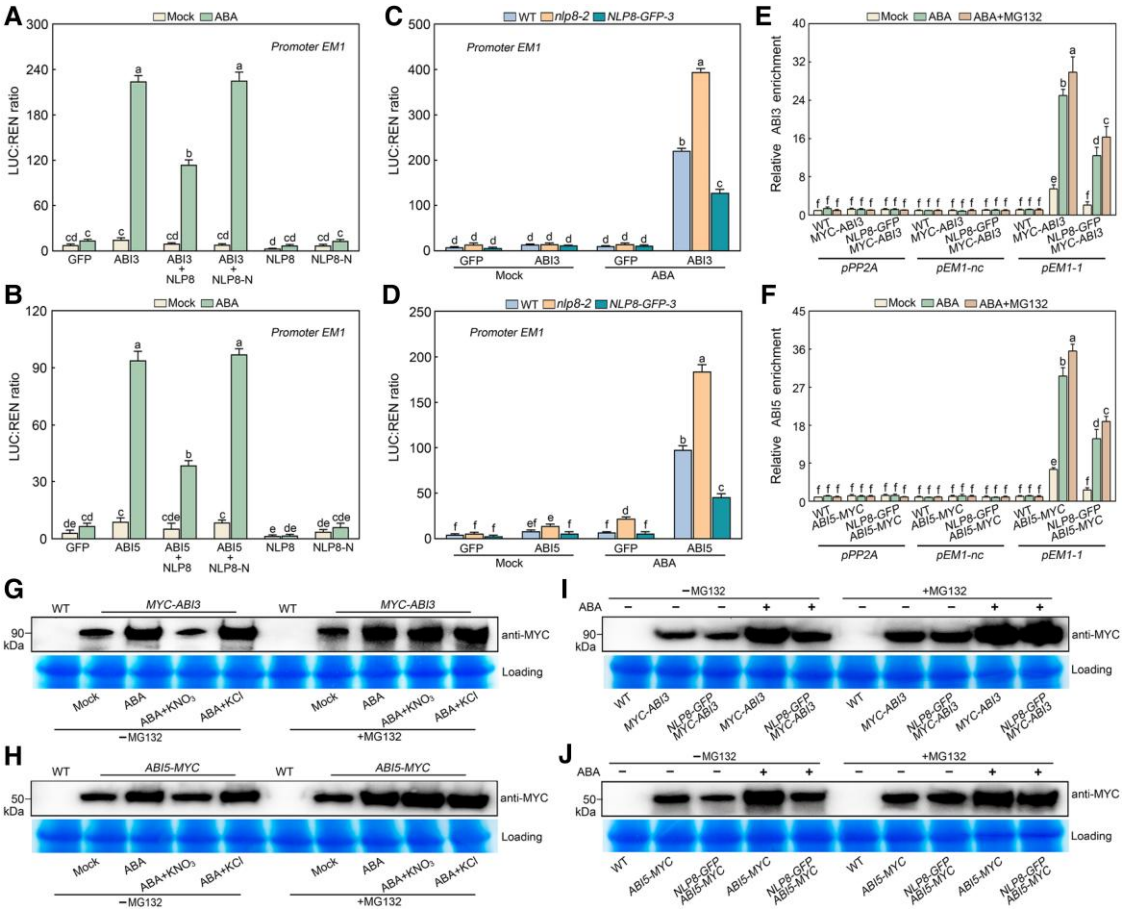

Figure 7. NLP8 represses the transcriptional functions and ABA-induced protein accumulation of ABI3 and ABI5. **A** and **B)** Transient dual-luciferase reporter assays showing that NLP8 antagonizes ABI3 **(A)** and ABI5 **(B)** to modulate *EM1* expression upon 5 μM ABA treatment. The N terminal of NLP8 without the C-terminal PB1 domain (NLP8-N, which does not interact with ABI3 and ABI5) exerts no effect on ABI3 and ABI5 functions. Values are means ± SD from 5 independent biological replicates using different batches of wild-type (WT) plants; each replicate was from different WT leaves of more than 50 plants. **C** and **D)** Transient dual-luciferase reporter assays showing that activation of the *EM1* promoter by ABI3 **(C)** and ABI5 **(D)** was enhanced in the *nlp8-2* mutant in response to 5 μM ABA. Values are means ± SD from 5 independent biological replicates using different batches of WT, *nlp8-2*, and *NLP8-GFP-3* plants; each replicate was from different leaves of more than 50 plants. **E** and **F)** ChIP-qPCR analysis of the enrichment of ABI3 **(E)** or ABI5 **(F)** in the promoter region of *EM1* (*pEM1-1*) in *MYC-ABI3*, *NLP8-GFP MYC-ABI3*, *ABI5-MYC*, or *NLP8-GFP ABI5-MYC*. Germinating seeds (2 d) treated with 1 μM ABA on water agar medium, with or without the 26S proteasome inhibitor MG132 pre-treatment (for 6 h) before the sample harvest, were used in ChIP assays. More than 500 germinating seeds for each sample were pooled for ChIP assays using an anti-MYC antibody. qPCR data from the ChIP assays with the *PP2A* (AT1G13320) promoter region sequence (*pPP2A*) were used as a negative control. The enrichment levels of ABI3 or ABI5 at a *EM1* promoter region without binding motif (i.e. *pEM1-nc*) were also detected and shown. Values are means ± SD from 5 independent biological replicates using different batches of seeds. Bars with different letters are significantly different from each other ($P < 0.05$). Data were analyzed by a two-way analysis of variance (ANOVA) using Tukey's honest significant difference (HSD) test. The statistical analyses described apply to all statistical analyses presented in this figure. **G** and **H)** Immunoblot analyzing the ABA-induced accumulation of MYC-ABI3 fusion protein in *MYC-ABI3* plants **(G)** and ABI5-MYC fusion protein in *ABI5-MYC* plants **(H)** in response to nitrate. Whole seedlings of 5-d-old WT, *MYC-ABI3*, and *ABI5-MYC* were pre-treated with 10 mM KNO_3 or KCl for 18 h and then treated with 100 μM ABA and 10 mM KNO_3 or KCl for 6 h (with or without 50 μM proteasome inhibitor MG132) before protein extraction. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. **I** and **J)** Immunoblot analyzing the ABA-induced accumulation of MYC-ABI3 fusion protein in *NLP8-GFP MYC-ABI3* plants **(I)** and ABI5-MYC fusion protein in *NLP8-GFP ABI5-MYC* plants **(J)**. Whole seedlings of 5-d-old WT, *MYC-ABI3*, *NLP8-GFP MYC-ABI3*, *ABI5-MYC*, and *NLP8-GFP ABI5-MYC* were treated with 100 μM ABA for 6 h (with or without 50 μM proteasome inhibitor MG132) before protein extraction. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. The accumulation of MYC-ABI3 or ABI5-MYC fused protein was detected by immunoblotting with an anti-MYC antibody (1:10,000). Experiments were repeated 3 times with similar results. LUC, firefly luciferase; REN, renilla luciferase; ABA, abscisic acid; MG132, carbobenzoxy-leu-leu-leucinal.

expressing *ABI3* or *ABI5* after the treatment with ABA (Fig. 7, A and B; Supplementary Figs. S10, H and I and S11, A and B). Collectively, these results support the notion that NLP8 attenuates the functions of ABI3 and ABI5 to modulate the expression of the downstream target genes, such as *EM1* or *EM6*, in response to ABA. Moreover, their physical interaction is crucial for the regulatory relationship.

Considering NLP8 and ABI3/ABI5 have the opposite effects on the ABA-induced expression of *EM1* and *EM6*, we wondered whether NLP8 directly modulates the expression levels of these two genes. In the yeast one-hybrid assays, NLP8 did not bind to the *EM1* and *EM6* promoters (Supplementary Fig. S12, A and B). To further clarify the regulatory effects of NLP8 on ABI3 and ABI5, we conducted chromatin immunoprecipitation (ChIP) assays to analyze whether the enrichment of ABI3 or ABI5 at the *EM1* promoter is modulated by NLP8 after an ABA treatment. Our ChIP analyses revealed that the enrichment of ABI3 at the *EM1* promoter region (i.e. *pEM1-1*; Supplementary Table S3) was lower in the germinating *NLP8-GFP MYC-ABI3* seeds (simultaneously overexpressing *NLP8* and *ABI3*) than in the *MYC-ABI3* seeds

following the ABA exposure, regardless of the absence or presence of the 26S proteasome inhibitor MG132 pre-treatment (for 6 h) before the sample harvest (Fig. 7E). Parallel experiments indicated that the enrichment of ABI5 at *pEM1-1* was considerably lower in the ABA-treated germinating seeds of *NLP8-GFP ABI5-MYC* (simultaneously overexpressing *NLP8* and *ABI5*) than in the corresponding control *ABI5-MYC* seeds (Fig. 7F). These results imply that NLP8 may negatively affect the accumulation of ABI3 or ABI5 in the promoter region of downstream target genes, such as *EM1*. To further verify this phenomenon, we generated recombinant His-ABI5 and His-NLP8 proteins in *Escherichia coli* and analyzed the influence of NLP8 on ABI5 binding activity to an oligonucleotide harboring two direct CACGTG G-box repeats (Pr in Supplementary Fig. S13A) using electrophoretic mobility shift assay (EMSA). As shown in Supplementary Fig. S13B, the protein–DNA complex with reduced migration was observed when His-ABI5 was incubated with the Pr DNA probes. As a control, no binding complex was detected when the G-box sequences in the probe were mutated from CACGTG to CACGAA (mPr; Supplementary Fig. S13, A and B), indicative of the specificity of ABI5 binding to the G-box sequences. Importantly, when His-ABI5, His-NLP8, and Pr DNA probes were combined in the binding reaction, we detected a band with considerably lower intensity (Supplementary Fig. S13B). This observation further supports the notion that NLP8 decreases the DNA-binding activity of ABI5.

Previous studies showed that the stability of ABI3 and ABI5 is strictly controlled by multiple regulators and signaling pathways at the post-translational level (Kobayashi et al. 2005; Zhang et al. 2005; Furihata et al. 2006; Fujii et al. 2007; Piskurewicz et al. 2008; Fujii and Zhu 2009; Lee et al. 2010; Seo et al. 2014; Li et al. 2023). These findings compelled us to examine whether nitrate and NLP8 also modulate the abundance of ABI3 and ABI5 during seed germination. We analyzed the ABI3 and ABI5 protein levels in *MYC-ABI3* and *ABI5-MYC* seedlings (5-d-old) following an exposure to 100 μM ABA (for 6 h) with or without KNO_3 (or KCl). In accordance with the findings of earlier studies (Lopez-Molina et al. 2001; Piskurewicz et al. 2008; Chen et al. 2012; Li et al. 2023), we observed that the MYC-ABI3 and ABI5-MYC fusion proteins were stabilized by ABA in the *MYC-ABI3* and *ABI5-MYC* seedlings, respectively (Fig. 7, G and H; Supplementary Fig. S14, A and B). Interestingly, the ABA-induced accumulation of the MYC-ABI3 and ABI5-MYC fusion proteins in seedlings decreased considerably in response to nitrate (Fig. 7, G and H; Supplementary Fig. S14, A and B). As a control, the addition of KCl had a relatively minor effect on the ABA-induced accumulation of MYC-ABI3 and ABI5-MYC (Fig. 7, G and H; Supplementary Fig. S14, A and B). However, in the presence of MG132, MYC-ABI3 (or ABI5-MYC) accumulated to similar levels in *MYC-ABI3* (or *ABI5-MYC*) seedlings after the ABA treatment with or without KNO_3 or KCl. To further confirm these observations, we also analyzed the MYC-ABI3 (or ABI5-MYC) protein levels in *MYC-ABI3* (or *ABI5-MYC*) seeds germinating (for 2 d) on agar water medium containing 1 μM ABA with or without KNO_3 (or KCl). Under both ABA and KNO_3 treatment, the accumulation of MYC-ABI3 (or ABI5-MYC) in germinating *MYC-ABI3* (or *ABI5-MYC*) seeds was obviously reduced compared with their appearance under ABA treatment alone or with KCl (Supplementary Fig. S15, A to D). Moreover, we also detected that the ABA-induced native ABI5 protein levels were dramatically reduced in germinating wild-type seeds treated with KNO_3 (Supplementary Fig. S15, E and F). In addition, the ABA-induced accumulation of native ABI5 was also decreased in germinating *prt6* mutant seeds in response to KNO_3 (Supplementary Fig. S15, E and F). These findings demonstrate that nitrate negatively modulates the ABA-induced accumulation of ABI3 and ABI5 proteins during seed germination.

Having elucidated the effect of nitrate on ABA-induced ABI3 and ABI5 accumulation, we then analyzed whether NLP8 is also involved in the regulation of ABI3 and ABI5 abundance. More specifically, we examined the accumulation of the MYC-ABI3 and ABI5-MYC fusion proteins in 100 μM ABA-treated (for 6 h) *NLP8-GFP MYC-ABI3* and *NLP8-GFP ABI5-MYC* seedlings (5-d-old), respectively. The MYC-ABI3 protein levels were lower in the ABA-treated *NLP8-GFP MYC-ABI3* seedlings than in the corresponding *MYC-ABI3* controls (Fig. 7I; Supplementary Fig. S14C). Similarly, ABI5-MYC was clearly less abundant in the *NLP8-GFP ABI5-MYC* seedlings than in the *ABI5-MYC* seedlings in response to ABA (Fig. 7J; Supplementary Fig. S14D). Notably, the NLP8-mediated degradation of MYC-ABI3 and ABI5-MYC was attenuated by the 26S proteasome inhibitor MG132 (Fig. 7, I and J). Taken together, these findings suggest that nitrate and NLP8 promote the degradation of ABI3 and ABI5 via the 26S proteasome pathway, thereby decreasing their suppressive effects on seed germination and early seedling establishment.

On the basis of the adverse effects of NLP8 on ABI3 and ABI5, we investigated whether the ABA responses of *MYC-ABI3* and *ABI5-MYC* plants were influenced by the overexpression of *NLP8* during seed germination. We first compared the *MYC-ABI3* and *NLP8-GFP MYC-ABI3* seed germination and cotyledon greening percentages in response to ABA. The progeny of *NLP8-GFP MYC-ABI3* had significantly higher seed germination and cotyledon greening percentages than *MYC-ABI3* on water agar medium containing 1 μM ABA (Fig. 8A), implying that the ABA hypersensitivity of *MYC-ABI3* decreased in response to the overexpression of *NLP8*. Similarly, the *NLP8-GFP ABI5-MYC* seeds were substantially less sensitive to ABA than the *ABI5-MYC* seeds during germination (Fig. 8A), indicating the overexpression of *NLP8* also attenuated the enhanced ABA signaling in the *ABI5-MYC* seeds. Furthermore, we examined the ABA sensitivity of *nlp8-2 MYC-ABI3* and *nlp8-2 ABI5-MYC* plants, which were obtained by crossing the *nlp8-2* mutant with the *MYC-ABI3* or *ABI5-MYC* plants, respectively. Compared with the *nlp8-2* and *MYC-ABI3* plants, the *nlp8-2 MYC-ABI3* plants had much lower seed germination and cotyledon greening percentages on medium containing ABA (Fig. 8, B and C). Likewise, the percentages of seed germination and cotyledon greening were lower for *nlp8-2 ABI5-MYC* than for *nlp8-2* and *ABI5-MYC* in the presence of ABA. Considered together, these results provide further genetic evidence that NLP8 inhibits ABI3 and ABI5 to modulate ABA responses during seed germination.

The critical adverse effects of NLP8 on ABI3 and ABI5 prompted us to further investigate the biological relevance of their interactions in modulating ABA signaling during seed germination. We generated transgenic plants (termed *HA-NLP8-N*) overexpressing a truncated form of NLP8 (i.e. NLP8-N with no interaction with ABI3 and ABI5; Fig. 3A) under the control of *Pro35S*. Phenotypic analyses showed that the progeny of *HA-NLP8-N* plants exhibited similar germination and cotyledon greening percentages upon ABA treatment as those of the wild-type seeds (Supplementary Fig. S16, A and B). This observation shows that overexpression of *NLP8-N* did not affect ABA signaling-related delayed seed germination. Considering together with the results that NLP8-N displayed no inhibitory impact on the transcriptional activation of ABI3 and ABI5 (Fig. 7, A and B; Supplementary Fig. S11, A and B), we speculated that the protein–protein interaction is crucial for NLP8 to antagonize ABI3 and ABI5 in ABA signaling during seed germination.

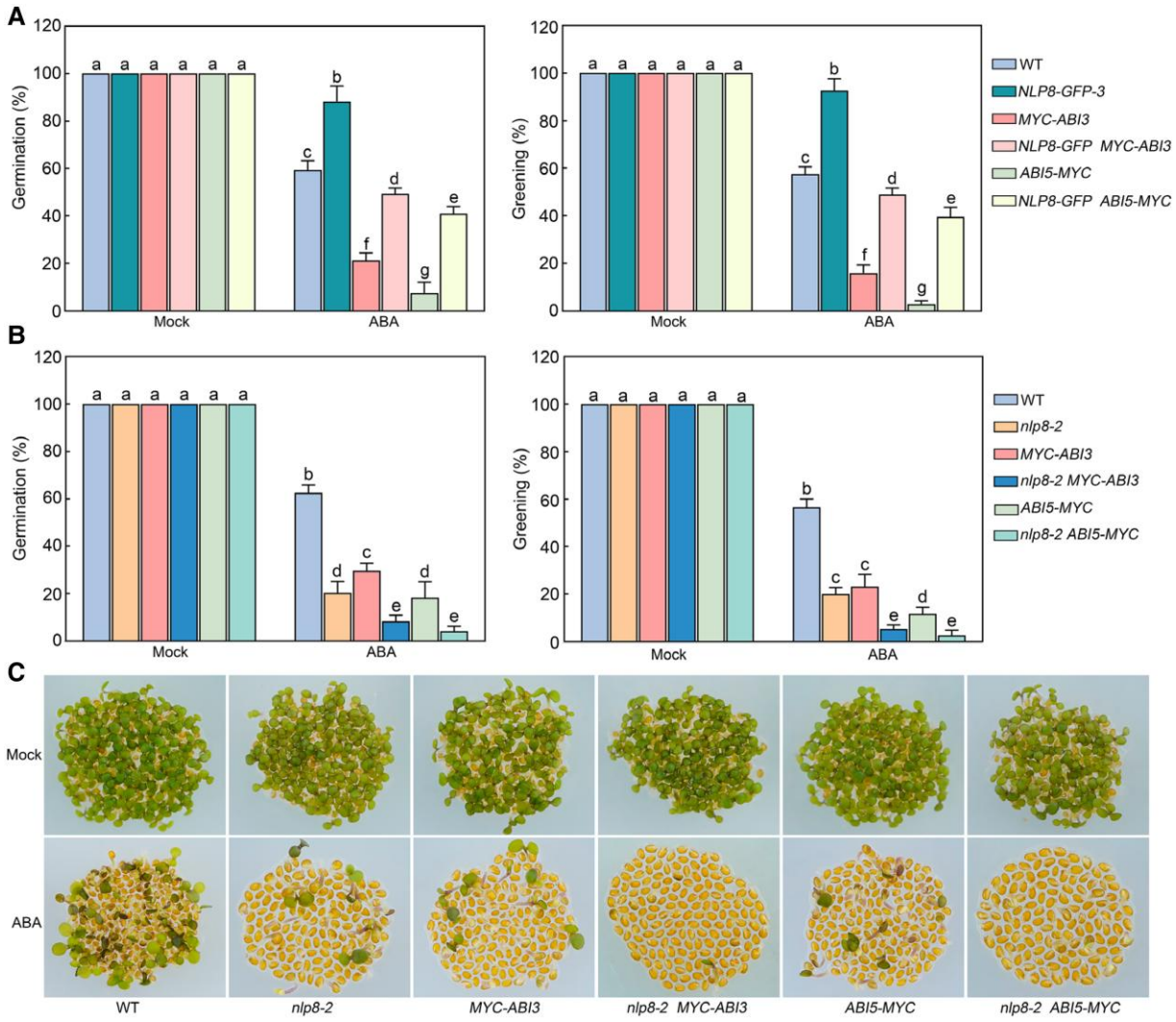

Figure 8. NLP8 antagonizes ABI3/ABI5-mediated ABA signaling during seed germination. **A)** Germination (Left) and cotyledon greening (Right) of the wild-type (WT), *NLP8-GFP-3*, *MYC-ABI3*, *NLP8-GFP MYC-ABI3*, *ABI5-MYC*, and *NLP8-GFP ABI5-MYC* on water agar medium supplemented with 1 μM ABA. Seed germination and cotyledon greening were scored 2.5 and 6 d after stratification, respectively. In the mock treatment, an equal volume of 10% (v/v) ethanol was added, and the values (i.e. 100%) indicated that all seeds germinated. **B)** Germination (Left) and cotyledon greening (Right) of WT, *nlp8-2*, *MYC-ABI3*, *nlp8-2 MYC-ABI3*, *ABI5-MYC* and *nlp8-2 ABI5-MYC* on water agar medium supplemented with 1 μM ABA. Seed germination and cotyledon greening were scored 2.5 and 6 d after stratification, respectively. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. The experiments were performed 5 times by analyzing different batches of seeds. Each batch of seeds was pooled from >80 individual plants. For each biological replicate, >120 seeds were examined. Values are means ± SD from 5 independent biological replicates using different batches of seeds. Bars with different letters are significantly different from each other ($P < 0.05$). Data were analyzed by a two-way analysis of variance (ANOVA) using Tukey's honest significant difference (HSD) test. The statistical analyses described apply to all statistical analyses presented in this figure. **C)** Seedlings of WT, *nlp8-2*, *MYC-ABI3*, *nlp8-2 MYC-ABI3*, *ABI5-MYC* and *nlp8-2 ABI5-MYC* 6 d after germination on water agar medium containing 1 μM ABA. ABA, Abscisic acid.

NLP8 overexpression largely represses the ABA hypersensitivity of the *nia1 nia2* and *prt6* mutants during seed germination

Earlier research showed that NO produced during the assimilation of nitrate negatively regulates ABA signaling during seed germination (Bethke et al. 2006; Mur et al. 2013; Gibbs et al. 2014; Duermeyer et al. 2018). Specifically, NO suppresses *ABI5* transcription through the PRT6-mediated N-end rule pathway and induces the degradation of ABI5 via the proteasome pathway (Gibbs et al. 2014; Albertos et al. 2015). Because nitrate can function independently of the NO pathway to repress ABA responses (Fig. 1; Supplementary Fig. S1), we wondered whether the overexpression of *NLP8* decreases the ABA hypersensitivity of the *nia1 nia2* or *prt6* mutants exhibiting impaired NO biosynthesis or signaling. For these analyses, we generated *NLP8-GFP nia1 nia2* and *NLP8-GFP prt6* plants by crossing *NLP8-GFP-3* with *nia1 nia2* or *prt6*. As expected, on the water agar medium containing 1 μM ABA, the seed germination and cotyledon greening percentages were higher for *NLP8-GFP-3*, but lower for *nia1 nia2* and *prt6*, than for the wild-type control (Fig. 9, A to D; Supplementary Fig. S17). Notably, the seed germination and cotyledon greening percentages were significantly higher for *NLP8-GFP nia1 nia2* than for *nia1 nia2* after the ABA treatment (Fig. 9, A and B; Supplementary Fig. S17A). Similarly, *NLP8-GFP prt6* had higher seed germination and cotyledon greening percentages than *prt6* on the water agar medium supplemented with ABA (Fig. 9, C and D; Supplementary Fig. S17B). Accordingly, the overexpression of *NLP8* considerably decreased the ABA hypersensitivity of the

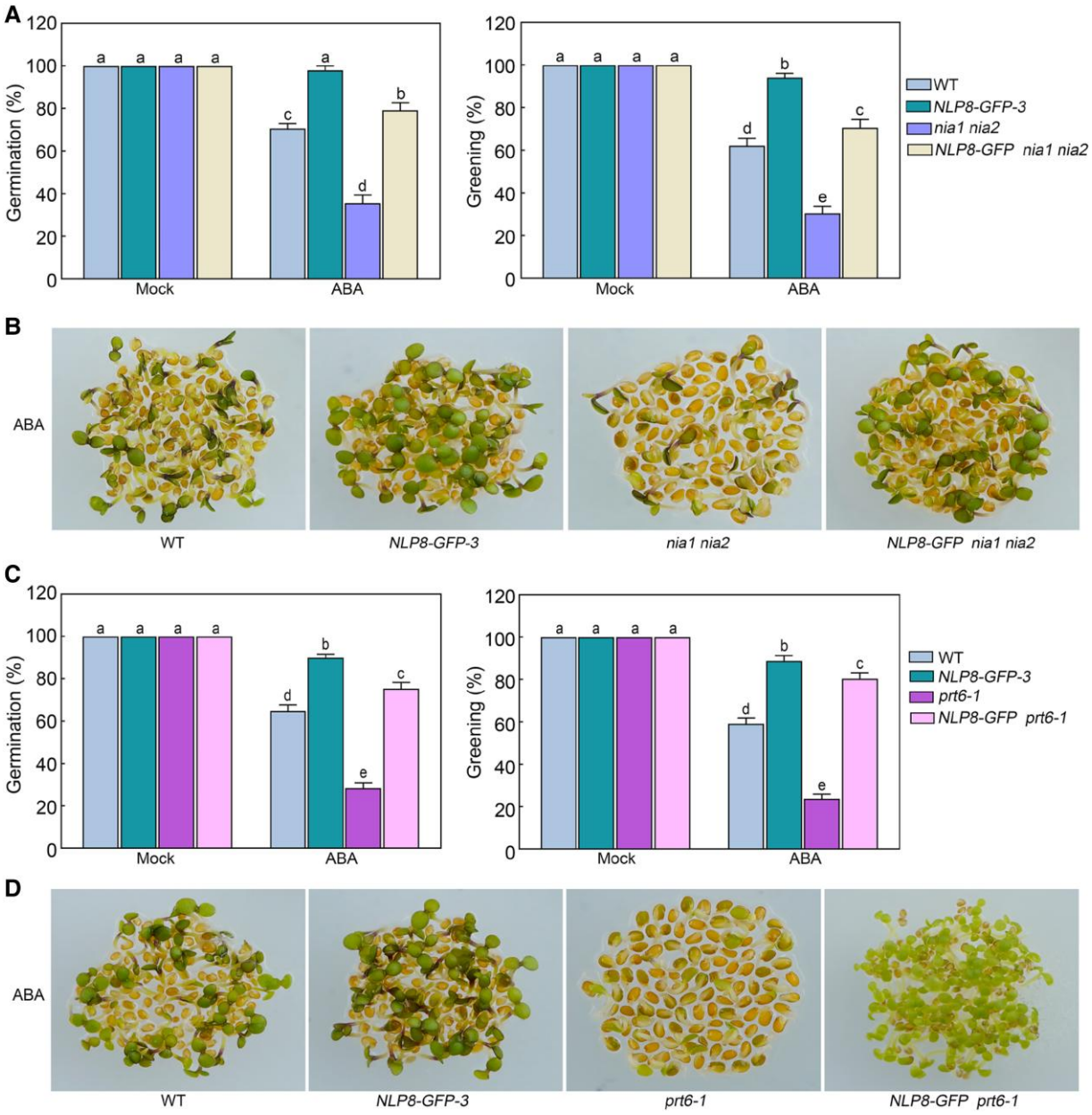

Figure 9. The ABA hypersensitivity of the *nia1 nia2* and *prt6* mutants was largely repressed by *NLP8* overexpression during seed germination. **A)** Germination (Left) and cotyledon greening (Right) of the wild-type (WT), *NLP8-GFP-3*, *nia1 nia2*, and *NLP8-GFP nia1 nia2* on water agar medium supplemented with 1 μM ABA. Seed germination and cotyledon greening were scored 3 and 6 d after stratification, respectively. In the mock treatment, an equal volume of 10% (v/v) ethanol was added, and the values (i.e. 100%) indicated that all seeds germinated. **B)** Seedlings of WT, *NLP8-GFP-3*, *nia1 nia2*, and *NLP8-GFP nia1 nia2* 7 d after germination on water agar medium containing 1 μM ABA. **C)** Germination (Left) and cotyledon greening (Right) of WT, *NLP8-GFP-3*, *prt6-1*, and *NLP8-GFP prt6-1* on water agar medium supplemented with 1 μM ABA. Seed germination and cotyledon greening were scored 2.5 and 6 d after stratification, respectively. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. **D)** Seedlings of WT, *NLP8-GFP-3*, *prt6-1*, and *NLP8-GFP prt6-1* 7 d after germination on water agar medium containing 1 μM ABA. The experiments were performed 5 times by analyzing different batches of seeds. Each batch of seeds was pooled from more than 80 individual plants. For each biological replicate, more than 120 seeds were examined. Values are means ± SD from 5 independent biological replicates using different batches of seeds. Bars with different letters are significantly different from each other ($P < 0.05$). Data were analyzed by a two-way analysis of variance (ANOVA) using Tukey's honest significant difference (HSD) test. The statistical analyses described apply to all statistical analyses presented in this figure. ABA, abscisic acid.

nia1 nia2 and *prt6* mutants during seed germination, further supporting our proposal that nitrate signaling can repress ABA responses independently of the NO pathway.

Discussion

Nitrogen is an essential macronutrient for plant growth and development. Nitrate is the main source of nitrogen available to plants in many natural systems and in agricultural soils (Crawford and Forde 2002; Vidal et al. 2020; Li et al. 2021; Jia et al. 2022). The seed germination of Arabidopsis and many other plants is enhanced under nitrate-sufficient conditions (Hilhorst et al. 1989; Fitter et al. 2002; Ali-Rachedi et al. 2004; Alboresi et al. 2005; Li et al. 2021). However, whether nitrate serves as a crucial regulatory cue in modulating ABA signaling during seed germination remains relatively undetermined. In this study, we

observed that the wild-type Arabidopsis seeds displayed higher germination and cotyledon greening percentages when both ABA and KNO_3 were present than when only ABA was present (Fig. 1, A to C). Further analyses revealed that nitrate attenuates ABA signaling via a mechanism that depends on a functional NLP8 (Fig. 5, A to D; Supplementary Fig. S8). Consistent with the involvement of NLP8 in mediating ABA responses, the expression of *NLP8* and the abundance of the encoding protein was enhanced by ABA (Supplementary Fig. S5, B to F). These observations imply nitrate and NLP8 have inhibitory effects on ABA signal transduction during seed germination. In line with these findings, nitrate and NLP8 can function independently of the NO signaling pathway to suppress ABA responses (Figs. 1 and 9; Supplementary Fig. S1). Further mechanistic investigations revealed that NLP8 physically associates with ABI3/ABI5 and exerts a negative influence on the transcriptional activation and ABA-induced accumulation of ABI3/ABI5 (Figs. 3, A to F and 7, A to J; Supplementary Figs. S14 and S15). Collectively, these findings indicate there may be a previously unknown signaling module in which the nitrate-associated NLP8 directly regulates the ABA-responsive ABI3 and ABI5. Importantly, these results also provide evidence that the crosstalk between nitrate and ABA pathways may occur through direct protein–protein interactions among core transcription factors.

Interestingly, the nitrate treatment is not a prerequisite for the NLP8–ABI3/ABI5 interactions and the NLP8 effects on ABI3/ABI5 (Figs. 3 and 7; Supplementary Figs. S3, S14, and S15). For example, the results based on the CoIP assays showed that NLP8 bound to ABI3 and ABI5 in plants in the absence of exogenous nitrate (Fig. 3, E and F; Supplementary Fig. S3). In accordance with these observations, even without the nitrate treatment, the *nlp8* mutants still had different responses to ABA during seed germination, compared with the wild-type plants (Fig. 4). Nevertheless, the nitrate exposure enhanced the interactions of NLP8 with ABI3/ABI5 and dramatically decreased the ABA-induced accumulation of ABI3/ABI5 (Fig. 7, G and H; Supplementary Figs. S4, D and E and S15). These regulatory actions are consistent with the inhibitory effects of nitrate on ABA signaling and its impact on *NLP8* overexpression-attenuated ABA responses during seed germination (Figs. 1 and 5). Notably, Liu et al. (2022) revealed that nitrate interacts directly with NLP7 via residues evolutionarily conserved among other NLP proteins and NLP7 orthologs, leading to a conformational change and enabling NLP7 to function as a transcription activator. Considering together with the finding that nitrate also stimulates the transcriptional function of NLP8 (Yan et al. 2016), further elucidation of how nitrate modulates NLP8 at the post-translational level may shed light on the molecular basis of nitrate/NLP8-mediated ABA signaling and seed germination. Furthermore, we demonstrated that the interactions between NLP8 and ABI3/ABI5 were enhanced by ABA, and the phosphorylation state of ABI5 was crucial for the augmented interaction with NLP8 (Supplementary Fig. S4). However, the precise mechanisms by which nitrate and ABA pre-treatments promote the associations of NLP8 with ABI3/ABI5 remain to be unraveled. For instance, future investigations should aim to clarify whether the heightened interaction following nitrate or ABA pre-treatment arises from a similar mechanism and whether the binding of nitrate by NLP8 is implicated in this process.

Accumulating research has demonstrated that ABI3 and ABI5 are strictly regulated through post-translational modifications (Kobayashi et al. 2005; Zhang et al. 2005; Dai et al. 2013; Singh et al. 2024). For example, in the presence of ABA, ABI5 is phosphorylated and stabilized by SnRK2, BIN2, and other related kinases (Kobayashi et al. 2005; Furihata et al. 2006; Fujii et al. 2007; Fujii and Zhu 2009; Nakashima et al. 2009; Hu and Yu 2014; Zhou et al. 2015). In contrast, several E3 ubiquitin ligases, such as KEG, PUB8/PUB35, and AIP2, facilitate the degradation of ABI3 and/or ABI5 through ubiquitination in the absence of ABA (Zhang et al. 2005; Stone et al. 2006; Miura et al. 2009; Lee et al. 2010; Liu and Stone 2010; Lyzenga et al. 2013; Seo et al. 2014; Du et al. 2024). Interestingly, we found that nitrate and NLP8 also promoted the proteasomal degradation of ABI3 and ABI5 (Fig. 7, G to J). Nevertheless, the biochemical mechanisms underlying the effects of nitrate and NLP8 on ABI3 and ABI5 accumulation remain to be investigated. On the basis of these findings and the importance of the above-mentioned kinases and E3 ubiquitin ligases for controlling ABI3 and/or ABI5 contents, future research should clarify whether (and how) the nitrate signal and the NLP8-mediated pathway affect these kinases or E3 ubiquitin ligases to modulate ABI3/ABI5 abundance or functions. For example, whether nitrate and NLP8 inhibit the ABA-induced phosphorylation of ABI3 and ABI5 should be analyzed. In addition, it is interesting to explore whether NLP8 indirectly recruits the TOPLESS co-repressor machinery, which may not interact with NLP8 (Supplementary Fig. S18), to suppress the transcriptional functions of ABI3 and ABI5. Further elucidating the structural basis of the physical associations involving NLP8, ABI3/ABI5, kinases, E3 ubiquitin ligases, or other related factors may increase our understanding of the molecular mechanisms responsible for the effects of NLP8 (and nitrate) on the accumulation or functions of ABI3 and ABI5. An earlier study demonstrated that the NO-insensitive *prt6* mutant is able to accumulate the group VII ETHYLENE RESPONSE FACTOR (ERF) transcription factors leading to increase *ABI5* expression during seed germination (Gibbs et al. 2014). Moreover, an NO treatment in *prt6* mutant background still produces the S-nitrosylation of ABI5 and its consequent proteasome degradation (Albertos et al. 2015). In the present study, the analysis of ABI5 protein levels showed that the ABA-induced accumulation of native ABI5 was reduced in germinating *prt6* mutant seeds upon KNO_3 treatment (Supplementary Fig. S15, E and F). Furthermore, the hemoglobin cycle of NO and nitrate is also functional in seeds (Wang and Hargrove 2013). These results add more complexity to the regulation of nitrate and NO on seed germination.

Similar to the *nlp8-2* seeds, the progeny of *chl1-5* (loss-of-function mutant lacking the nitrate sensor and transporter NRT1.1) (Ho et al. 2009) were more sensitive to the ABA treatment than the wild-type control (Supplementary Fig. S7, A and B). Accordingly, NRT1.1 may also negatively modulate ABA signaling during seed germination. Although *chl1-5* and *nlp8-2* had similar phenotypes after the ABA treatment, the seed germination and cotyledon greening percentages were much higher for *chl1-5* than for *nlp8-2* (Supplementary Fig. S7, A and B). One explanation for this phenotypic discrepancy is that in addition to NRT1.1, other potential nitrate sensor(s) may contribute to ABA signaling during seed germination. It is also possible that NLP8 functions as a putative nitrate sensor in the nitrate-repressed ABA signaling pathway. This possibility is supported by the results of a recent study by Liu et al. (2022) that showed NLP7 functions as a nitrate sensor in plants. More importantly, the nitrate-binding pocket of NLP7 has a conserved sequence and structure and has been found in other Arabidopsis NLP proteins (including NLP8), suggesting these NLP proteins serve as nitrate sensors (Liu et al. 2022). We also showed that NLP7 and NLP9 physically interacted with ABI3 and ABI5 in yeast (Supplementary Fig. S3A). However, unlike the progeny of *nlp8-2*, seeds of *nlp7-1* and *nlp9-1* mutants treated

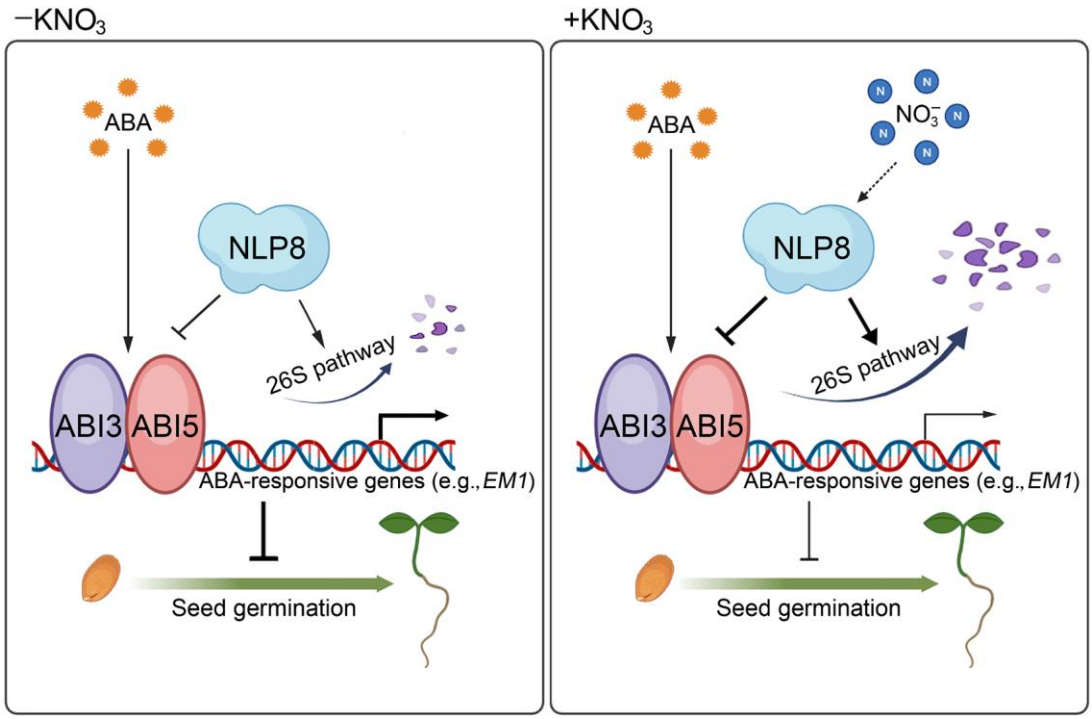

Figure 10. A simplified model involving the NLP8–ABI3/ABI5 interactions in nitrate-attenuated ABA signaling. When the concentration of ABA increases, the ABA-induced ABI3 and ABI5 positively regulate the expression of ABA-responsive genes and suppress seed germination. ABI3 and ABI5 physically associate with the nitrate-related NLP8 and their interactions are enhanced in the presence of nitrate. NLP8 interferes with the transcriptional functions of ABI3 and ABI5 to negatively mediate ABA signaling during seed germination. Furthermore, nitrate and NLP8 promote the degradation of ABI3 and ABI5 in a proteasome pathway-dependent manner. Pointed arrow indicates promotion or activation. Flat arrow, indicates inhibition or repression. Thick arrow indicates strong activation or inhibition. Thin arrow, indicates weak activation or inhibition. ABA, abscisic acid.

with ABA had germination and cotyledon greening percentages that were similar to those of the wild-type controls regardless of the absence or presence of KNO_3 (Supplementary Fig. S7). Hence, disrupting NLP7 or NLP9 alone apparently does not considerably affect ABA responses during seed germination. Nevertheless, the possibility that other NLP factors besides NLP8 also modulate ABA responses during seed germination and/or the subsequent establishment of seedlings cannot be ruled out completely. In line with this notion, the cotyledon greening after the ABA-treated *nlp8-2* seeds germinated was slightly enhanced by nitrate (Fig. 5B; Supplementary Fig. S7B). The potential regulatory relationships between other NLP proteins and ABA signaling components will need to be examined in future studies.

The modulation of ABI3/ABI5-mediated ABA responses during seed germination involves a complex network of transcriptional regulators that combine to establish and maintain appropriate ABA signaling levels. For example, the DELLA proteins, which are critical repressors of gibberellin signaling, interact with ABI3 and ABI5 to upregulate the expression of a subset of genes whose expression is induced by ABA and high temperatures, thereby inhibiting seed germination (Peng et al. 1997; Silverstone et al. 1998; Tyler et al. 2004; Lim et al. 2013; Xian et al. 2024). PSEUDO-RESPONSE REGULATOR5 (PRR5) and PRR7 function synergistically with ABI5 to integrate the circadian clock and ABA signaling pathways for the ABA-induced delayed seed germination (Yamamoto et al. 2003; Nakamichi et al. 2005, 2007, 2009; Yang et al. 2021). The AUXIN RESPONSE FACTOR10 (ARF10) and ARF16 transcription factors bind directly to ABI5 and stimulate its transcriptional activity to mediate the convergence of auxin, JA, and ABA signaling pathways (Mei et al. 2023). In addition, Zhao et al. (2019) demonstrated that the brassinosteroid-related transcription factor BES1 physically associates with ABI5 and adversely affects its function. Considered together, these previous findings and the results of the current study suggest that the intricate interplay among ABI3/ABI5 and other critical transcriptional components involved in different signaling pathways may contribute to specific and effective adaptative mechanisms that enhance stress tolerance, while minimizing the detrimental effects of ABA on seed germination and post-germinative growth. Future investigations will need to decipher the precise mechanisms underlying the potential interactions among ABI3/ABI5-binding transcriptional modulators and to elucidate the biological significance of these physical interactions. For example, whether and how NLP8 coordinates with DELLA, ARF10/ARF16, JAZ, or BES1 to fine-tune ABI3/ABI5-modulated signaling and mediate the integration of diverse signals should be clarified.

To further elucidate the molecular basis of the nitrate/NLP8-mediated suppression of ABA responses during seed germination in Arabidopsis, we propose a simplified model involving the NLP8–ABI3/ABI5 interactions (Fig. 10). When the concentration of ABA increases, the ABA-induced ABI3 and ABI5 positively regulate ABA signaling and suppress seed germination (Finkelstein and Lynch 2000; Lopez-Molina and Chua 2000; Lopez-Molina et al. 2001, 2002; Nakamura et al. 2001; Brocard et al. 2002). ABI3 and ABI5 physically associate with the nitrate-related NLP8 and their interactions are enhanced in the presence of nitrate (Fig. 3; Supplementary Fig. S4). NLP8 interferes with the transcriptional functions of ABI3 and ABI5 to negatively mediate ABA signaling during seed germination (Figs. 4 to 8). Furthermore, nitrate and NLP8 promote the degradation of ABI3 and ABI5 in a proteasome pathway-dependent manner (Fig. 7; Supplementary Figs. S14 and S15). Consistent with these findings, nitrate and NLP8 can

function independently of the NO pathway to regulate ABA responses (Figs. 1 and 9). Our results revealed the critical inhibitory effects of nitrate as a regulatory signal on ABA signaling during seed germination, while also providing mechanistic insights into the physical interactions and antagonistic relationships of NLP8 and ABI3/ABI5.

Materials and methods

Materials and plant growth conditions

Common chemicals were purchased from Shanghai Sangon (Shanghai, China), and Taq DNA polymerase was obtained from Takara Biotechnology (Dalian, China). The phytohormone ABA (catalog no. 862169-250MG) was purchased from Sigma-Aldrich (St Louis, MO, USA). The wild-type and mutant *Arabidopsis thaliana* plants used in this study were in the Columbia (Col-0) genetic background. Seeds of *chl1-5* (CS6384), *nlp7-1* (SALK_026134), *nlp9-1* (SALK_025839), and *prt6-1* (SALK_051088) were purchased from AraShare (www.arashare.cn). The *abi3-8* and *abi5-8* (Salk_013163C) mutants have been described previously (Nambara et al. 2002; Mei et al. 2023). The *nia1 nia2* (CS2356) mutant was kindly provided by Prof. Jianru Zuo (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences), and the *nlp8-2* (SALK_140298) mutant was kindly provided by Prof. Chunpeng Song (Henan University). The transgenic line *ABI5-MYC* (Chen et al. 2012; Hu and Yu 2014; Yang et al. 2021) was kindly provided by Dr. Chuanyou Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). To generate *NLP8-GFP* and *MYC-ABI3* transgenic plants, the full-length CDS sequences of *NLP8* (before the *GFP* tag sequences) and *ABI3* (behind the 3*MYC* tag sequence) were cloned into the binary vector pOCA30 in the sense orientation for the subsequent expression under the control of *Pro35S* (Hu et al. 2013). The *nlp8-2 abi3-8*, *nlp8-2 abi5-8*, *nlp8-2 abi3-8 abi5-8*, *nlp8-2 MYC-ABI3*, and *nlp8-2 ABI5-MYC* plants were generated via genetic crosses. The *NLP8-GFP-3* was crossed with *MYC-ABI3*, *ABI5-MYC*, *nia1 nia2*, and *prt6-1* to generate *NLP8-GFP MYC-ABI3*, *NLP8-GFP ABI5-MYC*, *NLP8-GFP nia1 nia2*, and *NLP8-GFP prt6-1*, respectively. Arabidopsis and *Nicotiana benthamiana* plants were grown in an artificial growth chamber set at 22 °C with a 16-h light (100 μE m^{-2} s^{-1}; white fluorescent bulbs, full light wavelength):8-h dark cycle.

Determination of seed germination and cotyledon greening

The germination of the wild-type and mutant seeds and the greening of the seedlings were analyzed as previously described (Hu et al. 2019; Yang et al. 2021). Freshly harvested seeds were stratified for 3 days at 4 °C and then germinated in an artificial growth chamber set at 22 °C with a 16-h light (100 μE m^{-2} s^{-1}; white fluorescent bulbs, full light wavelength):8-h dark cycle. Germination was determined on the basis of the appearance of the embryonic axis (i.e. radicle protrusion) as observed using a microscope. Seedling greening was determined on the basis of the appearance of green cotyledons. To analyze the effects of ABA on seed germination and cotyledon greening, the seeds were placed on water agar (0.6%) medium supplemented with ABA with or without KNO_3 (KCl as the control) in plates; deionized water was used for all phenotypic tests in this study. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. All experiments were conducted more than 5 times using different batches of seeds as biological replicates. Each batch comprised seeds pooled from >80 independent plants. More than 120 seeds were included in each biological replicate.

Total RNA extraction and RT-qPCR

Total RNA was extracted from the treated germinating seeds using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the RT-qPCR analysis was performed as described by Yang et al. (2021). Briefly, in a 20 μL reaction volume, oligo $(dT)_{18}$ primers and Moloney murine leukemia virus reverse transcriptase (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) were used to reverse transcribe 1.0 μg DNase-treated RNA. Next, 1.0 μL cDNA and the SYBR Premix Ex Taq kit (Takara) were used for the RT-qPCR analysis performed on the Roche LightCycler 480 real-time PCR machine. Target gene expression levels were calculated relative to the expression of At1g13320, which encodes a subunit of Ser/Thr Protein Phosphatase 2A (PP2A) and is stably expressed during seed germination (Czechowski et al. 2005). Five biological replicates per sample were used for the RT-qPCR analysis. The gene-specific RT-qPCR primers are listed in Supplementary Data Set 1.

Yeast two-hybrid assays

The full-length *ABI3* and *ABI5* coding sequences (CDSs) were inserted into pGBKT7 (Clontech) to generate the bait vectors (BD-ABI3 and BD-ABI5) containing the Gal4 DNA-binding domain. The full-length *NLP1*, *NLP5*, *NLP7*, *NLP8*, and *NLP9* CDSs were incorporated into pGADT7 (Clontech) to generate the prey vectors containing the Gal4 activation domain (AD-NLP1, AD-NLP5, AD-NLP7, AD-NLP8, and AD-NLP9, respectively). To further investigate the regions critical for the interactions, we truncated *ABI3* and *ABI5* sequences into multiple fragments and then inserted them into pGBKT7. Similarly, we truncated *NLP8* into multiple fragments and inserted them into pGADT7. The yeast two-hybrid assay was conducted using the mating protocol described in Clontech's Matchmaker Gold Yeast Two-Hybrid User Manual. To screen for protein interactions, yeast strain Y2HGold cells were co-transformed with the bait and prey vectors. Physical interactions were indicated by the ability of the cells to grow on dropout medium lacking Leu, Trp, His, and Ade with or without 3-aminotriazole at 4 days after plating. The primers used for cloning are listed in Supplementary Data Set 1.

BiFC assays

The cDNA sequences encoding an enhanced YFP fragment (cYFP) consisting of 64 C-terminal amino acids and a YFP fragment (nYFP) comprising 173 N-terminal amino acids were amplified by PCR and inserted into the pFGC5941 plasmid to generate pFGC-cYFP and pFGC-nYFP, respectively (Kim et al. 2008). The full-length CDS of *ABI5* and the full-length CDS of *ABI3* or sequence encoding the 416 N-terminal residues of ABI3 were cloned into pFGC-cYFP to produce an in-frame fusion with the C-terminal of cYFP (ABI5-cYFP, ABI3-cYFP, or $ABI3^{1–416}$-cYFP). The full-length CDS of *NLP5* and *NLP8* were inserted into pFGC-nYFP to generate an in-frame fusion with the N-terminal of nYFP (NLP5-nYFP and NLP8-nYFP). The resulting recombinant plasmids were inserted into *Agrobacterium tumefaciens* strain GV3101 cells, which were then infiltrated into *N. benthamiana* leaves as previously described (Walter et al. 2004). The leaves were examined for YFP and DAPI fluorescence at 40 to 52 h post-infiltration using the Fluoview FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). For the DAPI staining, the infiltrated leaves were stained with DAPI solution (10 mM) for 5 min before being examined.

The YFP signals were detected using an excitation wavelength of 488 nm (intensity, 24%; gains, 1) and an emission wavelength of 510 to 530 nm. The DAPI signals were detected using an excitation wavelength of 405 nm (intensity, 15%; gains, 1) and an emission wavelength of 420 to 440 nm. Experiments were performed at least 3 times using different batches of *N. benthamiana* plants. For each biological replicate, >12 *N. benthamiana* plants were infiltrated and more than 600 cells in the infiltrated area were analyzed. The primers used for cloning are listed in Supplementary Data Set 1.

CoIP assays

To confirm the NLP8–ABI3 and NLP8–ABI5 interactions, we extracted proteins from germinating seeds (2.5 d, with or without 0.5 μM ABA treatment) of transgenic Arabidopsis plants simultaneously overexpressing *NLP8* and *ABI3* (*NLP8-GFP MYC-ABI3*) or *NLP8* and *ABI5* (*NLP8-GFP ABI5-MYC*) under the control of *Pro35S*. Extraction buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 1 mM PMSF, and 1× Roche Protease Inhibitor Cocktail was used for the extraction of proteins from Arabidopsis plants. Immunoprecipitation experiments were completed using Protein A/G Plus agarose beads (Santa Cruz Biotechnology; catalog no. D1217) according to the manufacturer's protocol. Briefly, cell lysates were pre-cleared using Protein A/G Plus agarose beads, which were then added to the extraction buffer containing anti-GFP antibody (Sigma-Aldrich, catalog no. Ab6556) (1:250) for an overnight incubation at 4 °C. The beads were washed extensively with extraction buffer and then the co-immunoprecipitated proteins were detected by immunoblotting using the anti-MYC antibody (Sigma-Aldrich, catalog no. M4439; 1:10,000). The primers used for cloning are listed in Supplementary Data Set 1.

GUS staining

The putative *NLP8* promoter sequence (*ProNLP8*; 2,321 bp) was amplified by PCR using wild-type genomic DNA and gene-specific primers. The *ProNLP8-GUS* construct was cloned into the pOCA28 binary vector and introduced into wild-type plants. The dry seeds collected from *ProNLP8-GUS* transgenic lines were stripped of their seed coat prior to staining. The seeds from *ProNLP8-GUS* transgenic lines were placed on water agar medium with or without 1 μM ABA for 1, 2, or 3 days and then stained. The histochemical detection of GUS activity was performed as previously described (Chen et al. 2010). The primers used for cloning are listed in Supplementary Data Set 1.

Transient transactivation assays

The putative promoter sequences of *EM1* (2,000 bp) and *EM6* (1,273 bp) were amplified by PCR and cloned into the pGreenII 0800-LUC vector to produce the reporters (Hellens et al. 2005). Full-length or truncated *ABI3*, *ABI5*, *NLP8*, *NLP8-N* (truncated 1-670 amino acids coding sequences), and *GFP* CDSs were inserted into the pGreenII 62-SK vector to produce the effectors (Hellens et al. 2005). Different combinations of the recombinant plasmids were used for the transformation of the leaf mesophyll protoplasts of the wild-type, *nlp8-2* mutant, and *NLP8*-overexpressing transgenic plants as previously described (Sheen 2001). The transfected cells were cultured for 10 to 16 h with or without 5 μM ABA and then the relative LUC activity was examined using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA), which measures the activities of firefly LUC and the internal control *Renilla reniformis* LUC (REN). The primers used for cloning are listed in Supplementary Data Set 1.

Yeast one-hybrid assays

The Yeast One-Hybrid System Kit (Clontech) was used for yeast one-hybrid assays according to the manufacturer's instructions. The putative promoter fragments of *EM1* and *EM6* were cloned into the pAbAi vector to generate pAbAi-pEM1 and pAbAi-pEM6, which were linearized by *BstBI* and then inserted into Y1HGold yeast cells according to a polyethylene glycol/lithium acetate-based method. The AD-NLP8 construct was then incorporated into the cells carrying pAbAi-pEM1 or pAbAi-pEM6. The transformed cells were grown on SD/−Ura medium in plates for 3 days. The co-transformed cells were cultured on SD/−Leu medium supplemented with aureobasidin A (AbA, 200 μg/L) in plates for 3 days. Positive clones were detected for several yeast concentrations [dilutions ranging from 10^0 (OD_{600} = 1.0) to 10^{-3}]. The primers used for cloning are listed in Supplementary Data Set 1.

ChIP assays

The ChIP assays were performed essentially as previously described (Mukhopadhyay et al. 2008; Jiang et al. 2014). Briefly, the wild-type, *MYC-ABI3*, *NLP8-GFP MYC-ABI3*, *ABI5-MYC*, and *NLP8-GFP ABI5-MYC* germinating seeds (with or without a 2-d treatment with 1 μM ABA) were added to 1% formaldehyde (for cross-linking) and then their chromatin was isolated. Protein–DNA complexes were immunoprecipitated using the anti-MYC antibody. The precipitated DNA was purified using a PCR purification kit (Qiagen) and then subjected to a RT-qPCR analysis. To quantify the binding of ABI3 or ABI5 to DNA (i.e. target gene promoter), the RT-qPCR analysis was performed as previously described (Mukhopadhyay et al. 2008), with the *PP2A* (At1g13320) promoter selected as the endogenous control. Relative quantitative values were calculated using the $2^{-\Delta\Delta Ct}$ method (Mukhopadhyay et al. 2008) and then expressed as the DNA binding rate. The analyses were completed using the data of 5 biological replicates of different batches of seeds (>500 seeds per sample per replicate). The primers used for ChIP are listed in Supplementary Table S4.

EMSA

The full-length CDS of *ABI5* or *NLP8* was cloned into the expression vector pET-28(+). The recombinant plasmid was transformed into *E. coli* BL21 strain (TransGen Biotech). His-ABI5 and His-NLP8 were induced by 0.1 mM IPTG at 16 °C for 24 h. These expressed proteins were purified according to the manual provided by Novagen. The EMSA was performed using a Light Shift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions (Hu et al. 2019). The assays were performed 3 times with similar results. The primers used for cloning are listed in Supplementary Data Set 1.

Measurement of nitrate content

For nitrate content analyses, the seeds of wild-type, *nlp8-2*, *nlp8-Cas9*, *NLP8-GFP-3*, *NLP8-GFP-5*, *MYC-ABI3*, *nlp8-2 MYC-ABI3*, *ABI5-MYC*, *nlp8-2 ABI5-MYC*, *abi3-8 abi5-8*, *nlp8-2 abi3-8 abi5-8*, *chl1-5*, *nia1 nia2*, *NLP8-GFP nia1 nia2*, *prt6-1*, and *NLP8-GFP prt6-1* were germinated for 2 d on water agar medium containing 1 μM ABA with or without 1 mM KNO_3 or KCl. The collected seeds were added 1 M KOH and 60 mL milli-Q water per 2 g fresh weight of tissues, then the mixture ultrasonic extraction at 60 °C for 30 min. The material was centrifuged at 2,683 × *g* for 10 min at

R/T, then the supernatant was taken up in a 10 mL syringe, passed through a 0.22 μm filter and its nitrate content determined by HPLC (Thayer and Huffaker 1980; AQ-1100, Thermo Fisher, USA) using a AS-23 (strong anion exchanger) column (Supelco, USA). The analyses were completed using the data of 3 biological replicates of different batches of seeds (>2,000 seeds per sample per replicate).

Statistical analysis

Data were analyzed by performing an analysis of variance (ANOVA) using Tukey's honest significant difference (HSD) as a post hoc test. Statistically significant differences were defined as those with $P < 0.05$. Lowercase letters above the columns in the figures presented herein indicate significant differences ($P < 0.05$) among samples. The results of statistical analyses are provided in Supplementary Data Set 2.

Accession numbers

The genes discussed in this paper can be found in the Arabidopsis Genome Initiative database as follows: *EM1*, AT3G51810; *EM6*, AT2G40170; *RAB18*, AT5G66400; *ABI3*, AT3G24650; *ABI5*, AT2G36270; *NLP1*, AT2G17150; *NLP5*, AT1G76350; *NLP7*, AT4G24020; *NLP8*, AT2G43500; *NLP9*, AT3G59580; *NRT1.1*, AT1G12110; *NIA1*, AT1G77760; *NIA2*, AT1G37130; *PRT6*, AT5G02310, and *PP2A*, AT1G13320.

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Author contributions

Y.H. and Z.H. designed this study and wrote the article; Z.H., X.H., K.H., J.Y., T.X., C.Y., J.Z., J.D., Q.F., and Y.H. performed experiments or interpreted data; all authors approved the final article.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. Nitrate attenuates ABA responses independently of the NO pathway during seed germination.

Supplementary Figure S2. Nitrate reduces the ABA-induced expression of *ABI3* and *ABI5* during seed germination.

Supplementary Figure S3. The interactions between several NLP transcription factors and ABI3 or ABI5.

Supplementary Figure S4. The NLP8–ABI3/ABI5 interactions are responsive to ABA and nitrate.

Supplementary Figure S5. ABA induces *NLP8* expression during seed germination.

Supplementary Figure S6. The *nlp8-Cas9* mutant was generated using the CRISPR-Cas9 gene editing system.

Supplementary Figure S7. The ABA responses of *chl1-5*, *nlp7-1*, *nlp8-2*, and *nlp9-1* during seed germination.

Supplementary Figure S8. The responses of *nlp8-2* and *NLP8-GFP-3* plants to ABA and nitrate during seed germination.

Supplementary Figure S9. Seedlings of wild type (WT), *nlp8-2*, *abi3-8*, *nlp8-2 abi3-8*, *abi5-8*, *nlp8-2 abi5-8*, *abi3-8 abi5-8*, *nlp8-2 abi3-8 abi5-8* 5 days after germination on water agar medium.

Supplementary Figure S10. Schematic diagrams of the effectors and reporters used in the transient transactivation assays and the accumulation of ABI3, ABI5, NLP8, or NLP8-N in Arabidopsis mesophyll protoplasts upon ABA treatment.

Supplementary Figure S11. NLP8 represses the ability of ABI3 and ABI5 to mediate *EM6* expression.

Supplementary Figure S12. Yeast one-hybrid assays demonstrating NLP8 binding to the promoter regions of *EM1* and *EM6*.

Supplementary Figure S13. EMSA showing that NLP8 influences the DNA-binding activity of ABI5.

Supplementary Figure S14. Relative protein levels of MYC-ABI3 and ABI5-MYC.

Supplementary Figure S15. The ABA-induced accumulation of ABI3 and ABI5 in germinating seeds upon nitrate treatment.

Supplementary Figure S16. Overexpression of *NLP8-N* exerts little influence on ABA signaling-related delayed seed germination.

Supplementary Figure S17. Seedlings of wild type (WT), *NLP8-GFP-3*, *nia1 nia2*, *NLP8-GFP nia1 nia2*, *prt6-1*, and *NLP8-GFP prt6-1* 7 days after germination on water agar medium.

Supplementary Figure S18. Yeast two-hybrid assay showing that NLP8 and TOPLESS (TPL) or its close homologs (TPR) do not interact.

Supplementary Table S1. The ABA content of wild-type (WT), *nlp8-2*, and *NLP8-GFP-3* seeds and seedlings.

Supplementary Table S2. The nitrate content of wild-type (WT) and various mutant or transgenic seeds.

Supplementary Table S3. Promoter region of *EM1* (*pEM1-1*) and a negative control region (*pEM1-nc*) used to quantify ABI3 and ABI5 enrichment.

Supplementary Table S4. Primers used for ChIP analyses.

Supplementary Data Set 1. Primers used for cloning and RT-qPCR.

Supplementary Data Set 2. ANOVA tables.

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Data availability

All data supporting the findings of this study are available within the article and its supplementary materials.

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