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Arabidopsis COLD SHOCK DOMAIN PROTEIN 2 influences ABA accumulation in seed and negatively regulates germination

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ABSTRACT

The cold shock domain (CSD) is the most conserved nucleic acid binding domain and is distributed from bacteria to animals and plants. CSD proteins are RNA chaperones that destabilize RNA secondary structures to regulate stress tolerance and development. AtCSP2 is one of the four CSD proteins in Arabidopsis and is up-regulated in response to cold. Since AtCSP2 negatively regulates freezing tolerance, it was proposed to be a modulator of freezing tolerance during cold acclimation. Here, we examined the function of AtCSP2 in seed germination. We found that *AtCSP2*-overexpressing lines demonstrated retarded germination as compared with the wild type, with or without stress treatments. The ABA levels in *AtCSP2*-overexpressing seeds were higher than those in the wild type. In addition, overexpression of *AtCSP2* reduced the expression of an ABA catabolic gene (*CYP707A2*) and gibberellin biosynthesis genes (*GA20ox* and *GA3ox*). These results suggest that AtCSP2 negatively regulates seed germination by controlling ABA and GA levels.

1. Introduction

The cold shock domain (CSD) is a highly conserved nucleic acid-binding domain found in bacteria as well as animals and plants [1–4]. The CSD contains two consensus RNA binding motifs (RNP-1 and RNP2) that are essential for binding single-stranded DNA/RNA [5,6]. Bacterial cold shock proteins (CSPs) are composed of a single CSD and are involved in cold adaptation [7]. CSPs act as RNA chaperones that destabilize secondary structures in mRNAs [8–10]. At low temperature, RNA tends to form stable secondary structures that may inhibit RNA functions including translation and transcription [11]. Therefore, RNA chaperone activity is likely to be critical for RNA function at low temperature.

Plant CSD proteins contain an N-terminal CSD and a C-terminal glycine-rich region interspersed with various numbers of retroviral-like CCHC zinc fingers [4]. The first plant CSD protein characterized was wheat cold shock domain protein 1 (WCSP1), which accumulates in crown tissue during cold acclimation [12]. *WCSP1* mRNA is induced by cold treatment but not modulated by other environmental stresses [12]. WCSP1 has nucleic acid-binding activity [12,13] and can unwind nucleic acid duplexes [13]. Thus, WCSP1 likely functions as an RNA chaperone to destabilize RNA secondary structures during cold acclimation.

Genome analyses revealed that Arabidopsis contains four CSD protein genes (*AtCSP1–AtCSP4*) [2,4]. The expression of *AtCSP1*, *AtCSP2* and *AtCSP3* is induced by cold treatment [2,14–16]. AtCSP3 (At2g17870) is currently the best-studied plant CSD protein and is implicated in acquisition of freezing tolerance [16]. It has been demonstrated that C-repeat binding factors (CBFs) are transcription factors that positively regulate freezing tolerance through activation of their downstream genes (e.g., *COR*) [17–19]. However, expression of *CBF* and *COR* genes is not affected in the *atcsp3-2* knockout mutant, which suggests that AtCSP3 regulates freezing tolerance independent of the CBF pathway. Microarray analysis revealed that AtCSP3 influences the expression of stress-related genes but potential roles of these genes in freezing tolerance are unknown [16].

AtCSP2 (At4g38680) is the most highly expressed of the *AtCSP*s [20], and although *AtCSP2* expression is induced by cold treatment, AtCSP2 negatively regulates freezing tolerance during cold

Abbreviations: CSD, cold shock domain; CSP, cold shock protein.

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acclimation [14,21]. Specifically, AtCSP2 is involved in the negative regulation of *CBF* and *COR* gene expression during cold acclimation [21].

Expression of *AtCSP* genes is also regulated developmentally [20]. *AtCSP2* expression is detected in mature seed, shoot apex, root tip, anthers and pistils [14,15]. Overexpression of *AtCSP2* results in morphological and developmental alterations such as smaller plant size, late flowering and shorter silique length [21]. *AtCSP2* knockdown lines show early flowering and high rates of abnormal seed/embryo development [15]. The mechanism by which AtCSP2 affects developmental processes is currently unknown. However, it is possible that plant hormones such as gibberellin (GA) are involved [21].

In this work, we describe functional characterization of AtCSP2 during seed germination. We demonstrate that AtCSP2 negatively regulates seed germination through altering the expression of GA- and ABA-metabolism genes.

2. Materials and methods

2.1. Plant materials

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) was used as the wild type in this study. The *AtCSP2*-overexpressing lines (*35S:AtCSP2*-18 and -20) and mutants (*atcsp2-3 atcsp4-1*-#1 and #2) have been previously described [21]. To obtain seeds from these lines, seeds were first sown and germinated on Murashige and Skoog (MS) medium containing 2% sucrose at 22 °C with a 16-h photoperiod. Subsequently, 10-d-old plants were transferred to pots containing vermiculite and jiffy mix (Sakata Seed Corp., Japan) at a 3:1 ratio, and grown to maturity (22 °C, 16-h photoperiod).

2.2. Germination assays

All seed batches were harvested on the same day from plants grown side by side. Harvested seeds were dried for 2 weeks and then used for germination assays. Seeds were imbibed on MS medium with or without stratification (4 °C, 2-day, dark) and were then germinated at 22 °C under long-day conditions (16-h photoperiod). Emergence of visible radicles was used as a morphological marker for germination. Experiments were performed in triplicate, with 36 seeds counted for each measurement, and the results were averaged.

2.3. Quantification of ABA and GA

Extraction and purification of ABA, GA_1 and GA_4 were carried out according to Katsumata et al. [22] with some modifications. Samples (40 mg dry seeds) after stratification were homogenized with beads in 2-mL tubes with a Tissue Lyser (Qiagen) in 1 mL 80% (v/v) acetonitrile (MeCN) containing 1% (v/v) acetic acid (AcOH) with internal standards. d_6-ABA was obtained from Icon Isotopes, and d_2-GA_1 and d_2-GA_4 were obtained from OlChemIm (Olomouc, Czech Republic). After extraction for 1 h at 4 °C, samples were centrifuged at 4 °C for 10 min at 14,000×*g* and supernatants were collected. Samples were re-extracted twice with 1.5 mL 80% MeCN at 4 °C for 30 min and supernatants were collected after centrifugation. MeCN was removed using a SpeedVac (Thermo Fisher Scientific) and the acidified water extracts were loaded into Oasis HLB cartridges (Waters). After washing with water containing 1% AcOH, plant hormones were eluted with 80% MeCN containing 1% AcOH. MeCN was removed and samples were loaded into Oasis MCX extraction cartridges (Waters). After washing with water containing 1% AcOH, hormones were eluted with 80% MeCN containing 1% AcOH. MeCN was removed and samples were loaded on into Oasis WAX extraction cartridges (Waters). After washing with water containing 1% AcOH and successively by absolute methanol, hormones were eluted with 80% MeCN containing 1% AcOH. The fractions containing hormones were dried, dissolved in water containing 1% AcOH and analyzed by LC–MS/MS. Quantification of hormones by LC–MS/MS was performed as described previously [23]. Experiments were performed in triplicate, and results were averaged.

2.4. Total RNA extraction from seeds

Total RNA was extracted from seeds as previously described [24]. Routinely, 40 mg seeds were used for total RNA extraction. After extensive homogenization of seeds in liquid nitrogen, the powder was solubilized in 800 μL extraction buffer (100 mM Tris–HCl, pH 9.5; 10 mM EDTA, pH 8.0; 2% lithium dodecyl sulfate (w/v); 0.6 M NaCl; 0.4 M trisodium citrate; and 5% β-mercaptoethanol). Cell debris was removed by centrifugation at 12,000×*g* for 5 min at room temperature and RNAs were extracted from the aqueous phase. Chloroform:isoamylalcohol (CIA; 24:1) treatment was followed by treatment with phenol mixture (water-saturated phenol containing 35% (w/v) guanidine isothiocyanate and 0.2 M sodium acetate, pH 4.0). After a 3-min incubation, CIA corresponding to half the volume of the phenol mixture was added and the samples were centrifuged at 12,000×*g* for 5 min at 4 °C. The resulting supernatant was finally precipitated by addition of 0.6 volumes isopropanol. To remove genomic DNA, total RNA was treated with DNase I (Takara, Japan) following the manufacturer's instructions.

2.5. Quantitative real time RT-PCR (qRT-PCR)

First-strand cDNA synthesis was carried out using a High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. qRT-PCR analysis was performed with an ABI7500 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR master mix-Plus- (Toyobo, Japan). PCR was performed as follows: 95 °C for 1 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Transcripts were normalized to 18S rRNA. The specificity of the PCR was determined by melting curve analysis of the amplified products using the standard method installed in the system. The primers used are listed in Supplemental Table 1.

3. Results and discussion

We observed that *35S:AtCSP2* transgenic seeds tended to germinate slowly compared to wild type seeds. However, it was previously reported that overexpression of *AtCSP2* does not alter seed germination under non-stress conditions and actually accelerates germination under salt stress [25]. To address this discrepancy, we performed a detailed study of the function of AtCSP2 in seed germination. We first examined germination rates of wild type and *35S:AtCSP2* seeds that were harvested after side-by-side cultivation and stored under the same conditions. Germination of *35S:AtCSP2* was significantly delayed compared with the wild type under non-stratified conditions, although the ultimate germination rates of both plants were similar (Fig. 1A). After a stratification treatment, germination of both *35S:AtCSP2* and wild type seeds was accelerated (Fig. 1A). However, the delayed germination phenotype of *35S:AtCSP2* lines is maintained even after stratification (Fig. 1A).

Expression of *AtCSP2* is up-regulated during abiotic stresses such as cold and salt [14,21,25]. To examine the function of AtCSP2 in germination under abiotic stress conditions, we measured germination

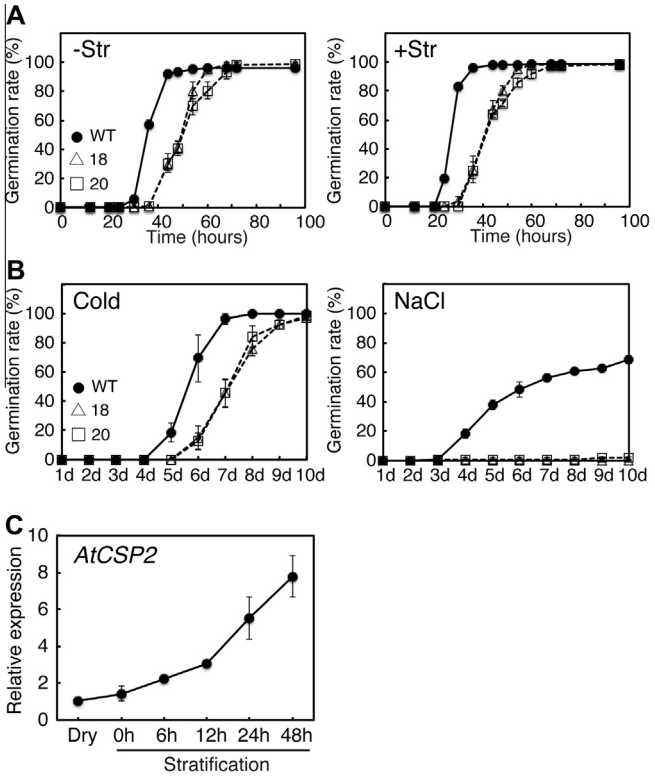

Fig. 1. AtCSP2 is a negative regulator of seed germination. (A) The germination of wild type (WT) and *35S:AtCSP2* seeds (lines 18 and 20) without (−Str) or with (+Str) stratification was scored at the indicated time. Data represent the means ± SE of three independent experiments. (B) The germination of stratified WT and *35S:AtCSP2* seeds under cold (8 °C) or 200 mM NaCl stress conditions. Data represent the means ± SE of three independent experiments. (C) Relative expression of *AtCSP2* in WT during stratification. qRT-PCR was performed in triplicate and means ± SD are shown. The value for WT dry seeds (Dry) was set to 1.

rates of *35S:AtCSP2* under cold and salt stress. When the seeds of wild type and *35S:AtCSP2* plants were germinated under cold (8 °C) conditions, *35S:AtCSP2* germination was substantially delayed as compared with that of wild type (Fig. 1B). The addition of NaCl (200 mM) to the medium completely blocked germination of *35S:AtCSP2* seeds through 10 d, while wild type displayed more than 60% germination under the same conditions (Fig. 1B). The ungerminated *35S:AtCSP2* seeds remained viable, as they germinated after being transferred to medium without salt (data not shown). This is in contrast with the previous report that overexpression of AtCSP2 promotes germination under salt stress [25].

We also characterized the germination phenotypes of *atcsp2* mutants. Since there is functional overlap between AtCSP2 and AtCSP4 [21], we utilized the *atcsp2-3 atcsp4-1* double mutant, in which expression of *AtCSP2* is reduced and that of *AtCSP4* is absent [21]. The *atcsp2-3 atcsp4-1* mutant seeds germinated slightly earlier than did the wild type in stratified conditions (Supplemental Fig. 1). However, difference of germination rate between mutant and wild type is not so clear in un-stratified conditions (Supplemental Fig. 1). It is thought that difference in germination rate between wild type and double mutant is limited since expression of *AtCSP2* is not completely dismissed in the double mutant [21]. In addition, another class of CSD protein, AtCSP1, also regulates seed germination negatively in a stratification dependent manner [26], suggesting a functional overlap between AtCSP2/4 and AtCSP1. No difference in germination timing was observed between wild type and the single mutants (*atcsp2-3* or *atcsp4-1*) (data not shown). Collectively, these data are consistent with AtCSP2 being a negative regulator of seed germination.

During cold acclimation in seedlings, *AtCSP2* expression is up-regulated [2,14,15,21]. However, overexpression of *AtCSP2* has a negative influence on freezing tolerance after cold acclimation [21]. These data suggest that AtCSP2 functions as a negative regulator that modulates proper levels of cold acclimation. We were therefore interested in determining if *AtCSP2* expression is induced during cold stratification. Expression analysis using quantitative real-time PCR (qRT-PCR) revealed that *AtCSP2* expression gradually increased during the 48-h timeframe of stratification (Fig. 1C). Accordingly, AtCSP2 might serve to attenuate germination pathways during stratification.

The germination potential of seeds is determined by the balance between the phytohormones ABA and GA [27]. To determine whether retarded germination of *35S:AtCSP2* seeds is associated with altered hormone accumulation, ABA and bioactive GA (GA_1 and GA_4) levels were determined in stratified seeds. The ABA levels in *35S:AtCSP2*-18 were about 2-fold higher than those in wild type (Fig. 2A). GA_1 and GA_4 were undetectable in both *35S:AtCSP2*-18 and wild type (data not shown), indicating that the GA levels are below the detection limit at this stage. These data suggest that increased levels of at least ABA in *35S:AtCSP2*-18 contribute to retarded germination.

To examine AtCSP2 promotion of ABA accumulation in more detail, we analyzed the effect of *AtCSP2* overexpression on the expression of ABA biosynthesis and catabolism genes during stratification. We performed qRT-PCR analysis of specific genes known to be correlated with dormancy maintenance (*ZEP*, *NECD6* and

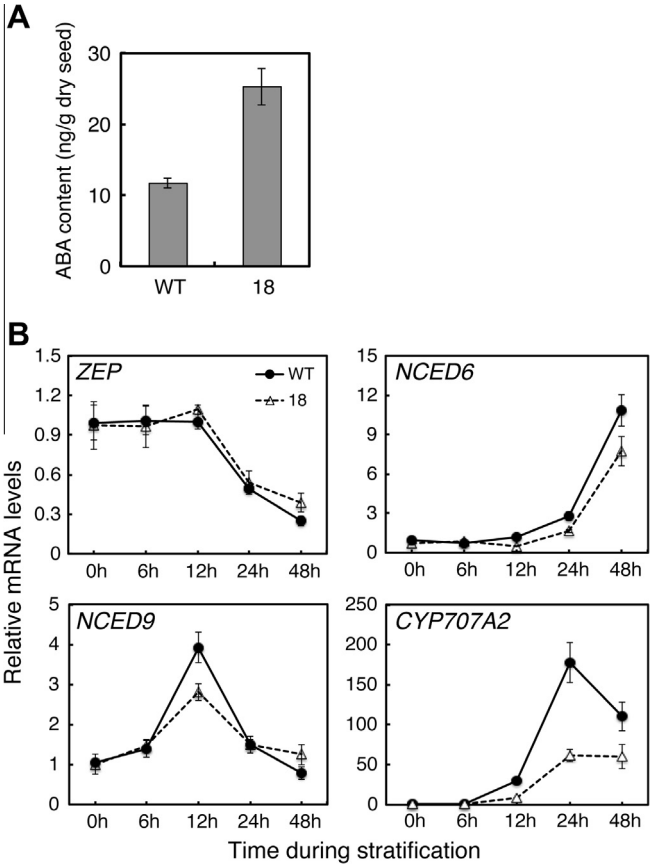

Fig. 2. Effect of *AtCSP2* overexpression on ABA metabolism. (A) ABA content in wild type and *35S:AtCSP2*-18 seeds after stratification (4 °C, 48 h, dark). Data represent the means ± SD of three independent experiments. (B) Relative expression of ABA biosynthesis (*ZEP*, *NCED6* and *NCED9*) and inactivation (*CYP707A2*) genes in WT and *35S:AtCSP2*-18 seeds during stratification. qRT-PCR was performed in triplicate and means ± SD are shown. The values for WT at 0 h were set to 1.

NCED9) and dormancy release (*CYP707A2*) via ABA metabolism in Arabidopsis [28–30]. There were no significant differences in transcript accumulation of *ZEP* and *NCED6*, *NCED9* between the wild type and *35S:AtCSP2*-18 (Fig. 2B). By contrast, *CYP707A2* expression showed differences in both overall level and timing. In wild type, *CYP707A2* transcript accumulation peaked at 24 h and decreased thereafter during stratification (Fig. 2B). The expression of *CYP707A2* in *35S:AtCSP2*-18 was reduced compared with that in wild type, and reached a peak at 24 h but then remained at that level at 48 h (Fig. 2B). Together, these data suggest that the higher level of ABA in stratified *35S:AtCSP2* seeds is due to down-regulation of ABA catabolism, rather than up-regulation of ABA biosynthesis.

Since bioactive GA in imbibed seeds is up-regulated by cold treatment (4 °C) in the dark [31], we also examined the expression patterns of GA metabolic genes (*GA20ox1*, *GA20ox2*, *GA20ox3* and *GA2ox2*) in *35S:AtCSP2*-18 during stratification. Transcripts of GA biosynthesis genes (*GA20ox1*, *GA20ox2* and *GA20ox3*) were markedly increased in both wild type and *35S:AtCSP2*-18 after 48 h stratification, although the expression of all three genes was lower in *35S:AtCSP2*-18 than in wild type (Fig. 3A). By contrast, expression of a GA-inactivating gene (*GA2ox2*) in *35S:AtCSP2*-18 was only slightly lower than in wild type (Fig. 3A). The down-regulation of GA biosynthesis genes could be indicative of less GA accumulation in *35S:AtCSP2* seeds, although, as noted above, the GA levels are below the threshold of detection at this stage. We also monitored the expression of GA metabolic genes in *35S:AtCSP2*-18 seeds transferred to normal growth conditions (22 °C, light) after 48 h stratification. Since GA3ox1 and GA3ox2 catalyze GA biosynthesis and are induced by light in imbibed seeds [32], we added these genes to the expression analysis. The transcripts of *GA20ox1*, *GA20ox2* and *GA20ox3* gradually declined in both wild type and *35S:AtCSP2*-18, but the expression in *35S:AtCSP2*-18 was lower compared with that in wild type (Fig. 3B). In wild type, *GA3ox1* transcript peaked at 12 h and decreased thereafter (Fig. 3B). By contrast, *GA3ox1* transcript accumulation in *35S:AtCSP2*-18 peaked at 6 h and was lower than that in wild type (Fig. 3B). *GA3ox2* expression peaked at 12 h in both wild-type and *35S:AtCSP2*-18; however, it was lower in *35S:AtCSP2*-18 (Fig. 3B). Expression of *GA2ox2* gradually declined after stratification and showed no significant difference between the wild type and *35S:AtCSP2*-18 (Fig. 3B). Collectively, these data suggest that AtCSP2 regulates the levels of ABA and GA in seeds by altering the expression of ABA and GA metabolism genes.

Our current data differ from those reported by Park et al. (2009), who suggested that *AtCSP2* is a positive factor in seed germination when salt stress is applied. By contrast, in our experiments, *35S:AtCSP2* seeds exhibited retarded germination under both non-stress and salt stress conditions (Fig. 1A and B). One possible explanation for this discrepancy is that differences in conditions during seed development, maturation or after-ripening may influence the seed germination properties. To address this, in the current study, we utilized an experimental setup designed to avoid any differences in the conditions experienced by wild type and *35S:AtCSP2* seeds (see Section 2), and our observations of germination effects are supported by expression data for GA and ABA metabolism genes, as well as the levels of endogenous ABA (Figs. 2 and 3). In addition, mutations in both *AtCSP2* and *AtCSP4* accelerate seed germination under our conditions (Supplemental Fig. 1). Therefore, we conclude that AtCSP2 negatively regulates seed germination by controlling ABA and GA metabolism.

It is unclear at present how AtCSP2 regulates the expression of ABA and GA metabolic genes in seeds (Fig. 4). An interactome analysis demonstrated that AtCSP3 interacts with different classes of proteins including nuclear polyA-binding proteins (PABNs) and DECAPPING PROTEIN 5 [33], suggesting possible regulatory roles

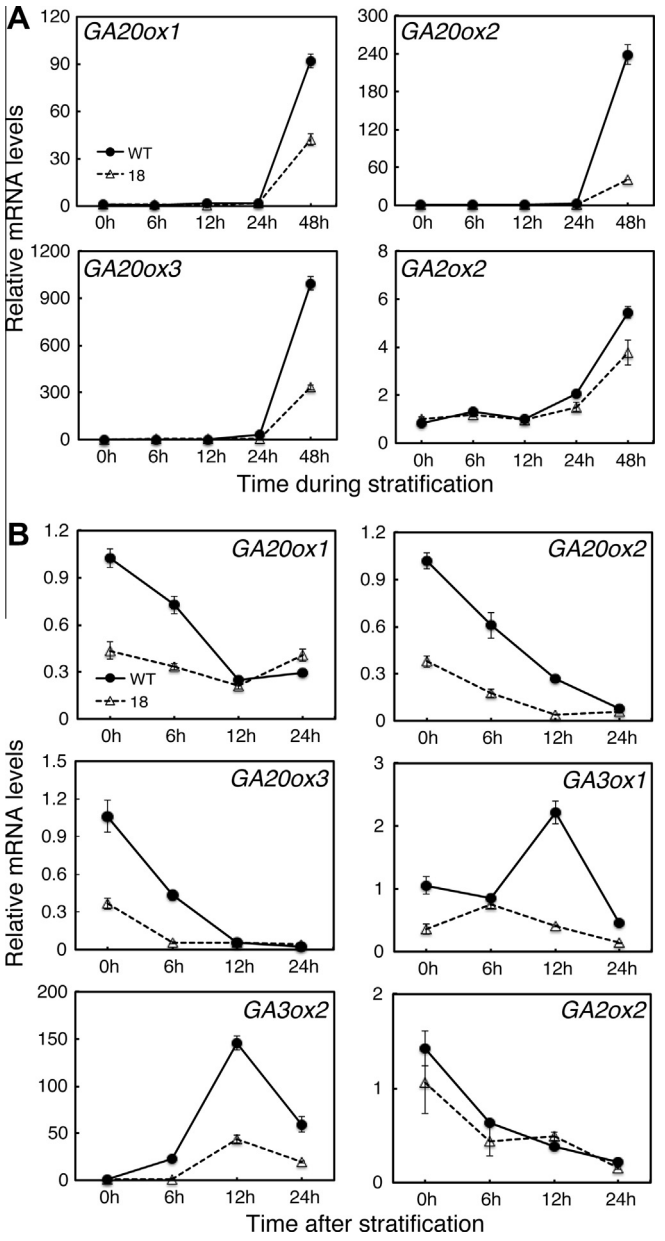

Fig. 3. Effect of *AtCSP2* overexpression on the expression of genes involved in GA metabolism. (A) The expression of GA biosynthesis (*GA20ox1*, *GA20ox2* and *GA20ox3*) and inactivation (*GA2ox2*) genes in WT and *35S:AtCSP2*-18 seeds during stratification. (B) The expression of GA biosynthesis (*GA20ox1*, *GA20ox2*, *GA20ox3*, *GA3ox1* and *GA3ox2*) and inactivation (*GA2ox2*) genes in WT and *35S:AtCSP2*-18 seeds after stratification under long-day conditions. qRT-PCR was performed in triplicate and means ± SD are shown. The values for WT at 0 h were set to 1.

for AtCSP3 in mRNA stability. In addition, preliminary data from yeast two-hybrid studies indicated that AtCSP2 also interacts with nuclear polyA-binding proteins, PABN2 and PABN3 (data not shown). SOMNUS (SOM), another negative regulator of seed germination, is a CCCH tandem zinc finger (TZF) protein that regulates seed germination by altering the expression of ABA and GA metabolism genes [34]. Although the molecular functions of TZF proteins are not known, some TZF proteins regulate RNA stability or processing by binding to RNA [35–39]. Like SOM, AtTZF1, 4, 5 and 6 are negative regulators of seed germination in Arabidopsis [40,41]. From these observations, it is possible to speculate that AtCSP2 regulates the stability of mRNAs during seed germination. Future identification of target mRNAs for AtCSP2 in seeds may

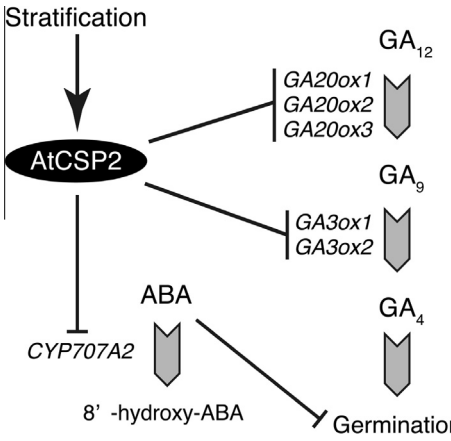

Fig. 4. A proposed model illustrating how AtCSP2 negatively regulates seed germination. AtCSP2 is induced during stratification and represses *CYP707A2* expression, which results in increased ABA content in seeds. AtCSP2 also represses GA biosynthesis genes (*GA20ox1*, *GA20ox2*, *GA20ox3*, *GA3ox1* and *GA3ox2*), resulting in inhibition of seed germination. Arrowheads and end lines indicate positive and negative regulation, respectively.

provide insight into the importance of RNA metabolism in the regulation of seed germination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.092.

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