FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in *Arabidopsis*

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The plant hormone abscisic acid (ABA) plays a wide range of important roles in plant growth and development, including embryogenesis, seed dormancy, root and shoot growth, transpiration, and stress tolerance. ABA and various abiotic stresses also activate the expression of numerous plant genes through undefined signaling pathways. To gain insight into ABA and stress signal transduction, we conducted a genetic screen based on ABA- and stress-inducible gene transcription. Here we report the identification of an *Arabidopsis* mutation, *fiery1* (*fry1*), which results in super-induction of ABA- and stress-responsive genes. Seed germination and postembryonic development of *fry1* are more sensitive to ABA or stress inhibition. The mutant plants are also compromised in tolerance to freezing, drought, and salt stresses. Map-based cloning revealed that *FRY1* encodes an inositol polyphosphate 1-phosphatase, which functions in the catabolism of inositol 1, 4, 5-trisphosphate (IP_3). Upon ABA treatment, *fry1* mutant plants accumulated more IP_3 than did the wild-type plants. These results provide the first genetic evidence indicating that phosphoinositols mediate ABA and stress signal transduction in plants and their turnover is critical for attenuating ABA and stress signaling.

[*Key Words*: Abscisic acid; cold stress; salt stress; inositol polyphosphate 1-phosphatase; IP_3; gene regulation]

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The growth and development of plants are profoundly affected by environmental conditions. Through evolution plants have acquired adaptive strategies to cope with adverse environmental conditions, such as freezing temperatures, drought, and salt stress. One important regulator that coordinates plant developmental programs with environmental stress responses is the plant hormone abscisic acid (ABA). ABA plays essential roles in many physiological processes, such as embryogenesis, seed dormancy, leaf transpiration, and stress tolerance (Koornneef et al. 1998; Leung and Giraudat 1998). Under normal physiological conditions, ABA content in plants is quite low. However, ABA level can dramatically increase during late stages of embryogenesis and when plants are subjected to drought or salt stress (Koornneef et al. 1998; Leung and Giraudat 1998).

Genetic analysis of ABA signal transduction has been conducted mainly by using the property of ABA inhibition of seed germination. Seed germination-based genetic screens have identified mutants affected in ABA biosynthesis or sensitivity. The latter include ABA-insensitive mutants (*abi*) and ABA-hypersensitive mutants. The *ABI1* and *ABI2* genes encode homologous type 2C serine/threonine protein phosphatases (for review, see Leung and Giraudat 1998). The two genes have overlapping functions during seed development, seed dormancy, and leaf transpiration. Consistent with observations from mutational analysis of the ABI1 protein (Sheen 1998), recent analysis of recessive *abi1* mutant alleles showed that *ABI1* is indeed a negative regulator of ABA signaling, as indicated by the enhanced sensitivity of the recessive mutants to ABA in seeds and in vegetative tissues (Gosti et al. 1999). The other three *ABI* genes, *ABI3*, *ABI4*, and *ABI5*, all encode putative transcription factors (Giraudat et al. 1992; Finkelstein and Lynch 2000) and their roles in regulating ABA responses are seed-specific. Interestingly, an independent genetic screen identified a glucose insensitive locus, *GIN6*, which was found to be identical to *ABI4* (Arenas-Huertero et al. 2000). Characterization of other *abi* mutants revealed that *ABI5* also participates in glucose signal transduction during postembryonic development (Arenas-Huertero et al. 2000).

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The other group of ABA-signaling mutants is represented by the *enhanced response to ABA* (*era*) mutations. *ERA1* encodes the β subunit of farnesyl transferase, implying that an as-yet-unknown protein(s) in ABA signaling needs to be farnesylated (Cutler et al. 1996). Recently, an additional *ERA* locus, *ERA3*, was found to be identical to *EIN2* (Ghassemian et al. 2000). *ein2* mutations also suppressed the sensitivity of *abi1-1* to ABA in germination (Beaudoin et al. 2000). Analysis of several ethylene response mutants showed that ethylene counteracts ABA signaling during seed germination; whereas it positively regulates ABA action in root growth (Beaudoin et al. 2000; Ghassemian et al. 2000).

In vegetative tissues, ABA and various abiotic stresses activate the expression of a large number of plant genes, which may play important roles in stress adaptation (Zhu et al. 1997). Recent molecular and genetic analysis suggests that stress-activated gene transcription is mediated by both ABA-dependent and ABA-independent signaling pathways. Analysis of stress-gene expression in ABA-deficient and ABA-insensitive mutants indicated that some stress genes are activated independently of ABA. These gene promoters contain a *cis*-acting DNA regulatory element, termed the dehydration-responsive element (DRE)/CRT, which responds to cold or osmotic stress but not to ABA (Yamaguchi-Shinozaki and Shinozaki 1994). In contrast, the ABA responsive element/complex (ABRE) found in these promoters mediates gene expression in response to ABA (Shen and Ho 1995).

We developed a system to screen for mutants with altered responses to ABA, drought, salt and/or cold stress by utilizing *Arabidopsis thaliana* plants that were engineered to emit bioluminescence in response to the phytohormone or to various abiotic stresses (Ishitani et al. 1997). The bioluminescent plants contain the firefly luciferase reporter gene under control of the *RD29A* promoter (*RD29A-LUC*) that contains both the DRE/CRT and the ABRE elements and is responsive to ABA, hyperosmotic stress, and cold. Here we present the identification of one mutation, *fiery1* (*fry1*), which results in dramatically enhanced expression of ABA- and stress-responsive genes when treated with ABA, low temperature, drought, or salt stress. Map-based cloning revealed that *FRY1* encodes an inositol polyphosphate 1-phosphatase, which functions in the catabolism of the second messenger inositol 1, 4, 5-trisphosphate. These results strongly suggest the inositol phosphate module as an early signaling module that functions in both ABA and environmental stress signaling pathways. Our work also reveals an important mechanism for the control of ABA and stress response amplitude and sensitivity by FRY1-mediated turnover of phosphoinositols.

Results

Isolation of Arabidopsis *mutants that exhibit super-induction of ABA- and stress-responsive bioluminescence*

Arabidopsis plants expressing the *RD29A-LUC* transgene were mutagenized with ethyl methane sulfonate (EMS) and mutants were screened for their aberrant bioluminescence in response to low temperature, osmotic stress, or ABA treatment (Ishitani et al. 1997). One group of mutants that exhibited enhanced luminescence expression when treated with either cold, NaCl, or ABA were isolated. Two allelic mutants were chosen for detailed characterization. Figure 1 presents the luminescence images of the wild type and mutant seedlings before treatment (Fig. 1B) and after being treated with cold (Fig. 1C), ABA (Fig. 1D), and NaCl (Fig. 1F). Because of its strong luminescence phenotype, the mutant was named *fiery1* (*fry1*). Quantitation of the luminescence intensity of the images indicated that the luminescence intensities in the *fry1-1* mutant are about three to four times higher than in the wild-type plants when treated with either cold, NaCl, polyethylene glycol (PEG), or ABA (Fig. 1G). Without stress treatment, *fry1* plants exhibited some basal *RD29A-LUC* expression; whereas the wild type did not (Fig. 1B). The low level of luminescence in untreated *fry1-1* mutant plants was likely induced by changes in humidity or other mild perturbations in the environment during experimental manipulation.

The *fry1* mutants were backcrossed with the wild-type plants. Luminescence analysis of the resulting F_1 and selfed F_2 progenies under cold or ABA treatments indicated that *fry1* is a recessive, single nuclear mutation (data not shown). Pairwise crosses among the mutants with similar enhanced luminescence phenotypes identified another *fry1* allele, *fry1-2* (data not shown). Both *fry1* mutants have leaves that are more serrated than wild-type leaves but otherwise do not show obvious developmental defects (data not shown).

Enhanced expression of ABA- and stress-responsive genes in fry1 *mutant plants*

To determine whether the expression of endogenous *RD29A* and other stress-responsive genes is affected by the *fry1* mutation, total RNA from *fry1-1* mutant, and wild-type plants treated with cold, NaCl, PEG, or ABA was analyzed by Northern hybridization. Consistent with the luciferase imaging results, the steady-state level of *RD29A* transcripts was higher in the mutant than in the wild type under any of the treatments (Fig. 2A).

The *fry1* mutation also increased the expression of other stress-responsive genes examined under low temperature, osmotic stress or ABA treatment (Fig. 2A). These genes include *KIN1* (GenBank accession no. X51474), *COR15A* (GenBank accession no. U01377), *ADH* (alcohol dehydrogenase; GenBank accession no. M12196), and *HSP70* (GenBank accession no. AF217459). Without ABA or stress treatment, there was also some basal expression of the endogenous *RD29A* gene in *fry1-1* mutant plants, consistent with the *RD29A-LUC* phenotype (Fig. 1B). We also note that in the *fry1* mutant treated with either ABA, NaCl, or PEG, the abundance of *HSP70* transcript is lower 1 h after treatment than 0.5 h after treatment (Fig. 2A). This is also true for *COR15A* expression in response to ABA or PEG.

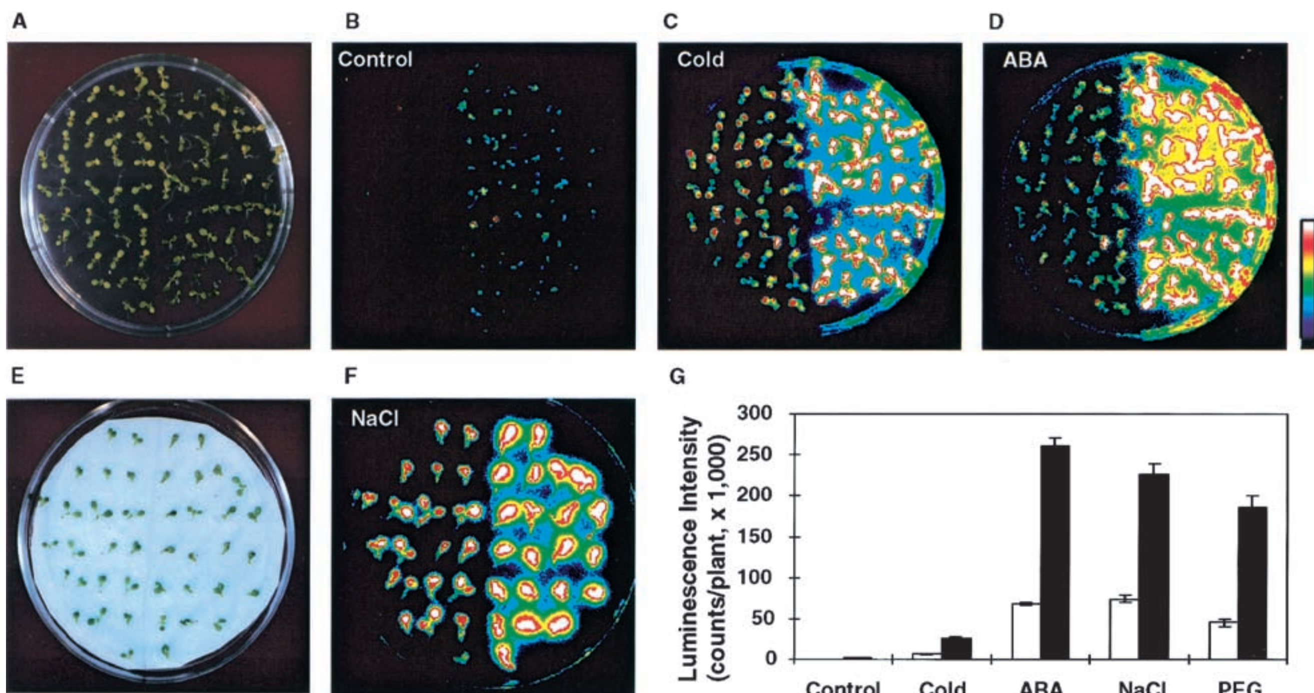

Figure 1. The *fry1* mutation enhances *RD29A-LUC* expression in response to cold, ABA, or hyperosmotic stress treatment. *RD29A-LUC* expression was quantitatively measured as luminescence intensity. (*A*) Wild-type (*left*) and *fry1-1* (*right*) seedlings grown in an agar plate. (*B*) Luminescence without stress or ABA treatment. (*C*) Luminescence after low-temperature treatment at 0°C for 24 h. (*D*) Luminescence after treatment with 100 µM ABA for 3 h. (*E*) Wild-type (*left*) and *fry1-1* (*right*) seedlings on filter paper saturated with 300 mM NaCl. (*F*) Luminescence of (*E*) after 3-h treatment. The color scale at right shows the luminescence intensity from dark blue (lowest) to white (highest). (*G*) Quantitation of the luminescence intensities of wild-type and *fry1-1* plants in (*B*) (control), (*C*) (cold), (*D*) (ABA), and (*F*) (NaCl). Also shown is 30% polyethylene glycol (PEG) treatment (for 5 h). Data represent means and standard errors ($n = 20$). Open bars, wild type; black bars, *fry1-1*.

Another ABA- and stress-regulated gene, *COR47* (GenBank accession no. X59814), exhibited strong constitutive expression in *fry1-1* mutant plants; whereas it was expressed only under induced conditions in the wild-type plants (Fig. 2A). Interestingly, *COR47* expression under ABA or the stress treatments was not substantially different between *fry1-1* and wild-type plants (Fig. 2A). As a control, the expression of an actin gene in the mutant and wild-type plants was also examined. The state-steady transcript levels of actin were not significantly regulated by ABA or the stress treatments and not substantially different between *fry1-1* and wild-type plants (Fig. 2A).

Because CBF/DBEB1 transcription factors are able to activate some of these stress-responsive genes and the expression levels of the transcription factors themselves are regulated by stress, we examined the expression of one of the CBF genes, *CBF2* (GenBank accession no. AF074601), which has the highest induction under low-temperature treatments. The results indicate that *CBF2* expression was induced by cold treatments and the expression levels are not substantially different between wild type and *fry1-1* for either 1.5 or 3 h of cold treatment (Fig. 2B). Whereas *CBF2* expression in wild type is transient and its transcript level decreased drastically after 6 h of cold treatment as reported (Medina et al. 1999; Lee et al. 2001), the expression in *fry1-1* is more sustained. Quantitation indicates that *CBF2* transcript level in *fry1* for a 6-h cold treatment was 1.8 times higher than that in the wild-type plants (Fig. 2B).

fry1 *mutation reduces the thresholds of gene induction by ABA and stress*

The luminescence images (Fig. 1) and RNA blot analysis (Fig. 2) indicated that *fry1* mutations significantly increase the amplitude of stress-regulated gene expression. To determine whether the sensitivity of *fry1* plants to low temperature, osmotic stress or ABA is also altered, we assayed the luminescence of the plants under different treatment dosages (Fig. 3A–C). At room temperature (23° +1°C) or 18°C, neither *fry1-1* nor wild-type seedlings showed any high-level *RD29A-LUC* expression. At 15°C. However, *fry1-1* plants exhibited a clear induction of *RD29A-LUC*; whereas the wild type still did not show any measurable expression. With further decreases in the treatment temperature, the wild-type plants began to show an induction of *RD29A-LUC* while the expression in *fry1-1* plants increased substantially (Fig. 3A). At 0°C, the luminescence intensity in *fry1-1* was ~5 times as high as that in the wild-type plants. With a further decrease in temperature (–5°C for 2 h), the luminescence intensity in *fry1-1* plants was ~20 times that of the wild-type level (data not shown).

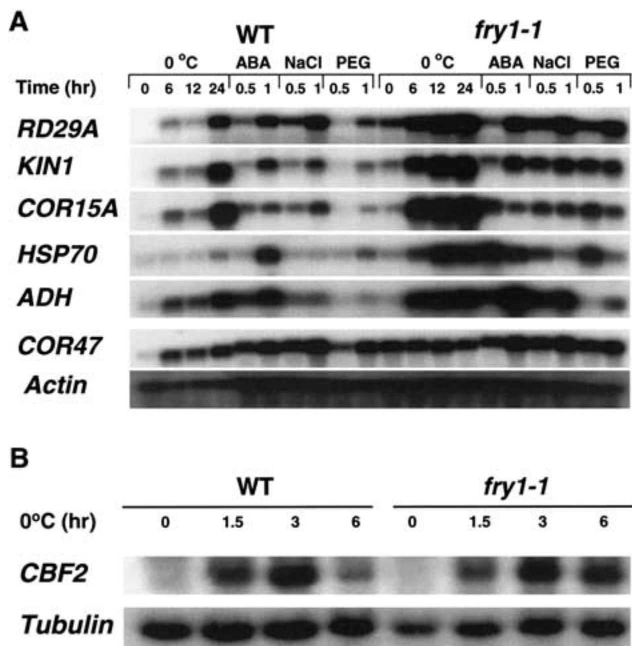

Figure 2. Transcript levels of stress-responsive genes in *fry1-1* and wild-type plants. Plants were treated for the indicated times with low temperature (0°C), 100 µM ABA, 300 mM NaCl, or 30% PEG. Actin and tubulin genes were used as loading controls. WT, wild type.

In the wild-type plants, treatment with 0.1 µM ABA did not induce obvious expression of *RD29A-LUC* (Fig. 3B). On the contrary, a significant expression of the reporter gene was induced by 0.1 µM ABA in *fry1-1* plants. In both wild-type and *fry1-1* plants, the level of *RD29A-LUC* expression increased with increases in ABA concentration. At 100 µM, the luminescence intensity in *fry1-1* plants is about four times higher than in the wild type. Similarly, the induction of *RD29A-LUC* was evident in *fry1-1* plants when treated with as low as 10 mM NaCl; whereas in the wild-type plants, an obvious induction only occurred at NaCl concentrations of 100 mM or higher (Fig. 3C). The highest expression was found at 300 mM NaCl with both wild-type and *fry1-1* plants. With further increases in NaCl concentration, the expression level decreased rapidly, probably due to stress damage to basic cellular functions (Fig. 3C).

The slopes of the dosage response curves for *fry1-1* mutant plants are substantially steeper than the corresponding ones for wild-type plants (Fig. 3A–C). This is true for all three treatments (i.e., low temperature, ABA, and NaCl). These results show that the enhanced stress and ABA responses seen in *fry1* mutants are results of increased sensitivity to the stimulations rather than simply proportional amplification with increasing stimulation strength.

Despite the increased sensitivity and higher response amplitude, the induction kinetics in *fry1-1* mutant plants is similar to that in the wild type (Fig. 3D–F). Time courses of low temperature, ABA, and NaCl responses are presented in Fig. 3D, 3E, and 3F, respectively. It can be seen that the levels of *RD29A-LUC* expression in *fry1-1* plants are consistently higher throughout the time courses (Fig. 3D–F).

Germination of fry1 *mutant seeds is more sensitive to ABA and osmotic stress*

In the absence of exogenous ABA, the germination of *fry1-1* mutant seeds was delayed in comparison with the wild type, reflecting an enhanced sensitivity of the mutant seeds to endogenous ABA (Fig. 4A,C). In the presence of 1 µM ABA, *fry1-1* seed germination was further delayed and the germination rate was reduced to <80% (Fig. 4B). In contrast, wild-type seeds reached near 100% germination rate in the presence of 1µM ABA (Fig. 4B). ABA dose-response curves show that lower concentrations of ABA also had more inhibition on germination of *fry1* seeds (Fig. 4C). Similarly, 50 mM NaCl reduced germination rate by 50% for *fry1-1* seeds but did not substantially affect the germination of wild-type seeds (Fig. 4E). In addition to a reduced rate of seed germination, ABA also inhibited the growth and the greening of cotyledons of *fry1-1* mutant seedlings. In the presence of 1 µM ABA in agar medium, although the radicles of most *fry1-1* seeds emerged, the seedlings failed to grow (Fig. 4D). These results indicate that *fry1-1* mutant plants are more sensitive to osmotic stress and ABA during seed germination and early seedling development.

fry1 *mutant plants are defective in cold acclimation*

The high sensitivity of *RD29A-LUC* expression to low temperature and the enhanced expression of cold-regulated genes in *fry1-1* plants may have an impact on cold acclimation (i.e., the development of freezing tolerance). We determined freezing-induced electrolyte leakage in detached leaves as an indicator of freezing-induced injury. Without cold acclimation, *fry1* leaves showed slightly more electrolyte leakage than the wild type. The temperatures at which 50% electrolyte leakage occurred (LT_{50}) were ~–2.5°C and –3.8°C for *fry1-1* and wild-type plants, respectively (Fig. 5A).

After a 7-d incubation at 4°C (i.e., cold acclimation), the freezing tolerance of wild-type plants increased significantly, as can be seen in the reduced electrolyte leakage (Fig. 5A). The LT_{50} for the cold acclimated wild-type plants dropped to ~–9.4°C (Fig. 5A). In contrast, *fry1-1* mutant plants showed only a slight increase in freezing tolerance after cold acclimation. The LT_{50} value for cold-acclimated *fry1-1* plants is ~–5.0°C, which is >4.4°C higher than that of the wild type.

fry1 *mutant seedlings are more sensitive to osmotic stress inhibition*

The sensitivity of *fry1* plants to osmotic stress was tested by growing seedlings on agar plates containing Murashige-Skoog (MS) nutrients supplemented with mannitol or NaCl. *fry1-1* Mutant seedlings were clearly more sensitive to NaCl (Fig. 5B) or mannitol stress (Fig. 5C). On medium containing MS nutrients only or MS plus 50 mM NaCl or 100 mM mannitol, both *fry1-1* and wild-type plants had healthy green cotyledons. On 100

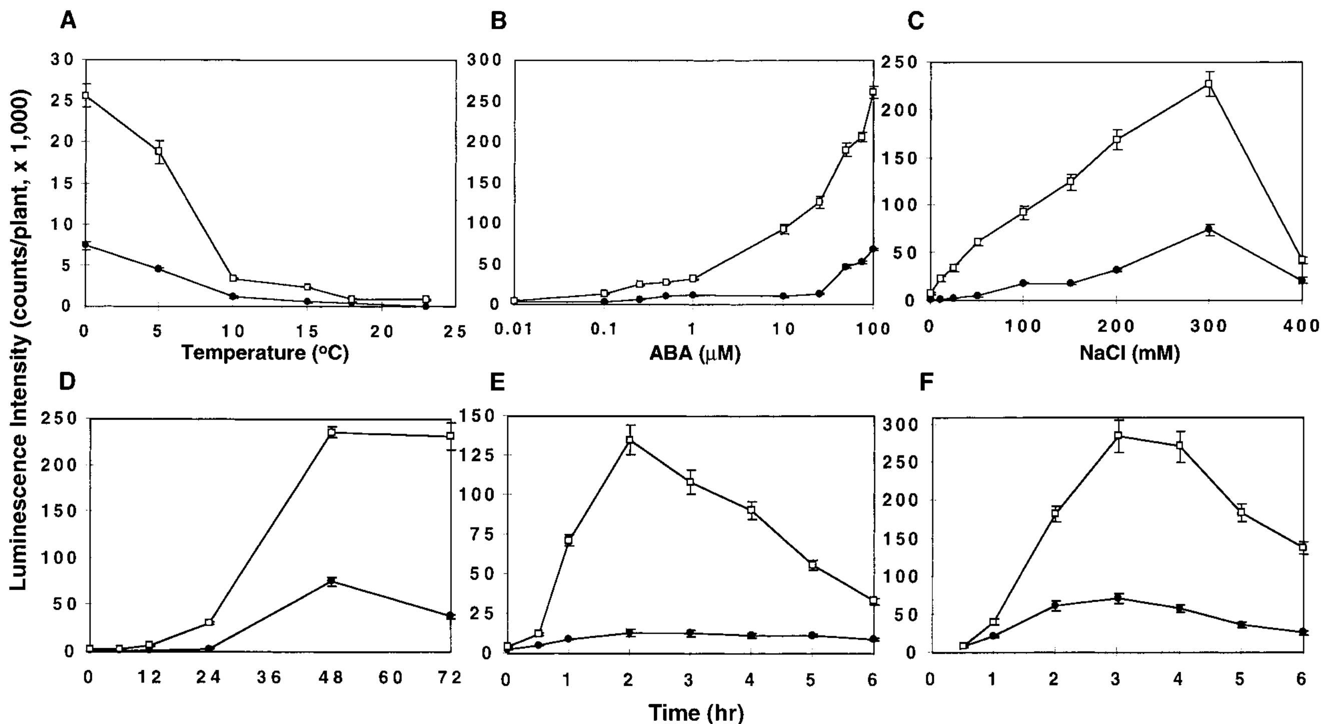

Figure 3. Dose-response (*A–C*) and time course (*D–F*) of *RD29A-LUC* induction by temperature (*A*,*D*), ABA (*B*,*E*) or salt stress (*C*,*F*) in wild-type and *fry1-1* plants. For the dosage response assay, the treatment durations were 24 h for the temperature treatment (*A*), 3 h for the ABA treatment (*B*), and 4 h for the NaCl treatment (*C*). For time-course analyses, the dosages were 0°C for the cold treatment (*D*), 100 µM ABA (*E*), and 300 mM NaCl (*F*). Data represent means and standard errors (n = 20). Closed circles, wild type; open squares, *fry1-1*.

mM NaCl medium, none of the wild-type plants had yellow cotyledons; whereas nearly 80% of *fry1-1* mutant plants had cotyledons that became yellowish (Fig. 5B). When the NaCl concentration was increased to 150 mM, the cotyledons of most *fry1-1* plants but not wild-type plants died (Fig. 5B). At 200 mM NaCl, almost all *fry1-1* seedlings were killed. However, ~90% of wild-type seedlings survived the 200 mM NaCl treatment (Fig. 5B). Similarly, high concentrations of mannitol caused substantial damage to *fry1-1* seedlings, as evidenced by the yellowing of cotyledons (Fig. 5C). The same concentrations of mannitol caused little or no visual damage to wild-type seedlings.

We treated *fry1-1* and the wild-type seedling with PEG to mimic drought stress. Electrolyte leakage was measured to quantify the extent of damage caused by the treatment. Under control (water only) or 10% PEG treatment, there was no difference in the percentage of electrolyte leakage between wild-type and *fry1-1* plants (Fig. 5D). However, with higher concentrations of PEG, significantly more electrolytes were lost from *fry1-1* plants than from the wild type. The results suggest that *fry1-1* plants suffer more damage to the cell membrane under drought stress.

Transpiration rates in detached shoots of wild type and *fry1-1* mutant plants were compared. No significant difference in transpirational water loss was found between *fry1-1* and wild-type plants (data not shown), suggesting that *FRY1* is not essential for stomatal regulation.

Genetic mapping of the fry1 *mutation*

To genetically map the *FRY1* gene, *fry1-1* mutant plants in the C24 ecotype were crossed with wild-type plants of the ecotype Columbia. From the segregating F_2 population, *fry1* mutant seedlings were identified by enhanced luminescence under cold and ABA treatments. A mapping survey of markers distributed throughout the five *Arabidopsis* chromosomes revealed that *FRY1* is linked with *nga129* near the bottom of chromosome V (Fig. 6A). We then localized *FRY1* to a region between the SSLP markers K19B1 and MSN2 (Fig. 6A). Fine mapping delimited *FRY1* to an ~160-kb region flanked by SSLP marker MBK5–64k and single nucleotide polymorphism marker MJH24–48k (Fig. 6A). Further mapping became very difficult due to a lack of recombination events.

To facilitate gene isolation, a large-scale T-DNA mutagenesis of the wild-type *RD29A-LUC* plants was carried out to isolate a tagged allele of *fry1*. We generated close to 50,000 transformants with an estimated average of 1.5 insertions/line. Among the T-DNA lines, one mutant was found to have phenotypes similar to those exhibited by *fry1* plants (i.e., enhanced *RD29A-LUC* expression under either cold, salt, or ABA treatment). This T-DNA mutant was crossed with *fry1-1* plants. The F_1 progenies were tested for bioluminescence in response to cold or ABA treatments and were found to be similar to the parental plants (data not shown). Therefore, this T-DNA mutant is allelic to *fry1-1* and is named *fry1-3*.

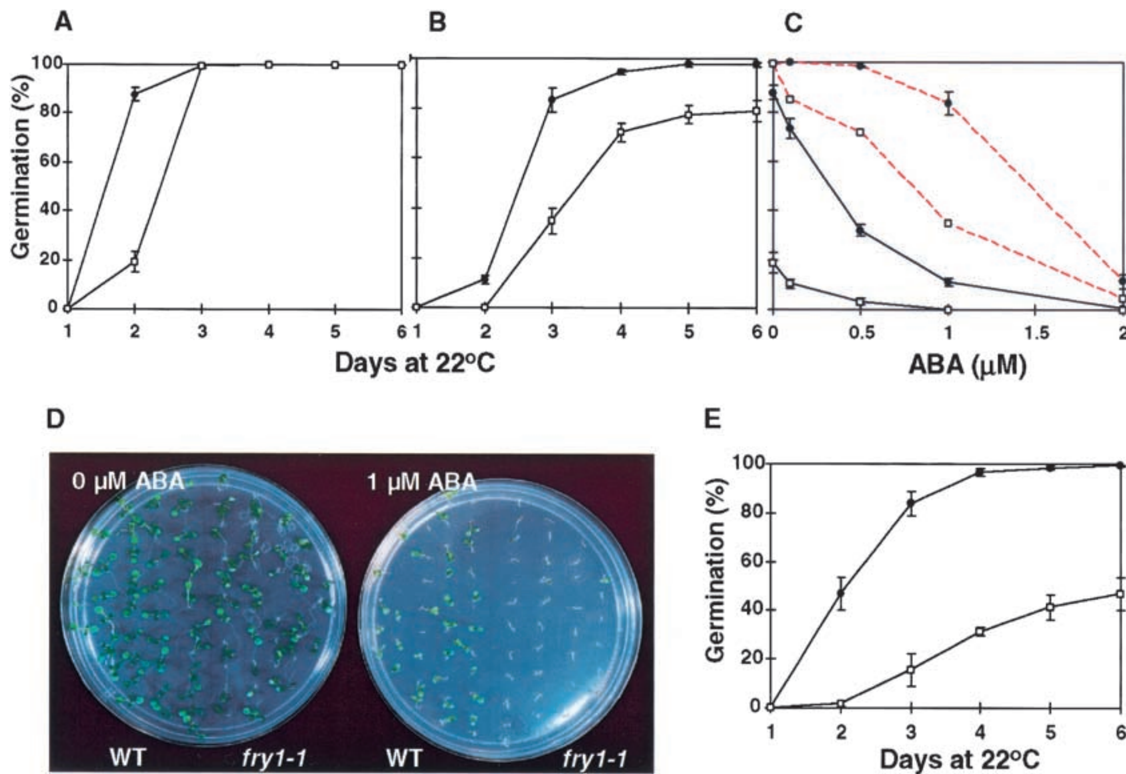

Figure 4. Seed germination and seedling development of wild type and *fry1-1* in response to ABA or NaCl. (*A–C*,*E*) Seeds on filter paper were incubated at 0°C for 48 h before being placed at 23°C for germination. (*A*) Germination in the absence of ABA (H_2O only); (*B*) Germination in the presence of 1.0 µM ABA; (*C*) Germination at day 2 (solid lines) and day 3 (broken lines) in the presence of different concentrations of ABA; (*D*) Inhibition of seedling development by ABA. Wild-type or *fry1-1* mutant seeds on MS agar plates supplemented with (plate on the right) or without (plate on the left) 1.0 µM ABA were incubated at 0°C for 48 h before being placed at 23°C for germination. The picture was taken 10 d after incubation at 23°C. (*E*) Germination in the presence of 50 mM NaCl. Results are means and standard errors (n = 3). Closed circles, wild type; open squares, *fry1-1*.

Genetic analysis showed that the luminescence phenotypes of *fry1-3* cosegregated with Basta resistance, suggesting that the *FRY1* gene was tagged in this T-DNA mutant.

Arabidopsis DNA flanking the T-DNA insertion in *fry1-3* plants was obtained by TAIL (thermal asymmetric interlaced) PCR (for left-border flanking DNA) and by plasmid rescue (for right-border flanking DNA; Weigel et al. 2000). The sequences of these flanking DNA fragments were found to match that of the predicted gene MBM17.8 on the P1 clone MBM17, consistent with the mapping location of *FRY1* (Fig. 6A).

The MBM17.8 gene was amplified by PCR from *fry1-1* and *fry1-2* mutant plants and sequenced. Single nucleotide mutations in the DNA sequence were found from both mutants that would result in changes in the predicated amino acid sequence (Fig. 6B). The wild-type MBM17.8 genomic DNA along with upstream sequence was cloned into a binary vector and transferred into *fry1-1* mutant plants via *Agrobacterium*-mediated in planta transformation. One hundred fifty independent T_1 transformants were tested for their ABA-responsive luminescence and 40 independent T_2 lines were tested for cold, NaCl, as well as ABA-responsive luminescence. All transformants tested showed wild-type *RD29A-LUC* expression patterns (data not shown). These results show that MBM17.8 is *FRY1*.

FRY1 *encodes a bifunctional enzyme with 3′(2′),5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities*

FRY1 cDNA was obtained by reverse transcriptase PCR and sequenced. BLAST searches indicated that the open reading frame *of FRY1* is identical to the *SAL1* gene that encodes a bifunctional enzyme with 3′(2′),5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities (Quintero et al. 1996). The *SAL1* gene was initially isolated because it was capable of increasing the salt tolerance of yeast cells when expressed in yeast (Quintero et al. 1996). SAL1 is similar to several Li^+ and Na^+-sensitive 3′(2′),5′-bisphosphate nucleotidases/inositol polyphosphate 1-phosphatases (INPP), such as the yeast MET22/HAL2, *Escherichia coli* CysQ, and INPPs from mouse, *Drosophila*, and human (Fig. 6C,D). Among this group of proteins, FRY1 shows the highest similarity with four other open reading frames in *Arabidopsis*, as well as DNPP1 from rice. Interestingly,

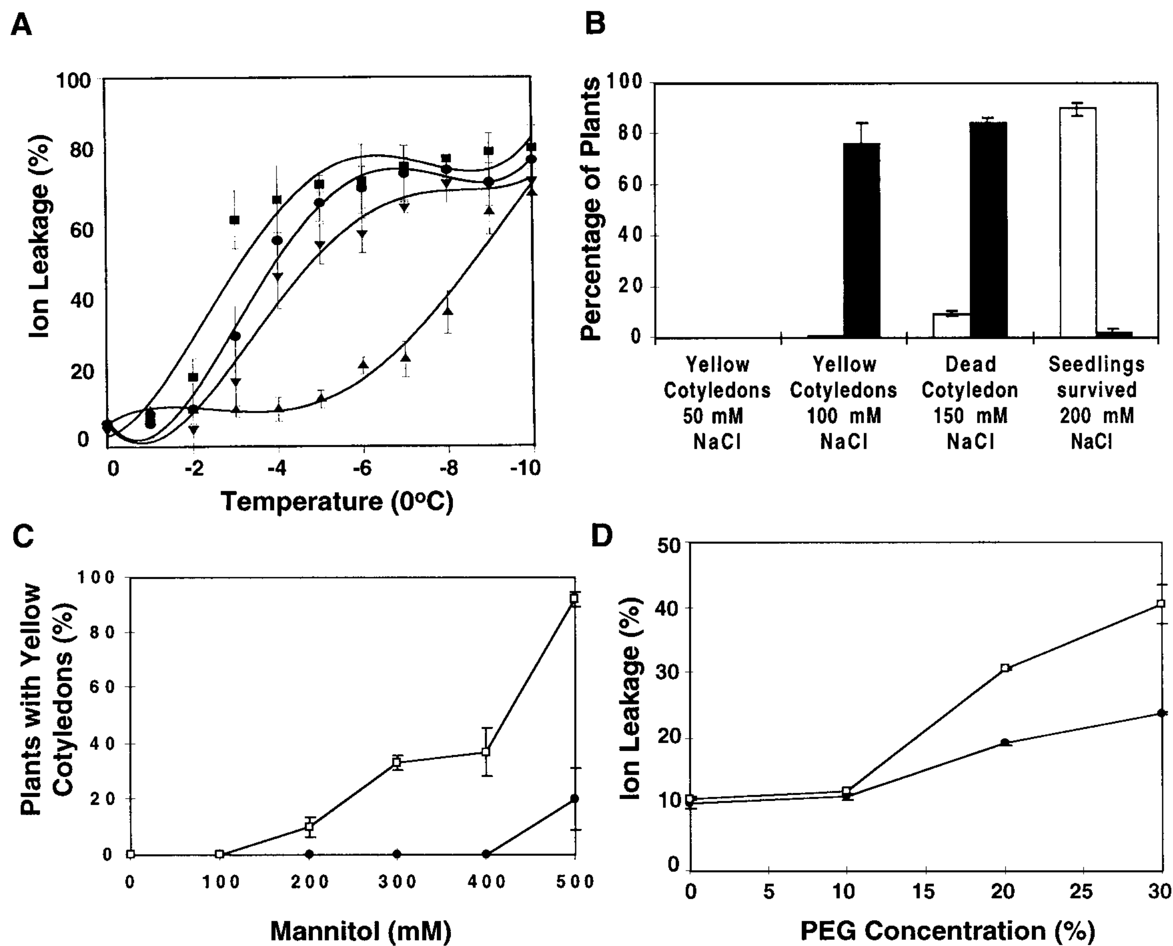

Figure 5. Compromised stress tolerance in *fry1* mutant plants. (*A*) Freezing-induced electrolyte leakage in *fry1-1* and wild-type plant leaves. Data are averages and standard deviations ($n = 3$). Wild type (solid square); *fry1-1* (solid circle); wild type, cold-acclimated (solid up arrow); *fry1-1*, cold-acclimated (solid down arrow). (*B–C*) *fry1-1* mutant seedlings suffer more damage as a result of hyperosmotic stress by NaCl or mannitol treatment. Shown are the percentages of seedlings with yellowish or bleached (dead) cotyledons on agar media supplemented with 50, 100, or 150 mM NaCl and the percentage of seedlings that survived 200 mM NaCl (*B*) and percentage of leaf damage on media supplemented with different concentrations of mannitol (*C*). Data are the means of three separate experiments each with 80 seedlings from wild type or *fry1-1*. (*D*) Ion leakage from seedlings treated with PEG. Data are means of four independent assays and standard errors. In *B*, *C*, and *D*, black symbols refer to wild type and open symbols refer to *fry1-1*.

FRY1 and two isologs, MBM17.9 (GenBank accession no. BAA96902) and MBM17.10 (GenBank accession no. Z83312), are arranged in tandem on chromosome V, presumably a result of duplications of a common ancestor. Phylogenetic analysis indicates that these plant isologs are more closely related to INPP1 from human and INPP from fruit fly than to others from lower organisms, such as CysQ from *E. coli* and MET22/HAL2 from yeast (data not shown).

In the *fry1-1* mutant, a G to A substitution in the sixth exon (Fig. 6B) creates a stop codon and thus truncates the protein at the beginning of the conserved α5 helix (Fig. 6D; York et al. 1995). The *fry1-2* mutation is also a G to A substitution (Fig. 6B) that results in the replacement of the invariant negatively charged residue E72 (glutamic acid) to a positively charged amino acid K (lysine), in the conserved α1 region (Fig. 6C; York et al. 1995). The EE consensus in the β1 sheet and the WD-X_{11}-GG motif in the α5 helix are both required for the coordination of metal ions and phosphate and for nucleophilic water activation (York et al. 1995). Therefore, the *fry1-1* mutation is most likely a null allele because the mutated protein is devoid of the α5 helix. In the *fry1-3* allele, a T-DNA is inserted between the fifth and sixth exons and thus led to the disruption of the gene. RNA analysis showed that the *FRY1* transcript is absent in the *fry1-3* allele (Fig. 7A). Thus, *fry1-3* mutation is also a null allele.

RNA blot analysis showed that *FRY1* is expressed in every organ examined although the transcript abundance is not the same in all plant tissues (Fig. 7A). The expression level of *FRY1* was not significantly affected by either stress or ABA treatment (Fig. 7B). *FRY1* expression was also examined in plants expressing the *FRY1::GUS* transgene. Among 21 independent transformants tested, 17 lines showed GUS activity. Consistent with RNA

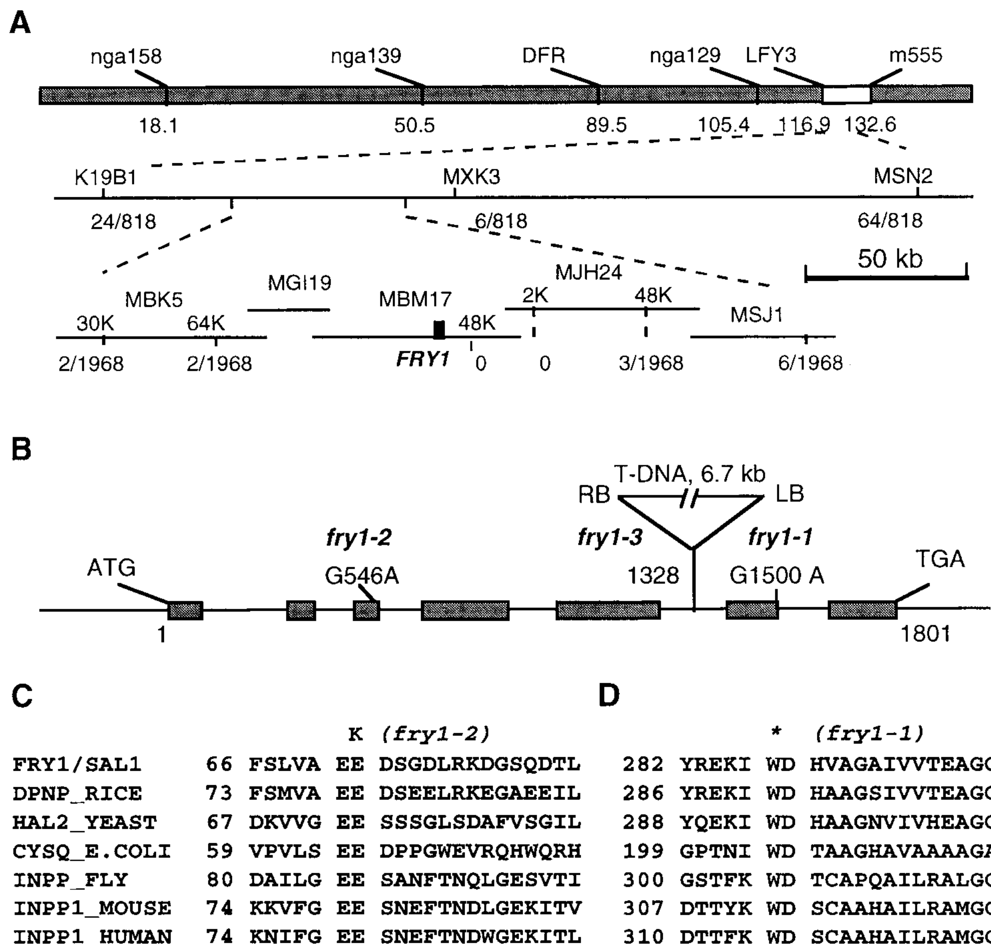

Figure 6. Positional cloning of the *FRY1*. (*A*) Physical mapping of *FRY1*. On the basis of analysis of 818 recombinant chromosomes, the *FRY1* locus was initially mapped to an 11-cM region on the low arm of chromosome V between the simple sequence length polymorphism (SSLP) markers K19B1 and MSN2. Further genetic mapping of 1968 chromosomes narrowed *FRY1* to a 160-kb region between the SSLP marker MBK5–64K and the single nucleotide polymorphism marker MJH24–48K. (*B*) Structure of the *FRY1* gene and positions of *fry1* mutations. Positions are relative to the translation initiation codon (position 36125, GenBank accession no. AB019227). Filled boxes indicate the open reading frame obtained by RT–PCR (GenBank accession no. AY034894) and lines between boxes indicate introns. (*C–D*) *FRY1* encodes a bifunctional enzyme with inositol polyphosphate 1-phosphatases and 3′(2′),5′-bisphosphate nucleotidases activities. Shown are amino acid alignments of FRY1 with other inositol polyphosphate 1-phosphatases (INPP) or 3′(2′),5′-bisphosphate nucleotidases (DPNP) at two highly conserved regions. (*C*) The EE motif in the β1 sheet. The *fry1-2* mutation changes the second glutamic acid residue (E) to a lysine (K). (*D*) The WD-X_{11}-GG motif in the α5 helix. The *fry1-1* mutation changes the tryptophan residue (W) to a stop codon. GenBank accession nos. are as follows: FRY1/SAL1, AY034894/Q42546; DPNP_rice, Q40639; MET22/HAL2_yeast, P32179; CYSQ_*Escherichia coli*, P22255; INPP_fly, AAC24856; INPP_mouse, P49442; and INPP1_human, NP_002185.

blot analysis, *GUS* reporter gene under control of the *FRY1* promoter was ubiquitously expressed in *Arabidopsis* seedlings (Fig. 7C–E). In leaves, *FRY1::GUS* expression was particularly evident in veins (Fig. 7C). It is also expressed in the primary root, root hairs (Fig. 7D), and stems (Fig. 7E; data not shown). In floral organs, *FRY1::GUS* expression was detected in sepals, petals, stamens, and carpels (Fig. 7E), with stamens showing the strongest expression (Fig. 7E).

To determine the impact of *fry1* mutation on FRY1 enzyme activity, recombinant FRY1-GST fusion protein was tested against 3′-phosphoadenosine 5′-phosphate (PAP). Whereas wild-type FRY1 showed a strong activity toward PAP (11.7 ± 0.74 µmole P/h per mg protein, *n* = 3), *fry1-1* mutant protein was entirely inactive, consistent with the prediction that *fry1-1* is a null mutation. FRY1/SAL1 protein has been shown to be able to hydrolyze inositol 1, 4-bisphosphate [Ins(1,4)P_2] and inositol 1, 3, 4-trisphosphate [Ins(1,3,4)P_3] with activities about one-third of that against PAP (Quintero et al. 1996), yet its activity against inositol 1, 4, 5-trisphosphate (IP_3) was not reported. We measured the activities of wild-type FRY1 on IP_3 and found that FRY1 had a low activity against IP_3 (0.47 ± 0.11 µmole P/h per mg protein), which is ~4% that of the activity against PAP. Interestingly, mutant *fry1-1* protein completely lost this activity against IP_3.

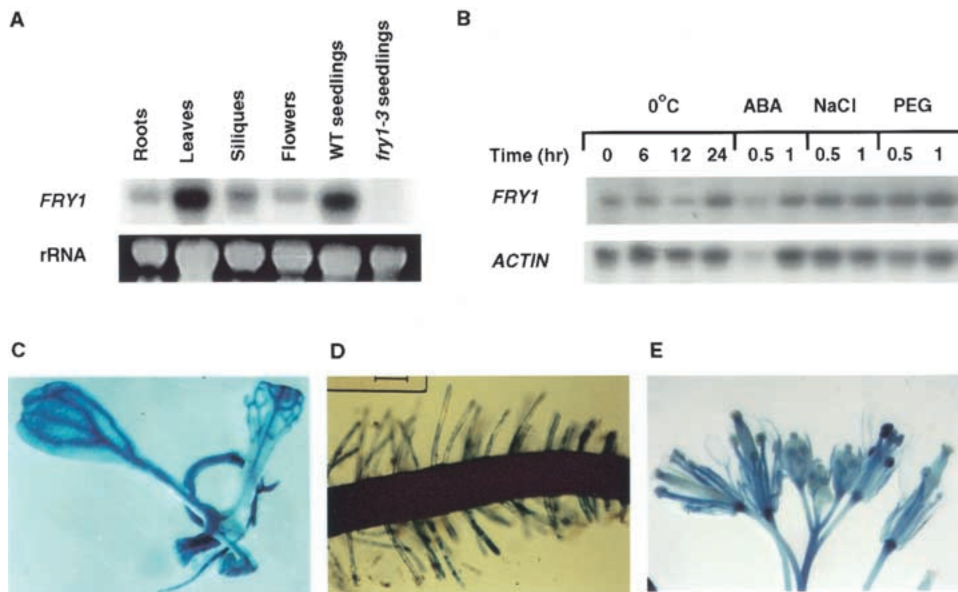

Figure 7. Expression of the *FRY1* gene. (*A*) *FRY1* transcript was detected in each plant organ examined and was absent in *fry1-3* mutant plants due to T-DNA disruption. Total RNA (20 µg) from indicated plant parts or from 7-day-old wild-type (WT seedlings) and *fry1-3* (T-DNA allele) seedlings were resolved on 1.3% agarose-formaldehyde gel and blotted onto a nylon membrane. The membrane was probed with ^{32}P-labeled *FRY1* cDNA. (*B*) The expression of *FRY1* transcript in wild-type seedlings was not substantially regulated by stress treatment. rRNA or ACTIN was used as loading controls. (*C–E*) Expression of *FRY1::GUS* in seedling (*C*), root and root hairs (*D*), and in floral organs (*E*).

Increased IP_3 accumulation in fry1 *mutant plants*

The inositol polyphosphate 1-phosphatase activity of FRY1/SAL1 suggests that it participates in phosphoinositide signaling. Although FRY1 and its homologs in other organisms were shown to have both nucleotidase activity and inositol phosphatase activities, in multicellular organisms, FRY1 homologs mainly function as an inositol phosphatase. In animal cells, FRY1/SAL1 homologs are specific to the phosphoinositide signaling system and are primary targets of therapeutic action of Li^+ treatment in patients for manic-depressive disease (Majerus 1992). The inositol phosphatase activity was proposed to mediate IP_3 breakdown by dephosphorylating the IP_3 catabolic intermediates, Ins(1,4)P_2 and Ins(1,3,4)P_3 (Majerus 1992). As *fry1* mutant plants grow normally with inorganic sulfate as the sole sulfur source and there is no sign of sulfur deficiency (data not shown), the nucleotidase activity of FRY1 in sulfate assimilation seems to be dispensable. Thus, we hypothesized that FRY1 mainly functions as an inositol polyphosphate 1-phosphatase that mediates the catabolism of IP_3.

To gain insight into the role of FRY1 in IP_3 metabolism in vivo, IP_3 content was determined in wild-type and *fry1-1* seedlings treated with 100 µM ABA. In wild-type seedlings, ABA induced a transient increase in the level of IP_3, with the level of IP_3 after 1 min nearly 10 times that of untreated control plants (Fig. 8). Ten min after ABA treatment, IP_3 level returned to pretreatment levels. At 30 min after ABA treatment, the IP_3 level was not significantly different from untreated control. *fry1-1* mutant seedlings had a high level of IP_3 even in the absence of exogenous ABA treatment (Fig. 8). This constitutive level of IP_3 is likely responsible for the low constitutive expression of *RD29A-LUC* (Fig. 1B) and the endogenous *RD29A* and *COR47* transcripts (Fig. 2) in *fry1* mutant plants. In response to ABA treatment, the IP_3 level in the mutant did not increase substantially until about 30 min. Except at 1 min after ABA treatment, *fry1-1* mutant plants had significantly higher levels of

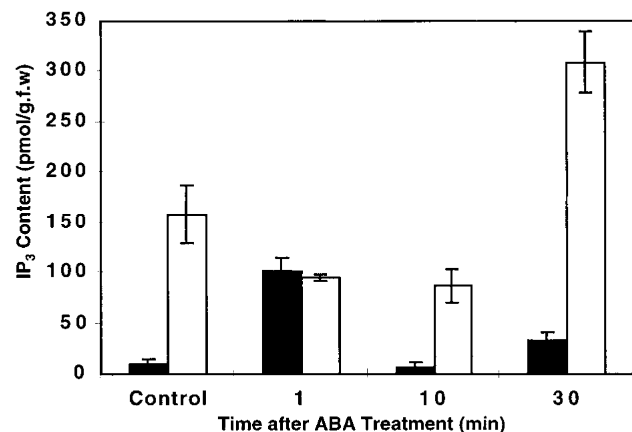

Figure 8. Changes in the level of inositol 1, 4, 5-trisphosphate (IP_3) in wild-type and *fry1-1* plants in response to ABA treatment. Rosette plants grown in soil were sprayed with 100 µM ABA, leaves were then sampled at the indicated times, and IP_3 measured using a receptor-binding assay as described in Materials and Methods. Error bars represent standard errors (n = 3). Closed bars, wild type; open bars, *fry1-1.*

IP_3 than wild-type plants at all the other time points (Fig. 8). These results are consistent with *fry1* mutant plants having altered inositol polyphosphate 1-phosphatase activity and support our hypothesis that *fry1* mutations impair the mechanism for attenuating ABA and abiotic stress responses by blocking IP_3 turnover.

Discussion

Previous genetic screens based on the inhibitory effect of ABA on seed germination have successfully identified several genetic loci important in ABA signaling, which in turn affect plant stress responses (for review, see Koornneef et al. 1998). In the present study, we used a reporter-gene approach to identify mutations that directly affect vegetative plant responses to stress and ABA. The *fry1* mutants thus isolated show enhanced *RD29A-LUC* reporter gene expression in response to ABA or stress treatments. RNA blot analysis showed that the expression of the endogenous *RD29A* and other stress-responsive genes such as *KIN1*, *COR15A*, *HSP70*, and *ADH* was also significantly increased by the mutation under low temperature, osmotic stress, or ABA treatment (Fig. 2A). For reasons that are still not clear, the mutation altered the expression kinetics of some of the genes such as *HSP70* and *COR15A*. *fry1* Mutations also significantly lowered the excitatory threshold of gene induction by stress or ABA (Fig. 3A–C). Previous studies on the regulation of the diverse stress-responsive genes and our data of different expression profiles of these genes (Fig. 2) indicate that these genes are activated by different signaling pathways. The fact that *FRY1* affects the expression of all these genes in one way or another suggests that it plays an important regulatory role in an early step connecting these different signal transduction pathways. Consistent with the early signaling role of *FRY1*, *CBF2* induction by cold in *fry1* mutant plants is more sustained (Fig. 2B), although this altered *CBF2* expression kinetics by itself may not be sufficient to explain the elevated downstream gene expression.

Mutations in *FRY1* not only increased the expression of stress-responsive genes, but also render the mutant plants more sensitive to ABA and to damage by low temperature, drought, or salt stress. Germination of *fry1* seeds is more sensitive to ABA (Fig. 4A–C). ABA also inhibits early seedling development of *fry1*; whereas at the same concentrations it does not significantly inhibit the development of the wild-type seedlings (Fig. 4D). Freezing stress causes more damage to *fry1* than it does to the wild-type plants. Upon freezing treatment, *fry1* plants lost more electrolytes (Fig. 5A). Likewise, *fry1* plants also showed a higher sensitivity to osmotic stresses. On NaCl media, *fry1* plants were more damaged than were wild-type plants, as indicated by increased leaf injuries and reduced survival rate in the mutant (Fig. 5B). The increased sensitivity to mannitol (Fig. 5C) indicated that *fry1* plants are sensitive to general osmotic stress. Accordingly, *fry1* mutant plants are less tolerant to drought stress as indicated by increased electrolyte leakages upon treatment with PEG (Fig. 5D).

These phenotypes of *fry1* mutant plants clearly reflect important functions of wild-type *FRY1* in ABA and stress responses. To our surprise, *FRY1* encodes a bifunctional phosphatase with both 3′(2′), 5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities. Although mutants with lesions in *FRY1*-like genes in *E. coli*, yeast, and more recently in *Drosophilia* have been isolated, gene expression studies in these mutants have not been reported. The *E. coli cysQ* (Neuwald et al. 1992) and yeast *met22/hal2* mutants (Gläser et al. 1993) were unable to reduce sulfate and need reduced or organic form of sulfur (e.g., methionine or cysteine) to grow. Additionally, the yeast *met22/hal2* was also more sensitive to salt stress presumably as a result of the accumulation of PAP, which inhibits sulfate reduction and RNA processing (Gläser et al. 1993; Dichtl et al. 1997). FRY1/SAL1 was able to complement yeast *met22/hal2* mutants for their methionine auxotrophy and Li^+/Na^+ sensitivity (Quintero et al, 1996). The facts that *fry1* mutant plants are able to use inorganic sulfate as the sole sulfur source and do not show any sulfur-deficiency symptoms suggest that FRY1 is not required for sulfate reduction in *Arabidopsis*, perhaps because there are five *FRY1*-like genes in the *Arabidopsis* genome (data not shown). Among *Arabidopsis* FRY1-like proteins, the product encoded by AT4g05090 (GenBank accession no. CAB81051) on chromosome IV, is most closely related to MET22/HAL2 (data not shown), suggesting that the AT4g05090 product likely functions in sulfate reduction in *Arabidopsis*. Despite their sequence similarities, FRY1-like proteins in the unicellular organisms, *E. coli* and yeast do not have inositol polyphosphate 1-phosphatase activities (Neuwald et al. 1992; Murguia et al. 1995), contrasting with those in multicellular organisms in which 1-phosphatase activity has been shown both in plants and in animal systems (e.g., Quintero et al. 1996; York et al. 1995). Phylogenetic analysis also reveals that FRY1 is more closely related to its animal homologs than to either the bacterial or yeast counterparts (data not shown). Therefore, the defect in phosphoinositol 1-phosphatase function is most likely responsible for the *fry1* mutant phenotypes.

It has been established that phosphoinositides are involved in many signal transduction processes in animal systems. A generic scheme depicts that the activation of receptors activates specific phospholipases that function in the breakdown of phosphotidylinositol 4,5-bisphosphate (PIP_2), generating the second messengers inositol 1,4,5-trisphosphate and diacylgercerol. Inositol 1,4,5-trisphosphate (IP_3) was shown to trigger Ca^{2+} release from internal stores in many cell types. In plants, exogenous IP_3 was shown to release Ca^{2+} from vacuolar vesicles or isolated vacuoles (e.g., Schumaker and Sze 1987) and to mediate transient increases in cytosolic Ca^{2+} (e.g., Allen et al. 1995). Both IP_3 and Ca^{2+} have been implicated in ABA and environmental stress responses (for reviews, see Munnik et al. 1998; Sanders et al. 1999). In response to ABA treatment, there is a transient increase in IP_3 in guard cell protoplasts of *Vicia faba* (Lee et al. 1996). IP_3 transients have also been observed in

cells in response to hyperosmotic stress (Heilmann et al. 1999). These results and our finding that *FRY1* encodes an enzyme with inositol polyphosphate 1-phosphatase activity led us to hypothesize that the role of FRY1 is in the attenuation of ABA and stress responses by controlling the turnover of the second messenger IP_3. That is, the initial perception of ABA or abiotic stress by plants results in a transient increase in IP_3. The amplitude of this signal is attenuated by inositol polyphosphate 1-phosphatase mediated breakdown of IP_3. This does not occur in *fry1* mutants, which results in sustained IP_3 and perhaps other inositol polyphosphate levels and enhanced expression of stress-responsive genes. To test this hypothesis, we determined IP_3 levels in wild-type and *fry1* mutant seedlings. Our results show that *fry1* mutants accumulated higher levels of IP_3 when treated with ABA (Fig. 8), indicating that *FRY1* does play a role in the catabolism of IP_3 in plants. Interestingly, the basal IP_3 content in *fry1* mutant plants before ABA treatment was also significantly higher than that in the wild type (Fig. 8), which may have resulted from mild stress on the growth medium. This basal level of IP_3 may be responsible for the constitutive *RD29A-LUC* expression shown in Figure 1B and the constitutive expression of several stress-responsive genes in the absence of stress or ABA treatment (Fig. 2).

Given the important role of IP_3 in cell signaling, the level of this molecule, not surprisingly, is tightly regulated. The biochemical pathways of IP_3 catabolism have been intensively studied in vitro in animal systems. It is known that there are two major routes that mediate IP_3 breakdown. These are the 5-phosphatase pathway and the 3-kinase pathway, resulting in the accumulation of $Ins(1,4)P_2$ and inositol 1,3,4,5-tetraphosphate [$Ins(1,3,4,5)P_4$] intermediates, respectively (Majerus et al. 1992). $Ins(1,3,4,5)P_4$ can be further dephosphorylated by 5-phosphatases to generate inositol 1,3,4-trisphosphate [$Ins(1,3,4)P_3$]. In animal cells, inositol polyphosphate 1-phosphatase (IPP) was shown to hydrolyze the intermediate products of the IP_3 degradation pathways, $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$, at the 1-position (Majerus et al. 1992). FRY1/SAL1 was also able to hydrolyze both of these two inositol polyphosphates (Quintero et al. 1996). Although IPP isoforms can hydrolyze IP_3 directly in certain cell types, in many other cells, the 1-phosphatase does not have this activity to directly hydrolyze IP_3 (Majerus et al. 1992). Our assay of FRY1 activity against IP_3 indicated that the recombinant protein had about 4% activity on IP_3 relative to its activity on PAP, and impressively, *fry1-1* mutation also abolished this activity. It is not known whether FRY1 needs some cofactors for full activity against IP_3 or whether this low activity of FRY1 against IP_3 as measured in vitro has any biological significance. Further clarification of these uncertainties in the future would shed light on the biochemical specificity of FRY1 function. Nonetheless, the 1-phosphatase activity of FRY1 toward $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$ would inevitably affect the catabolism of IP_3 as the accumulation of these intermediates would prevent the IP_3 degradation pathway from going forward and result in the slowdown of IP_3 degradation. It should be pointed out, however, that an enhanced accumulation of IP_3 alone might not account for all the enhanced expression of stress-responsive genes and the altered stress sensitivity in the mutant. The reasons are twofold. First, from the IP_3 degradation pathways, one can predict that a defect in FRY1 might also result in the accumulation of $Ins(1,3,4,5)P_4$. $Ins(1,3,4,5)P_4$ is also a signal in animal cells that gates Ca^{2+} influx into the cell and may also potentiate the effect of IP_3 in releasing Ca^{2+} from internal stores (Shears 1998). Studies also suggested that $Ins(1,3,4)P_3$ is a strong inhibitor of Ins (3,4,5,6)-1 kinase; whereas $Ins(3,4,5,6)P_4$ is an inhibitor of Ca^{2+}-regulated Cl^- secretion (Shears 1998). Thus, $Ins(1,3,4)P_3$ may indirectly regulate cellular ion homeostasis in vivo (Shears 1998). In yeast, FRY1/SAL1 increases salt tolerance of the yeast transformants probably by increasing the efflux of Li^+/Na^+ (Quintero et al. 1996). This process may involve phosphoinositide signaling because inhibition of phospholipase C reduces Li^+ efflux (Quintero et al. 1996). Therefore, *fry1* mutations may cause the disruption of ion homeostasis that is required for cell adaptation to various stresses. Second, a disruption of FRY1 activity is likely to alter the dynamics of more highly phosphorylated inositols such as IP_5 and IP_6, signals that have been shown to affect mRNA export and gene expression (e.g., Odom et al. 2000). In plants, it was recently reported that ABA elicits a rapid increase in IP_6 level, and IP_6 was shown to inhibit Ca^{2+}-dependent inward K^+ currents in guard cells of *Solanum tuberosum* and *Vicia faba* (Lemtiri-Chlieh et al. 2000).

Although phosphoinositols have been implicated by biochemical and molecular studies to play roles in stress and ABA signal transduction, our results provide the first genetic evidence that phosphoinositols mediate gene regulation by cold, drought, and salt stress, as well as by ABA. Mutations in *FRY1* result in significantly higher induction of ABA and stress-regulated gene expression (Figs. 1,2), indicating that the wild-type FRY1 protein attenuates these signaling pathways. This early attenuation mechanism is an important aspect of signal transduction to prevent overactivation of downstream pathways. Moreover, this attenuation mechanism is also critical to maintain resistance to the abiotic stresses since *fry1* mutant plants are compromised in stress tolerance (Fig. 5). This indicates that a deregulated IP_3 metabolism can have a dramatic negative effect on plant tolerance to environmental stresses, despite an enhanced expression of stress-responsive genes (Fig. 2). These findings differ from some transgenic studies where enhanced expression of stress-responsive genes in plants overexpressing CBF/DREB1 transcription factors increased plant tolerance to various abiotic stresses (e.g., Jaglo-Ottosen et al. 1998; Kasuga et al. 1999). These apparent discrepancies may result from some as-yet-unknown determinants. It is notable that FRY1 manifests its effect in early steps of signal transduction. Hence, in addition to the signaling roles of other phosphoinositols and their regulation of ion-channel activities as discussed above, the defect in early steps of signaling may impair some

yet-unknown signaling branches that might be required for stress tolerance.

Materials and methods

Isolation of fry1 *mutants*

Transgenic *Arabidopsis thaliana* of the C24 ecotype expressing the *RD29A-LUC* transgene (referred to as the wild type) were obtained by *Agrobacterium*-mediated transformation (Ishitani et al. 1997). The wild-type *RD29A-LUC* plants were mutagenized by ethyl methanesulfonate (Ishitani et al. 1997) or by transformation with *Agriobacterium tumefaciens* strain GV3101 carrying the pSKI015 activation tagging vector (Weigel et al. 2000). M_2 or T_2 seeds were planted on 0.6% agar plates containing full strength MS salt base (JRH Biosciences) and 1-week-old seedlings were screened for abnormal *LUC* expression in response to low temperature, ABA, or osmotic stress with a thermoelectrically cooled CCD camera. For ABA treatment, (±)-*cis*, *trans*-abscisic acid in H_2O was sprayed uniformly on the leaves of seedlings and the plants were incubated at room temperature under cool-white light before luminescence imaging. For NaCl or PEG treatment, seedlings on MS plates were transferred to filter paper saturated with MS solution supplemented with 300 mM NaCl or 30% of polyethylene glycol (molecular mass 6000). For luminescence imaging, plants were sprayed uniformly with 1 mM luciferin in 0.01% Triton X-100 and then kept in the dark for 5 min before imaging. All images were acquired with 5-min exposure time. The luminescence intensity of each seedling was quantified with the WinView software.

RNA analysis

Ten-day-old seedlings grown on MS agar plates were treated with either low temperature, ABA, NaCl, or PEG. Respective treatment conditions were as stated in the text. Total RNA from control or treated plants was extracted and analyzed as described (Ishitani et al. 1998). Gene-specific probes were as described (Ishitani et al. 1998; Lee et al. 2001).

Germination assay

One hundred seeds from wild-type and *fry1-1* plants in triplicates were placed on filter paper saturated with distilled water or different concentrations of ABA or NaCl and incubated at 0°C for 48 h before being placed at room temperature under cool-white light for germination. Seeds were considered as germinated when radicles completely penetrated the seed coat. Germination was scored daily up to 10 d after being placed at room temperature.

Stress tolerance

For salt or drought tolerance assay, wild-type and *fry1-1* seeds were planted on MS agar plates for germination. Two days after germination, 80 seedlings from each line were carefully transferred to a new MS agar plate supplemented with different concentrations of NaCl or mannitol. There were three replicates for each treatment. After growing for ten days in the treatment media, plants with yellowish (i.e., damaged) or dead cotyledons were scored. The fresh weight and dry weight of the seedlings were measured. For freezing tolerance assay, *fry1-1* and wild-type seeds were sown in soil. For cold acclimation treatment, plants at the rosette stage were placed at 4°C under white fluorescent light for one week before sampling the leaves for freezing tolerance assay. Fully developed rosette leaves were used to determine freezing-caused electrolyte leakage as previously described (Ishitani et al. 1998).

To measure ion leakage in seedlings induced by PEG treatment, 1-week-old wild-type and *fry1-1* seedlings growing in MS agar plates were carefully removed from the plate and placed in solutions containing different concentrations of polyethylene glycol (PEG) (molecular mass 6000) for 5 h. After the treatment, seedlings were rinsed briefly in distilled water and immediately placed in a tube with 3 mL H_2O. The tube was then agitated for 3 h before electrolyte content was measured. Four replicates of each treatment were conducted.

IP_3 assay

Soil-grown wild-type and *fry1-1* plants at the rosette stage were sprayed with 100 µM ABA and leaves were excised at 1, 10, and 30 min after ABA treatment and immediately frozen in liquid nitrogen. After being ground in liquid nitrogen, the samples were extracted with 20% perchloric acid and supernatants collected after centrifugation at 2000 *g* for 15 min at 4°C. The supernatants were neutralized to pH 7.5 with KOH, and then the IP_3 content was measured by the bovine adrenal binding protein assay using a $[^3H]IP_3$ assay kit following manufacturer's instructions (Amersham Pharmacia Biotech).

Cloning of FRY1

For genetic mapping of the *fry1* mutation, *fry1-1* was crossed with wild-type plants of the Columbia ecotype. The resulting F_1 plants were allowed to self and homozygous *fry1* mutants in the segregating F_2 population were selected based on their cold- and ABA-hypersensitive luminescence. Mapping of the mutation was carried out as described previously (Lee et al. 2001). For the T-DNA tagged allele, *fry1-3*, plant flanking DNA was cloned as described (Weigel et al. 2000). *FRY1* cDNA was obtained by using reverse transcriptase-PCR (RT–PCR) and cloned into pCR2.1-TOPO cloning vector (Invitrogen) and sequenced. For phylogenetic analysis of FRY1-like proteins, the neighbor-joining method was used to construct the tree using the GENETYX 8.0 program (Software Development Co., Tokyo, Japan).

For complementation assay, a genomic fragment containing the *FRY1* open reading frame along with 1070 bp of sequence upstream the translation initiation codon (corresponding to position 36125, GenBank accession no. AB019227) was amplified from wild-type genomic DNA and inserted into the *Pst*I–*Kpn*I sites of the binary vector pCAMBIA1200. The plasmid was transferred to *fry1-1* mutant via *Agrobacterium*-mediated transformation. One hundred fifty T_1 transformants were selected based on hygromycin resistance and then transferred to soil to grow to maturity. Seedlings of the T_2 generation were treated with cold or ABA and the luminescence images were taken as described above.

A *FRY1* promoter fragment from 1287-bp to 42-bp upstream the translation initiation codon was amplified from genomic DNA prepared from the wild-type seedlings and inserted into *Bam*HI–*Hin*dIII sites of the binary vector pCAMBIA1381. The resulting construct was transferred into wild-type plants via *Agrobacterium*-mediated in planta transformation. The T_1 seedlings were stained with X-Gluc for 12 h, followed by incubating in 70% ethanol to remove chlorophyll.

FRY1 recombinant protein and enzyme activity assay

Wild-type and *fry1-1* mutant cDNAs were obtained by reverse transcriptase-PCR using mRNA isolated from wild-type and

fry1-1 plants, respectively. The resulting PCR products were cloned into pCR2.1-TOPO cloning vector (Invitrogen) and confirmed by sequencing. The plasmids were partially digested with *Eco*RI and *Sma*I and the desired inserts were cloned inframe into the bacterial expression vector pGEX-2TK. The resultant expression constructs were transferred into *E. coli* BL21 cells. After induction with 0.1 mM isopropyl β-D-thiogalactopyranoside, the cells were harvested and proteins extracted by ultrasonication. Crude extracts were purified with the Gluthathione Sepharose 4B system (Pharmacia Biotech). Enzyme activity against 3′-phosphoadenosine 5′-phosphate (PAP) was assayed at 30°C as described (Murguia et al. 1995). For the assay of activity against IP_3, inositol 1, 4, 5-trisphosphate (Sigma) was first treated with anion exchanger resin (Whatman International, Maidstone, England) to remove free phosphate and the enzyme activity was measured as described (Murguia et al. 1995).

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