The Arabidopsis RNA Binding Protein with K Homology Motifs, SHINY1, Interacts with the C-terminal Domain Phosphatase-like 1 (CPL1) to Repress Stress-Inducible Gene Expression

Jiafu Jiang*, Bangshing Wang, Yun Shen, Hui Wang, Qing Feng, Huazhong Shi*

Department of Chemistry and Biochemistry, Center for Chemical Biology, Texas Tech University, Lubbock, Texas, United States of America

Abstract

The phosphorylation state of the C-terminal domain (CTD) of the RNA polymerase II plays crucial roles in transcription and mRNA processing. Previous studies showed that the plant CTD phosphatase-like 1 (CPL1) dephosphorylates Ser-5-specific CTD and regulates abiotic stress response in Arabidopsis. Here, we report the identification of a K-homology domain-containing protein named SHINY1 (SHI1) that interacts with CPL1 to modulate gene expression. The shi1 mutant was isolated from a forward genetic screening for mutants showing elevated expression of the luciferase reporter gene driven by a salt-inducible promoter. The shi1 mutant is more sensitive to cold treatment during vegetative growth and insensitive to abscisic acid in seed germination, resembling the phenotypes of shi4 that is allelic to the cpl1 mutant. Both SHI1 and SHI4/CPL1 are nuclear-localized proteins. SHI1 interacts with SHI4/CPL1 in vitro and in vivo. Loss-of-function mutations in shi1 and shi4 resulted in similar changes in the expression of some stress-inducible genes. Moreover, both shi1 and shi4 mutants display higher mRNA capping efficiency and altered polyadenylation site selection for some of the stress-inducible genes, when compared with wild type. We propose that the SHI1-SHI4/CPL1 complex inhibits transcription by preventing mRNA capping and transition from transcription initiation to elongation.

Introduction

In eukaryotes, gene transcription includes several co-transcriptional processes such as mRNA 5’ capping, splicing, and polyadenylation. These co-transcriptional processes are executed by protein enzymes and factors that are recruited to the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II (Pol II) during gene transcription. The factors to be recruited to the CTD are determined by the phosphorylation patterns of the highly conserved tandem repeats (YSSPSYSSPS) in the CTD that is regulated by site-specific CTD kinases and phosphatases [1]. Phosphorylation status of the Ser-2 and Ser-5 in the heptapeptide repeat of the CTD is thought to be important for the co-transcriptional processes and recycling of the RNA polymerase II. 5’ capping of the nascent transcript occurs shortly after the transcription initiation and requires CTD phosphorylation at the Ser-5 by the general transcription factor TFIH [1,2], while phosphorylation of the Ser-2 residues by the positive transcription elongation factor b (P-TEFb) is believed to promote transcription elongation, splicing, and 3’ end processing [3]. Recycling of Pol II requires dephosphorylation of the CTD and several CTD phosphatases are known to function in this process. In yeast, SCP1 [4,5] and Ssu72 [6] are for the dephosphorylation of Ser-5, while FCP1 is for the dephosphorylation of Ser-2 [7]. However, in Encephalitozoon cuniculi, Fcp1 dephosphorylates both Ser-5 and Ser-2 [8].

The Fcp1 protein has a conserved N-terminal Fcp1 homology (FCPH) region with the DXDX (T/V) signature motif important for its catalytic activity. In Arabidopsis, the FRY2/CPL1 protein also possesses the FCPH domain at its N-terminus that is essential for its CTD phosphatase activity. FRY2/CPL1 dephosphorylates Ser-5 rather than Ser-2 in the CTD repeat polypeptides in vitro [9,10]. In addition, the FRY2/CPL1 contains two dsRNA binding domains at its C-terminus that may be important for its association with RNA. FRY2/CPL1 was initially identified in genetic screenings in Arabidopsis for mutants showing altered expression of luciferase reporter gene driven by the stress-inducible promoter Rd29A. The fyu2 mutant was recovered from an EMS mutagenized population [11] and the cpl1 mutant was identified from a T-DNA insertion mutagenized population [12]. Mutations in FRY2/CPL1 resulted in significant increases in the expression of luciferase reporter gene and other stress-responsive genes, indicating that FRY2/CPL1 is a repressor for stress inducible genes. fyu2/cpl1 mutant plants do not show apparent growth and development phenotypes under normal growth conditions except that the mutants flower later than wild type [11,12]. However, fyu2 mutants
Author Summary

Plants, including important economic crops, frequently grow under unfavorable conditions that largely reduce their production potential. Plants respond to these stress conditions by adjusting physiological status resulting from changes in gene expression. Many genes that are repressed at normal growth conditions are activated in response to stresses. The presented work here attempted to answer the question as to how stress inducible genes are repressed at normal growth conditions. We established a genetic system to identify genes that are essential for such repression and found a protein complex that plays crucial roles in regulating stress inducible gene expression. Our work also revealed important molecular processes that are modulated by the identified protein complex. These findings will help our understanding about gene expression and regulation in general and the molecular mechanisms governing plant stress response in particular.

Results

Identification of the Shi1 Locus

In an attempt to identifying regulators of stress-inducible genes, we established a forward genetic screening for mutants showing elevated expression of the luciferase reporter gene driven by a salt inducible promoter from the sulfotransferase gene \( \text{AtSOT12} \) (At2g03760). The \( \text{AtSOT12} \) gene expression is highly induced by salt and osmotic stress according to our previous finding [28] and a microarray analysis [29]. To test whether the \( \text{AtSOT12} \) promoter is a salt-inducible promoter, a chimeric gene consisting of the firefly luciferase gene driven by the \( \text{AtSOT12} \) promoter was transformed into Arabidopsis ecotype Columbia-0. A homozygous transgenic line showing normal morphology, growth and development was selected for further study. Both Northern hybridization and luciferase imaging revealed that the luciferase expression is highly induced by salt stress (Figure S1) in the homozygous line, indicating that the \( \text{AtSOT12} \) promoter is indeed a salt inducible promoter. Seeds of the homozygous transgenic line (referred to as wild type) were mutagenized with EMS and the M2 seeds from 20 pools were subjected to mutant screening using the luciferase imaging system. A number of mutants showing higher expression of luciferase after salt stress treatment were identified. These mutants were named shiny (shi in short) mutants because of their bright luminescence imaging. One shiny mutant, designated \( \text{shi}1 \), was studied in the present paper.

As shown in Figure 1A, the \( \text{shi}1 \) mutant displayed an elevated luciferase expression comparing with the wild type under both normal condition and after salt treatment. Quantitative analysis of luminescence intensity further confirmed that luciferase activity in the \( \text{shi}1 \) seedlings was much higher than that in the wild type with or without NaCl treatment (Figure 1B). To determine whether the increased luciferase activity in \( \text{shi}1 \) mutant was due to increased luciferase transcript level, RNA blot analysis was carried out. Figure 1C shows that the luciferase gene transcript was not detectable in both wild-type and \( \text{shi}1 \) mutant plants without stress treatment, whereas \( \text{shi}1 \) mutant exhibited substantially higher luciferase transcript level than the wild type after 200 mM NaCl treatment for 5 hours. Quantification of the transcript levels indicated that the fold change of luciferase transcript is much lower than the fold change of luciferase activity (Figures 1B and 1D), which suggests that the increased transcription level of luciferase gene only partly contributes to the increase luciferase activity in \( \text{shi}1 \) mutant.

The \( \text{shi}1 \) Mutation Alters ABA and Cold Responsiveness

After extensive phenotyping of the \( \text{shi}1 \) mutant in response to different abiotic stresses and plant hormones, the \( \text{shi}1 \) mutant was found to be more resistant to ABA in seed germination and more...
sensitive to low temperature during vegetative growth (Figure 2). The germination rate of \textit{shi1} seeds at 7 d was reduced to 84.0% relative to 35.8% of the wild-type seeds in the presence of 0.5 mM ABA. When the concentration of ABA increased to 1.0 mM, the germination rate of \textit{shi1} decreased further to 53.5% comparing with 17.9% of the wild-type seeds (Figure 2B). Under normal growth conditions, \textit{shi1} plants essentially resembled the wild type plants in growth and development (Figure 2C). However, when growing under cold condition at \(\sim 4^\circ C\) with 16 h light/8 h dark, the \textit{shi1} mutant plants showed yellow leaves and smaller rosette comparing with the wild-type plants (Figure 2C). The flowering \textit{shi1} plants exhibited apparent smaller size comparing with the flowering wild type plants when growing under such cold condition (Figure 2C). We also tested other stress treatments, such as ABA, NaCl, LiCl, mannitol and UV-light on the root growth and the \textit{shi1} mutant showed similar response to these stress conditions with wild type.

Expression of Stress-Responsive Genes in \textit{shi1} Mutant

To determine the effects of the \textit{shi1} mutation on stress-responsive gene expression, we selected a set of genes, including \textit{CBF3}, \textit{COR} genes and \textit{DREB2A} that have been used as marker genes for cold, ABA and salt response [11]. The transcript levels of these stress-responsive genes were analyzed by using RNA blot. As shown in Figure 3, \textit{CBF3} transcript levels were higher in the \textit{shi1} mutant than in wild type plants after cold treatments. At the time point of 12 hours of cold treatment, the \textit{shi1} mutant still showed strong induction of \textit{CBF3} gene expression, while wild type did not. \textit{CBF2} expression levels were similar between the \textit{shi1} and wild type after cold treatments, while \textit{shi1} mutant exhibited slightly higher \textit{CBF2} transcript levels than the wild type plants when treated by NaCl or ABA. The \textit{shi1} mutation had opposite effect on the expression of two \textit{COR} (cold-responsive) genes, \textit{COR15A} and \textit{COR47}. The expression of \textit{COR15A} was substantially lower in the \textit{shi1} mutant than in the wild type, while the expression of \textit{COR47} was substantially higher in the \textit{shi1} mutant than in the wild type under some tested stress conditions (Figure 3). The major differences in induced gene expression of \textit{CBF3}, \textit{COR15A} and \textit{COR47} were further verified by using quantitative RT-PCR (Figure S2). These data indicate that SHI1 is involved in the regulation of abiotic stress responsive genes.

Figure 1. Characterization of the \textit{shi1} mutant. (A) Luciferase imaging of 7-day-old seedlings with or without 200 mM NaCl treatment. (B) Quantitative measurements of luciferase activity. Values are means ± SD (n = 10). (C) Northern blot showing luciferase and \textit{AtSOT12} gene transcript levels. Tubulin is shown as a loading control. (D) Quantitative measurement of luciferase transcript levels by quantitative RT-PCR. Values are means ± SD (n = 3). doi:10.1371/journal.pgen.1003625.g001
Map-Based Cloning of the SHI1 Locus

Genetic analysis verified that the shi1 mutation is a single nuclear recessive mutation. To determine the molecular identity of the SHI1 gene, map-based cloning was employed using F2 seeds of the shi1 mutant crossed with Landsberg erecta wild type as a mapping population. Mutant seedlings from the F2 mapping population were selected and the shi1 mutation was mapped with simple sequence length polymorphism (SSLP) markers. The mutation was first mapped to the Chromosome 5 and then further narrowed down to between the BAC clones MNB8 and MFH8 (Figure 4A). After sequencing several candidate genes within this region, a nucleotide change of G1494A was found in the At5g53060 gene in the shi1 mutant. This nucleotide change resulted in an amino acid substitution of E369K in the third KH domain of this KH domain-containing protein (Figure 4A). To confirm whether the mutation in At5g53060 is responsible for the phenotype of shi1 mutant, the shi1 mutant was crossed with two T-DNA lines SALK_143161 and SALK_001448 with T-DNA insertions in the SHI1 gene. The F1 seedlings resulted from these genetic crosses displayed elevated luciferase expression comparing with the wild type (Figure 4B). For molecular complementation assay, a genomic fragment containing the SHI1 open reading frame along with 1910 bp promoter sequence upstream of the translation start codon (corresponding to position 21518401, Chromosome 5) was amplified from the BAC clone MINB8 and introduced into the shi1 mutant by using the Agrobacterium-mediated floral dip transformation method [30]. The wild type SHI1 gene recovered the luciferase expression level of the shi1 mutant to the level in the wild type (Figure 4B), which further supports that the shi1 mutation in At5g53060 gene is indeed responsible for the shi1 mutant phenotype.

Expression Pattern of the SHI1 Gene

The expression of the SHI1 gene in plant was analyzed by using promoter-GUS assay and RNA blot analysis. SHI1 is expressed in roots, leaves, flowers, and siliques, but its expression is low in stems (Figures 5A and 5B). SHI1 is a nuclear-located protein, which was revealed by examining the SHI1-GFP fusion protein expressed in a transgenic Arabidopsis plant (Figure 5C). The expression of SHI1 gene was sharply reduced by treatments with high concentrations of salt such as NaCl, NaNO3, KCl and LiCl, hyperosmotic stress treatment with sorbitol, and treatments with ABA, low or high pH (Figure 5D). However, cold treatment did not significantly change the SHI1 expression level (Figure 5D). Interestingly, the effect of NaCl treatment on the SHI1 expression appears to have a clock rhythm. SHI1 gene expression was quickly and sharply reduced by NaCl treatment for 10 min and this inhibition lasted up to 2 hours of NaCl treatment. The SHI1 expression level gradually recovered to the control level after salt treatment from 3 to 6 hours. After 12 hours NaCl treatment, the
SHI1 transcript was again reduced to very low level and then recovered substantially after 24 hours of NaCl treatment (Figure 5D).

SHI1 Interacts with FRY2/CPL1
To better understand the molecular function of SHI1 in Arabidopsis, a yeast two-hybrid screen for SHI1-interacting proteins was performed. 18 independent clones representing FRY2/CPL1 cDNA showing interaction with SHI1 bait protein were identified from the prey cDNA library (TAIR Cat. No. CD4-30). Among the 18 clones, three different sizes of cDNAs were obtained: the longest cDNA corresponding to 476–967 aa sequence, the medium sized cDNA corresponding to 533–967 aa sequence, and the shortest cDNA corresponding to 666–967 aa sequence of the C-terminus of the FRY2/CPL1 protein. The full length protein of FRY2/CPL1 was also confirmed to interact with SHI1 in the yeast two-hybrid system (Figure 6B).

Protein deletion analysis was used to pinpoint the regions responsible for SHI-FRY2/CPL1 interaction. As shown in Figure 6A, the first KH domain and the third KH domain in the SHI1 protein could interact with FRY2/CPL1. The shi1 mutation of Glu389 to Lysine change in the third KH domain disrupted the interaction of SHI1 with FRY2/CPL1, suggesting that the formation of SHI1-FRY2/CPL1 protein complex is essential for SHI function and loss-of-function of shi1 mutation is probably due to disruption of such a complex formation. Deletion analysis also revealed that the first dsRNA binding motif is required for the interaction of FRY2/CPL1 with SHI1 (Figure 6B).

Figure 3. Detection of stress-inducible gene expression in wild type, shi1 and shi4 mutants. (A) Northern blot showing the transcript levels of stress-inducible genes in wild type, shi1 and shi4 mutants. Tubulin is shown as a loading control. (B) Quantitative measurements of the relative signal strengths compared with the signal strength in the wild type control. The signal strengths were measured by Imaging J. Each signal strength was normalized with the signal strength of the corresponding tubulin.

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Figure 4. Map-based cloning of the SHI1 locus. (A) Identification of the shi1 mutation. Recombination events from 1858 chromosomes analyzed for each marker are shown. The locations of the shi1 mutation of G1494A and two T-DNA insertions are shown. (B) Complementation tests for the shi1 mutation. Left panel, genetic complementation between the shi1 mutation and the T-DNA knockouts; Right panel, molecular complementation of the shi1 mutation by the wild type SHI1 gene.

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Direct physical interaction between SHI1 and FRY2/CPL1 was determined with protein pull-down assay. The amino acid sequence including the first dsRNA binding domain (666–855 aa) of the FRY2/CPL1 was fused with 6XHIS tag (HIS-cFRY2) and synthesized by using an in vitro translation system. GST-SHI1 tagged protein was expressed and purified from *E. coli* for in vitro pull-down assay. As shown in Figure 6C, the 35S-methionine radioactive labeled HIS-cFRY2 was pulled down together with GST-SHI1, which confirms a physical interaction between SHI1 and FRY2/CPL1. Interaction of SHI1 with FRY2/CPL1 in plant cells was further established by using a split luciferase complementation assay [31] and split YFP complementation assay. The SHI1 protein was fused with the N-terminal portion of the luciferase and the FRY2/CPL1 protein was fused with the C-terminal part of the luciferase and co-expressed in Arabidopsis protoplasts. Luciferase activity measurements indicated that co-expression of these two fusion proteins generated significantly higher luciferase activity than all controls tested (Figure 6D), which suggests that SHI1 can interact with FRY2/CPL1 in plant cells. Split-YFP assay also confirmed the interaction of SHI1 with FRY1 in plant protoplasts (Figure S3). Furthermore, co-immunoprecipitation assay was carried out to determine in planta interaction of these two proteins. Transgenic plants expressing FLAG-SHI1 and FRY2-c-TAP tagged proteins were created and homozygous transgenic lines were generated. The F1 plants resulting from the cross between FLAG-SHI and FRY2-c-TAP plants were used for protein isolation and co-immunoprecipitation. Figure 6E shows that SHI1 and FRY2/CPL1 could mutually co-precipitate in plants, which strongly suggests that these two proteins can form a protein complex in plant for gene regulation.

**Identification of shi4 as an Allele of the fry2/cpl1**

The *shi4* mutation was first located in the chromosome 4 and then narrowed down to the region between the BAC clones F7K2 and F7J7. In this region, the FRY2/CPL1 gene (*At4g21670*) was previously identified as an important regulator of stress-responsive genes [11,12,32]. Sequencing of the FRY2/CPL1 gene in the *shi4* mutant determined an allelic mutation of a G to A transition in the second exon resulting in an E116K substitution in the FRY2/CPL1 protein (Figure 7A). This recessive loss-of-function mutation in FRY2/CPL1 was also identified in a forward genetic screening for mutations altering wounding-induced gene expression [32].

The *shi4* mutant displayed elevated luciferase expression resembling the *shi1* and the previously reported *fry2/cpl1* mutants [11,12]. Besides, the *shi4* mutant also exhibited cold sensitive
SHINY1 and Gene Repression

A

Interaction
BD FRY2

SC-T/L
X-Gal
SC-T/LH +3-AT

E389K

1-652aa
1-575aa
1-411aa
1-323aa
1-174aa
141-652aa
246-652aa
392-652aa
465-652aa
246-411aa
392-575aa
246-575aa

shl1

B

Interaction
BD SHI1

SC-T/L
X-Gal
SC-T/LH +3-AT

727 790 856 920 967

1-967aa
666-967aa
726-967aa
791-967aa
855-967aa
921-967aa
666-855aa
666-791aa
666-726aa

C

MW Anti-His Anti-GST Pull-down assay
kDa 1 2 3 4 5 6 7 8

97.5

26
28

D

RLU

Low
High

Mock
Nluc-SHI1
Cluc-FRY2
Nluc-SHI1
Cluc-FRY2

E

IP with Flag AB
IP with Rabbit IgG

WT Flag-SHI1 X
FRY2-CTAP F1

Anti CBP

Input Anti Flag

Input
The phenotype similar to that of the _shi1_ mutant (Figure 7B). Analysis of SHI4-GFP fusion protein revealed a nuclear localization of SHI4 (Figure 7C). Study of the expression of stress inducible genes in _shi4_ indicated that both _shi4_ and _shi1_ mutations affected the expression of those cold, osmotic and ABA inducible genes in a similar way (Figure 3). Taken together, these results further support that SHI1 and SHI4/FRY2/CPL1 form a functional complex in Arabidopsis to regulate the expression of stress responsive genes.

**SHI1 and SHI4/FRY2/CPL1 Involves in mRNA Capping and Polyadenylation Site Selection**

The FRY2/CPL1 protein has been shown to specifically dephosphorylate the Ser-5 at the CTD repeat of the RNA polymerase II.
polymerase II [9,10]. The Ser-5 phosphorylation is known to be required for recruiting the capping enzyme and stimulating mRNA capping [1], while dephosphorylation of the Ser-5 by CTD phosphatase has been shown to decrease mRNA capping [33]. To determine whether SHI1 and SHI4/FRY2/CPL1 are involved in mRNA capping, two methods were used to analyze the ratio of capped mRNA in total mRNA of individual genes. The first method was designed based on RNA Ligation Mediated Rapid Amplification of cDNA Ends (RLM-RACE) with modifications in which qRT-PCR was used instead of RACE. The total RNA was treated with Calf Intestinal Alkaline Phosphatase to remove free 5’ phosphate in the uncapped mRNA to prevent ligation of these mRNAs with the RNA adapter. The total RNA was then treated with Tobacco Acid Pyrophosphatase to remove the cap of the capped mRNA followed by a ligation of a RNA adapter with the treated RNA population. Only the de-capped mRNAs could be ligated with the RNA adapter. The RNA was reverse transcribed into cDNAs using random primers, and the ratio of capped transcripts in total transcripts of selected genes was determined by real-time PCR. This method was used to determine the relative mRNA capping ratio of five selected genes including the luciferase transgene, AtSOT12, Atg5g25280 (a constitutively higher expressed gene in shi1 and shi4 that was found from our unpublished microarray data, Figure S4), COR15A, and COR47. As shown in Figure 8A, shi1 mutant displayed substantially increased mRNA capping ratio of all five tested genes, while shi1 mutant exhibited differential regulation of capping event in these five genes. For the LUC transgene and the endogenous stress-inducible gene COR47, shi1 mutant showed increases in capping ratios comparable to that in shi4 mutant. However, the shi1 mutation did not alter the capping ratio of the endogenous AtSOT12 mRNA and only caused marginal increases in capping ratios of Atg5g25280 and COR15A. These results suggest that SHI1/FRY2/CPL1 is the major player in modulating mRNA capping and SHI1 protein is involved in capping of some mRNAs by partnering with SHI4.

The second method was a 5’RACE-based method to determine the 5’-m7G cap that can be reverse transcribed into a C in the first strand of the cDNA [34]. This method was first verified by detection of capped mRNA captured by the Arabidopsis cap-binding protein eIF4E (At4g18040) and uncapped mRNA incapable of binding by eIF4E. The Arabidopsis eIF4E was fused with GST and expressed and purified from E. coli. The binding specificity and kinetics of this fusion protein with capped mRNA were analyzed according to the previously published protocol [35]. The GST fused Arabidopsis eIF4E was found to specifically bind with capped mRNA and the binding constant is 0.18 nM (Figure S5). Purified GST-eIF4E proteins were incubated with Arabidopsis total RNA and capped mRNAs associated with the fusion protein were pulled down with the resin against the GST tag. 5’RACE results showed that 92% of the eIF4E-bound luciferase mRNAs have a 5’-cap that was reverse transcribed into the cDNA, while approximately 80% of the unbound luciferase mRNA did not show 5’ cap. This result supports that the 5’RACE based method is a reliable method for mRNA 5’ cap detection. Therefore, the 5’RACE-based method was used to detect the absolute ratio of capped transcripts in total transcripts of the selected genes. Figure 8B shows that the ratios of capped transcripts in total transcripts of luciferase, AtSOT12, and Atg5g25280 genes in shi1 mutant were significantly higher than that in the wild type, while shi4 mutant showed significantly higher capping ratio of the luciferase mRNA, but exhibited no change in the endogenous AtSOT12 mRNA capping and slight increase in Atg5g25280 mRNA capping when compared with wild type. These results are consistent with the results showing in Figure 8A and strongly support that SHI1 and SHI4/FRY2/CPL1 act to negatively regulate mRNA capping.

To determine whether SHI1 and SHI4/FRY2/CPL1 are involved in other co-transcriptional processes, a 3’RACE analysis was carried out to pinpoint the polyadenylation sites of the five selected genes transcripts. Major polyadenylation sites were found in four of the five genes, and the AtSOT12 gene transcripts exhibited a dispersed pattern of polyadenylation sites mainly downstream of the putative polyadenylation signal sequences (Figure 9A). Two major polyadenylation sites were found in the luciferase mRNAs; one (designated 1st PA) is located at the 21st position upstream of the canonical polyadenylation signal (PA) sequence AAUAAA and the other (2nd PA) at the position of the 13th nucleotide downstream of the cPA (Figure 9A). In wild type, polyadenylation at the 1st and 2nd PA sites were 44.4% and 33.3%, respectively. In shi1 and shi4 mutants, however, polyadenylation at the 1st PA site was reduced to about 20%, while polyadenylation at the 2nd PA site was increased to about 60% (Figure 9B). AtSOT12 transcripts did not show a major polyadenylation site, but more than 70% of the AtSOT12 transcripts in wild type displayed polyadenylation downstream of the cPAs. This ratio was significantly increased in the shi1 and shi4 mutants (Figure 9B). Both shi1 and shi4 mutations also strongly affected polyadenylation site selection in COR47 transcripts. Polyadenylation at the major PA site of COR47 in shi1 and shi4 mutants reduced remarkably when compared with that in wild type (Figure 9B). shi1, but not shi4 mutant showed significant reduction in polyadenylation at the major PA site of Atg5g25280, and both mutants did not affect polyadenylation site selection of COR15A (Figure 9B). These results indicate that the shi1 and shi4 mutations have profound influence on PA site selection of the LUC transgene and also affect some endogenous genes. SHI1 is likely to be involved in some, but not all genes that are regulated by SHI1, as suggested by both capping and PA site selection analyses shown in Figure 8 and Figure 9.

**Discussion**

Gene regulation is central for growth, development and adaptation to environmental changes in all living organisms. In plants, many genes are environmentally regulated, and proper response of these genes is critical for stress response and tolerance. Stress-inducible genes must be repressed or silenced at normal growth conditions but can be readily activated upon stress treatments. In the past decades, intensive studies have been focused on the activation mechanisms of inducible genes in response to stress conditions. Consequently, a number of cis-elements and transacting proteins required for gene induction have been identified [36]. For instance, the regulatory element DRE (dehydration responsive element) was identified from the drought, salt and cold inducible promoters and found to be recognized by the transcription factors DREB1/CBF and DREB2 [37–39]. However, little attention has been paid to the repression mechanisms of stress inducible genes at normal growth conditions. In this study, we utilized a luciferase-based mutant screening system to identify repressor proteins for stress inducible genes. We uncovered two repressor proteins, SHI1 and SHI4/FRY2/CPL1 that can form a complex and repress gene expression by modulating transcription and co-transcriptional processes. Interestingly, the SHI1 protein was also identified as a negative regulator of heat-inducible genes in a recent study using forward genetic screening for mutations affecting luciferase expression driven by the cold-inducible CBP2 promoter [40]. Thus, SHI1 protein might be a general repressor for some of the stress-
Figure 8. Detection of 5’ capping of the transcripts of LUC, AtSOT12, At5g25280, COR15A, and COR47 in wild type, shi1 and shi4 mutants. (A) Relative capping ratios of the five selected genes transcripts determined by using the RLM-qRT-PCR method. Values are shown as mean ± SD (n = 3). (B) Percentages of the capped mRNA of three selected genes determined by using the 5’ RACE based method.

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inducible genes. The cold and ABA responsive phenotypes of shi1 and shi4 protein could be attributed to mis-regulation of those cold and/or ABA-responsive genes as shown in Figure 3.

SHI1 protein belongs to the family of KH domain containing proteins comprising of 26 members in Arabidopsis. The nuclear localization of SHI1 (Figure 5C) indicated that SHI1 functions in a nuclear-based process. Moreover, we found that SHI1 can interact with the CTD phosphatase FRY2/CPL1, a previously characterized protein that dephosphorylates the Ser-5 at the CTD of the RNA polymerase II [9,10]. Thus, it is conceivable that the SHI1 may modulate the CTD phosphorylation status through interacting with FRY2/CPL1, thereby regulating transcription and co-transcriptional processes. Functional interaction between SHI1 and FRY2/CPL1 was evidenced by the following findings. First, our genetic screening also recovered a fry2/cpl1 allele named shi4, and shi4 exhibited luciferase imaging and stress response phenotypes similar to the shi1 mutant (Figure 7). Second, SHI1-FRY2/CPL1 interaction was verified by several protein interaction techniques including yeast two-hybrid assay, pull-down assay, split-luciferase assay and in planta Co-IP analysis (Figure 6). These analyses indicate that SHI1 directly interacts with FRY2/CPL1 in plant cells. Third, the shi1 mutation disrupted the interaction between SHI1 and FRY2/CPL1 (Figure 6), which suggests that SHI1 requires FRY2/CPL1 for its function. Forth, shi1 and shi4 mutants exhibited very similar expression patterns of stress inducible genes (Figure 3). These evidences strongly support that SHI1-FRY2/CPL1 forms a functional complex to regulate gene expression.

Both SHI1 and FRY2/CPL1 contain domains for interaction with nucleic acids. SHI1 contains five KH domains that have been considered as RNA or single stranded DNA binding domains, while FRY2/CPL1 possesses two dsRNA binding domains. Although both domains were proposed to interact with nucleic acids, our deletion assay for SHI1-FRY2/CPL1 interaction revealed that the third KH domain in SHI1 and the first dsRNA binding domain in FRY2/CPL1 are required for such interaction. This indicates that both KH domain and dsRNA binding domain can mediate protein-protein interaction. Interestingly, we found that, in addition to the third KH domain, the first KH domain in SHI1 is also sufficient to interact with FRY2/CPL1 (Figure 6A). The deletion analysis showing in the Figure 6A also raised a possibility of intramolecular interactions within the SHI1 protein, we carried out a yeast two-hybrid assay for each KH domain. We found that the KH2 can interact with both KH1 and KH3, and KH3 alone can activate the reporter gene expression in yeast cells (Figure S6). Self-activation of the reporter gene expression by the KH3 indicates that the KH3 may be able to interact with and recruit the transcription machinery to the promoter for transcription. These results suggest that SHI1 protein may undergo structural rearrangement upon binding with FRY2/CPL1. This structural rearrangement may promote SHI1 interactions with chromatin and components within the transcription machinery, thus acting as a bridging protein to recruit FRY2/CPL1 to the transcription initiation site through its KH domains that are capable of interacting with nucleic acids and proteins.

SHI1-FRY2/CPL1 complex is involved in mRNA 5’-capping, which is supported by our findings shown in Figure 8. This is consistent with the facts that FRY2/CPL1 functions as a Ser-5 specific CTD phosphatase [10] and the Ser-5 phosphorylation is required for recruiting 5’ capping enzymes [1]. Based on our results, we propose that, at normal growth conditions, the SHI1
The CTD and compromising subsequent 5' initiation to elongation due to dephosphorylation of the Ser-5 in the transcription initiation site of the stress inducible promoter.

The translation efficiency of the luciferase mRNA is higher in the mutants than in the wild type (Figure 9) suggests that lacking a functional SHI1 or SHI4 in the mutants may have caused formation of a complex not only with low repression activity but also lacking components that are needed for polyadenylation site selection. These hypotheses need to be further verified. Identification of the components within the repressor complex involving SHI1-FRY2/CPL1 will greatly help our understanding about gene repression, which deserves further study.

**Materials and Methods**

**Plant Materials, Mutant Isolation, and Growth Conditions**

The firefly luciferase ORF fragment was released from the RD29A-LUC construct by digestion with Hind III and Sma I and inserted into pCAMBIA1381Z at Hind III and Pmal I by replacing the original GUS gene to form pCAMBIA1381Z-LUC. The promoter and the entire 5'-UTR of At2g03760 was amplified using Pfu polymerase (Stratagene) with a forward primer cccgagaggctacagccttacgtc and a reverse primer aaaa caaaggtgaacagctgacatatcaacat. A homozygous line (named T3 9-1) showing normal growth and development and induced luciferase expression by salt stress treatment was selected as the parental line (referred to as wild type) for mutagenesis using ethyl methane sulfonate (EMS). Seedlings of the M2 generation from the EMS-mutagenized seeds were screened for mutants with altered luciferase expression with or without NaCl treatment by using a charge-coupled device camera (DU434-BV, Andor Technology, Connecticut) according to the previously published protocol [41]. The putative mutants were transferred to soil and subjected to a re-screening process to eliminate the false positives.

**Cold and ABA Tolerance Assays**

To determine the sensitivity of seed germination to ABA, the seeds were sterilized for 15 min in 20% Clorox bleach with 0.05% Triton X-100, washed 3–5 times with sterilized water, suspended in 0.3% low melting point agarose and incubated at 4°C for 2 days. The seeds were then planted onto half MS agar medium with different concentrations of ABA and incubated at room temperature under 16 h/8 h fluorescent light cycle. Germination was scored when cotyledon was emerged. The test of plant sensitivity to cold conditions was carried out by transferring two-week old seedlings growing in soil into a 4°C cold room and incubated under 16 h/0 h light cycle.

**Mapping and Identification of the SHI1 and SHI4 Locus**

For map-based cloning, mutants were crossed with wild-type Landsberg erecta and the F1 plants were selfed to generate the F2 seeds. The F2 seedlings showing higher luciferase expression were selected for SSLP marker-assisted genetic mapping. The mutations were identified by sequencing candidate genes. For genetic complementation of the shi1 mutation, two T-DNA insertion lines SALK_143161 and SALK_001448 of the SHI1 were obtained from the Arabidopsis Biological Resource Center (ABRC) [42]. The firefly luciferase ORF fragment was released from the RD29A-LUC construct by digestion with Hind III and Sma I and inserted into pCAMBIA1381Z at Hind III and Pmal I by replacing the original GUS gene to form pCAMBIA1381Z-LUC. The promoter and the entire 5'-UTR of At2g03760 was amplified using Pfu polymerase (Stratagene) with a forward primer cccgagaggctacagccttacgtc and a reverse primer aaaa caaaggtgaacagctgacatatcaacat. A homozygous line (named T3 9-1) showing normal growth and development and induced luciferase expression was obtained by Agrobacterium-mediated transformation using the floral dip method [30]. A homozygous line (named T3 9-1) showing normal growth and development and induced luciferase expression was obtained by Agrobacterium-mediated transformation using the floral dip method [30].
mutant and the T2 transgenic lines were used for luciferase imaging.

Promoter-GUS and Subcellular Localization Analysis

The promoter region of the \textit{SHI1} gene with 1847 bp upstream of the translation start codon was amplified using from the BAC clone MINB8 using \textit{Pfu} polymerase (Stratagene) and inserted into \textit{Bomh} I and \textit{Pst} I sites of the binary vector pCAMBIA1301\textsubscript{Z} to create a transcriptional fusion of the \textit{SHI1} promoter with the GUS reporter gene. The resulting construct was transferred into Columbia-0 wild-type plants by the floral dip method [30]. The T2 seedlings were stained with X-Gluc staining buffer (10 mM Tris pH 7.0, 10 mM EDTA, 0.1% Triton X-100 and 2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) for 12–24 hours at 25°C, followed by incubating in 70% ethanol to remove chlorophyll. For protein subcellular localization assay, the ORF of the \textit{SHI1} gene was recombined from pENTR1A into the PmDC45 vector [43] to create SHI1-GFP fusion and the ORF of FRY2 was recombined into pEarleyGate105 [44] for FVR2-GFP fusion. The fusion constructs were transferred into Arabidopsis by the floral dip method [30] and the T2 transgenic plants were used to examine the location of the GFP fluorescence using confocal microscope.

Gene Expression Assay

For gene expression study, wild-type and \textit{shi1} mutant seed plants were planted on half MS agar medium. Ten-day-old seedlings were subjected to NaCl, ABA and cold treatments as previously described [11]. To study the expression of \textit{SHI1}, 10-day-old wild type seedlings were subjected to different stress conditions following the method described by Chung et al. [45] and different parts of plants were collected from soil-growing plants. Total RNA was extracted from the seedlings and analyzed by RNA blotting. The DNA probes for the tested genes were PCR-amplified by using the following primers: \textit{SHI1}, atggagagatctagatccaagagaaactctactcactactcagattgacctgctggagtagacattctcaccaactg; \textit{COR15A}, aagaaaagctcagacggtgactgtgg; and \textit{actin2} for RNA blotting using the following primers: \textit{COR47}, cgatagtcgtttccatttttgt and ttgctagattcgaagtagagtagaagtgtttcatggt with \textit{Sac I} restriction site (underlined), the reverse primers are cgatagtcgtttccatttttgt and ttgctagattcgaagtagagtagaagtgtttcatggt for \textit{Sac I} restriction site (underlined). DNA probes were radioactively labeled using \textit{[\textsuperscript{32}P]} dCTP using the Prime-It II Random Primer Labeling Kit (Stratagene).

Quantitative RT-PCR to determine the expression levels of \textit{LUC}, \textit{CBF3}, \textit{COR15A} and \textit{COR47} was carried out as follows: 2 \textmu g of total RNA isolated with Plant RNA Purification Reagent Kit (Stratagene). The PCR fragments were cloned into the Gateway entry vector pENTR1A (Invitrogen), then recombined into the destination vectors pDEST-GADT7 (prey vector) and pDEST-GGBK7 (bait vector) [40] using \textit{Lg} clonase kit (Invitrogen). Self-activation test verified that the bait pGBKKT7–SHI1 plasmid has no autonomous activity of the reporter genes in the yeast strain Y190. For screening, yeast cells harboring the bait construct were transformed with the cDNA library and plated onto synthetic high-stringency selection medium lacking tryptophan, leucine and histidine supplemented with 23 mM 3-amino-1, 2, 4-triazole (Sigma). The putative positive cDNA clones were further confirmed by the \textit{\beta}-galactosidase assay and tested for specificity by co-transformation into Y190, either alone or in combination with the empty pDEST-GGBK7 vector. The cDNA inserts from positive clones were sequenced using a Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and the ABI 3100 DNA sequencer.

Deletion analysis was used to determine the domains required for interaction between \textit{SHI1} and FRY2 in the yeast strain Y190. For a series deletions of the carboxyl terminus of \textit{SHI1}, the forward primer is acgcgtcagacggtgactgtgg; and \textit{Sal I} restriction site (underlined), the reverse primers are cggaatctcggagtagacattctcaccaactg with \textit{Sal I} restriction site (underlined); \textit{CBF3}, taaaactcagattattatttccattt and aggagccacgtagagggcc; \textit{COR47}, cgatagtcgtttccatttttgt and ttgctagattcgaagtagagtagaagtgtttcatggt with \textit{Sac I} restriction site (underlined); \textit{actin2}, with \textit{Sac I} restriction site (underlined). For the Baroxyl terminus deletions of FRY2, the forward primer is acgcgtcagacggtgactgtgg; and \textit{Sal I} restriction site (underlined), the reverse primers are cggaatctcggagtagacattctcaccaactg with \textit{Sal I} restriction site (underlined); \textit{CBF3}, taaaactcagattattatttccattt and aggagccacgtagagggcc; \textit{COR47}, cgatagtcgtttccatttttgt and ttgctagattcgaagtagagtagaagtgtttcatggt with \textit{Sac I} restriction site (underlined); \textit{actin2}, with \textit{Sac I} restriction site (underlined); \textit{CBF3}, taaaactcagattattatttccattt and aggagccacgtagagggcc; \textit{COR47}, cgatagtcgtttccatttttgt and ttgctagattcgaagtagagtagaagtgtttcatggt with \textit{Sac I} restriction site (underlined); \textit{actin2}, with \textit{Sac I} restriction site (underlined).

The ORF of SHI1 without start codon was amplified with the primers cgaggtgagatctagatccaagaaactctactcactactcagattgacctgctggagtagacattctcaccaactg with restriction site \textit{Bomh} I (underlined) and cggaggtgagatctagatccaagaaactctactcactactcagattgacctgctggagtagacattctcaccaactg with restriction site \textit{Eco R I} (underlined) using \textit{Pfu} polymerase (Stratagene) and was cloned into pGEX2T to create a GST-SHI1 fusion construct. The resulting plasmid was transformed into \textit{E. coli} Rosetta-gami 2 host strains (Novagen) and individual colonies
were inoculated in LB medium and grown at 37°C overnight. The cultures were then diluted to approximately OD600 of 0.1 and grown at 37°C until they reached OD600 0.5–1.2. The protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside for an additional 16–24 h at 30°C on a shaker. Bacterial cells were pelleted and then resuspended in the lysis buffer (50 mM Tris–HCl pH 8.0; 100 mM NaCl and 1 mM EDTA). After sonication and centrifugation, the recombinant protein in the supernatant was purified by using GST Bind Resin (Novagen).

The fragment of FYR2 encoding the first dsRNA binding domain (666–853 aa) in the pENTR1A vector was recombined into the pDEST17 vector to generate a HIS-tagged protein under the control of the T7 promoter. The plasmid was used for in vitro transcription-coupled translation in the presence of [35S]-methionine using the TNT Quick Coupled Translation/Translation Systems (Promega) according to the manufacturer’s instructions. The purified GST-SHI1 and in vitro translated peptides of FYR2 were analyzed by protein gel blotting using anti-GST and anti-HIS antibodies, respectively. Pull-down experiments were performed as follows. GST (as negative control) or GST-SHI1 protein was immobilized to GST Bind Resin (Novagen), which was incubated with [35S]-labeled peptides of FYR2 (HIS-cFYR2) for 30 min at room temperature in 100 μL of the binding buffer (20 mM Tris–HCl, pH 7.2, 10 mM MgCl₂, and 2 mM DTT). After extensive washing with PBS buffer, the beads were resuspended in 50 μL of the protein loading buffer and the proteins were resolved in 12% SDS-polycrylamide gel. The gels were dried and placed in direct contact with the X-ray film (Kodak) in dark at room temperature for autoradiography.

Interaction of SHI1 and FYR2 in Plants

For split luciferase (Renilla reniformis) complementation assay, the SHI1 ORF was amplified with a forward primer acgcgatagattgagagacagttagctgcatgcaacaagtaattc with elimination stop codon using Pfu polymerase (Stratagene). The PCR fragment was digested with Sal I and EcoR V. The SHI1 ORF was then recombined into pDUExAn6 vector and the FYR2 ORF in the pENTR1A vector was recombined into pDUEX6 vector [31]. For split YFP complementation assay, the SHI1 ORF was amplified with a forward primer cgccgatagattgagagacagttagctgcaacaagtaattc and a EcoR I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse primer cgccgatagattgagagacagttagctgcaacaagtaattc with a Xho I restriction site (underlined) and a reverse prime...
5’RACE using GoTaq DNA Polymerase (Promega). The PCR products were further amplified with an adaptor primer ctagtctgctaacga-3 and the nested luciferase gene specific primer gcaggcttcagctgcttcatg-3. The PCR fragments were then cloned into pGEM-T Easy vector (Promega) and sequenced using a Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems) on the ABI 3100 DNA Sequencer following a standard protocol. At least 20 independent clones were sequenced and the additional G at the very end of each cDNA was counted as from a capped mRNA.

**Determination of Polyadenylation Sites**

The polyadenylation sites of the mRNAs were determined by using 3’ RACE method. Briefly, 2 µg of total RNA isolated with Plant RNA Purification Reagent (Invitrogen) was reverse transcribed using the avian myeloblastosis virus (AMV) reverse transcriptase (Promega) and oligo dT(15). The cDNA corresponding to the 3’ end of the target gene mRNA was then synthesized by PCR amplification using the adaptor primer ctagtctgctaacga-3 and the target gene specific primer (LUC: tttgggagccaaagagctc; AtSOT12: tgctacaagtgagaatgtaacac; At5g25280: gttacagctgctatgagcataa; COR15A: ccacaacagggcagatgg; COR47: tctacacgcttagcagca). The PCR product was then used as template for a nested PCR amplification using the adaptor primer ctagtctgctaacga-3 and the polyadenylation sites were scored according to the sites linking with the PolyA.

**Supporting Information**

**Figure S1** Detection of salt-induced expression of the luciferase transgene driven by the AtSOT12 promoter. (A) Luciferase activity measurements of the homozygous line expressing AtSOT12-LUC transgene. Values are means ± SD (n = 3). (B) Northern blot showing induction of the luciferase transgene and AtSOT12 gene by NaCl treatment. Tubulin is shown as a loading control. Ctrl. Control without salt treatment; NaCl, 200 mM NaCl for 5 h. (TIF)

**Figure S2** Quantitative RT-PCR showing the transcript levels of stress-inducible genes in wild type (WT), shi1 and shi4 mutants in response to different abiotic stress treatments. Cold, 0°C for 12 h; NaCl, 200 mM NaCl for 12 h; ABA, 100 µM ABA for 3 h. Values are mean ± SD (n = 3). (TIF)

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