Repression of stress-responsive genes by FIERY2, a novel transcriptional regulator in *Arabidopsis*

Liming Xiong*, Hojoung Lee*, Manabu Ishitani*, Yuko Tanaka*, Becky Stevenson*, Hisashi Koiwa†, Ray A. Bressan†, Paul M. Hasegawa†, and Jian-Kang Zhu*‡

*Department of Plant Sciences, University of Arizona, Tucson, AZ 85721; and †Center for Plant Environmental Stress Physiology, 1165 Horticulture Building, Purdue University, West Lafayette, IN 47907-1165

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Low temperature, drought, and high salinity induce the expression of many plant genes. To understand the mechanisms for the transcriptional activation of these genes, we conducted a reporter gene-aided genetic screen in *Arabidopsis*. Seven allelic mutations in the FIERY2 (*FRY2*) locus result in significant increases in the expression of stress-responsive genes with the DRE/CRT (drought-responsive/C-repeat) cis element but non-DRE/CRT type stress-responsive genes were less affected. The specific regulation of DRE/CRT class of genes by FRY2 appears to be caused by repression of stress induction of the upstream CBF/DREB transcription factor genes. *fry2* mutants show increased tolerance to salt stress and to abscisic acid during seed germination but are more sensitive to freezing damage at the seedling stage. *FRY2*/*CPL1* encodes a novel transcriptional repressor harboring two double-stranded RNA-binding domains and a region homologous to the catalytic domain of RNA polymerase II C-terminal domain phosphatases found in yeast and in animals that regulate gene transcription. These data indicate that FRY2 is an important negative regulator of stress gene transcription and suggest that structured RNA may regulate hormone and stress responses in plants as it does in animals.

In response to adverse environmental conditions such as low temperature, drought, and salinity, plants activate a large number of genes that usually are not expressed under normal growth conditions (1–5). Overexpression of these genes by manipulating their cognate transcriptional activators results in increased tolerance to various stress conditions such as cold, salt, and drought (6, 7), which supports the premise that the products of these genes are beneficial to plants under stress. For future progress in the genetic manipulation of stress tolerance, it is critical to have a better understanding of the mechanisms responsible for activation of these stress-responsive genes.

One approach that has contributed significantly to our understanding of gene activation is to study their promoter elements and to search for transcription factors that bind to these elements. Such studies have discovered several classes of cis elements in the promoters of stress-responsive genes. The phytohormone abscisic acid (ABA)-responsive element is responsible for gene activation by ABA (8–11). On the other hand, the DRE/CRT (drought-responsive/C-repeat) element is responsible for gene induction by drought, salt, and low temperature (9, 12). CBF/DREBs, a family of AP2/ERF class transcription factors, were found to bind to the DRE/CRT element and confer low temperature or drought/salt stress-induced gene expression (12, 13). In contrast, ABF/AREBs, a family of basic leucine zipper class of transcription factors, bind to ABA-responsive element and confer ABA-responsive gene expression (14, 15).

To understand the mechanisms by which plants perceive stress stimuli and transmit the signals to cellular machinery to activate gene expression, we have used a reporter gene approach to screen for mutations that impair stress signaling (16). Here, we present the characterization and cloning of a new genetic locus, *FIERY2* (*FRY2*). Recessive mutations in *FRY2* result in super-induction of the DRE/CRT class of stress-responsive genes, suggesting that FRY2 negatively regulates stress and ABA activation of these genes. FRY2 acts upstream of CBF/DREB transcription factors and has important roles in regulating stress tolerance and ABA responsiveness during seed germination. *FRY2* encodes a novel transcriptional repressor showing a limited homology to yeast and human C-terminal domain (CTD) phosphatases that recently were found to be involved in gene transcription and pre-mRNA processing. *FRY2* is identical to *CPL1* independently identified by Koiwa *et al.* (17) and is thus referred to as *FRY2*/*CPL1*. The identification of *FRY2*/*CPL1* provides clues to the mechanisms of gene transcription under environmental stresses. The feature of double-stranded RNA-binding domains (DSRMs) in FRY2/CPL1 also suggests the possibility that structured RNA may regulate plant stress responses as it does in animals.

Materials and Methods

Plant Materials, Mutant Isolation, and Growth Conditions. *Arabidopsis thaliana* plants in the C24 ecotype expressing *RD29A-LUC* (referred to as wild type) were obtained by *Agrobacterium*-mediated transformation as described (16). Seeds from this line were mutagenized with ethyl methanesulfonate, and seedlings of the M_2 generation were screened for mutants with altered luminescence expression under cold, ABA, or NaCl treatment by using a charge-coupled device camera (Princeton Instruments, Trenton, NJ) (16). Stress treatments for luminescence analysis were conducted as described (16). Briefly, cold treatment was conducted by incubating 7-day-old seedlings growing in MS (Murashige and Skoog salt base; JRH Biosciences, Lenexa, KS) agar plates at 0°C for the indicated time period before image analysis. ABA treatment was conducted by spraying 100 μM ABA on leaves and incubating the seedlings under light for 3 h. NaCl treatment was conducted by incubating seedlings onto filter paper saturated with 300 mM NaCl. Luminescence intensities of each seedling were quantified with the WINVIEW software provided by the camera manufacturer.

Stress and ABA Tolerance Assays. The sensitivity of seed germination to ABA and NaCl was assayed on filter paper saturated with ABA or NaCl solutions as described (18). The imbibed seeds on filter paper first were incubated at 0°C for 2 days before being incubated at room temperature (22 ± 2°C) under white light for germination. Germination (i.e., radical emergence) was scored daily up to 10 days. Seedling sensitivity to freezing temperatures was assayed by measuring freezing-induced electrolyte leakage from leaves of rosette stage as described (19). Cold acclimation

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Abbreviations: ABA, abscisic acid; DRE/CRT, drought-responsive/C-repeat; CTD, C-terminal domain; RNPII CTD, RNA polymerase II CTD; DSRM, double-stranded RNA-binding domain(s); TAR, transactivation responsive; TRBP, TAR RNA-binding protein; MS, Murashige and Skoog salt base.

‡To whom reprint requests should be addressed. E-mail: jkzhu@ag.arizona.edu.

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was conducted by incubating plants at 4°C under light for 4 days before the electrolyte leakage assay. Root growth on NaCl or ABA medium was assayed by transferring 7-day-old seedlings to MS plates with 1.2% agar supplemented with various concentrations of ABA or NaCl. New root elongation was measured at designated time intervals as stated in the text.

Genetic Analysis, Mapping, and Cloning of the *FIERY2* Locus. The nature of the *fry2* mutations was analyzed by crossing the mutants with the wild-type plants. The F_1 and F_2 progenies were scored for stress and ABA-induced luminescence. To test the allelism, *fry2* mutants were crossed reciprocally and the F_1 progenies were analyzed for cold and ABA-induced luminescence. To clone *FRY2*, *fry2–1* was crossed with the *Arabidopsis* Columbia ecotype and the F_2 progeny from self-pollinated F_1 were used to select mapping samples with *fry2* luminescence phenotypes under cold and ABA treatments. Genomic DNA extracted from these seedlings served as templates for PCR-based mapping by using simple sequence polymorphism markers as described (18).

RNA Analysis. For gene expression study, wild-type and *fry2* seeds were planted on separate halves of the same MS agar plates. One-week-old seedlings then were subjected to stress or ABA treatment as described above. Total RNA was isolated from the seedlings and analyzed by RNA blotting as described (19). To study the expression of *FRY2*, different parts of plants were harvested from soil-growing plants and total RNA extracted and analyzed similarly. The full-length *FRY2* cDNA probe was obtained by reverse-transcriptase PCR. Probes for the stress responsive genes have been described previously (19, 20).

Results

Identification of the *FIERY2* Locus. The *RD29A* (also known as *COR78* or *LTI78*) gene is induced strongly by cold, drought, salt, and ABA (21, 22). Although the function of its product is unknown, the promoter of this gene is well characterized and contains both the ABA-responsive element and DRE/CRT elements (9, 12). For genetic analysis, *Arabidopsis* plants (referred to as wild type) that express a chimeric gene consisting of the firefly luciferase cDNA driven by the *RD29A* promoter (*RD29A-LUC*) were mutagenized, and mutants with deregulated expression of the transgene were isolated (16). A group of mutants were found to have significantly higher expression of luminescence under stress and ABA treatments. Because of their extremely high luminescence intensities, the mutants were named *fiery* (*fry*). The *FRY1* locus has been cloned and found to encode an inositol polyphosphate 1-phosphatase, providing genetic evidence that stress signaling and ABA signaling involve phosphoinositide second messengers (18). A second locus, *FRY2*, is the subject of the present study.

The *fry2* mutants exhibit a much-enhanced luminescence relative to the wild type when treated with cold, salt, or ABA at a wide range of dosages and treatment times (Fig. 1 *C*, *D*, and *F* and data not shown). Under the nonstress condition, there is virtually no luminescence in either wild type or *fry2–1* mutants (Fig. 1*B*). Quantitation of the luminescence intensities for plants shown in Fig. 1 *B–D* and *F* indicates that the levels of luminescence in *fry2–1* seedlings are about 11, 8, and 28 times higher than those in the wild type when treated with cold, ABA, and NaCl, respectively (Fig. 1*G*).

The *fry2* mutation not only increases the response amplitudes of the luminescence, but it also greatly reduces the thresholds of luminescence induction by stress and ABA. Fig. 1*H* presents an example of the induction of *RD29A-LUC* by different temperatures. At room temperatures (22 ± 2°C), both wild-type and *fry2–1* seedlings had virtually no luminescence. However, at 18°C, *fry2–1* showed a significant luminescence expression, the expression level being higher than that of the wild-type plants treated at 5°C. With further decreases in temperature, the luminescence in *fry2–1* increased substantially. For example, at 0°C, the intensity in *fry2–1* is more than 35 times higher than that in the wild type (Fig. 1*H*).

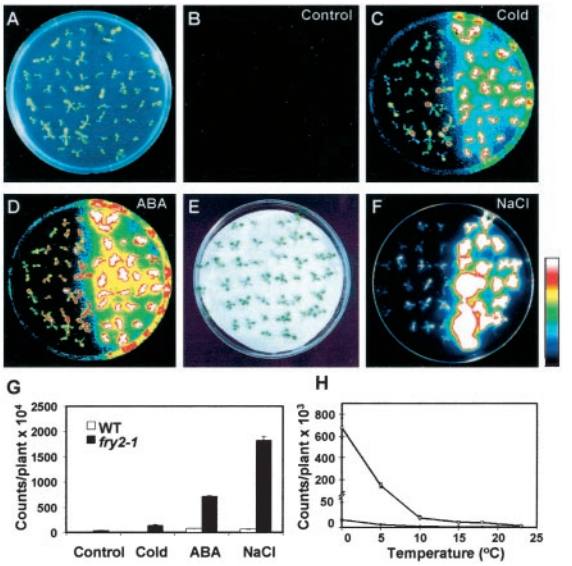

Fig. 1. Luminescence phenotypes of wild-type and *fry2* plants. One-week-old wild-type and *fry2–1* mutant plants were either untreated or treated with cold, ABA, or NaCl before taking images (wild type on the left and *fry2–1* on the right). The color scale at right shows the luminescence intensity from dark blue (lowest) to white (highest). (*A*) Morphology of seedlings growing in an agar plate. (*B*) Luminescence without stress treatment (Control). (*C*) Luminescence after cold treatment (0°C, 48 h). (*D*) Luminescence after ABA treatment (100 μM, 3 h). (*E*) Morphology of wild-type and *fry2–1* seedlings on filter paper for NaCl treatment. (*F*) Luminescence after NaCl treatment (300 mM, 3 h). (*G*) Quantitation of the luminescence intensity in *B* (Control), *C* (Cold), *D* (ABA), and *F* (NaCl). (*H*) Luminescence in wild type (closed symbols) and *fry2–1* (open symbols) treated at the indicated temperature for 24 h. Note different scales in *G* and *H*. Error bars in *G* and *H* represent SE ($n = 20$).

Similarly, high luminescence was observed in a dozen mutants isolated in our screen. Allelism tests found six new *fry2* alleles, which were named as *fry2–2* through *fry2–7*. Quantitation of luminescence expression in these mutants indicates that the average luminescence intensities in these mutants are 11–20, 8–11, and 15–21 times higher than those in the wild type under cold, ABA, and NaCl treatments, respectively. The intensities for the nonstress control treatment are only 1.6–4.1 times higher in *fry2* mutants than in the wild type (data not shown).

The *fry2* mutants each were crossed with the wild type. The F_1 progenies exhibited a wild-type luminescence expression in response to cold and ABA, indicating that the *fry2* mutations are recessive. The F_2 populations segregated at an ≈3:1 ratio of wild type to mutants in luminescence expression, which suggests that the *fry2* mutations occurred in a single nuclear gene.

Expression of the Endogenous *RD29A* and Other Stress-Responsive Genes in *fry2*. To ascertain whether the high luminescence in *fry2* mutants is a result of altered *RD29A-LUC* expression or post-translational regulation of the luciferase enzyme, we conducted RNA blot analysis by using the *LUC* gene as a probe. We found that the *LUC* transcript was at a very low level in the wild-type plants regardless of the treatments. In contrast, the *LUC* transcript was detected at high levels in *fry2–1* under the stress or ABA treatments, but not in the untreated control (Fig. 2). *LUC* transcript levels in *fry2* plants correlated with the dosage or duration of the stress or ABA treatments, suggesting that the

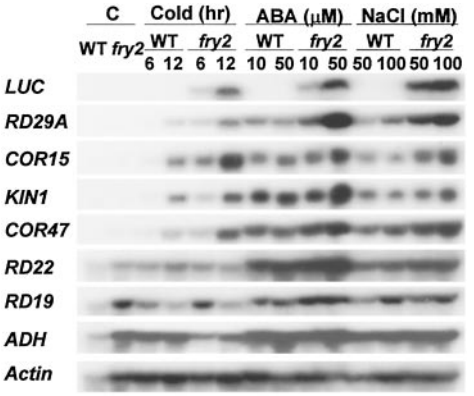

Fig. 2. Transcript levels for stress-responsive genes in wild-type and *fry2* plants. Total RNA was extracted from 7-day-old plants without stress (control) or with cold (0°C, 6 or 12 h), ABA (10 or 50 μM, 3 h), or NaCl (50 or 100 mM, 3 h) treatment. Twenty micrograms of total RNA was loaded in each lane. An actin gene was used as the loading control.

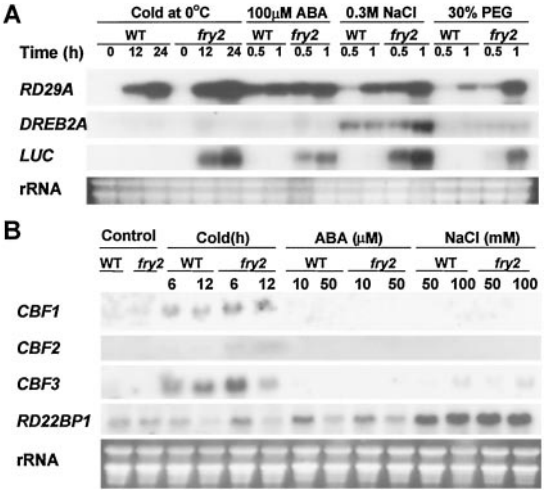

Fig. 3. Expression of several stress-responsive transcription factor genes in wild-type and *fry2–1* plants. Total RNA was extracted from 7-day-old plants without stress or with the indicated stress treatment. Twenty micrograms of total RNA was loaded in each lane. Ribosomal RNA (ethidium bromide staining) was used as a loading control. (*A*) Gene expression in response to stress treatments at different time points. Treatment time periods are Control (time 0, no treatment); Cold (0°C, 12 or 24 h); ABA (100 μM, 0.5 or 1.0 h); NaCl (300 mM, 0.5 or 1.0 h); and polyethylene glycol (PEG, 30% concentration, molecular weight = 6,000, 0.5, or 1.0 h). (*B*) Gene expression in response to stress treatments at different dosages. Stress dosages are C, Control; Cold (0°C, 6 h or 12 h); ABA (10 or 50 μM, 3 h); and NaCl (50 or 100 mM, 1 h).

high luminescence in *fry2* plants is caused by increased *RD29A-LUC* gene expression.

To examine whether the expression of the endogenous *RD29A* is affected similarly by the *fry2* mutation, the same RNA blot was probed with *RD29A*. Fig. 2 shows that *RD29A* gene also was induced to higher levels in *fry2–1* than in the wild type when treated with cold, ABA, or NaCl. Higher induction of *RD29A* in *fry2–1* was found at all dosages or durations of the stress or ABA treatment (Fig. 2).

The expression of several other stress-responsive genes also was examined in the *fry2–1* mutant. The transcript levels of *COR15A*, *KIN1*, and *COR47* were substantially higher in *fry2–1* than in the wild type after cold, ABA, or NaCl treatment (Fig. 2). The difference is more evident under the longer duration of cold (12 h) treatment or under higher dosages of ABA (50 μM) or NaCl (100 mM). The transcript levels of two non-DRE/CRT stress-responsive genes, *RD22* and *RD19*, were less affected by the *fry2* mutation (Fig. 2). The transcript level of the *ADH* gene was lower in *fry2* under cold treatment, but was unaffected under ABA or NaCl treatment (Fig. 2). These data indicate that the *fry2* mutation mainly may enhance the expression of the DRE/CRT class of stress-responsive genes.

The expression of the DRE/CRT class of stress-responsive genes is positively regulated by the upstream CBF/DREB transcription factors. The CBF/DREB genes also are induced by stresses (12, 13). The enhanced expression of DRE/CRT genes in *fry2* plants prompted us to ask whether the effect may be a consequence of increased expression of the upstream transcription factors. RNA blot analysis showed that the expression of the drought-specific transcription factor gene *DREB2A* was at a higher level in *fry2–1* than in the wild type after 1 h of NaCl treatment (Fig. 3*A*). The transcription factors CBF/DREB1s activate genes specifically under low temperature. Fig. 3*B* shows that the expression levels of *CBF1*, *CBF2*, and *CBF3* were all higher in *fry2–1* than in the wild type under a 6-h cold treatment and that the higher levels of transcripts of *CBF1* and *CBF2* in *fry2–1* persisted at 12 h of cold treatment.

The *fry2* Mutation Alters Stress Tolerance and ABA Responsiveness. Because the DRE/CRT class of genes is implicated to play roles in the development of stress tolerance, we tested the stress and ABA responses of *fry2* mutants from seed germination through seedling growth. In the presence of NaCl at a concentration of as low as 25 mM, the germination of wild-type seeds was delayed whereas that of *fry2* was not affected (data not shown). At 50 mM NaCl, the germination rates for *fry2* were consistently higher than those for the wild type. For example, at day 6 after imbibition, the germination rate of *fry2–1* seeds was 42% higher than that of the wild type (Fig. 4*A*). Nevertheless, both wild-type and *fry2–1* seeds were unable to germinate when NaCl concentrations were at 100 mM or higher (data not shown).

In the presence of exogenous ABA in the filter paper, the germination of both wild-type and *fry2–1* seeds was inhibited significantly. However, the germination of *fry2–1* seeds was consistently less inhibited by the same concentrations of ABA than that of wild-type seeds (Fig. 4*B*). When ABA levels were higher than 2.0 μM, the germination of both *fry2–1* and wild-type seeds was inhibited completely (data not shown). On agar plates supplemented with 0.5 μM ABA, most of the wild-type seeds germinated (i.e., radicals emerged) but were incapable of developing green cotyledons. In contrast, all *fry2–1* seeds germinated and developed green cotyledons and true leaves (Fig. 4*C*).

The NaCl and ABA tolerance of *fry2–1* at the germination stage was not observed at the seedling stage. Only a slightly enhanced tolerance to NaCl in terms of root elongation and fresh weight gain was observed in *fry2–1* relative to wild-type plants (data not shown). Root elongation of *fry2–1* on agar plates supplemented with ABA in fact was inhibited more than that of the wild-type seedlings. This increased ABA sensitivity was particularly clear at ABA concentrations of 5 and 10 μM (Fig. 4*D*).

To evaluate freezing tolerance, *fry2–1* and wild-type seedlings were assayed for their electrolyte leakage under freezing temperatures. Without cold pretreatment (nonacclimated), both wild-type and *fry2–1* seedlings were equally vulnerable to freezing by this criterion (Fig. 4*E*). After a 4-day cold treatment (cold acclimation), both wild type and *fry2–1* acquired increased tolerance to freezing. However, the gain in freezing tolerance was less in *fry2–1* than in the wild type. The biggest difference was found at −5°C, where the ion leakage rate in *fry2–1* was 70% higher than that in wild-type plants (Fig. 4*F*).

To test the freezing sensitivity of these plants directly, wild-type and *fry2–1* seedlings in soil (Fig. 4*G*) were pretreated at 4°C

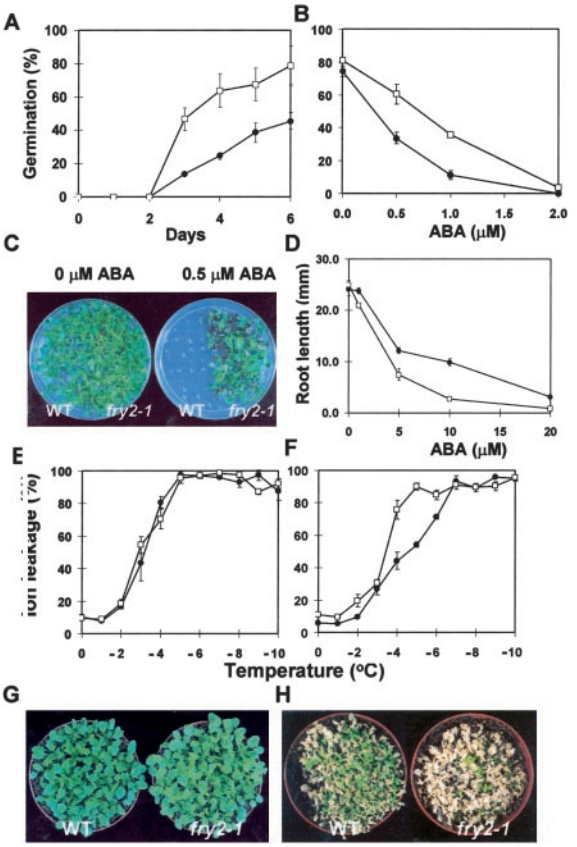

Fig. 4. Altered responses to stress and exogenous ABA in *fry2*. Closed symbols, wild type; open symbols, *fry2–1*. (*A*) Seed germination rates on filter paper saturated with 50 mM NaCl in relation to incubation times (days after being incubated at 22°C). (*B*) Seed germination on filter paper in the presence of ABA. The germination was scored at day 4 after being incubated at 22°C. Seeds in *A* and *B* were incubated at 0°C for 2 days before being incubated at 22°C for germination. Data are means of three replicates (each with 100 seeds for each line) ± SE. (*C*) Seedling development on agar plates with or without 0.5 μM ABA. The picture was taken 3 weeks after imbibition. (*D*) Root growth (elongation) on agar plates supplemented with ABA. One-week-old seedlings growing on the surface of MS plates were transferred to MS plates supplemented with the indicated concentrations of ABA. New root growth was measured 10 days after the transfer. Data are means ± SE ($n = 20$). (*E*) Electrolyte leakage from wild-type and *fry2–1* leaves without cold acclimation. (*F*) Electrolyte leakage from wild-type and *fry2–1* leaves after 4-day cold acclimation. Data in *E* and *F* are means ± SE ($n = 4$). (*G*) Morphology of wild-type and *fry2–1* plants growing in soil. (*H*) Morphology of wild-type and *fry2–1* plants after freezing treatment. The plants were cold-acclimated at 4°C for 1 week before being incubated at −7°C for 5 h. After the freezing treatment, plants were moved to a growth chamber (22 ± 2°C) and the picture was taken 5 days later.

for 7 days, and the seedlings then were exposed to −7°C for 5 h. After the freezing treatment, plants were allowed to recover in a growth chamber at 22°C. Most of the wild-type plants survived the freezing stress, whereas the majority of *fry2–1* seedlings were killed (Fig. 4*H*). The result further suggests that *fry2* mutant plants are more susceptible to freezing damage.

Map-Based Cloning of the *FRY2* Locus. The *fry2* mutant phenotypes suggest that wild-type FRY2 is a key regulator of stress and ABA-responsive gene transcription and stress and ABA tolerance. To isolate the *FRY2* gene, a map-based cloning strategy was used. Initial mapping with 46 samples placed the *FRY2* locus in the middle of chromosome IV. Fine mapping with more than 500 mapping samples delimited *FRY2* to an interval covered by

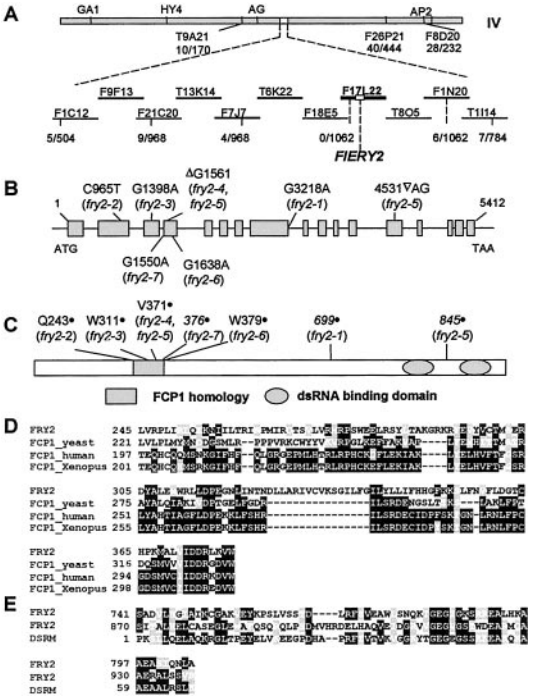

Fig. 5. Positional cloning of the *FRY2* locus and domain structure of the FRY2 protein. (*A*) Fine mapping of the *FRY2* locus and the identification of *FRY2* gene. Numbers indicate the number of recombinant chromosomes over total number of chromosomes examined. Candidate gene sequencing indicated that *FRY2* is localized on the clone F17L22. (*B*) Structure of the *FRY2* gene and the position and nature of *fry2* mutations. Filled boxes indicate exons, and lines between boxes donate introns. Positions are relative to the translation initiation codon. (*C*) Domain structure of the FRY2 protein and the position and nature of predicted *fry2* mutations. The FCP1 homology and two DSRMs are indicated. Numbers indicate the positions relative to the first methionine. Italicized numbers indicate predicted amino acid positions resulting from splicing variations, insertions, or deletions. (*D*) Comparison of the FCP1 homology domain with FCP1 proteins from other organisms. GenBank accession numbers are as follows: FRY2, T05842; FCP1_yeast, NP_014004; FCP1_Xenopus, AAK27686; and FCP1_human, NP_004706. (*E*) Alignment of the DSRM domain with conserved DSRM sequence (Smart domain accession no. 00358). In *D* and *E*, amino acids are shaded in black to indicate identity and in gray to indicate similarity. Dotted lines indicate gaps that are introduced to maximize alignment.

four bacterial artificial chromosome clones (Fig. 5*A*). With a marker on clone F17L22, no recombinant was detected of 1,062 chromosomes surveyed, suggesting that *FRY2* may be close to this marker (Fig. 5*A*). Because the *fry2* mutant has more alleles than any other mutants isolated in our screen, we reasoned that *FRY2* likely is a very large gene. We thus searched for candidate genes with relatively large genomic sizes and with a potential role in transcriptional regulation from the *Arabidopsis* annotation database (www.arabidopsis.org). DNA sequencing with *fry2–1* in a candidate gene, F17L22.130 (5,412 bp of predicted genomic sequence) on bacterial artificial chromosome clone F17L22, found a single nucleotide change that would disrupt a predicted splicing donor recognition site. We then sequenced this gene from all other *fry2* mutants and found mutations in each of the alleles (Fig. 5*B*).

All of the *fry2* mutations (Fig. 5*B*) would result in changes in the predicted protein. The multiple mutant alleles in F17L22.130 provide definitive evidence that it is the *FRY2* gene. After *FRY2* was cloned, we found out that *FRY2* is identical to the *AtCPL1* (for CTD-phosphatase-like 1) gene independently isolated by Koiwa *et al.* (17) through screening T-DNA mutagenized *RD29A-LUC* plants. The *cpl1* mutant plants had luminescence

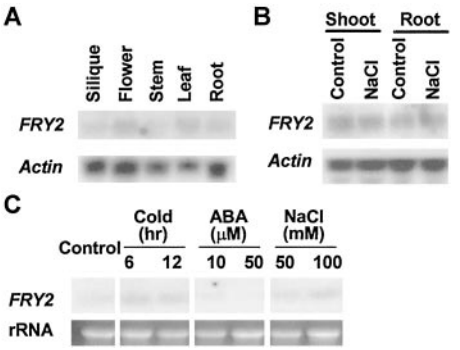

Fig. 6. Expression patterns of the *FRY2* gene. (*A*) The expression of *FRY2* in different parts of plants. (*B*) The expression of *FRY2* in roots and shoots with or without 200 mM NaCl treatment. (*C*) *FRY2* expression as affected by cold, ABA, and NaCl treatments. An actin gene or ribosomal RNA (ethidium bromide staining) was used as a loading control.

phenotypes similar to but weaker than *fry2* mutants (17), presumably because the T-DNA was inserted in an intron, and, thus, some of the transcripts still might be spliced correctly.

***FRY2* Encodes a Novel Transcriptional Repressor.** The coding sequence of *FRY2/CPL1* consists of 17 exons and 16 introns (Fig. 5*B*). The ORF is predicted to encode a protein of 995 aa with an estimated molecular mass of 112 kDa. Database searches revealed that FRY2/CPL1 has significant sequence homology with only a few unknown proteins in plants, including hypothetical proteins in *Arabidopsis* (T45967, 42% identity and 55% similarity) and rice (BAB63701, within a stretch of 491 aa, the identity is 55% and similarity is 69%). Although the overall sequence of FRY2/CPL1 does not show significant similarity with any nonplant proteins, there is a stretch of 138 aa from position 243 to 380 that shows considerable similarity with a group of proteins named FCP1 from nonplant eukaryotes (Fig. 5*D*). FCP1 proteins are RNA polymerase II CTD (RNPII CTD) phosphatases (23). The FRY2/CPL1 FCP1 homology domain shows 28% identity and 43% similarity with yeast FCP1 and 28% identify and 41 to ≈42% similarity with human or *Xenopus* FCP1 homologs (Fig. 5*D*). Additionally, the FRY2/CPL1 protein has two prototype DSRMs. Both DSRMs are highly homologous to the DSRMs in many other dsRNA-binding proteins such as ribonuclease III in diverse organisms and TAR RNA-binding protein 2 (transactivation responsive RNA-binding protein) in humans (Fig. 5*E*).

Each of the *fry2* lesions was predicted to result in a nonsense mutation either directly or indirectly (Fig. 5*C*). Thus, all these mutations are severe lesions and are predicted to be null.

***FIERY2* Is Expressed Ubiquitously at a Low Level.** The expression of the *FRY2/CPL1* gene in different plant parts and its regulation were studied with RNA blot analysis. Fig. 6*A* shows that *FRY2/CPL1* is expressed at very low levels in all plant parts examined including roots, leaves, stems, flowers, and siliques. There is no difference in *FRY2/CPL1* expression between control and NaCl-treated shoot or root samples (Fig. 6*B*). In young seedlings, *FYR2/CPL1* expression is not regulated substantially by periods of cold shock or different concentrations of NaCl (Fig. 6*C*). Interestingly, the low expression level of *FRY2* appears to be reduced further by ABA treatment (Fig. 6*C*).

Discussion

In the present study, we identified, characterized, and cloned the *FIERY2* locus that controls the expression of the DRE/CRT class of genes. *fry2* mutations not only greatly increase the expression of the DRE/CRT class of stress-responsive genes such as *RD29A*, *COR15A*, *KIN1*, and *COR47* on stress and ABA treatments, they also alter physiological responses to stress and ABA in the mutants. The germination of *fry2* mutant seeds is more resistant to inhibition by ABA or NaCl. However, *fry2* seedlings appear to be more sensitive to ABA and to freezing damage, which is similar to that which was observed for several other mutants with enhanced expression of stress-responsive genes. These other mutants include *fry1* (18), *sad1* (24), and *hos1* (19, 20).

Importantly, *fry2* mutants differ from *fry1* or *sad1* in that the germination of *fry1* or *sad1* seeds is more sensitive to ABA inhibition (18, 24) but that of *fry2* is more tolerant. Additionally, the gene expression patterns in *fry1* and *fry2* are different. In *fry1*, the expression of several non-DRE/CRT class genes such as *ADH* also is enhanced, whereas in *fry2* only the DRE/CRT genes are significantly up-regulated. This is probably because *FRY1*, which regulates phosphoinositol messengers, functions early in stress and ABA signaling (18), whereas *FRY2* may function relatively late in the pathway.

It is known that DRE/CRT genes are activated by CBF/DREB transcription factors. In *fry2* mutants, the expression of these transcription factor genes is induced to levels higher than in the wild type. In contrast, the expression of an unrelated transcription factor, *RD22BP1*, which is induced by NaCl treatments (25) (Fig. 3*A*), is not significantly affected by *fry2*, suggesting that *FRY2* may specifically control the CBF/DREB regulon. It is likely that FRY2 suppresses the expression of the CBF/DREB transcription factor genes rather than directly regulates the downstream DRE/CRT genes. Alternatively, FRY2 may function with the CBF/DREB-associated transcription complex, given the relatively small changes in *CBF* transcript abundance as compared with the significant levels of transcripts for downstream target genes.

FRY2 encodes a novel protein with two DSRMs and a conserved domain with similarity to the RNPII CTD phosphatase FCP1 found in yeast and in animals. It is identical to the *CPL1* locus independently identified by Koiwa *et al.* (17). RNPII, which is responsible for the transcription of eukaryotic protein-coding genes, is regulated by both environmental and developmental cues (26). Recent studies in yeast and in animal cells suggest that the phosphorylation of the CTD is a focal control point in gene transcription and is the target of a handful of cellular proteins and their respective pathways (26). The phosphorylation status regulates transcription initiation vs. elongation and thereby controls the recycling of RNPII (27). CTD phosphorylation also affects all pre-mRNA processing including capping, splicing, and 3′ processing (28). Although many kinases are capable of phosphorylating CTD, only the FCP1 phosphatase is dedicated to dephosphorylate CTD, and, therefore, the null *fcp1* mutant in yeast is lethal (23, 29). However, FRY2/CPL1 might not be a genuine FCP1 because of the lack of other conserved domains such as the BRCT domain found in all known FCP1 proteins. Additionally, our gene expression data suggest that the *fry2* mutation affects the expression of only a subset of stress-responsive genes but not general gene expression, which is different from the conditional *fcp1* mutations in yeast, where global gene expression is impaired (29).

Consistent with a specific role of FRY2 in stress responses, all *fry2* mutants are viable and appear healthy at normal growth conditions. The C-terminal domain (including parts of the DSRMs) of FRY2 shows 25% identity and 43% similarity to the TAR (transactivation responsive) RNA-binding protein (TRBP) found in human (AAA36765) and in mouse (AAB38885). TRBP binds to HIV-1 TAR RNA, a short stem-loop structure found at the 5′ end of nascent HIV-1 transcripts, and inhibits the IFN-induced protein kinase R activity on long terminal repeat expression through protein–protein interactions (30). Protein

kinase R is a dsRNA-activated protein kinase that is also activated by growth factors and stress signals, and its activity negatively regulates protein synthesis and many other cellular signals under stress (31). Thus, TRBP could be a translational repressor as was suggested previously for the mouse TRBP (32). Similar to the inhibition by FCP1 on RNPII-mediated Tat transactivation (33), TRBP also may affect gene transcription. It is very unusual that FRY2 has both FCP1- and TRBP-like regions, which makes FRY2 a unique protein in all eukaryotes. This structural feature may bestow on FRY2/CPL1 a transcriptional repressor function that regulates plant responses to cellular stress, comparable to the function of protein kinase R in the regulation of gene transcription and mRNA translation under stress conditions (31). The presence of the FCP1-like catalytic domain suggests that FRY2/CPL1 may possess some enzymatic functions. Indeed, Koiwa *et al.* (17) demonstrated that CPL1/FRY2 recombinant protein exhibits a clear phosphatase activity. Because FRY2/CPL1 contains dsRNA-binding domains, it is tempting to speculate that dsRNA might be a regulator of the enzymatic activity of FRY2/CPL1. The activity of protein kinase R in human cells is regulated by dsRNA (31). With the findings that human 7SK snRNA is a regulator in repressing general and HIV-1 transcription by association with positive elongation factor of RNPII CTD (34, 35), it is thought that RNA–protein interaction may be one major control point regulating stress-related responses (34). Several recent studies in plants have identified components in mRNA processing such as the cap-binding protein ABH1 (36) and Sm-like snRNP protein SAD1 (24) as being specifically involved in ABA and stress responses. In this study, our identification of FRY2 as a putative dsRNA-binding protein adds exciting evidence for the involvement of structured RNAs as potential signal intermediates in regulating stress and hormone signaling in plants.

The dsRNA-binding domains of FRY2/CPL1 appear to be essential, because the *fry2–1* mutant protein that has these domains truncated off is not different phenotypically from other null alleles. Many proteins with DSRM(s) have been identified. In *Arabidopsis*, the dsRNA-binding protein HYL1 was found to negatively regulate ABA and other hormonal responses through unknown mechanisms (37). The transcript abundance of *HYL1*, which is much higher than that of *FRY2*, is down-regulated by exogenous ABA. Interestingly, the *FRY2* transcript level also appears to be down-regulated by ABA (Fig. 6*C*).

Although the understanding of the exact modes of action of the FRY2/CPL1 protein awaits future studies, the evidence presented in this study strongly suggests that FRY2/CPL1 is a transcriptional repressor for the DRE/CRT genes, probably through the repression of the stress induction of CBF/DREB transcription factor genes. Future elucidation of the precise mechanisms of FRY2/CPL1 action will significantly advance our understanding of the regulation of gene expression in response to ABA and environmental stresses.

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