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Nucleocytoplasmic Trafficking of the Arabidopsis WD40 Repeat Protein XIW1 Regulates ABI5 Stability and Abscisic Acid Responses

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Running title: XIW1 regulates ABA responses
Short summary: XIW1 was identified as a substrate for the nuclear transport receptor XPO1, which is exported from the nucleus in an XPO1-dependent manner. In the presence of ABA, XIW1 accumulates in the nucleus, where it interacts with ABI5 and promotes ABI5 stability and ABA responses.
ABSTRACT

WD40 repeat-containing proteins (WD40 proteins) serve as versatile scaffolds for protein-protein interactions, modulating a variety of cellular processes such as plant stress and hormone responses. Here, we describe a WD40 protein, XIW1 (for XPO1-Interacting WD40 protein 1), that positively regulates the Abscisic acid (ABA) response in Arabidopsis. XIW1 is located in the cytoplasm and nucleus. It interacts with the nuclear transport receptor XPO1 and is exported by XPO1 from the nucleus. Mutation of XIW1 reduces the induction of ABA-responsive genes and the accumulation of ABA Insensitive 5 (ABI5), leading to ABA-insensitive phenotypes during seed germination and seedling growth, and decreased drought stress resistance. ABA treatment upregulates the expression of XIW1, and both ABA and abiotic stresses promote the nuclear accumulation of the XIW1 protein. In the nucleus, XIW1 interacts with ABI5, and loss of XIW1 results in rapid proteasomal degradation of ABI5. Taken together, these data suggest that XIW1 is a nucleocytoplasmic shuttling protein that has a positive role in ABA responses by interacting with and maintaining the stability of ABI5 in the nucleus.

Keywords: WD40 protein, ABA signaling, nuclear export, ABI5 stability, XPO1
INTRODUCTION

Plants respond and adapt to abiotic and biotic stimuli through various physiological and metabolic processes. The signal transduction cascades underlying these responses depend on gene regulation at several levels such as transcription, translation, post-translational modifications, and the selective import or export of some proteins to and from the nucleus (Shinozaki and Yamaguchi-Shinozaki, 2007; Meier and Somers, 2011; Zhu, 2016). For example, the nucleocytoplasmic trafficking of *Arabidopsis* COP1 (Constitutive Photomorphogenesis 1) regulates photomorphogenesis and seed germination. In the dark, COP1 localizes to the nucleus and acts as a repressor of photomorphogenesis by suppressing the expression of light-induced genes in hypocotyl cells. Light facilitates the nuclear export of COP1 to the cytosol and therefore promotes photomorphogenesis (von Arnim and Deng, 1994; Lau and Deng, 2012). Another typical example is the nucleocytoplasmic trafficking of brassinazole-resistant 1 (BZR1), a transcription factor involved in brassinosteroid (BR) signaling. In the presence of BR, the dephosphorylation of BZR1 facilitates its import to the nucleus, where it activates a plethora of BR-responsive genes. Conversely, in the absence of BR, phosphorylation of BZR1 promotes its binding to 14-3-3 proteins, causing its cytoplasmic retention and inactivation (Bai et al., 2007; Ryu et al., 2007).

Nucleocytoplasmic trafficking of proteins is mediated by nuclear transport receptors (NTRs), of which importin β was the first identified (Gorlich et al., 1995). In *Arabidopsis*, 17 genes encoding importin β-like NTRs are divided into nuclear import receptors and nuclear export receptors (Merkle, 2011). Some of these genes have been functionally characterized, e.g. the *Arabidopsis* nuclear import receptor SAD2 (Sensitive to ABA and Drought 2) mediates the nuclear import of the transcription factor MYB4, consequently altering ABA sensitivity and UV-B responses (Verslues et al., 2006; Zhao et al., 2007). The nuclear export receptor HST/Exportin 5 plays a crucial role in miRNA biogenesis (Bollman et al., 2003), while PSD/Exportin-T functions in tRNA export (Hunter et al., 2003). *Arabidopsis* exportin 1
(XPO1), an importin β-like NTR, mediates the nuclear export of proteins containing leucine-rich nuclear export signals (Haasen et al., 1999). Two closely related XPO1 genes, namely XPO1A (At5g17020) and XPO1B (At3g03110), have been identified in Arabidopsis. Single mutants of xpolα and xpolβ appear normal; however, the xpolα xpolβ double mutant is gametophyte lethal, suggesting that the function of XPO1 is essential for plant development (Blanvillain et al., 2008).

In humans and yeast, XPO1/CRM1 (Chromosomal Maintenance 1) has been reported to facilitate the transport of large numbers of proteins from the nucleus to the cytoplasm and thus participates in various biological processes (Fukuda et al., 1997; Hutten and Kehlenbach, 2007; Kirli et al., 2015). More than 700 proteins in yeast and 1,050 proteins in human cells have been identified as substrates of CRM1. Some of these proteins function in translation, ribosome biogenesis, mRNA degradation, vesicular transport, autophagy, and cellular regulatory circuits (Kirli et al., 2015). In Arabidopsis, nucleocytoplasmic shuttling of serine/arginine-rich splicing factors (i.e. RSZp22 and SRSF1 subfamily splicing factors) is partially controlled by the CRM1/XPO1-dependent nuclear export pathway (Rausin et al., 2010; Stankovic et al., 2016). However, the information on XPO1 substrates in plants is still very scanty.

In this study, we identified a transducin/WD40 repeat-like superfamily protein (AT1G15470) as a potential substrate for XPO1. This WD40 protein interacts directly with XPO1 and is exported from the nucleus by XPO1. Nucleocytoplasmic partitioning of WD40 protein is regulated by ABA and abiotic stresses. Furthermore, the WD40 protein interacts with ABI5 and helps to stabilize ABI5 in the nucleus, therefore positively regulates the ABA response.
RESULTS

XIW1 interacts with XPO1A
To identify the substrates of XPO1 in Arabidopsis, we performed co-immunoprecipitation (co-IP) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using XPO1Apro:XPO1A-MYC/xpo1a-3 transgenic plants (Figure 1A and Supplemental Figure 2). Proteins extracted from Col-0 (as control) and XPO1A-MYC transgenic plants were precipitated with anti-MYC antibodies. A large number of candidate XPO1A-associated proteins were identified in the anti-MYC immunoprecipitates (Supplemental Table 1). Among these candidates, we focused on a transducin/WD40 repeat-like superfamily protein (AT1G15470). This protein was named as XIW1 (XPO1-Interacting WD40 protein 1).

To confirm the interaction between XIW1 and XPO1A, we crossed XPO1Apro:XPO1A-MYC and XIW1-GFP/xiw1 transgenic plants. Co-IP assays showed that XPO1A could be co-precipitated by XIW1 (Figure 1B). Additionally, the direct association of these proteins was demonstrated by yeast two-hybrid assays, as well as by bimolecular fluorescence complementation (BiFC) assays upon transient expression of the proteins in Nicotiana benthamiana (Figure 1C and 1D).

XIW1 is exported from the nucleus in an XPO1-dependent manner
Since XPO1A directly interacts with XIW1, we wondered whether XPO1A may mediate the nuclear export of XIW1. To test this, a T-DNA insertion mutant xpo1a-3 was crossed with XIW1-GFP/xiw1 transgenic plants. Unexpectedly, the GFP signal did not accumulate in the nucleus of the xpo1a background plants (Supplemental Figures 3A and 3B). Later, we found that XPO1B also interacts with XIW1 (Supplemental Figures 3C and 3D) and that the expression of XPO1B is increased in the xpo1a mutant (Supplemental Figures 3F), indicating that XPO1B may compensate for the function of XPO1A in the nuclear export of XIW1. As the xpo1a xpo1b double mutant is gametophyte lethal (Blanvillain et al., 2008), leptomycin B
(LMB), a specific inhibitor of XPO1/CRM1 (Kudo et al., 1999), was used to test the relevance of the XPO1 function for the nuclear export of XIW1. When 35S:XIW1-GFP was transiently expressed in *N. benthamiana* leaves, GFP fluorescence was mainly detected in the cytoplasm under normal conditions, whereas it accumulated in the nucleus after LMB treatment (Figure 1E). This result was confirmed in the XIW1-GFP/xiw1 transgenic plants, where LMB treatment enhanced the nuclear accumulation of XIW1-GFP (Figure 1F). These results suggest that the nuclear export of XIW1 is XPO1-dependent.

XPO1-mediated nuclear export requires the presence of a nuclear export signal (NES) in the target protein. Using a published analysis tool (http://prodata.swmed.edu/LRNes/predictNES/LocNES.php) (Xu et al., 2015), we identified an NES in the XIW1 sequence (310-LVAAEVVRKAESLRI-325). To test the relevance of this signal, we made point mutations within the NES (L323A and I325A) to render it non-functional, generating XIW1^mNES^. Yeast two-hybrid assays showed that this NES is required for the interaction between XPO1A and XIW1 (Figure 1C). Transiently expressing XIW1^mNES^-GFP in *N. benthamiana* leaves and observation in the XIW1^mNES^-GFP/xiw1-1 transgenic plants showed that XIW1^mNES^-GFP accumulated mostly in the nucleus (Figure 1E and 1G), indicating that the NES sequence is crucial for the export of XIW1 from the nucleus.

**Characterization and expression analysis of XIW1**

The XIW1 gene has a 1,002-bp-long coding region encoding a protein with 333 amino acid residues. Sequence analysis using the SMART motif search program (http://smart.embl-heidelberg.de/) predicted seven WD40 domains in XIW1 (Figure 2A and Supplemental Figure 4). XIW1 is evolutionarily conserved from lower to higher plants. Phylogenetic analysis of XIW1 proteins revealed two clusters in higher plants, suggesting the divergence between monocotyledons and dicotyledons (Supplemental Figure 5).

Quantitative RT-PCR analysis showed that XIW1 is ubiquitously expressed and has a relatively higher expression in seedlings and the inflorescence of mature plants (Figure 2B).
Analysis of *XIWI*::GUS transgenic plants confirmed that *XIWI* is mainly expressed in germinating seeds, seedlings, and inflorescences (Figure 2C). The expression of *XIWI* decreased following seed germination but up-regulated when exposed to exogenous ABA (Figure 2D), implying that *XIWI* may play a role in regulating seed germination and ABA responses in *Arabidopsis*.

**XIWI regulates seed germination, seedling growth, and drought stress resistance**

To test whether the loss of *XIWI* function may lead to an altered response to ABA, a T-DNA insertion mutation *xiw1-1* with abolished *XIWI* expression was obtained from ABRC (Supplemental Figure 6). Difference in seed germination was not detected between *xiw1-1* and Col-0; however, in the presence of 0.5 μM ABA, *xiw1-1* exhibited higher seed germination rate and cotyledon greening than Col-0 (Figures 3A-3C). To assess the effect of *XIWI* on ABA-mediated post-germination seedling growth, seeds of *xiw1-1* and Col-0 were allowed to germinate on 1/2 MS medium and then transferred to medium containing 10 μM ABA. As shown in Figures 3D and 3E, the ABA-mediated inhibition of primary root growth was milder in the *xiw1-1* mutant (Figures 3D and 3E). Besides, we found that *xiw1* displayed decreased sensitivity to NaCl and mannitol during seed germination and early post-germination growth (Supplemental Figure 7).

We also tested whether *XIWI* affect drought stress resistance. For this experiment, 3-week-old *xiw1-1* and Col-0 seedlings grown in soil were exposed to drought stress by withholding irrigation. Compared with Col-0, the *xiw1-1* plants showed drought-hypersensitive phenotypes. Only 20% of the *xiw1-1* plants while more than 90% of the Col-0 plants survived after withholding water for 12 d and re-watering for 3 d (Figures 3F and 3G). Water loss analysis in detached leaves indicated that the rate of water loss in *xiw1-1* was higher than in Col-0 (Figure 3H). These results suggest that *XIWI* acts as a positive regulator of ABA sensitivity, regulating seed germination, primary root growth, and drought stress resistance.
To confirm that the ABA-insensitive phenotype was caused by the loss of XIW1, we transformed the \textit{XIWI} coding region driven by the 35S promoter into \textit{xiw1-1}. Two independent transgenic lines with enhanced XIW1 mRNA and protein levels were tested (Supplemental Figure 6B). As expected, the ABA-insensitive phenotype of \textit{xiw1-1} during seed germination and post-germination growth was masked in the complemented lines (Figures 3A-3E), and the complementation also enhanced drought stress resistance (Figures 3F and 3G).

\textbf{XIW1 regulates the expression of ABA responsive genes}

To understand the role of XIW1 in the ABA response pathway, we measured the expression of several typical ABA-responsive genes in \textit{xiw1-1} and Col-0. Although the expression levels of \textit{ABI5}, \textit{RD29A} and \textit{RD29B} increased in both \textit{xiw1-1} and Col-0 after ABA treatment, the increment in expression was lower in \textit{xiw1-1} than Col-0 (Figure 4A).

To analyze the global transcript changes in \textit{xiw1}, three independent biological replicates of 10-d-old Col-0, \textit{xiw1-1}, and \textit{abi5-8} seedlings were subjected to RNA sequencing analysis under mock conditions and ABA treatment. Compared to Col-0, 2,450 differentially expressed genes (DEGs) in \textit{xiw1-1}, and 1,220 DEGs in \textit{abi5-8} were identified under mock conditions (Fold Change $\geq$ 2, FDR < 0.01). Similarly, 2,468 DEGs in \textit{xiw1-1}, and 2,591 DEGs in \textit{abi5-8} were found in the presence of ABA (Figure 4B, Supplemental Data Sets 1). To assess the similarity of the DEGs between \textit{xiw1} and \textit{abi5}, we plotted common and unique genes using Venn diagrams. 862 overlapping genes (representing 35.2% of DEGs in \textit{xiw1-1}, and 70.7% in \textit{abi5-8}) under mock conditions, and 1,719 overlapping genes (representing 69.7% of DEGs in \textit{xiw1-1} and 66.3% in \textit{abi5-8}) after ABA treatment were observed (Figure 4C). In addition, the transcriptome analysis showed that only 540 genes (representing 2.0% of all genes) and 172 genes (0.6%) were differentially expressed between \textit{xiw1} and \textit{abi5} under mock conditions and ABA treatment, respectively (Figure 4B, Supplemental Data Sets 1).
The RNA sequencing analysis also revealed that 4,109 genes in Col-0 (representing 15.5% of all genes), 2,647 in \textit{xiw1-1} (10.0%), and 2,784 in \textit{abi5-8} (10.5%) were differentially expressed (Fold Change $\geq 2$, FDR < 0.01) between mock conditions and ABA treatment (Figure 4D and Supplemental Data Sets 2). Venn analysis showed that 1,198 genes were up-regulated in all three genotypes in the presence of ABA (Supplemental Data Sets 2). Among these, genes classified in group I (61.6% of the 1,198 up-regulated genes) showed lower expression levels in \textit{xiw1-1} and \textit{abi5-8} than Col-0. This group includes a large number of ABA signaling genes, i.e. \textit{SnRK2.2}, \textit{SnRK2.6}, \textit{ABRE2/ABF2}, as well as ABA-responsive genes, i.e. \textit{RD29A}, \textit{RD29B}, and \textit{RAB18} (Figure 4E and Supplemental Data Sets 2). These results are consistent with the qRT-PCR analysis described above (Figure 4A). Genes classified in group II (23.0%), on the other hand, displayed higher expression levels in both \textit{xiw1-1} and \textit{abi5-8} compared to Col-0 after ABA treatment (Figure 4E and Supplemental Data Sets 2). Taken together, these results indicate that the \textit{xiw1} and \textit{abi5} mutations induce similar changes in the transcriptome, both in terms of the number of altered transcripts and the amplitude of ABA-regulated transcription levels.

\textbf{XIW1 regulates ABA responses in ABI5 dependent manner}

Notably, ABI5 transcripts were lower in the \textit{xiw1} plants than the Col-0 wild type in the presence of ABA (Figure 4A). Furthermore, immunoblot analysis showed that the abundance of ABI5 protein in \textit{xiw1-1} plants was lesser than Col-0 upon ABA treatment (Figure 5A), suggesting that the mRNA and protein expression levels of ABI5 were regulated by XIW1.

ABI5 is reported to be a key regulator in ABA signaling that controls seed germination and seedling development (Brocard et al., 2002; Nakashima and Yamaguchi-Shinozaki, 2013). To investigate whether XIW1 regulates ABA response depending on ABI5, we crossed \textit{ABI5}-overexpressing plants (\textit{ABI5-OE}) with \textit{xiw1-1}. Compared to \textit{xiw1-1}, the crossed plants \textit{ABI5-OE} \textit{xiw1-1} showed increased ABA sensitivity and decreased seed germination rate and cotyledon greening (Figure 5B and 5C). Next, we overexpressed \textit{XIWI} (\textit{XIWI-OE}) in the
abi5-8 backgrounds plants. In the presence of ABA, XIW1-OE plants showed higher ABI5 expression and lower seed germination and cotyledon greening rate than Col-0 (Figures 5C and 5D, Supplemental Figure 8B). XIW1-OE abi5-8 plants, however, exhibited an ABA-insensitive phenotype (Figures 5D and 5E). These findings indicate that XIW1 regulates ABA-mediated seed germination in an ABI5-dependent manner.

**ABA facilitates the nuclear retention of XIW1**

XIW1 is a nucleocytoplasmic protein that localizes mainly in the cytoplasm under normal plant growth conditions (Figure 6A and 6B), while ABI5 functions in the nucleus. To understand how XIW1 participates in the ABA response, we investigated the nucleocytoplasmic trafficking of XIW1. In the presence of ABA, XIW1-GFP accumulated in the nucleus both in *N. benthamiana* transiently expressing XIW1-GFP and in the *XIW1-GFP/xiw1* transgenic plants (Figure 6A and 6B). We next isolated the nuclei and the cytoplasm of *XIW1-GFP/xiw1* seedlings to analyze the nucleocytoplasmic partitioning of XIW1. Immunoblot analysis showed that ABA treatment increases the relative protein abundance of XIW1 in the nucleus (Figure 6C). Similarly, exogenous ABA facilitated the accumulation of XIW1 in the nucleus of stomata cells of the *XIW1-GFP/xiw1* transgenic leaves (Figure 6C).

Since ABA is important for plant responses to various environmental stresses, we tested the effects of environmental stresses on the nucleocytoplasmic shuttling of XIW1. We found that abiotic stresses such as salt stress (NaCl), osmotic stress (mannitol) and oxidative stress (H$_2$O$_2$) also promoted the nuclear accumulation of XIW1 (Supplemental Figure 9). The data indicate that the nuclear retention of XIW1 by ABA may be a common mechanism for plants to respond to these abiotic stresses.

To understand the potential physiological function of XIW1 retention in the nucleus, we examined ABA sensitivity and drought stress resistance using *XIW1mNES/xiw1* transgenic plants, where XIW1 was confirmed to be predominantly localized to the nucleus (Figure 1G).
In the presence of ABA, the \( \text{XIW1}^{mNES} / \text{xiw1} \) seeds exhibited lower cotyledon greening rates than Col-0 (Figures 6E and 6F). Drought stress assays showed that increased expression of XIW1 in the nucleus enhanced drought stress resistance (Figures 6G).

**XIW1 interacts with ABI5 and promotes its stability**

Since XIW1 affects ABI5 protein abundance, and both XIW1 and ABI5 are located in the nucleus in the presence of ABA, we wondered if there is a direct interaction between XIW1 and ABI5. Yeast two-hybrid assays showed that XIW1 directly interacts with ABI5 through its WD40 domains (Figure 7A). Additionally, glutathione S-transferase (GST) pull-down assays showed that ABI5-His was precipitated by XIW1-GST (Figure 7B), indicating a physical interaction between XIW1 and ABI5 \textit{in vitro}. The interaction was also supported by the BiFC assays in \textit{N. benthamiana}, which showed that interaction occurs predominantly in the nucleus in the presence of ABA (Figure 7C). Co-IP assays indicated that ABI5 was co-precipitated by anti-GFP antibodies using \textit{XIW1-GFP/xiw1} transgenic plants pretreated with ABA (Figure 7D).

Previous reports have shown that ABI5 is degraded via the ubiquitin-26S proteasome pathway, and that several WD40 proteins are involved in this process (Seo et al., 2014; Yu et al., 2015). We, therefore, were interested to know if XIW1 affects ABI5 degradation. For this experiment, we treated Col-0 and \textit{xiw1-1} seeds with 5 mM ABA for 3 d, washed out the ABA, and grew the seeds on filter paper soaked with the protein synthesis inhibitor cycloheximide (CHX). The ABI5 protein levels in Col-0 and \textit{xiw1-1} were reduced after the removal of ABA. Compared to Col-0, ABI5 degradation was faster in \textit{xiw1-1} at the indicated time points (Figure 7E). We also treated the seeds with CHX and the proteasome inhibitor MG132 during the ABA washout. Immunoblot analysis showed that ABI5 abundance did not significantly decrease in Col-0 and \textit{xiw1-1} after ABA removal (Figure 7E), indicating that ABI5 degradation is dependent on the 26S proteasome. Furthermore, the cell-free degradation assay showed that ABI5 degradation in \textit{xiw1-1} was accelerated compared to its degradation in
Col-0, and that this degradation was inhibited in the presence of MG132 (Figure 7F). Taken together, these data support the hypothesis that XIW1 promotes ABI5 stability by negatively regulating the proteasomal degradation of ABI5.

**DISCUSSION**

In plants, WD40 proteins are abundant, interact with diverse proteins, and participate in a variety of biological processes, including anthocyanin biosynthesis, cell wall formation, plant development, and immunity (van Nocker and Ludwig, 2003; Xu and Min, 2011; Guerriero et al., 2015; Miler et al., 2016). In addition, many WD40 proteins have been reported to be involved in stress responses through the negative regulation of ABA signaling in *Arabidopsis*. For example, *Arabidopsis* RACK1 (receptor for activated C kinase 1) is a WD40 protein that mediates multiple hormonal responses and developmental processes. Loss of function of RACK1 results in hypersensitivity to ABA and salt stress during seed germination (Chen et al., 2006; Guo et al., 2009). *Arabidopsis* MSI1 (multicopy suppressor of *ira* 1) belongs to a family of histone-binding WD40-repeat proteins. MSI1 associates with HDA19 (histone deacetylase 19) and forms a complex that binds to the chromatin of ABA receptor genes and fine-tunes ABA signaling and drought stress resistance through repressing the expression of the ABA receptor genes (Alexandre et al., 2009; Mehdi et al., 2016).

XIW1 is a typical WD40 domain-containing protein (Figure 2). Interestingly, unlike most of the reported WD40 proteins which are characterized as negative regulators of ABA signaling, we found that XIW1 positively regulates ABA responses. A loss of function *xiw1* mutant displayed an insensitive phenotype to ABA during seed germination and decreased drought stress resistance (Figure 3). As a key regulator in ABA response, ABI5 plays critical roles in various ABA-dependent biological processes, such as seed dormancy and germination, plant vegetative growth and development, and plant stress responses (Brocard et al., 2002; Nakashima and Yamaguchi-Shinozaki, 2013; Skubacz et al., 2016). In this study,
we found that the loss of XIW1 leads to decreased ABI5 transcript and protein levels (Figure 4A and 5A). Transcriptome data revealed that the \textit{xiw1} and \textit{abi5} mutations induce similar changes in response to ABA (Figure 4B-4E). Genetic analysis showed that XIW1 regulates ABA-mediated seed germination in an ABI5-dependent manner (Figure 5B-5E). Based on these results, we conclude that the positive regulation of ABA responses by XIW1 is mediated, at least in part, by ABI5.

XIW1 mainly localizes in the cytosol under normal conditions, while ABI5 functions in the nucleus. How XIW1 affects ABI5 expression remains unknown. XIW1 was identified as a substrate of XPO1A, a nuclear transport receptor (Figure 1), implying that XIW1 might be a nucleocytoplasmic shuttling protein. In plants, regulation of protein nucleocytoplasmic trafficking by hormones and environmental stresses is widely reported, i.e. salt stress stimulates while ethylene suppresses the nuclear export of COP1, thereby controlling seed germination via COP1-mediated downregulation of HY5 and ABI5 (Yu et al., 2016); cold stress activates the phosphorylation of 14-3-3 proteins by CRPK1 (cold-responsive protein kinase 1) and facilitates their nuclear import, thus negatively regulating freezing tolerance (Liu et al., 2017). In this study, we found that the nucleocytoplasmic partitioning of XIW1 is regulated by ABA and abiotic stresses. Both exogenous ABA and oxidative stresses promoted the rapid accumulation of XIW1 in the nucleus (Figure 6, Supplemental Figure 9). In \textit{Saccharomyces cerevisiae}, oxidative stress-induced disulfide bond formation and masking the NES of a protein by conformational changes is an important mechanism for protein accumulation in the nucleus (Kuge et al., 2001; Wood et al., 2004). To test whether a similar mechanism exists in plants, we mutated all seven cysteine residues in the XIW1 protein and transiently expressed the mutated plasmids in \textit{N. benthamiana}. This cysteine-free version of XIW1, nevertheless, retained its nuclear localization ability when treated with ABA (Supplemental Figure 10). Interestingly, we found that mutation in Cys260 results in constitutive accumulation of XIW1 in the nucleus (Supplemental Figure 10), which may imply a potential mechanism for the nuclear retention of XIW1.
Although we found that loss of *XIWI* affects *ABI5* transcription levels upon ABA treatment (Figure 4A), lower ABI5 protein levels in *xiw1* by ABA cannot be explained exclusively by transcriptional regulation, as we also found that XIW1 directly affects the degradation rate of ABI5 (Figure 7E and 7F). Moreover, previous reports have described that the ABI5 protein is necessary for the proper accumulation of the *ABI5* transcript due to autoregulation of this gene (Brocard et al., 2002; Seo et al., 2014). Our results also revealed that XIW1 interacts with ABI5 predominantly in the nucleus in the presence of ABA (Figure 7A-D). Since ABI5 is a member of the bZIP/ABRE family, we tested the interactions between XIW1 and several related TFs in this family (Kim et al., 2002), including ABA responsive element binding factor 1 (ABF1), ABF2/AREB1, ABF3 and ABF4/AREB2. Yeast two-hybrid assays showed no obvious interactions (Supplemental Figure 11), suggesting that XIW1 may specifically act on ABI5 to regulate ABA response.

In *Arabidopsis*, ABI5 stability and activity is regulated by its degradation via the ubiquitin-26S proteasome pathway (Yu et al., 2015). Several proteins are involved in ABI5 degradation. The RING-type E3 ligase KEEP ON GOING (KEG) is responsible for the cytoplasmic degradation of ABI5 in the absence of ABA (Stone et al., 2006; Liu and Stone, 2013). Whereas, a group of DCAF (DDB1-CUL4 associated factor) proteins, i.e. ABD1/DCAF1, DWA1, DWA2, DWA3, and ASG2 acting as substrate-recognition receptors for CULLIN4-based ubiquitin ligases, are involved in ABI5 degradation in the nucleus (Zhang et al., 2008; Lee et al., 2010; Lee et al., 2011; Seo et al., 2014; Dutilleul et al., 2016). Besides, post-translational modifications of ABI5 such as phosphorylation, ubiquitination, sumoylation, and nitrosylation have effects on ABI5 stability (Yu et al., 2015). For example, the S-nitrosylation of ABI5 at cysteine-153 facilitates its degradation via CULLIN4 and KEG E3 ubiquitin ligases (Albertos et al., 2015). Our findings provide new insight into the regulation of ABI5 stability. In the absence of ABA, *XIWI* has low expression, and the XIW1 protein is mainly accumulated in the cytosol, maintaining normal ABI5 levels. In the presence of ABA, *XIWI* is induced and the XIW1 protein mainly accumulates in the nucleus,
where it interacts with ABI5 and protects it from proteasomal degradation, thereby promoting ABA responses (Figure 7G). Interestingly, both XIW1 and DCAF proteins belong to the family of WD40 domain-containing proteins, even though they play opposite roles in the regulation of ABI5 stability. How XIW1 and DCAF proteins coordinately regulate the stability of ABI5 upon the integration of environmental stimuli still needs to be addressed.
METHODS

Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Col-0 was used as the wild-type. The T-DNA insertion mutants *xpo1a-3* (SALK_078639), *xiw1-1* (SALK_075873), and *abi5-8* (SALK_013163) were obtained from the ABRC (http://www.arabidopsis.org). The genotyping primers were used as described in Supplemental Table 2. Seeds were stratified for at least 2 d at 4°C and germinated at 22°C in a standard growth chamber with a 14-h/10-h light/dark cycle. 10-d-seedlings grown on 1/2 MS plates were transplanted into the soil in a phytochamber with a 16-h/8-h light/dark cycle at 22°C. The *XPO1A-MYC XIW1-GFP*, *ABI5-OE xiw1-1*, and *XIWI-OE abi5-8* plants were generated by crossing, and homozygous lines were identified and used for Co-IP or genetic analyses.

Plasmid construction and plant transformation

The complemented plasmids were constructed in Gateway-compatible vectors system (Earley et al., 2006). To generate the complemented lines of *xpo1a-3*, genomic DNA of XPO1A containing native promoter fragment was amplified and cloned into the pENTR-TOPO entry vector (Invitrogen), and then recombined into the pEarleyGate 302 vector to form *XPO1Apro:XPO1A-MYC* plasmid. The constructs were introduced into *Agrobacterium* strain GV3101 for *xpo1a-3* transformation using the floral dip method (Clough and Bent, 1998). To complement *xiw1-1*, *XIWI* coding region was amplified and cloned into the pENTR-TOPO vector, subsequently recombined into the pEarleyGate 103 vector to generate *35S:XIWI-GFP*. The constructs were transformed into *xiw1-1* to generate complemented transgenic plants (*XIWI-GFP/xiw1*). The *35S:XIWI-GFP* plasmids were also transformed into Col-0 to generate *XIWI* over-expressing transgenic plants (*XIWI-OE*). The *35S:XIW mNES-GFP* plasmids (carrying L323A and I325A mutations in XIW1) were transformed into *xiw1-1* to generate NES mutation plants (*XIWmNES-GFP/xiw1*). For histochemical analysis of XIW1, a
1627 bp promoter fragment of \textit{XIW1} was amplified from Col-0 genomic DNA, and then cloned into GUS expression vector pBI101 (Clontech). The \textit{XIW1pro:GUS} construct was transformed into Col-0. All of the primers and cloning sites for vector construction are listed in Supplemental Table 2.

\textbf{Seed germination and root length assays}

Seeds were germinated and grown on 1/2 MS medium with or without 0.5 $\mu$M ABA (Sigma-Aldrich), if not otherwise indicated in the text. The percentage of germinated seeds and green cotyledons were recorded, and the seedlings were photographed at the specified time points. 3-d-seedlings were transferred onto fresh MS plates with or without 10 $\mu$M ABA and grown for an additional 7 d for root length assays.

\textbf{Drought stress and water loss analysis}

For drought stress treatment, 3-week-old seedlings were treated by withholding watering for 12 d, and then the plants were tested for survival rates and photographed at 3 d after rewaterning. For water loss analysis, ten rosette leaves were detached from 4-week-old seedlings and weighed at the indicated times.

\textbf{GUS histochemical analysis}

The T3 transgenic seedlings or tissues were harvested and incubated in freshly prepared buffer containing 5-bromo-4-chloro-3-indolyl-$\beta$-D-glucuronic acid for 4 h at 37°C in the dark, and then washed with 70% ethanol. Three independent transgenic lines were analyzed and the representative images were photographed.

\textbf{Gene expression analysis and RNA-sequencing}

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen), and then digested with DNase I to eliminate genomic DNA contamination. 1 $\mu$g RNA was used for reverse
transcription with a SuperScript first-strand synthesis system (Invitrogen). Transcript levels of selected genes were detected by SYBR Green Supermix according to the manufacturer’s instructions (Bio-Rad). *Arabidopsis ACTIN2* gene was used as an internal control to normalize different samples. Primer sequences used for qRT-PCR are given in Supplemental Table 2.

10-day-old Col-0, *xiw1-1* and *abi5-8* seedlings treated with or without 50 µM ABA for 4 h were used for the RNA-sequencing analysis. Total RNA was isolated using TRIzol reagent (Life Technologies) and treated with DNase I (Amersham). RNA sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina (NEB) according to manufacturer’s instruction. The libraries were sequenced on a flow cell using an Illumina HiSeq4000 sequencing platform. FPKM values (Reads Per Kilobase of exon model per Million mapped reads) were used for the estimation of gene expression levels. DESeq package (Anders and Huber, 2010) was used to evaluate differentially expressed genes (DEGs) between Col-0, *xiw1-1* and *abi5-8* seedlings. The DEGs between different samples were restricted with FDR <0.01 and the absolute value of log2 Ratio ≥ 1. Three biological replicates were used for each sample. All the data about gene expression profiles were submitted to Gene Expression Omnibus, NCBI (https://www.ncbi.nlm.nih.gov/sra/SRP155277).

**Transient expression and GFP Fluorescence Assay**

For transient expression, the *XIWI* coding region was amplified and cloned into pCAMBIA1300-GFP driven by the 35S promoter, and the plasmids were transformed into epidermal *N. benthamiana*. The GFP signal was visualized under confocal microscope (Leica sp5, Germany) after 2 days of infiltration. For assays of the nucleocytoplasmic partitioning of XIWI, the transformed *N. benthamiana* leaves were immersed in the solutions with 40 nm LMB, 100 µM ABA, 150 mM NaCl, 300 mM mannitol, and 0.3% H2O2 for 4 h. Similarly, 5-day-old *XIWI-GFP/xiw1* transgenic plants were treated by the exogenous reagents as
described above for 4 h, and GFP signal in root or hypocotyl cells was visualized using confocal microscopy.

**IP/MS and Co-IP analysis**

As XPO1A is ubiquitously expressed and has a higher expression in the inflorescence (Supplemental Figure 1A), 2-week-old seedlings or floral tissues of *XPO1Apro:XPO1A-MYC/xpo1a-3* transgenic plants under normal growth conditions were used for IP analysis. Proteins were isolated with the extraction buffer containing 50 mM Tris pH 7.6, 150 mM NaCl, 5 mM MgCl$_2$, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 1 mM PMSF, and 1x protease inhibitor cocktail (Roche). The dynabeads conjugated with MYC antibody (Millipore) were used for immunoprecipitation. Affinity purification and LC-MS/MS analysis were performed as described by Lang et al. (2015). *XPO1A-MYC* and *XIWI-GFP* crossed plants or *XIWI-GFP* plants were used for the Co-IP assay of XPO1A-XIWI and XIW1-ABI5, respectively. The agarose beads conjugated with GFP antibody (Sigma-Aldrich) were used to immunoprecipitate XIW1, and the complex was detected by anti-MYC and anti-ABI5 (Agrisera) antibodies, respectively.

**Yeast Two-Hybrid Assay**

*Saccharomyces cerevisiae* strain Y2HGold (Clontech) was used for co-transformation of the AD and BD constructs. Full-length or truncated XIW1 was cloned into pGAD-T7 vector to generate AD-XIW1 plasmid, and full-length of XPO1A and ABI5 were cloned into pGBK-T7 to generate BD-XPO1A and BD-ABI5 plasmids, respectively. A series of diluted co-transformed Y2H Gold culture was spotted onto SD plates lacking Trp and Leu or lacking Trp, Leu, His and Ade, and incubated at 30°C for 3 days to observe yeast growth. All of the primers and cloning sites for construction are listed in Supplemental Table 2.

**BiFC**
The coding region of XPO1A and ABI5 were cloned into the binary BiFC vectors pSPYNE173 to produce the XPO1A-YFP\(^{N}\) and ABI5-YFP\(^{N}\) plasmids, respectively. The coding region of XIW1 was cloned into pSPYCE (M) to produce the XIW1-YFP\(^{C}\). Constructs including empty vectors were introduced into the *Agrobacterium* strain GV3101 and then infiltrated on *N. benthamiana* leaves. YFP signals were observed using confocal microscopy after 2 days of infiltration. All of the primers for construction are listed in Supplemental Table 2.

**GST-pull down**

The coding region of XIW1 was cloned into pGEX-4T-1 to obtain XIW1-GST recombinant protein, and the coding region of ABI5 was cloned into pET28a to obtain ABI5-His recombinant protein. Glutathione beads containing XIW1-GST were incubated with Fd-His or ABI5-His proteins in 1×PBS buffer at 4 °C for 2 h. The beads were washed 5 times with wash buffer (1x PBS, 0.1% Triton X-100). Proteins retained on the beads were analyzed by immunoblot with an anti-GST or anti-His antibody.

**Cell Fractionation Assays**

10-day-old *XIWI1-GFP/xiw1* transgenic plants were treated with or without 50 µM ABA for 4 h for cell fractionation assays. Preparation of nuclear and cytoplasmic fractions was performed using CelLytic PN Isolation/Extraction kit according to the manufacturer’s instructions (Sigma). Briefly, 1 g leaves were ground to a fine powder and exacted in 2 ml Nuclei Isolation Buffer (NIA buffer, 50 mM Tris pH 8.0, 2 mM EDTA, 20% glycerol, 1 mM DTT, 0.1% Triton X-100, and 1 x protease inhibitor cocktail). Homogenate was filtered and then centrifuged for 10 min at 1,260 g. The pellet was resuspended in 1 ml NIA buffer with 1 x Protease Inhibitor Cocktail and 0.2% Triton X-100. 0.5 ml lysate was saved as total protein (T), and 0.5 ml lysate was further centrifuged for 10 min at 12,000 g. The supernatant was saved as a cytoplasmic protein (C), and the pellet was resuspended with 0.5 ml Nuclei PURE
Storage Buffer and saved as a nuclear protein (N). XIW1 protein was detected using an anti-GFP antibody. Anti-cFBPase (Agrisera) and anti-H3 antibodies (Agrisera) were used as cytosolic and nuclear markers, respectively. Image J software was used for the quantification of bands in the immunoblot analysis.

**Protein degradation assays**

For *in vivo* ABI5 protein level assays (Figure 5A), 7-d-old seedlings of Col-0 and *xiw1* were cultured for 4 h in liquid MS medium containing 50 µM ABA with or without addition of 50 µM MG132. *In vivo* ABI5 degradation assays were performed according to Seo et al., (2014) with some modifications. Briefly, 2-d germinated Col-0 and *xiw1-1* seeds were treated with 5 µM ABA in liquid MS medium for 3 d. The seeds were washed 3 times with liquid MS medium containing 300 µM CHX or 300 µM CHX and 50 µM MG132 and then grown on filter paper. Samples were harvested for protein extraction at the indicated time points. ABI5 was detected with anti-ABI5 antibodies. Actin was used as a control. *In vitro* cell-free protein degradation assay was performed as described (Kong et al., 2015). Purified ABI5-His recombinant proteins were incubated with total proteins extracted from Col-0 and *xiw1-1* plants in the presence of 1 mM ATP at 25 °C for different times. The anti-His antibody was used to detect the ABI5 protein level by immunoblot analysis. Actin protein was used as a loading control.

**SUPPLEMENTAL INFORMATION**

Supplementary information includes 11 figures, 2 tables and 2 data sets.

**AUTHOR CONTRIBUTIONS**

G.Z. and J.K.Z. conceived and designed this work. X.X., W.W. and G.J. performed most of
the experiments; Y.X., H.H., J.C. and Y.C. contributed to the molecular cloning and transformation work; C.G.D., S.K.M., and X.P. oversaw the entire study. G.Z. and J.K.Z. wrote the manuscript.

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REFERENCES


Figure legends

Figure 1. XIW1 Directly Interacts with XPO1A and Is Nuclear Exported by XPO1.

(A) Mass spectrometry to identify XPO1A co-precipitated proteins. MYC-tagged XPO1A transgenic plants were used for co-immunoprecipitation by anti-MYC. Proteins were detected by LC-MS/MS.

(B) In vivo co-IP assay of XPO1A and XIW1. Transgenic Arabidopsis plants of MYC-tagged XPO1A crossed with GFP-tagged XIW1 were used to detect the interaction between XPO1A and XIW1. The anti-GFP affinity matrix was used for immunoprecipitation and anti-MYC was used for immunoblot analysis.

(C) Yeast two-hybrid assay of the interactions between XPO1A and XIW1. The XPO1A protein is fused to the GAL4 binding domain (BD), the XIW1 protein and its truncated or NES-mutated proteins are fused to GAL4 activation domain (AD). WD represents the WD40 domain indicated in Figure 2A and Supplemental Figure 4. NES, nuclear export signal.

(D) Confirmation of positive interactions by BiFC assay. XPO1A-EYFP_N and XIW1-EYFP_C fusion proteins were co-expressed in N. benthamiana, and YFP was imaged after 2 d of infiltration. Scale bar, 50 µm.

(E and F) Nuclear export of XIW1 is inhibited by LMB. 35S:XIW1-GFP and 35S:XIW1^{mNES}-GFP plasmids were transiently expressed in N. benthamiana for 2 d. The transformed N. benthamiana leaves (E) or 5-d-old XIW1-GFP/xiw1 transgenic seedlings (F) were incubated with or without 40 nm LMB for 4 h, respectively. The GFP signals in N. benthamiana epidermis cells or Arabidopsis root tip cells were observed by confocal microscopy. DAPI staining was used as a nuclear marker. Scale bar, 50 µm.

(G) Point mutations in NES facilitate nuclear retention of XIW1. The GFP signals in XIW1-GFP/xiw1 and XIW1^{mNES}-GFP/xiw1 hypocotyl cells were observed by confocal microscopy. Scale bar, 50 µm.
Figure 2. Schematic Structure and Expression Pattern of XIW1.

(A) Protein structure of XIW1 depicting the predicted WD40 region. The WD40 domains were predicted using the SMART motif search program (http://smart.embl-heidelberg.de/). NES, nuclear export signal

(B) The expression of XIW1 in various organs was analyzed by quantitative RT-PCR. R, root; YL, young leaf; St, stem; RL, rosetta leaf; I, inflorescence; Si, silique.

(C) Histochemical analysis of XIW1. The GUS gene driven by XIW1 promoter was expressed in germinating seed (a), 2-d-old seedlings (b), 4-d-old seedlings (c), 14-d-old plants (d), inflorescence (e), pistil (f), stem (g) and silique (h). Scale bars = 0.2 mm (a,b,f) and 1 mm (c,d,e,g,h).

(D) Quantitative RT-PCR analysis of XIW1 expression in germinating Col-0 wild-type seeds (Left) or in the presence of 0.5 μM ABA for 3 d (Right).

In (B) and (D), data are means of three biological replicates ±SD, and the asterisk in (D) indicates a significant difference between the absence and presence of ABA treatment (Student’s t-test, P<0.01).

Figure 3. XIW1 Regulates Seed Germination, Seedling Growth, and Drought Stress Resistance.

(A-C) The seed germination rate and the cotyledon greening rate of Col-0, xiw1-1, and xiw1 complemented lines in response to ABA. The germination rate (A) was calculated in the absence or presence of 0.5 μM ABA for 7 d, seedlings were photographed (B) and the greening rate was scored (C) at 5 d.

(D and E) Post-germination seedling growth of Col-0, xiw1-1 and xiw1 complemented lines in response to ABA. Seeds were germinated for 3 d and then transferred to medium containing 10 μM ABA. Primary root length (D) was counted and photographs (E) were taken at 7 d after transfer.

(F) Drought resistance assays of Col-0, xiw1-1 and xiw1 complemented lines. 3-week-old
plants were subjected to soil drought stress by withholding water for 12 d. The plants were photographed after re-watering for 3 d.

(G) The survival rate after drought stress as described in (F).

(H) Relative water loss in Col-0 and xiw1-1. 4-week-old plants were used for water loss assay in detached leaves.

In (A), (C), (D), (G) and (H), data are means of three biological replicates ±SD (n = 60 for A and C, n = 10 for D and H, n = 27 for G), and the asterisks indicate a significant difference between the mutants and wild-type Col-0 receiving the same treatment (Student’s t-test, P<0.01).

Figure 4. Effects of the xiw1 mutations on the global gene expression profile.

(A) Expression of ABA-responsive genes in Col-0 and xiw1 seedlings. 10-d-old Col-0 and xiw1-1 seedlings were transferred to liquid MS medium with or without 50 µM ABA for 4 h. mRNA levels were determined by quantitative RT-PCR analysis. The results were obtained from three replicates and the values represent means ±SD. Asterisks indicate a significant difference compared to Col-0 wild type (Student’s t-test, P < 0.01).

(B) The transcriptomes of the indicated genotypes were analyzed by RNA sequencing. The six comparisons of the transcriptome data of each genotype (arrows) identified different numbers of DEGs. 10-d-old Col-0, xiw1-1, and abi5-8 seedlings under mock conditions or 4 h of 50 µM ABA treatment were subjected to RNA sequencing analysis. The results are shown from three biological replicates.

(C) Venn diagram shows the shared and specific DEGs between xiw1 and abi5 under mock conditions and ABA treatment. Red represents the DEGs between xiw1 and Col-0, blue represents abi5 and Col-0.

(D) Comparison of global transcriptional response in the presence of ABA in Col-0, xiw1 and abi5 seedlings. All changes are given as log2-fold values compared with mock conditions (no ABA treatment). Blue represents a non-significant change, while a significant change (Fold
Change ≥ 2, FDR < 0.01) appears in red. The number of up-regulated and down-regulated genes is given for each genotype.

(E) Differential expression patterns for the shared up-regulated genes induced by ABA in Col-0, xiw1 and abi5. Group I: genes with lower expressions in xiw1-1 and abi5-8 than Col-0 after ABA treatment; Group II: genes with higher expressions in xiw1-1 and abi5-8 than Col-0 after ABA treatment.

**Figure 5. XIW1 Regulates ABA Responses Depending on ABI5.**

(A) Immunoblot analysis of ABI5 protein levels in Col-0 and xiw1 plants. 7-d-old seedlings were treated with 50 µM ABA or 50 µM ABA plus 50 µM MG132 for 4 h. Total proteins were extracted for western blot analysis. Actin protein was used as a loading control.

(B and C) Overexpressing ABI5 suppresses xiw1 ABA-insensitive phenotype. Seeds of Col-0, xiw1-1, ABI5-OE and ABI5-OE xiw1-1 were plated on 1/2 MS medium containing 0.5 µM ABA for 7 d (B). Germination rate was calculated at 4 d after imbibition, the greening rate was scored at 7 d (C). Data in (C) are means of three biological replicates ±SD (n = 50). Asterisks indicate a significant difference compared to xiw1-1 (Student’s t-test, P < 0.01).

(D and E) abi5 mutation reduced ABA sensitivity in XIW1 overexpressing plants. Seeds of Col-0, abi5, XIW1-OE and XIW-OE abi5 were plated on 1/2 MS medium containing 0.5 µM ABA for 7 d (D). Germination rate was calculated at 4 d after imbibition, the greening rate was scored at 7 d (E). Data in (E) are means of three biological replicates ±SD (n = 50). Asterisks indicate a significant difference compared to XIW1-OE plants (Student’s t-test, P < 0.01).

**Figure 6. Effects of ABA on the Nucleocytoplasmic Partitioning of XIW1.**

(A and B) Effects of ABA on the nucleocytoplasmic partitioning of XIW1 in N. benthamiana (A) and XIW1-GFP/xiw1-1 transgenic plants (B). The N. benthamiana leaves transiently expressing 35S:XIW1-GFP and 5-d-old XIW1-GFP/xiw1 transgenic plants were immersed in
liquid MS medium with or without 100 µM ABA for 4 h before the fluorescence detection. GFP signals were observed by confocal microscopy. Scale bar, 50 µm.

(C) Immunoblot analysis of XIW1 protein in the presence of ABA treatment. 7-d-old XIWI-GFP/xiw1 transgenic plants were treated with or without 100 µM ABA for 4 h. Total (T), nuclear (N) and cytosol (C) proteins were separated by Celllytic PN Isolation/Extraction kit (Sigma), and XIW1 proteins were detected by immunoblot analysis with an anti-GFP antibody. Anti-cFBPase and anti-H3 antibodies were used as cytosolic and nuclear markers, respectively. The protein levels were quantified using Image J software (National Institutes of Health). The ratios of total protein anti-GFP/anti-H3 or anti-GFP/anti-cFBPase values were set to 1.0.

(D) Effects of ABA on the subcellular localization of XIW1 in the stomatal cells of XIWI-GFP/xiw1 transgenic leaves. 5-d-old transgenic plants were treated with 40 nm LMB, 100 µM ABA and 150 mM NaCl for 4 h, respectively. GFP signals were observed by confocal microscopy. Scale bar, 10µm.

(E and F) Overexpressing XIW1 in the nucleus enhanced ABA sensitivity during seed germination. Col-0, xiw1-1 and XIWI<sub>mNES</sub>/xiw1 transgenic plant seeds were plated on 1/2 MS medium containing 0.5 µM ABA for 5 d (E). The greening rate was scored at 5 d (F). Data in (F) are means of three biological replicates ±SD. Asterisks indicate a significant difference compared to Col-0 (Student’s t test, P < 0.01).

(G) Overexpressing XIW1 in the nucleus enhanced drought stress resistance. 3-week-old Col-0 and XIWI<sub>mNES</sub>/xiw1 transgenic plants were subjected to soil drought stress by withholding water for 18 d. The plants were photographed after re-watering for 5 d.

**Figure 7. XIW1 Interacts with ABI5 and Maintains ABI5 Stability.**

(A) Yeast two-hybrid assay of the interactions between XIW1 and ABI5. The ABI5 protein is fused to the GAL4 binding domain, the XIW1 protein and its truncated proteins are fused to the GAL4 activation domain. WD represents the WD40 domain indicated in Figure 2A.
(B) GST pull-down assay of XIW1 and ABI5. Fd-His, ABI5-His and XIW1-GST were expressed in *E. coli*. Purified proteins were used for the pull-down assay. Fd-His and ABI5-His proteins were detected with anti-His antibodies, and XIW1-GST protein was detected with anti-GST antibodies. Fd (Ferredoxin) protein was used as a control.

(C) BiFC assay of the interactions between XIW1 and ABI5. *N. benthamiana* leaves co-expressing ABI5-EYFP^N^ and XIW1-EYFP^C^ fusion protein were cultured for 2 d, and then treated with or without 50 µM ABA for another 4 h. YFP signals were observed by confocal microscope. DAPI staining was used as a nuclear marker. Scale bar, 50µm.

(D) Co-IP assay of XIW1 and ABI5. 10-d-old Col-0 and *XIW1-GFP/xiw1* transgenic plants were treated with 50 µM ABA for 4 h. Anti-GFP antibodies were used for immunoprecipitation, and anti-ABI5 antibodies were used for immunoblot analysis.

(E) *In vivo* ABI5 degradation assays. Col-0 and *xiw1-1* seeds were treated with 5 µM ABA for 3 d, washed out the ABA with liquid MS medium containing 300 µM CHX (top) or 300 µM CHX and 50 µM MG132 (bottom). Samples were harvested for protein extraction at indicated time points. ABI5 was detected with an anti-ABI5 antibody. Actin was used as a control.

(F) *In vitro* cell-free ABI5 degradation assays. Recombinant purified ABI5- His was incubated with total protein extracted from Col-0 and *xiw1* mutant treated with or without 50 µM MG132. ABI5 was detected with an anti-His antibody. Actin was used as a control.

(G) Model for XIW1 positively regulating ABA response. XIW1, a nucleocytoplasmic protein is exported from the nucleus in an XPO1-dependent manner. XIW1 mainly localizes in the cytosol in basal conditions, maintaining normal ABI5 levels. In the presence of ABA, XIW1 is induced and the XIW1 protein accumulates in the nucleus, where it interacts with ABI5 and protects it from proteasomal degradation, thereby promoting ABA responses.
A

<table>
<thead>
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<th>kDa</th>
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<tr>
<td>50</td>
<td>a-ABi5</td>
<td>a-ABi5</td>
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<tr>
<td>45</td>
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B

Col-0 ABI5-OE xiw1-1

ABI5-OE xiw1-1

MS 0.5 μM ABA

C

Seed germination %

MS 0.5μM ABA

Green rate %

MS 0.5μM ABA

D

Col-0 XIW1-OE abi5-8

abi5-8 XIW1-OE

MS 0.5 μM ABA

E

Seed germination %

MS 0.5μM ABA

Green rate %

MS 0.5μM ABA
A) XIW1-GFP, DAPI, Merged, Bright images for -ABA and +ABA conditions.

B) XIW1-GFP, DAPI, Merged, Bright images for -ABA and +ABA conditions.

C) Western blot analysis for -ABA and +ABA conditions, showing a-GFP, a-cFBPase, and a-H3 bands.

D) XIW1-GFP images under different treatments: CK, LMB, ABA, NaCl.

E) Phenotypic assays for Col-0, xiw1-1, and XIW1nNES lines under -ABA and +ABA conditions.

F) Graph showing green rate% for -ABA and +ABA conditions.

G) Before and after re-watering assays for Col-0, XIW1nNES #1, #2, and XIW1nNES #1 lines.