Phytocystatins (PhyCYSs) are plant-specific proteinaceous inhibitors that are implicated in protein turnover and stress responses. Here, we characterized a PhyCYS from Arabidopsis thaliana, which was designated AtCYS5. RT-qPCR analysis showed that the expression of AtCYS5 in germinating seeds was induced by heat stress (HS) and exogenous abscisic acid (ABA) treatment. Analysis of the expression of the β-glucuronidase reporter gene under the control of the AtCYS5 promoter showed that AtCYS5 expression during seed germination was induced by HS and ABA. Constitutive overexpression of AtCYS5 driven by the cauliflower mosaic virus 35S promoter led to enhanced HS tolerance in transgenic Arabidopsis, which was characterized by higher fresh weight and root length compared to wild-type (WT) and knockout (cys5) plants grown under HS conditions. The HS tolerance of AtCYS5-overexpressing transgenic plants was associated with increased insensitivity to exogenous ABA during both seed germination and post-germination compared to WT and cys5. Although no HS elements were identified in the 5′-flanking region of AtCYS5, canonical ABA-responsive elements (ABREs) were detected. AtCYS5 was upregulated in ABA-treated protoplasts transiently co-expressing this gene and genes encoding bZIP ABRE-binding factors (ABFs and AREB3). In the absence of ABA, ABF1 and ABF3 directly bound to the ABREs in the AtCYS5 promoter, which activated the transcription of this gene in the presence of ABA. These results suggest that an ABA-dependent pathway plays a positive role in the HS-responsive expression of AtCYS5 during seed germination and post-germination growth.

Keywords: abscisic acid, cis-element, gene expression, transcription factor, transgenic plants

INTRODUCTION

Plant cystatins, or phytocystatins (PhyCYSs), are proteinaceous inhibitors of the papain-like (C1A) and legumain (C13) families of plant cysteine proteases (CPs) (MEROPS peptidase database; http://merops.sanger.ac.uk; Christoff et al., 2016). Members of these CP families play many roles in plant growth and development, including seed germination (van der Hoorn 2008), together with the specific endogenous PhyCYSs that regulate CP activity (Szewińska et al., 2016).

The establishment of seed germination and early seedling growth is strongly influenced by various unfavorable environmental conditions, which induce stress responses, therefore negatively affecting these processes. The joint action of papain-like CPs and legumains plays a key role in the degradation of reserve proteins (Zakharov et al., 2004), and their activity is inhibited by PhyCYSs under unfavorable conditions (Julían et al., 2013), including drought (Rodriguez et al., 2010), heat (Je et al., 2014), high alkalinity (Sun et al., 2014), high salinity (Tan et al. 2016), and low temperature stress.
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(Zhang et al., 2008), as well as osmotic imbalance (Bae et al., 2010), but their complex interactions make it difficult to establish clear connections between a particular stress and the corresponding response at the level of CPs and PhyCYSs (Kidric et al., 2014).

The transcriptional regulation of CPs and PhyCYSs is determined by cis-elements located in the promoters of these genes and by the expression of the corresponding transcription factors. The transcripts of several CP and PhyCYS genes accumulate in imbibed seeds exposed to unfavorable conditions, and the precise control of their expression is important in regulating germination rates in response to stress (Huang and Xu, 2008). For example, the expression of barley PhyCYS Hv-CPI in the aleurone layer is regulated by two Dof (DNA-binding one zinc finger) transcription factors (Martinez et al., 2005), and in Arabidopsis, two Dof family members, DAG1 and DAG2, have opposite effects on seed germination (Gabriele et al., 2010). Arabidopsis PhyCYS3 (AtCYS3), which is induced by both drought and cold treatment, contains a 9 bp dehydration-responsive element (DRE) in its promoter sequence (Seki et al., 2002). The role of DREs in regulating PhyCYS expression is exemplified by AtCYS3 (syonymous with AtCYS5), which is expressed in Arabidopsis seedlings exposed to drought, salt, and cold stress (Zhang et al., 2008). The regulation of the Arabidopsis PhyCYS4 gene, AtCYS4, in response to heat stress (HS) occurs via DRE-binding factor 2C (DREB2C), and DREB2C acts as a transcriptional activator of this thermotolerance-related gene during seed germination (Je et al., 2014).

The rate of seed germination is also influenced by the contents of abscisic acid (ABA), which increase in response to various stresses and negatively affect germination and early seedling growth (Vermi et al., 2016). ABA-responsive genes, including PhyCYSs, have multiple cis-elements known as ABA-responsive elements (ABREs) in their promoters. The basic leucine zipper transcription factors (bZIPs) ABRE-binding proteins (AREBs)/ABRE-binding factors (ABFs) can bind to ABRE, resulting in the upregulation of ABA-responsive genes, including CPs and PhyCYSs (Szewińska et al., 2016). The findings that AREBs and/or ABFs are induced by abiotic stress and that plants overexpressing these factors exhibit enhanced stress tolerance further confirm the importance of these proteins and, hence, ABA in abiotic stress responses (Fujita et al., 2005).

In the present study, we investigated the activity of the Arabidopsis PhyCYS5 (AtCYS5) gene during seed germination and post-germination growth under HS and high ABA conditions. AtCYS5 expression was modulated by HS and ABA, and plants overexpressing AtCYS5 exhibited enhanced ABA insensitivity during seed germination and early seedling growth, correlating directly with the ability of Arabidopsis plants to tolerate HS. Finally, we showed that AtCYS5 is transcriptionally regulated and activated by bZIP transcription factors, suggesting that these factors might be components of the ABA signaling pathway from HS to AtCYS5 induction.

MATERIALS AND METHODS

Plant materials and growth conditions

The Arabidopsis thaliana L. Heynh wild-type (WT) and transgenic seeds used in this study were in the Columbia (Col-0) background. The seeds were stratified at 4°C for 3 days in the dark and grown in soil or in vitro on phytohormone-free MS medium (MSO; 1% sucrose, 0.25% Phytagel, pH 5.8) at 22°C under a 16 h light/8 h dark photoperiod with 10 μE m⁻² s⁻¹ light (Song et al., 2016).

A T-DNA insertional mutant containing a single T-DNA insertion in AtCYS5 (At5g47550) was identified in the SALK T-DNA collection (SALK, 149928C). To identify mutants homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant seedlings and subjected to PCR genotyping using the following AtCYS5 primer sets: AtCYS5 P1 forward (5’-TCTAGAATGACTAGTAGGCTGCCTTCCCTT-3’), P2 reverse (5’-AGGTACAAAGAGCGGTTACATGTTAAAAGC-3’), T-DNA P3 right (5’-TGGGAAACCCGGCCGTGTACCCCAACTT AAT-3’), and P4 left primer (5’-GTGATGTCGATCTAGTGGCCCATCG-3’) (Supplementary Fig. 1A).

Reverse-transcription quantitative PCR (RT-qPCR)

To analyze the expression of AtCYS5 in response to HS or exogenous ABA treatment, stratified WT Col-0 seeds were germinated on MSO at 37°C or on MS-ABA5 medium (MSO + 5 μM ABA) at 22°C. Total RNA was isolated from imbibed seeds at various time points using TRizol reagent (Invitrogen, USA). Complementary DNA was synthesized using SuperScript II RNase H-reverse transcriptase (Thermo Fisher Scientific, USA), and RT-qPCR analysis was performed using AtCYS5-specific primers (P1 forward and P2 reverse; Supplementary Fig. 1A) as described by Hwang et al. (2010). Relative AtCYS5 transcript levels were determined using ImageJ software (http://rsb.info.nih.gov/ij).

Histochemical GUS staining and fluorometric GUS assays

To monitor the activity of the AtCYS5 promoter during seed germination, a fragment of AtCYS5 (-1542 to +58 bp relative to transcriptional start site) encompassing the promoter region of AtCYS5 (-1542 to -1), the 5′-untranslated region (+1 to +31), and the translational start site ATG with nine amino acids (+32 to +58) was obtained by PCR amplification of Arabidopsis genomic DNA using primers AtCYS5P1F (5′-AAGCTTATGGAACCGTGAGTGTGGAGGT-3′) and AtCYS5P1R (5′-ACCCGGGGAGAAAGAAGAAGGACGACTTACCTGTC-3′). PCR amplicons were cloned into the vector pGEM-T Easy (Promega, USA) and sequenced to confirm the fidelity of amplification (Supplementary Fig. 2). The putative AtCYS5 promoter sequence was excised from the pGEM-T Easy vector with HindIII/BamHI and subcloned into the same sites of pCambia1381 (Marker Gene Technol., USA). The respective construct (PAtCYS5::GUS) was introduced into Agrobacterium tumefaciens strain GV3101, which was used to produce transgenic Arabidopsis plants by the floral dip method (Clough and Bent, 1998).

GUS activity in transgenic Arabidopsis plants was analyzed by histochemical staining using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc; Duchefa, The Netherlands) as described by Jefferson et al. (1987). Imbibed seeds were harvested and immediately fixed for 30 min in ice-cold 90% acetone (Vanderbeld and Snedden...
2007), rinsed with water, and incubated in 50 mM sodium phosphate buffer (pH 7.0), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 0.2% Triton X-100 containing 1 mM X-gluc. The histochemical reaction was performed in the dark at 37℃ for 12 h, after which the samples were transferred to 70% ethanol to remove the chlorophyll. Digital images were obtained under a stereoscope (Olympus SZX12, Japan).

Quantitative measurement of GUS activity in protein extracts was performed using the fluorogenic substrate 4-methylumbelliferylβ-D-glucuronide (4-MUG; Sigma-Aldrich, USA). Protein extracts were isolated by grinding the tissues in extraction buffer (50 mM sodium phosphate [pH 7.0], 10 mM EDTA, 0.1% SDS, 0.1% Triton X-100, 10 mM β-mercaptoethanol, and 25 μg/ml phenylmethylsulfonfyl fluoride), followed by centrifugation (12,000 rpm for 10 min). The supernatants were combined with extraction buffer containing 0.8 mM 4-MUG, and GUS activity at various time points was determined in triplicate and calculated as pmoles 4-methylumbelliferone (4-MU)/min/mg protein as described by Chen et al. (2010).

Generation of AtCYS5-overexpressors

To investigate the effect of AtCYS5 on seed germination and seedling growth in vivo, AtCYS5-overexpressing transgenic lines were generated as described by Song et al. (2014). AtCYS5 cDNA was isolated from 2-day-old Col-0 seedlings by RT-PCR using primers AtCYS5 P1 forward and P2 reverse (Supplementary Fig. 1A). PCR amplicons were cloned into the pGEM-T Easy vector (Promega) and sequenced to confirm the fidelity of amplification. The AtCYS5 cDNA was excised from the pGEM-T Easy vector with XbaI/KpnI and subcloned into the same sites of pCambia1301 (Marker Gene Technol.). The respective construct (35S:AtCYS5) was introduced into Agrobacterium tumefaciens strain GV3101 and used to transform Arabidopsis plants as previously described (Clough and Bent, 1998). Homozygous T3 generation plants were obtained as described by Koo et al. (2002).

To examine the inhibitory activity of AtCYS5 in AtCYS5-overexpressors, CP activity measurements in AtCYS5-overexpressors were performed as described by Hong et al. (2007).

Germination and seedling growth measurements

AtCYS5-overexpressors, an AtCYS5 knockout line (cys5), and untransformed WT seeds were collected from fully mature silique s of dehydrated plants of the same age for seed germination and seedling growth assays (Je et al., 2014). Stratified seeds (>100 seeds per replicate) were germinated on MS medium containing 2% sucrose, and the plates were transferred to a growth chamber (EYELA, Japan) at 22 or 50℃ and incubated for various periods of time. Germination was scored by microscopy based on radical emergence (Hwang et al., 2009).

To measure seedling growth, stratified seeds treated with HS at 50℃ for 115 min or normal temperature conditions (22℃) were germinated for 7 days prior to fresh weight and primary root length measurements.

To compare the effects of ABA on germination and seedling growth in various plants, seeds from AtCYS5-overexpressors, cys5, and WT were germinated on MS medium with or without 0.5 or 0.7 μM ABA (Sigma). The plates were transferred to a growth chamber set at 22℃. Germination rates at various time points and seedling growth at 7 days were scored as described above.

Transient promoter activation assay

Transcriptional activators of the AtCYS5 promoter were identified using the reporter plasmid construct PAtCYS5::GUS. The effector plasmids used in the assay contained ABF1, ABF2, ABF3, ABF4, ABI5, or AREB3 cDNAs fused in-frame at their C-termini to the green fluorescent protein (GFP) gene (Je et al., 2014). Each effector chimeric gene was inserted between the CaMV35S promoter and the nopalin synthase terminator in pCambia1381 (Marker Gene Technol.) for constitutive expression of the cDNA (Fig. 5A). A PJD300 plasmid expressing luciferase (LUC) driven by the CaMV35S promoter was used for normalization of gene expression in samples with different transformation efficiencies (Park et al., 2004). The reporter and effector plasmids were introduced into Arabidopsis leaf protoplasts (Je et al., 2014), and GUS activity was analyzed as described by Jefferson et al. (1987).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described by Je et al. (2014), using double-stranded (ds)-oligonucleotide probes corresponding to a fragment of the AtCYS5 promoter spanning the G-box (wG; CACGTG) and two C-box (wC1 and wC2; CACGTC) sequences (Fig. 6A). The ABRE CACGTG/CACCGGCAC-3' for wC1+wG (wC1 [right side] and wG [left side] sequences are underlined) and 5'-CATAACACCGTCATTGCGG-3' (mutations in wG are indicated by lowercase letters) were used to transform Arabidopsis plants as previously described (Kim et al., 2004). The sequences of the six oligonucleotide probes used in EMSA are as follows: WT C1 and G-box (wC1+wG) and WT C-box 2 (wC2), which correspond to the native promoter fragments, with the sequences 5'-CTGCCACACGTCATGC-3' (mutations in wG are indicated by lowercase letters): mG, 5'-CTGCCACACGTCATGC-3' (mutations in wC1+wG are indicated by lowercase letters): mC1+mG, 5'-CTGCCACACGTCATGC-3' (mutations in wC1+wG are indicated by lowercase letters): mC1+mG, 5'-CTGCCACACGTCATGC-3' (mutations in wC1+wG are indicated by lowercase letters): mG. P-labeling of probe and purification of recombinant proteins were carried out as described by Lim et al. (2007).

RESULTS AND DISCUSSION

AtCYS5 transcript levels increase during seed germination

To determine whether AtCYS5 might also function in HS responses, we isolated total RNA from germinating Arabidopsis seeds under HS conditions. AtCYS5 transcript levels peaked at 12 h in stratified seeds imbibed under both normal (22℃) and HS conditions (37℃) (Fig. 1A). However, at 22℃, AtCYS5 transcript levels sharply decreased after 12 h,
Fig. 1. AtCYS5 expression in imbibed seeds increases under mild heat stress and exogenous ABA treatment. (A) Imbibed wild-type Col-0 Arabidopsis seeds were germinated at 22℃ and 37℃ and exposed to 5.0 μM ABA (under 22℃) for the indicated time periods. RT-qPCR analysis (30 cycles) was performed on total RNA; ethidium bromide-stained gels are shown. Actin2 (At3g18780) transcript levels are shown as the loading control. (B) Quantitative analysis of signal intensity in the ethidium bromide-stained gels shown in (A), as measured with ImageJ software. Values represent mean ± SD, and values prior to treatment (0 h) were set at 1. Statistically significant differences between the exposure times are indicated by asterisks (n = 3; * P < 0.01 by Student’s t-test). (C) Histochemical localization of GUS expression in transgenic Arabidopsis plants carrying the GUS coding region fused to the AtCYS5 promoter (PAtCYS5:GUS). Seeds were germinated at 22℃ and 37℃ and exposed to 1.0 μM ABA at 22℃ for the indicated periods of time. Scale bar is 100 μm. (D) GUS activity over time was determined by quantitative fluorometric assays using germinated seeds. The effect of mild heat shock or exogenous ABA treatment was investigated in seeds. The data presented are the mean ± SD of three independent experiments (* P < 0.01 by Student’s t-test).

Overexpression of AtCYS5 increases seed germination and seedling growth

To confirm the function of AtCYS5 in seed germination and seedling growth in vivo, we created AtCYS5 overexpression transgenic lines (35S:AtCYS5; Supplementary Fig. 1B). To determine the copy numbers of AtCYS5 in the transgenic plants, T1 plants were self-pollinated and the progeny (T2) were allowed to segregate on selection medium. Following self-pollination of the T2 lines, we selected two T3 homozygous lines (35S:AtCYS5-L6 and -L7) containing a single insertion (Supplementary Fig. 1C). PCR amplicons encompassing the T-DNA insertion site were not detected for the cys5 mutant (Supplementary Fig. 1C); however, when the P2 primer and the T-DNA primer (P4) were used, amplicons were detected (Supplementary Fig. 1D). PCR genotyping revealed that the cys5 mutation resulted in the complete loss of AtCYS5 expression. Accordingly, the endogenous CP inhibitory activity of L6 and L7 was...
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**Fig. 2. Comparison of seed germination rates in transgenic AtCYS5 plants.** Time course of germination (in hours after imbibition) for freshly harvested seeds of untransformed wild-type (WT), AtCYS5 overexpression lines (35S:AtCYS5-L6, 35S:AtCYS5-L7), and AtCYS5 knockout line (cys5). (A) Seeds were surface-sterilized, plated on MSO medium, and stratified in the dark at 4°C for 3 d before shifting the plates to a growth chamber. Percent radical emergence was measured at 12 h intervals after shifting to a growth chamber set at 22°C. (B) Imbibed seeds were treated at 50°C for 115 min as shown and shifted to a growth chamber set at 22°C. (C) Imbibed seeds were plated on MSO containing 1.0 μM ABA and stratified in the dark at 4°C for 3 d before shifting the plates to a growth chamber set at 22°C. Bars represent mean ± SD (n = 3, *P < 0.01 by Student’s t-test).

higher than that of WT and cys5 (Supplementary Fig. 1E). We imbibed seeds from the two independent AtCYS5 overexpression lines, the knockout mutant, and untransformed WT, stratified them for 3 d in the dark at 4°C, and allowed them to germinate under long-day conditions at 22°C and 50°C to investigate the effect of temperature on germination. Under normal growth conditions (22°C), we detected little difference among the four lines (Fig. 2A). Approximately 20% lower germination rates were observed in WT and cys5 seeds at 24 h after imbibition compared to L6 and L7, but by 36 h, the germination rate of all four lines was 100%. The similarity of phenotype to WT in cys5 may be due to functional redundancy of other AtCYSs in *Arabidopsis* genome. However, under HS conditions, the germination rate was strongly reduced in WT and cys5 seeds, with only 30-50% of the seeds having germinated by 84 h compared to 80% in the 35S:AtCYS5 transgenic lines (Fig. 2B). Therefore, the increased germination rate in 35S:AtCYS5 transgenic seeds indicates that AtCYS5 helps improve germination under HS conditions.

To investigate the effect of ABA, an inhibitor of seed germination, on 35S:AtCYS5 seed germination, we incubated WT and transgenic seeds on MS medium containing 1.0 μM ABA (Fig. 2C). At 22°C, there was no significant difference in germination rate between WT and 35S:AtCYS5 transgenic seeds, whereas the germination rate was lower in cys5, with 70% of the seeds having germinated by 36 h compared to 100% for WT and 35S:AtCYS5 seeds. Collectively, these results strongly suggest that AtCYS5 is involved in promoting germination under HS and ABA treatment.

Overexpressing AtCYS5 improves HS tolerance during post-germination growth

Since seeds of the AtCYS5 overexpressors exhibited increased germination under HS compared to WT, we investigated whether the overexpression of AtCYS5 is associated with increased HS tolerance during post-germination as well. We compared two morphological phenotypes (fresh weight and primary root length) in WT and AtCYS5 transgenic *Arabidopsis* plants, including an AtCYS5 overexpressor and a knockout mutant, by subjecting imbibed seeds to HS for 115 min and observing the plants after a 7 day recovery period at 22°C (Fig. 3). Under normal growth conditions (22°C), the AtCYS5 transgenic plants exhibited no difference in morphology or growth compared to WT. However, while the growth of both WT and the transformants was inhibited under HS conditions, this inhibition was much more severe in WT and cys5 than in the AtCYS5 overexpressors (Fig. 3A). For example, under HS conditions, the fresh weight was reduced by approximately 55% for the overexpressors and 95% for WT. The reduction in fresh weight was much more severe in cys5 than in WT (Fig. 3B). Primary root growth was altered in a similar manner (Fig. 3C). Measurements of fresh weight and root length, which reflect the level of damage due to HS, indicated that seedling growth was reduced in plants from HS-exposed seeds compared to the untreated controls in all lines examined. However, the AtCYS5 overexpression lines had higher fresh weights and longer roots than WT or cys5 under HS (Figs. 3B and 3C). These results indicate that AtCYS5 overexpression increased the resistance to HS during the post-germination period.
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**Fig. 3. Comparison of seedling growth in transgenic AtCYS5 plants.** Phenotypes are shown for 7-day-old untransformed wild-type (WT), AtCYS5 overexpression lines (35S:AtCYS5-L6, 35S:AtCYS5-L7), and AtCYS5 knockout line (cys5). (A) Imbibed seeds were treated at 50°C for 115 min, and photographs were taken after 7 days of recovery at 22°C. Scale bar is 1 cm. (B) Fresh weights of 7-day-old WT and transgenic seedlings. (C) Quantitative analysis of primary root lengths of 7-day-old seedlings from each transgenic line. Values represent mean ± SD (n = 3, *P < 0.01 by Student’s t-test).

**Fig. 4. Seedling growth of transgenic AtCYS5 plants in response to ABA.** (A) Seeds of untransformed wild-type (WT), two independent 35S:AtCYS5 lines (35S:AtCYS5-L6, -L7), and the AtCYS5 knockout line (cys5) were plated on MSO agar medium containing 0.5 μM (middle panel) or 0.7 μM ABA (right panel). The photographs were taken 7 days after sowing. Scale bar is 1 cm. (B) Fresh weights of 10-day-old WT and transgenic seedlings. (C) Quantitative analysis of primary root length in 10-day-old seedlings from each transgenic line. Values represent mean ± SD (n = 3, *P < 0.05, **P < 0.01 by Student’s t-test).

Overexpression of AtCYS5 accelerates post-germination growth in plants from ABA-treated seeds
HS increases ABA levels in plant cells (Toh et al., 2008). Therefore, the increased germination in 35S:AtCYS5 transgenic seeds under HS conditions might have been due to the insensitivity of the seeds to increased ABA levels. We reasoned that overexpression of AtCYS5 may also lead to increased post-germination growth under ABA treatment. To test this hypothesis, surface-sterilized WT, 35S:AtCYS5, and cys5 seeds were plated on medium supplemented with 0.5 or 0.7 μM ABA, imbibed, stratified, and allowed to germinate, and the plants were photographed 7 days later. In the absence of ABA, the AtCYS5 transgenic plants exhibited no difference in morphology or growth compared to WT. However, as the concentration of ABA increased, the growth inhibition became more severe in both WT and transgenic plants, but this effect was much more severe in WT and cys5 than in the AtCYS5 overexpressors (Fig. 4A). For example, the fresh weight of the overexpressors was reduced by approximately 55% in response to 0.5 μM ABA and 70% in response to 0.7 μM ABA treatment. By contrast, in WT, the fresh weight was reduced by 90% and 98% in response to 0.5 and 0.7 μM ABA treatment, respectively (Fig. 4B). The reduction in fresh weight was much more severe in cys5 than in WT. Primary root growth was altered in a similar manner (Fig. 4C). Measurements of fresh weight and root length showed that seedling growth was proportional to the ABA concentration in the medium for all lines but was 2-5 fold higher in the 35S:AtCYS5 transgenic lines than in WT or cys5 (Figs. 4B and 4C). These results suggest that AtCYS5...
reduces the sensitivity of plants to ABA during seed germination and post-germination growth.

**ABF1 and ABF3 transactivate AtCYS5 expression in Arabidopsis protoplasts**

ABA- and stress-responsive cis-acting elements are usually found in the promoter regions of stress-inducible genes (Zhang et al., 2005). Thus, we analyzed the upstream region (-1542 bp upstream of the transcription start site) of AtCYS5 to identify putative ABA- or HS-responsive cis-acting elements. The AtCYS5 promoter contains three putative ACGT-containing ABREs identical to the well-characterized motifs present in inducible promoters that respond to ABA and/or abiotic stress (Zhang et al., 2005), whereas no HS element (HSE) was identified (Supplementary Fig. 2). The induction of gene expression by HS does not always require a HSE (Je et al., 2014). For example, Arabidopsis ABRE sequences (CCACGTGG) are important for transcriptional activation in response to a variety of stress conditions, including heat (Je et al., 2014; Toh et al., 2008). Therefore, the absence of a HSE in the AtCYS5 promoter suggests that some other cis-elements in the promoter, such as ABREs, along with their cognate binding proteins, might be involved in the thermoregulation of AtCYS5 expression.

ABREs represent a subset of the C- and G-box sequences \([CAGT(C/G)]\) present in the promoter regions of many light-regulated genes (Giuliano et al., 1988). The AtCYS5 promoter contains three putative ABREs containing C- and G-boxes (Supplementary Fig. 2). C- and G-box-containing ABREs are the binding sites for the ABFs, ABA-insensitive 5 (ABI5), and AREBs, i.e., group A bZIP transcription factors involved in ABA-dependent signaling in Arabidopsis (Jakoby et al., 2002). Thus, we focused on members of these transcription factor families to explore the importance of the ABREs in the AtCYS5 promoter. We evaluated the ability of bZIps to regulate AtCYS5 transcription via these motifs by performing transient promoter activation assays in Arabidopsis leaf protoplasts. Effector constructs were designed for constitutive overexpression of each full-length ABF, ABI5 and AREB3, whereas the reporter construct consisted of the AtCYS5 promoter (-1542 to +58 bp; Supplementary Fig. 2) fused upstream of GUS (\(P_{AtCYS5}:GUS\)). The pJD300 plasmid was used to normalize effects arising from variations in transformation efficiency between samples (Park et al., 2004). Both constructs were mixed and co-transformed into Arabidopsis leaf protoplasts (Fig. 5A). Co-expression of the transcription factors did not transactivate the expression of the GUS reporter gene, whereas the addition of ABA increased GUS/LUC activity approximately 5- to 7-fold, compared to ABA-treated samples that received no effector plasmid (Fig. 5B). These results suggest that bZIP transcription factors function as transcriptional activators of AtCYS5 expression in the presence of ABA and that the overproduction of bZIP factors alone is not sufficient to induce the expression of AtCYS5.

**Fig. 5. Group A bZIps activate the transcription of AtCYS5 in the presence of ABA.** (A) Schematic representation of the effector and reporter constructs used for the transient promoter activation assays. The effector constructs used to express the bZIP factor tagged with green fluorescent protein (GFP) contained the CaMV35S promoter \((35S)\) fused to the entire ORF of the test proteins (except the stop codon), followed by the nopaline synthase gene terminator \((nos-T)\). The bZIP cDNAs encoded ABF1, 2, 3, and 4, ABI5, and AREB3. The reporter construct \((P_{AtCYS5}:GUS)\) contained the GUS gene inserted between the AtCYS5 promoter \((-1542 \text{ to } +58 \text{ bp})\) and \(P_{AtCYS5}:GUS\). T The pJD300 plasmid was used to normalize effects arising from variations in transformation efficiency between samples (Park et al., 2004). Both constructs were mixed and co-transformed into Arabidopsis leaf protoplasts (Fig. 5A). Co-expression of the transcription factors did not transactivate the expression of the GUS reporter gene, whereas the addition of ABA increased GUS/LUC activity approximately 5- to 7-fold, compared to ABA-treated samples that received no effector plasmid (Fig. 5B). These results suggest that bZIP transcription factors function as transcriptional activators of AtCYS5 expression in the presence of ABA and that the overproduction of bZIP factors alone is not sufficient to induce the expression of AtCYS5.
ABF1 and ABF3 bind directly to the AtCYS5 promoter
Since in the presence of ABA, the expression of AtCYS5 was activated by bZIP transcription factors in the transient promoter activation assays (Fig. 5), we compared the ability of bZIP factors to physically bind to the C- and G-boxes in the AtCYS5 promoter via EMSA (Fig. 6A). When 32P-labeled oligonucleotide probes (wC1+wG or wC2) corresponding to the native AtCYS5 promoter fragment were incubated with GST alone (control) or purified recombinant GST-bZIP fusion proteins, bands with retarded mobility were observed in the EMSA only for GST-ABF1 and GST-ABF3 (Fig. 6 and 7), indicating that ABF1 and ABF3 physically bound to both the wC1+wG and wC2 probes. By contrast, ABF2, ABF4, ABF5, and AREB3 did not bind to C- or G-box in the AtCYS5 promoter (Supplementary Fig. 3). The addition of unlabeled wC1+wG or wC2 to the 32P-wC1+wG or 32P-wC2 binding reaction reduced binding of ABF1 to the probes in competition assays (Fig. 6B and 6C). The addition of unlabeled competitor mC1+wG or wC2+mG to the 32P-wC1+wG binding reaction reduced the binding of ABF1 to the probes to a similar degree (Fig. 6B), indicating that ABF1 binds equally to wC1 and wG (Fig. 6C). The addition of unlabeled mC1+mG and mC2 did not reduce the binding of 32P-wC1+wG or 32P-wC2. Interestingly, the signal generated by the binding of ABF1 to wC1 was stronger than that using wG, providing evidence that ABF1 binds more efficiently to the C-box than the G-box (Fig. 6D).

In the case of ABF3, the addition of unlabeled wC1+wG or wC2 to the 32P-wC1+wG or 32P-wC2 binding reaction reduced binding of ABF3 to the probes (Figs. 7A and 7B). Unlike for ABF1, the addition of unlabeled competitor mC1+wG to the 32P-wC1+wG reaction did not reduce the binding of ABF3 to 32P-wC1+wG, but the addition of competitor wC1+mG to 32P-wC1+wG reduced the binding of ABF3 to the probes (Fig. 7A). These results indicate that ABF3 binds strongly to wC1 but very weakly to wG. The addition of unlabeled mC2 to the 32P-wC2 binding reaction did not reduce the binding of ABF3 to the probes (Fig. 7B), indicating that ABF3 can also bind to wC2. The signal generated by the binding of ABF3 to wG was stronger than that with wC1, suggesting that ABF3 binds more efficiently to the G-box than the C-box (Fig. 7C). Finally, the binding activity of wC2 was between that of wG and wC1 (Figs. 7B and 7C). These observations indicate that ABF1 and ABF3 bind to the C2, C1, and G regions of the AtCYS5 promoter but with differential binding abilities.

In conclusion, group A bZIPs ABF1 and ABF3 regulate AtCYS5 expression through the ABRE-mediated signaling pathway in the presence of ABA. The upregulated expression of AtCYS5 increases thermotolerance during seed germination and seedling growth. These findings suggest that AtCYS5 might be useful in molecular breeding aimed at increasing plant tolerance to HS during seed germination and post-germination growth.
Fig. 7. EMSA of ABRE-binding activity of ABF3. (A) $^{32}$P-labeled wC1+wG was used as a probe, and unlabeled wC1+wG, mC1+wG, wC1+mG, and mC1+mG fragments were used as competitors. (B) $^{32}$P-labeled wC2 was used as a probe, and unlabeled wC2 and mC2 fragments were used as competitors. (C) $^{32}$P-labeled wC1+mG and mC1+wG were used as probes, and unlabeled wC1+mG and mC1+wG fragments were used as competitors. Shown are autoradiograms of gels used to analyze binding reactions of the indicated composition, where minus (-) indicates omission, plus (+) indicates addition, and “++” indicates twice the amount of a component compared to +.
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