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The *sfr6* Mutation in Arabidopsis Suppresses Low-Temperature Induction of Genes Dependent on the CRT/DRE Sequence Motif

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The *sfr* mutations, which result in sensitivity to freezing after cold acclimation, define genes that are required for freezing tolerance. We tested plants homozygous for mutations *sfr2* to *sfr7* for cold-induced gene expression and found that *sfr6* plants were deficient in cold-inducible expression of the genes *KIN1*, *COR15a*, and *LTI78*, which all contain the C repeat/dehydration-responsive element (CRT/DRE) motif in their promoters. Similarly, *sfr6* plants failed to induce *KIN1* normally in response to either osmotic stress or the application of abscisic acid. In contrast, cold-inducible expression of genes *CBF1*, *CBF2*, *CBF3*, and *ATP5CS1*, which lack the CRT/DRE motif, was not affected. The freezing-sensitive phenotype that defines *sfr6* also was found to be tightly linked to the gene expression phenotype. To determine whether the failure of cold induction of CRT/DRE–containing genes in *sfr6* was due to altered low-temperature calcium signaling, cold-induced cytosolic-free calcium ($[Ca^{2+}]_{cyt}$) elevations were investigated in the *sfr6* mutant, but these were found to be indistinguishable from those of the wild type. We discuss the possibilities that CRT/DRE binding proteins (such as CBF1) require activation to play a role in transcription and that the SFR6 protein is a vital component of their activation.

INTRODUCTION

Hardy plants, such as Arabidopsis, develop freezing tolerance only when exposed to cool temperatures. This adaptive change, occurring on a time scale of days to weeks, is known as cold acclimation (Thomashow, 1994). The phenomenon of cold acclimation has provided an avenue for investigation of freezing tolerance by allowing comparison of the physiology and molecular biology of acclimated and nonacclimated plants. Expression of a number of genes is induced during cold acclimation (reviewed in Thomashow, 1990, 1998; Hughes and Dunn, 1996). The products of some of these genes, for example, cold-responsive *COR15a*, have been demonstrated to play a protective role in freezing stress (Artus et al., 1996), whereas the role of others, for example, low-temperature-induced *LTI78* (Mäntylä et al., 1995), also known as *COR78* (Horvath et al., 1993), and *RD29A* (Yamaguchi-Shinozaki and Shinozaki, 1994), has not been determined. It is thought that the process of cold acclimation requires the coordinate expression of a whole battery of cold-induced genes (Jaglo-Ottosen et al., 1998).

Classic genetics offers an orthogonal approach for determining genes essential to freezing tolerance. Xin and Browse (1998) selected Arabidopsis mutants that showed elevated freezing tolerance in the absence of cold acclimation. The first mutation described, *eskimo1*, elevates the level of the compatible osmolyte proline. Conversely, Warren et al. (1996) identified Arabidopsis mutants deficient in freezing tolerance even after cold acclimation; these are known as the *sfr* (for sensitive to freezing) mutants. One of these, *sfr4*, showed depressed levels of sugars, which are, like proline, compatible osmolytes (McKown et al., 1996).

One of the earliest reported events in plant responses to low temperature is an immediate elevation in the concentration of cytosolic free calcium ($[Ca^{2+}]_{cyt}$; Knight et al., 1991, 1996). This controls downstream events, including changes in protein phosphorylation (Monroy et al., 1993, 1998) and ultimately the expression of cold-inducible genes (Monroy and Dhindsa, 1995; Knight et al., 1996; Polisensky and Braam, 1996; Tähtiharju et al., 1997), and has been implicated in the acquisition of freezing tolerance through cold acclimation (Monroy et al., 1993; Monroy and Dhindsa, 1995).

Many of the Arabidopsis cold-responsive or *COR* genes are regulated by the C repeat/dehydration-responsive element (CRT/DRE), which is a *cis*-acting element, containing the CCGAC motif, which activates transcription in response to both low temperature and water deficit (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). These genes include *KIN1* (Kurkela and Franck, 1990), *COR15a* (Hajela et al., 1990), and *LTI78* (Nordin et al., 1991), all of

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which contain CRT/DRE motifs within their promoters. The CRT/DRE is activated by the transcription factor CBF1 (for CRT/DRE binding factor; Stockinger et al., 1997). Other related CRT/DRE binding factors also are implicated in mediating expression of cold- and drought-inducible genes (Liu et al., 1998). The overexpression of the CBF1 protein causes expression of a number of *COR* genes in the absence of a cold stimulus and can cause plants to become freezing tolerant (Jaglo-Ottosen et al., 1998). However, some cold-inducible Arabidopsis genes (including *CBF1* itself and the closely related homologs *CBF2* and *CBF3*; Gilmour et al., 1998) do not contain a CRT/DRE and therefore do not depend on CBF1 or other CRT/DRE binding proteins (Liu et al., 1998) for their regulation.

In this study, our goal was to determine whether any of the *sfr* mutants showed aberration in low-temperature signaling that might possibly account for their freezing-sensitive phenotype. This approach has identified *sfr6* as being of particular interest, and we present data relating to altered control of *COR* gene expression in the *sfr6* mutant.

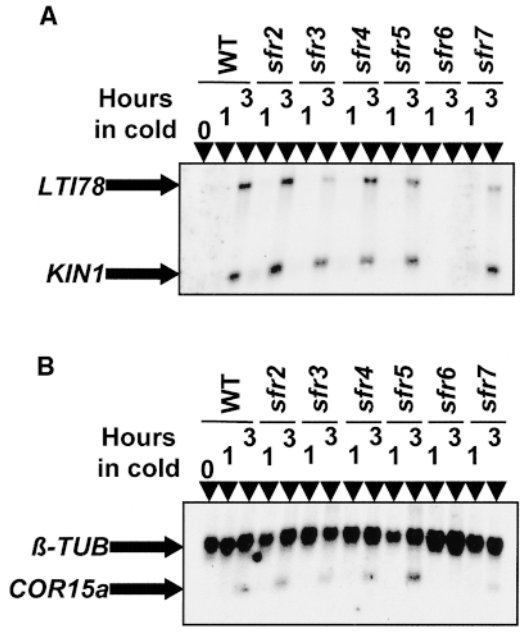

Figure 1. Cold-Induced *COR* Gene Expression in Arabidopsis *sfr* Mutants.

Wild-type Col (WT), *sfr2*, *sfr3*, *sfr4*, *sfr5*, *sfr6*, and *sfr7* plants were treated at 5°C for the length of time indicated (0, 1, or 3 hr). After this time, plant tissue was harvested, and total RNA was extracted and size fractionated on an RNA gel. After blotting onto a membrane, the membrane was hybridized simultaneously with labeled DNA probes.
(A) Hybridization with *LTI78* and *KIN1*.
(B) The same membrane was hybridized with DNA probes for *COR15a* and β-tubulin (*β-TUB*).
Hybridizing bands corresponding to each transcript are marked with arrows and tagged *LTI78*, *KIN1*, *COR15a*, and *β-TUB*, respectively.

RESULTS

Cold-Induced *COR* Gene Expression in Mutants *sfr2* to *sfr7*

Six *sfr* mutants were tested for *COR* gene expression in response to low temperature. The expression levels of three genes were compared with those seen in the wild type (ecotype Columbia [Col]) after 0, 1, or 3 hr at 5°C. RNA gel blot analysis revealed that in six of the mutants, as occurred in the wild type, induction of *KIN1* and *LTI78* (Figure 1A) and *COR15a* (Figure 1B) gene expression occurred at relatively low levels after 1 hr at 5°C. After 3 hr at this temperature, significant levels of expression were seen (Figures 1A and 1B). However, cold-induced expression was not detectable in *sfr6* plants after 1 or 3 hr (Figures 1A and 1B). Similar results were obtained in quantitative reverse transcription–polymerase chain reaction (RT-PCR) experiments that used primers described in Methods (data not shown). Expression of β-tubulin was monitored as a control in this and all subsequent experiments to verify that comparable levels of RNA were present in wild-type and mutant samples.

To repeat and extend this result, we again compared *KIN1* expression in *sfr6* and the wild type by using an additional time point and also examined its expression at a second temperature. Low or undetectable levels of *KIN1* gene expression were detected by using RNA gel blot analysis of *sfr6* treated at 5°C for 1, 2, or 3 hr, whereas in the wild type, levels of expression reproducibly increased over time (Figure 2A). Induction was also deficient in *sfr6* plants but readily detectable in the wild type after treatment at 10°C for 3 hr. Failure of *sfr6* to express *KIN1* in response to 3 hr of cold treatment could be due either to a complete failure in expression or simply to a delay in the induction of *KIN1* expression. Therefore, we conducted another experiment in which plants were treated at 4°C for up to 48 hr. RNA gel blot analysis revealed that a small amount of *KIN1* expression was detectable in *sfr6* samples after 6 hr and that expression increased thereafter up to 24 hr. *KIN1* expression in the mutant was consistently and significantly much lower than that seen in wild-type plants at every time point (Figure 2B). However, it appeared that the kinetics of response were similar in the wild type and *sfr6*, with both reaching a maximum in transcript levels between 24 and 48 hr after cold treatment (Figure 2B).

Linkage of Expression Deficiency to Freezing Sensitivity

Because a deficiency in *COR* gene induction might be responsible for impairing freezing tolerance, it already seemed likely that the observed expression deficiency was caused by the *sfr6* mutation. To test this supposition, we looked for

cosegregation of expression deficiency with freezing sensitivity. A homozygous *sfr6* line in a Col background was crossed to wild-type Landsberg *erecta* (L*er*-0). Plants of the F_1 generation, heterozygous for the mutant *sfr6* allele, showed no deficiency in cold-induced expression of *KIN1* (Figure 3, lane 3; cold-induced expression in both L*er*-0 and wild-type Col is shown in lanes 1 and 2 for comparison). This result established that, like the freezing-tolerance phenotype (Warren et al., 1996), the gene expression phenotype of the line was recessive.

Among F_2 plants from the cross, we identified individuals homozygous for *sfr6* by freeze testing their progeny. Additional progeny of the identified *sfr6*/*sfr6* F_2 individuals then were grown and tested for the cold inducibility of *KIN1.* The progeny of each *sfr6* homozygote showed a deficiency in cold-inducible expression similar to that of the original *sfr6* line (two examples are shown in Figure 3, lanes 7 and 8). In addition, the original *sfr6* line was backcrossed to wild-type Col and also crossed with a line that was homozygous for an aequorin transgene (see sections below) (Knight et al., 1991). F_2 individuals homozygous for *sfr6* again were identified (by freeze testing), and their progeny were tested for cold-induced *KIN1* gene expression. In all cases, we observed a deficiency of cold-induced expression comparable to that of the original line (examples are shown in Figure 3, lanes 4 to 6). Similar results were obtained by quantitative

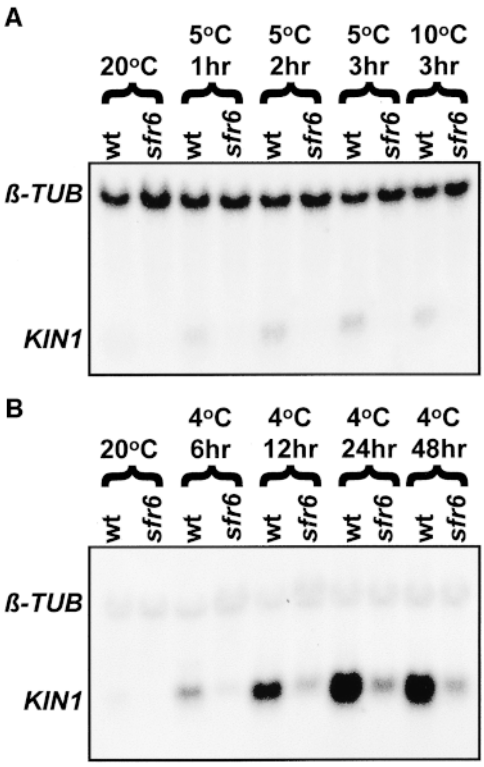

Figure 2. Time Course of Cold Induction of *KIN1* Gene Expression in Arabidopsis *sfr6* Mutants.

(A) Wild-type Col (wt) or *sfr6* plants were treated in water at 5, 10, or 20°C for the length of time indicated.

(B) Seedlings grown on Murashige and Skoog agar plates were placed in a growth chamber at 4°C for the length of time indicated. Plants were harvested immediately after removal of the plate from the chamber. Total RNA was extracted and size fractionated on an RNA gel. After blotting onto membrane, the membrane was hybridized simultaneously with labeled DNA probes for *KIN1* and β-tubulin. Hybridizing bands corresponding to both transcripts are tagged *KIN1* and *β-TUB*, respectively.

β-TUB, β-tubulin.

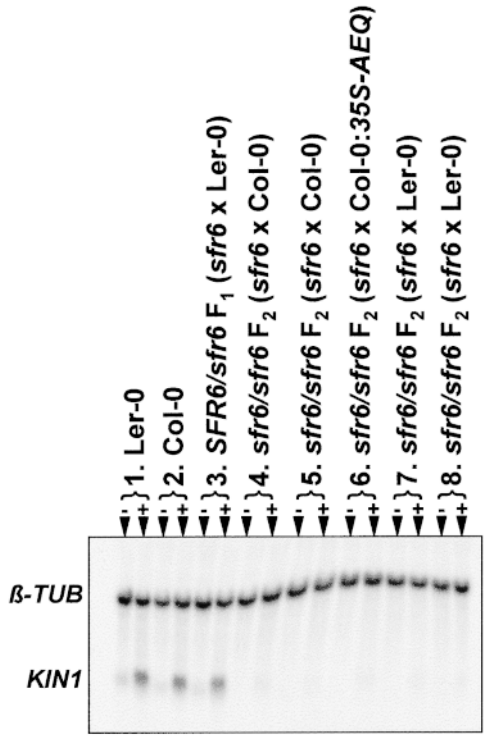

Figure 3. Linkage of the *COR* Gene Expression Deficiency Phenotype in Arabidopsis *sfr6* Mutants.

Each plant line was treated at either 20 or 5°C for 3 hr (labeled [−] and [+], respectively). After this time, plant tissue was harvested and total RNA extracted and size fractionated on an RNA gel. After blotting onto membrane, the membrane was hybridized simultaneously to labeled DNA probes for *KIN1* and β-tubulin (*β-TUB*). Hybridizing bands corresponding to both transcripts are tagged *KIN1* and *β-TUB*, respectively. Lane numbering indicates plant lines used, and they are as follows: lanes 1, wild-type L*er*-0; lanes 2, wild-type Col; lanes 3, F_1 *SFR6*/*sfr6* heterozygote line from a cross between *sfr6* and wild-type L*er*; lanes 4 and 5, two F_2 *sfr6*/*sfr6* homozygote lines from a cross between *sfr6* and wild-type Col; lanes 6, F_2 *sfr6*/*sfr6* homozygote line from a cross between *sfr6* and transgenic Col expressing the aequorin (*AEQ*) transgene; lanes 7 and 8, two F_2 *sfr6*/*sfr6* homozygote lines from a cross between *sfr6* and wild-type L*er.*

RT-PCR by using primers described in Methods (data not shown). Overall, we saw cosegregation of expression deficiency with freezing sensitivity in all of the 13 F_2 individuals that we tested, representing 26 nonrecombinant gametes. This indicated that the expression phenotype was tightly linked to *sfr6* (95% probability that separation was <11 centimorgans [cM]). These data strongly suggested that the expression-deficient phenotype was due to the *sfr6* mutation.

KIN1 Gene Expression in Response to Drought Stimuli

The *LTI78*, *KIN1*, and *COR15a* genes also are expressed in response to drought signals (Hajela et al., 1990; Kurkela and Franck, 1990; Mäntylä et al., 1995). The cold and drought signaling pathways leading to expression of these genes use many of the same components (Ishitani et al., 1997). Both involve transient increases in $[Ca^{2+}]_{cyt}$ (Knight et al., 1996, 1997; C. Podmore, H. Knight, and M.R. Knight, unpublished results) and can involve the phytohormone abscisic acid (ABA; Kurkela and Franck, 1990; Nordin et al., 1991; Baker et al., 1994; Ishitani et al., 1997), and both stresses activate the expression of CRT/DRE binding proteins (Liu et al., 1998). Therefore, we compared the effects of osmotic stress (by the addition of mannitol) and ABA (the levels of which are elevated during exposure to drought) (Lång et al., 1994) on *COR* gene expression in wild-type plants and the *sfr6* mutant. Expression of *KIN1*, shown by RNA gel blot analysis, was detectable in *sfr6* after a 3-hr treatment with 0.44 M mannitol (Figure 4, lane 4) or 100 μM ABA (lane 8); however, the extent of induction was much less than in the wild type (cf. lane 8 to lane 7 and lane 4 to lane 3). Similar results were obtained by quantitative RT-PCR by using the primers described in Methods, and expression patterns similar to those obtained with *KIN1* in response to mannitol and ABA (Figure 4) were obtained with *COR15a* and *LTI78* (data not shown).

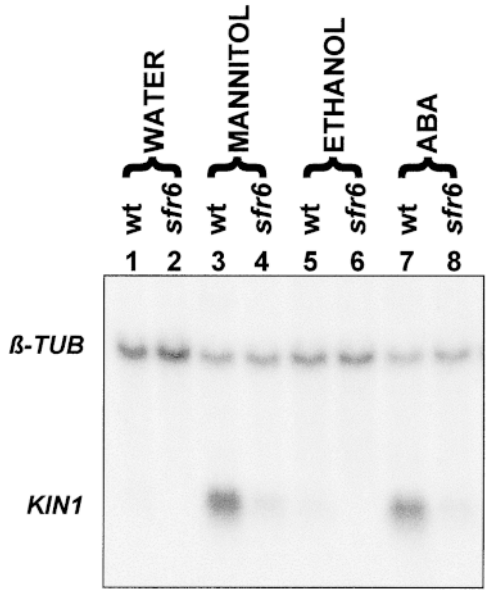

Figure 4. Osmotic Stress–Induced and ABA-Induced *KIN1* Expression in Arabidopsis *sfr6* Mutants.

Wild-type Col (wt; lanes 1, 3, 5, and 7) or *sfr6* (lanes 2, 4, 6, and 8) plants were treated with water (lanes 1 and 2), 0.44 M mannitol (lanes 3 and 4), 0.1% (v/v) ethanol (v/v control treatment for ABA; lanes 5 and 6), or 100 μM ABA (lanes 7 and 8). Treatments were for 3 hr. After this time, plant tissue was harvested, and total RNA was extracted and size fractionated on an RNA gel. After blotting onto a membrane, the membrane was hybridized simultaneously with labeled DNA probes for *KIN1* and β-tubulin (*β-TUB*). Hybridizing bands corresponding to both transcripts are tagged *KIN1* and *β-TUB*, respectively.

Measurement of Cold-Induced $[Ca^{2+}]_{cyt}$ Elevations in *sfr6*

Both cold- and drought-induced *COR* gene expression was deficient in *sfr6* (Figures 1 to 4). Both cold and drought signal transduction pathways involve a transient $[Ca^{2+}]_{cyt}$ elevation (Knight et al., 1996, 1997), which controls cold- and drought-induced gene expression, including *KIN1* and *LTI78* (Knight et al., 1996, 1997; Tähtiharju et al., 1997). Thus, it seemed possible that the deficiency of *sfr6* in gene expression was due to lack of a normal $[Ca^{2+}]_{cyt}$ elevation in response to cold or drought. Therefore, we measured cold-induced $[Ca^{2+}]_{cyt}$ elevations in *sfr6* and compared them with those in wild-type plants. We previously have used luminometry and luminescence imaging of Arabidopsis and tobacco plants expressing cytosolic aequorin to measure $[Ca^{2+}]_{cyt}$ transients in response to environmental stimuli (Knight and Knight, 1995; Campbell et al., 1996; Knight et al., 1996, 1997). To apply this technique to *sfr6* plants, we made use of a plant line in the Col background that was homozygous for *sfr6* and contained an aequorin transgene; the construction of this line was described above, and it had been confirmed as deficient in cold-induced *KIN1* expression (Figure 3, lanes 6).

Eight-day-old seedlings of this line were used to measure $[Ca^{2+}]_{cyt}$ responses to cold in an *sfr6* background. Both wild-type and *sfr6* plants expressing aequorin were cooled on a Peltier element, and cold-induced $[Ca^{2+}]_{cyt}$ elevations were compared using aequorin luminescence imaging (Campbell et al., 1996). A two-stage cooling protocol was performed, ramping from 20 to 10°C (Figure 5A), holding briefly at 10°C, and then ramping from 10 to 0°C (Figure 5B). In response to both periods of temperature reduction, an increase in luminescence from the wild type and mutant was observed immediately after the onset of cooling (Figure 5D). The responses of wild-type and *sfr6* plants in both tempera-

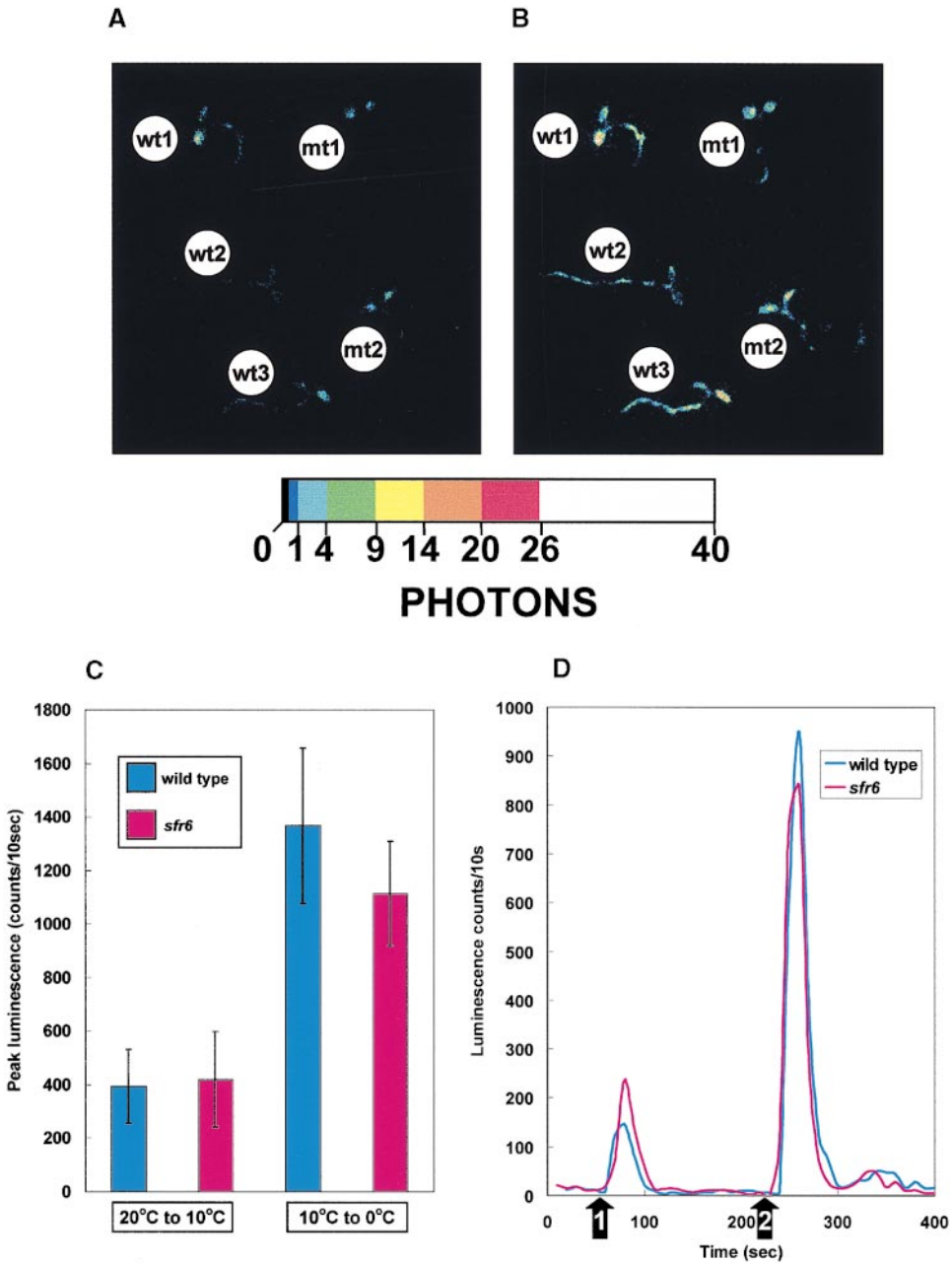

Figure 5. Cold-Induced $[Ca^{2+}]_{cyt}$ Response of Arabidopsis *sfr6* Mutants.

Wild-type Col and *sfr6* seedlings were cooled on a Peltier element from 20 to 10°C, followed by cooling from 10 to 0°C. Calcium-dependent luminescence from aequorin was imaged using a highly sensitive photon-counting camera.

(A) An integrated luminescence image taken during cooling from 20 to 10°C with three wild-type seedlings (labeled wt1 to wt3) and two *sfr6* seedlings (labeled mt1 and mt2). Luminescence was integrated for 60 sec and corresponds to 60 to 120 sec on the graph in **(D)**.

(B) An image similar to the one in **(A)** captured during cooling from 10 to 0°C, corresponding to 230 to 290 sec on the graph in **(D)**. Labeling in **(B)** is the same as in **(A)**. Luminescence images are shown in pseudocolor, with the color scale representing different numbers of photon counts (scale bar is shown below **[A]** and **[B]**).

(C) The average peak luminescence counts achieved per seedling during both cooling regimes. Error bars indicate standard error of the mean (n = 6 for the wild type; n = 5 for *sfr6*).

(D) Time course of cold-induced luminescence for a typical single wild-type and single *sfr6* seedlings during the two-stage cooling. Numbered arrows indicate the start of cooling from 20 to 10°C (1) and 10 to 0°C (2).

ture ranges did not differ significantly, either in magnitude of response (Figure 5C) or the relative sizes of the first and second peaks of Ca^{2+}-dependent luminescence (Figure 5D).

Cold-Induced Expression of *CBF1*, *CBF2*, and *CBF3*

The transcription factor CBF1 (also known as DREB1B) controls cold-induced gene expression of a number of *COR* genes, including *KIN1*, *COR15a*, and *LTI78*, by interaction with CRT/DRE motifs in the promoters of these genes (Jaglo-Ottosen et al., 1998). Two other CBF1-like proteins, CBF2 and CBF3 (also known as DREB1C and DREB1A), also bind the CRT/DRE motif, and the *CBF2* and *CBF3* genes are themselves expressed in response to cold (Gilmour et al., 1998). Because expression from CRT/DRE–containing promoters was deficient in the *sfr6* mutant, it seemed possible that the CBF1/2/3::CRT/DRE interaction was affected by the *sfr6* mutation. One way in which this might occur is if the expression of the *CBF1*, *CBF2*, and *CBF3* genes themselves was suppressed.

Therefore, we examined expression of the *CBF1*, *CBF2*, and *CBF3* transcripts (DREB1B, DREB1C, and DREB1A; Liu et al., 1998) by using RT-PCR. This method was preferable to RNA gel blot analysis for this particular assay, because it allowed us to distinguish between expression of the three *CBF* homologs with high sequence similarity. Expression of *CBF1* to *CBF3* was strongly induced after 3 hr at 5°C in both the wild type and the *sfr6* mutants and reached similar levels in both (Figure 6A). This result indicated that the signal transduction pathway leading to *CBF* transcription was unimpaired in the *sfr6* mutant, and that *sfr6* must affect either a component in the signaling pathway downstream of *CBF* transcription or a component in an independent pathway that is simultaneously required for *COR* gene expression.

Cold-Induced Expression of *AtP5CS1*

Two genes in Arabidopsis encode Δ^1-pyrroline-5-carboxylate synthetase (Strizhov et al., 1997), the key regulating enzyme in the pathway leading to proline production (Savouré et al., 1995), which is expressed in response to water stress and to low temperature (Savouré et al., 1997). One of these genes, *AtP5CS2* (also known as *P5CSB*), contains a CRT/DRE motif in its promoter, whereas the *AtP5CS1* (also known as *P5CSA*) gene lacks this element. As shown in Figure 6A, cold-induced expression of *CBF1*, *CBF2*, and *CBF3* was normal in *sfr6.* Because the promoters of all three of these genes lack CRT/DRE motifs (Gilmour et al., 1998), it became a possibility that the gene expression deficiency of *sfr6* was limited to genes containing CRT/DRE elements. To investigate this further, we assayed the cold inducibility of *AtP5CS1* in *sfr6* and wild-type plants. Expression was analyzed using RT-PCR (to distinguish between the closely related *AtP5CS1* and *AtP5CS2* genes). After 3 hr at 5°C, similar levels of *AtP5CS1* transcripts were observed in the wild-type and *sfr6* mutants (Figure 6B). These data supported the evidence from *CBF1*, *CBF2*, and *CBF3* expression studies that the failure to express the *COR* genes *LTI78*, *COR15a*, and *KIN1* is related to the presence of CRT/DRE elements in their promoters.

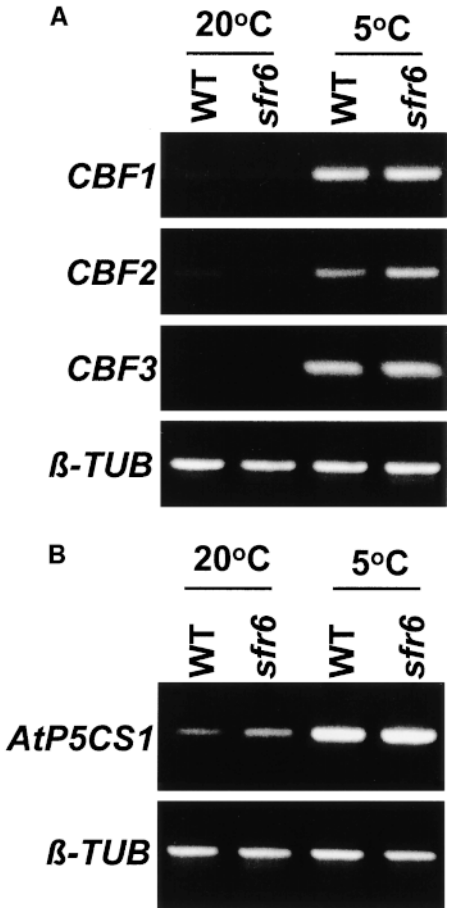

Figure 6. Cold-Induced Expression of *CBF1*, *CBF2*, *CBF3*, and *AtP5CS1* in Arabidopsis *sfr6* Mutants.

Wild-type Col (WT) or *sfr6* plants were treated at either 5 or 20°C for 3 hr, as indicated. After this time, plant tissue was harvested, total RNA was extracted, and cDNA was synthesized. This cDNA was used as template for RT-PCR by using gene-specific primers.

(A) Amplified DNA fragments from RT-PCR by using primers specific for *CBF1*, *CBF2*, *CBF3*, and β-tubulin (*β-TUB*).

(B) Amplified DNA fragments from RT-PCR by using primers specific for *AtP5CS1* and β-tubulin.

Amplified DNA fragments were visualized on standard ethidium bromide–stained agarose gels. DNA bands corresponding to transcripts are tagged *CBF1*, *CBF2*, *CBF3*, *β-TUB*, and *AtP5CS1*, respectively.

Map Location of *SFR6*

A cross between *sfr6* (Col) and wild type (L*er*-0) was described above. Eight F_2 plants from this cross were genotyped by freeze testing their progeny. DNA representative of each F_2 plant was isolated from additional pooled progeny and analyzed for molecular markers mapping at 22 points distributed throughout the genome. Such analysis indicated the presence of *SFR6* on chromosome IV, with linkage to marker nga8 at >99% probability. At the same time, three-point analysis eliminated all potential locations on other chromosomes. Thirty additional F_2 plant genotypes at *SFR6* were determined and analyzed for molecular markers in the region of nga8. Three-point analysis mapped *SFR6* between markers *GA1* (at 16.6 cM) and nga8 (at 24.2 cM; Figure 7). This region is well separated from all previously mapped cold-inducible genes (Thorlby et al., 1999), including *CBF1*, *CBF2*, and *CBF3.*

DISCUSSION

In recent years, our understanding of cold signal transduction in plants has improved. Numerous cold-induced genes have been identified (Cattivelli and Bartels, 1990; Hughes and Dunn, 1996; Thomashow, 1998), and a role is emerging for Ca^{2+} in early cold-signaling events controlling subsequent gene expression (Monroy and Dhindsa, 1995; Knight et al., 1996; Tähtiharju et al., 1997). Such information is of particular relevance to the phenomenon of cold acclimation—the process whereby plants gain freezing tolerance. Cold acclimation occurs when hardy plants, such as Arabidopsis, are exposed to low positive temperatures; during this process, many cold-induced genes are expressed (Hughes and Dunn, 1996; Thomashow, 1998). It appears likely that expression of these genes is necessary for freezing tolerance to increase; however, the functions of very few have been identified. Mutants deficient in cold acclimation provide a useful resource for investigation of the necessary components to complete this process (Warren et al., 1996).

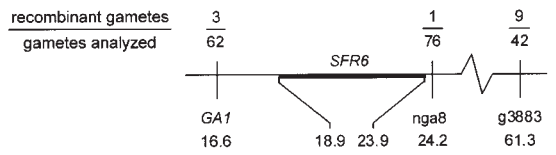

Figure 7. Mapping of *SFR6*.

The region containing *SFR6* at 95% probability is denoted by the thick bar. The positions of GA1, nga8, and g3883 are derived from the recombinant inbred map (August 1998 release) published by the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk). Numbers below the map indicate distances in centimorgans.

In this study, we investigated cold-induced gene expression in six *sfr* mutants that are impaired in cold acclimation–induced freezing tolerance (Warren et al., 1996). Our investigation revealed that the *sfr6* mutant line failed to express three different cold-inducible genes, namely, *LTI78*, *COR15a*, and *KIN1*, at normal wild-type levels (Figures 1 and 2), and genetic linkage of the expression phenotype to *sfr6* suggests that the expression deficiency is a consequence of the *sfr6* mutation (Figure 3). This provides a plausible explanation for the impairment of freezing tolerance in *sfr6* plants. It has been shown that expression of cold-inducible genes is sufficient to elevate levels of freezing tolerance in Arabidopsis (Jaglo-Ottosen et al., 1998; Liu et al., 1998); our result suggests that such expression is not only sufficient but necessary. It would be expected that the low levels of *COR* gene transcripts observed in response to cold treatment of *sfr6* would be reflected in reduced levels of COR polypeptides.

Previous work suggested that cold-induced polypeptide profiles were similar in both the wild-type and *sfr6* mutants (McKown et al., 1996). However, this study identified only three polypeptides whose abundance increased in response to cold acclimation and whose molecular masses were 8, 45, and 50 kD (McKown et al., 1996). None of these corresponds in size to the products of the *COR* genes whose expression we have measured. Therefore, we have no evidence showing that the impairment of *KIN1*, *COR15a*, and *LTI78* gene expression in *sfr6* mutants during cold acclimation leads to reduced accumulation of KIN1, COR15a, and LTI78 polypeptides. This is an important issue to be addressed in the future. The fact that the accumulation of three unidentified polypeptides during cold acclimation occurs unabated in *sfr6* mutants (McKown et al., 1996) means either that reduced *COR* expression in *sfr6* has a minimal long-term effect or that these three polypeptides are the products of genes that do not contain CRT/DRE motifs. We subsequently focused on *sfr6* for further study of the signal transduction pathways linking cold perception with gene expression in an attempt to identify which component was deficient in the mutant.

To ascertain whether the deficiency in the *sfr6* mutant was a failure to perceive specifically the cold stimulus, we tested the response of *sfr6* to other stimuli that elevate *COR* gene expression, namely, osmotic stress and the application of ABA. The *sfr6* mutant responded to these signals with only relatively weak expression of *KIN1* compared with the wild type, indicating that the drought induction of these genes also was impaired (Figure 4).

Cold-Induced $[Ca^{2+}]_{cyt}$ Elevation in *sfr6*

Low-temperature stimulation results in a rapid elevation of $[Ca^{2+}]_{cyt}$, due mostly to an influx of Ca^{2+} from extracellular sources (Knight et al., 1991, 1996). In alfalfa cell suspensions, this has been shown to be necessary for expression of acclimation-specific genes and for a gain in freezing tolerance to

occur (Monroy and Dhindsa, 1995). In Arabidopsis, this $[Ca^{2+}]_{cyt}$ elevation has been demonstrated to be necessary for full expression of *KIN1* (Knight et al., 1996; Tähtiharju et al., 1997). Therefore, it appeared possible that the lack of expression of a number of *COR* genes in the *sfr6* mutant could be due to a failure in the cold-induced $[Ca^{2+}]_{cyt}$ elevation. It has been speculated that the cold-induced $[Ca^{2+}]_{cyt}$ elevation constitutes the primary sensing mechanism for low temperature (Minorsky, 1989), and if this is so, a failure in this early event in *sfr6* could have explained the deficiency in expression of a number of different cold-inducible genes (Figure 1).

We previously have used transgenic Arabidopsis expressing the Ca^{2+}-dependent luminescent protein aequorin to measure cold-induced $[Ca^{2+}]_{cyt}$ elevations in whole intact plants (Knight et al., 1991, 1996; Campbell et al., 1996). Therefore, we made genetic crosses between aequorin-expressing plants and *sfr6* mutants to measure cold-induced $[Ca^{2+}]_{cyt}$ transients in aequorin-expressing *sfr6* progeny. When aequorin-expressing *sfr6* plants were compared with Col wild-type plants expressing aequorin, no difference in cold-induced $[Ca^{2+}]_{cyt}$ elevations was seen. Responses were measured both to a temperature drop from 20 to 10°C and from 10 to 0°C to ascertain whether the *sfr6* mutation affected sensitivity to temperatures in a particular range (Figure 5). The fact that normal cold-induced $[Ca^{2+}]_{cyt}$ elevations were seen indicated that there was no failure in cold sensing (Minorsky, 1989) but rather a failure in one of the downstream components between cold sensing and gene expression.

The expression of at least three different *COR* genes was reduced in the *sfr6* mutant, suggesting that the mutation was not simply a mutation in one of these genes. This conclusion was borne out by mapping of the *sfr6* mutation, which mapped remotely from any of the three genes tested (Figure 7). Our data suggest that the *sfr6* mutation therefore was most likely causing a failure in a component common to the cold-induced signal transduction pathways leading to expression of each of these genes.

Suppression of Cold-Induced Gene Expression in *sfr6* Mutants Is Dependent on the CRT/DRE

The CRT/DRE is common to the promoters of the *LTI78*, *COR15a*, and *KIN1* genes of Arabidopsis and controls their expression in response to low-temperature and dehydration stresses (Yamaguchi-Shinozaki and Shinozaki, 1994). The DRE (a 9-bp conserved sequence; TACCGACAT) and the CRT (TGGCCGAC) both contain a core sequence CCGAC, which has been designated as the low-temperature-responsive element (Jiang et al., 1996). During cold treatment, CBF binds to the CRT/DRE element of the promoter and induces *COR* gene expression (Stockinger et al., 1997). When *CBF1* was overexpressed in Arabidopsis under the control of a constitutive promoter, induction of *COR* genes, including *LTI78*, *COR15a*, and *KIN1*, occurred in the absence of cold stimulation, and plants were constitutively acclimated (Jaglo-Ottosen et al., 1998). The genes encoding all three CBF proteins (also known as DREB1 proteins) are themselves normally cold inducible, so we tested their expression in the *sfr6* mutant. Their cold inducibility was not impaired in *sfr6* (Figure 6A). This indicated that the failure in *sfr6* could lie between the transcription of the *CBF* genes and the expression of *COR* genes, such as *LTI78*, *COR15a*, and *KIN1*, and perhaps be related to a lack of functional CBF protein. This is not due to mutation in the *CBF1*, *CBF2*, and *CBF3* genes themselves because *sfr6* maps elsewhere (Figure 7). Alternatively, normal cold-induced *COR* gene expression may require flux through two separate pathways, one leading to the production of CBF protein and the other facilitating the activation of *COR* transcription by CBF.

The unimpaired expression of *CBF1*, *CBF2*, and *CBF3* in the *sfr6* mutant (Figure 6A) is significant not only in terms of pinpointing the possible lesion as a component acting after *CBF* gene transcription. We noted that unlike the other *COR* genes tested, the *CBF* genes are distinguishable for not containing a CRT/DRE element in their promoters. This observation provided support for the idea that the lesion in the *sfr6* mutant corresponds to a specific failure to transcribe CRT/DRE-controlled genes. We tested this hypothesis further by examining the cold-induced expression of *AtP5CS1*, a drought- and cold-inducible gene encoding the key enzyme in the biosynthetic pathway leading to proline production (Savouré et al., 1997). Unlike many other *COR* genes (but similar to *CBF1*, *CBF2*, and *CBF3*), the *AtP5CS1* gene does not contain a CRT/DRE element in its promoter. We found cold-induced expression of *AtP5CS1* to be normal in the *sfr6* mutant (Figure 6B), further strengthening the hypothesis that the *sfr6* mutation specifically affects the transcription of CRT/DRE–containing genes and does not affect the induction of cold-regulated genes that use different transcription factors. Formally, it is possible that the gene expression phenotype of *sfr6* is not due to altered transcription but to altered transcript processing or stability. However, a post-transcriptional effect would require differential handling of transcripts according to sequences not present in those transcripts (i.e., transcripts generated from promoters containing CRT/DRE elements would need to be controlled differently at the post-transcriptional level than other *COR* genes), which seems unlikely. It is also formally possible that the specificity of the *sfr6* effect could be due to promoter elements other than CRT/DRE that are present in the three CRT/DRE–containing promoters but that are absent in the four CRT/DRE–deficient promoters that we tested. Again, this seems improbable; thus, impairment of CRT/DRE transcriptional activation by CRT/DRE binding factors remains our preferred hypothesis to explain the *sfr6* phenotype.

Interestingly, osmotic stress– and ABA-induced expression of CRT/DRE–containing genes also was inhibited in the *sfr6* mutant. This suggests that the *sfr6* lesion affects pathways leading to the CRT/DRE box in promoters not only via

CBF1/2/3 but also via DREB2A and DREB2B, which are CRT/DRE binding factors postulated to be involved only in drought-induced expression of CRT/DRE–containing genes (Liu et al., 1998).

Role for *sfr6*

At which point within the pathway leading to cold-induced gene expression might SFR6 act? SFR6 clearly is not required for inducibility of *CBF1*, *CBF2*, and *CBF3* gene expression but is needed for the responsiveness of the targets of the CBF1 proteins. This implies that SFR6 potentiates the action of the CBF proteins. Such an interaction would be consistent with the conclusion of several recent studies—that stress responses result from a complex of intersecting control pathways (Ishitani et al., 1997; Liu et al., 1998). SFR6 does not appear to be required for recognition of the cold stimulus per se because induction of *COR* gene expression does occur in *sfr6* in response to cold, albeit at a very low level (Figure 2B). Indeed, it seems most likely that low-temperature perception occurs via cold-induced $[Ca^{2+}]_{cyt}$ elevation, which appears to be normal in *sfr6.* SFR6 is thus more likely to be involved in regulation of the magnitude of *COR* gene expression, and this may explain why unstimulated levels of expression are also low in the mutant. The *sfr6* mutation has pleiotropic effects on pigmentation (McKown et al., 1996) and fertility (G.J. Warren, unpublished data), and such effects are manifested in plants grown without exposure to low temperatures. The effects suggest that the role of *SFR6* is not limited to the transduction of the low-temperature signal.

In conclusion, the *sfr6* mutation inhibits expression of a number of *COR* genes in response to cold or drought signals, but its effect appears to be limited to those genes that have a CRT/DRE in their promoter. The expression of these genes is activated by the *cis*-acting factor CBF1 and related CRT/DRE binding proteins. These data would be consistent with the implication that the SFR6 product potentiates the action of these CRT/DRE binding proteins to allow cold- and drought-inducible gene expression.

METHODS

Plant Materials and Chemicals

All experiments were performed using seedlings (*Arabidopsis thaliana*, homozygous *sfr6*, and wild-type Columbia [Col]) grown on 0.8% (w/v) agar plates containing full-strength Murashige and Skoog nutrient medium (Murashige and Skoog, 1962), as previously described (Knight et al., 1997). Seedlings were 7 to 8 days old at the beginning of experiments.

Abscisic acid (ABA) was obtained from Sigma and was dissolved in ethanol at a concentration of 100 mM. Mannitol (AnalaR grade) was purchased from BDH (Poole, UK).

Measurement of Gene Expression by Using Reverse Transcription–Polymerase Chain Reaction and RNA Gel Blot Hybridization

Approximately 20 to 25 mg of 7-day-old wild-type or mutant Arabidopsis seedlings (grown as described above) was placed in 50-mL conical plastic tubes containing 25 mL of water at ambient temperature. The conical tubes then were placed in water baths of the appropriate temperature for 1, 2, or 3 hr. For the 48-hr time-course experiment, 7-day-old seedlings grown on Murashige and Skoog agar plates were placed in a growth chamber at 4°C under a light regime, as described above. Plates were removed from the growth chamber after 6, 12, 24, or 48 hr at 4°C or 48 hr at 20°C and harvested immediately. In other experiments, seedlings were removed from agar plates and floated on solutions of mannitol (0.44 M), ABA (100 μM), ethanol control for ABA treatment (0.1% v/v), or water for 3 hr. Total RNA was prepared from whole seedling tissue by using RNeasy plant RNA minipreps (Qiagen, Dorking, UK).

For RNA gel blot hybridizations, RNA samples (10 μg per lane) were electrophoresed through 1.0% agarose (Life Technologies, Paisley, UK) formaldehyde gels (Sambrook et al., 1989). RNA was transferred to nylon membranes (Boehringer Mannheim) by capillary action. Blots were prehybridized and hybridized in 50% formamide at 42°C. Blots were washed twice in each of the following successively: 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.1% SDS, followed by 1 × SSC and 0.1% SDS, and finally 0.1 × SSC and 0.1% SDS at 42°C. Probes for *KIN1* and low-temperature-induced *LTI78* were prepared from the products of polymerase chain reaction (PCR) by using specific primers, as described previously (Knight et al., 1996, 1997). Probes for cold-responsive *COR15a* and for β-tubulin were prepared in the same way, using primers with the following sequences (Genosys, Cambridge, UK): cor15a forward, 5′-CTCCTCCTTTCATTTCCAAA-3′; cor15a reverse, 5′-AGAATGTGACGGTGACTGTG-3′; tubulin forward, 5′-CCTGATAACTTCGTCTTTGG-3′; and tubulin reverse, 5′-GTGAACTCCATCTCGTCCAT-3′.

Probes were labeled using ^{32}P-CTP and DNA labeling beads (Pharmacia Biotech, St. Alban's, UK). ^{32}P-labeled blots were visualized either by autoradiography or by using a Bio-Rad PhosphorImager.

Reverse transcription–PCR (RT-PCR) of *AtP5CS1* and *CBF1* was performed using oligonucleotide primers designed to amplify part of the coding sequence of *AtP5CS1*, as given previously (Knight et al., 1997), tubulin (oligonucleotides as above), or *CBF1*, *CBF2*, and *CBF3* (sequence given below) to give a PCR product from a cDNA template that corresponded to a region of the gene spanning at least one intron (with the exception of *CBF1*, for which this was not possible), as described previously (Knight et al., 1997). cDNA for RT-PCR was synthesized from 1 μg of total RNA, which was combined with 1 μL of oligo(dT) (500 μg/mL) in a total volume of 12 μL. The mixture was heated to 70°C for 10 min and quick chilled on ice to denature the RNA. To this mixture was added 4 μL of 5 × first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$) and 2 μL of 0.1 M DTT, 1 μL 10 mM deoxynucleotide triphosphates, and 1 μL (200 units) of Superscript II (RNase H− reverse transcriptase; Life Technologies). cDNA synthesis was performed by incubating at 42°C for 50 min, and the enzyme was inactivated at 70°C for 15 min.

The cDNA was diluted to a concentration of 1:100 for use in the

PCR, and 10 μL of this diluted cDNA was used in a total reaction volume of 50 μL. The cDNA was amplified under the following conditions: 94°C for 5 min, 60°C for 5 min, and 72°C for 5 min, followed by 27, 31, or 33 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for tubulin, *CBF1*, and *AtP5CS1*, respectively. The number of cycles was optimized for each specific primer pair. Control PCR was performed using β-tubulin cDNA amplification under the same conditions. Ten microliters of the PCR products was loaded and electrophoresed on 1% agarose Tris-borate-EDTA gels and visualized by ethidium bromide fluorescence.

We used the following primers: cbf1/2/3 forward, 5′-CCTTATCCAGTTTCTTGAAACAGAG-3′; cbf1 reverse, 5′-CGAATATTAGTAACTCCAAAGCGAC-3′; cbf2 reverse, 5′-CACTCGTTTCTCAGTTTTACAAAC-3′; and cbf3 reverse, 5′-GACCATGAGCATCCGTCGTCATATGAC-3′.

$[Ca^{2+}]_{cyt}$ Measurements in *sfr6* and the Wild Type

Arabidopsis seedlings were grown on Murashige and Skoog agar plates, as described above. Reconstitution of aequorin was performed in vivo, essentially as described previously (Knight et al., 1991), by floating seedlings on water containing 2.5 μM coelenterazine in the dark overnight at 20°C.

Calcium-dependent aequorin luminescence was imaged using an intensified CCD (charge-coupled device) camera (Campbell et al., 1996) (model EDC-02), with a camera control unit (HRPCS-2) and image acquisition and processing software (IFS216), all from Photek (St. Leonards-on-Sea, UK). The equipment was cooled from an ambient temperature to 10°C and then to 0°C by using a water-cooled Peltier element incorporated into the assembly.

DNA Preparation

DNA was obtained by using the following protocol, which is a modified version of a published method (Edwards et al., 1991). Unopened buds at the tip(s) of one to 12 racemes were ground for 10 sec in a microcentrifuge tube. Four hundred microliters of 200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS, pH 7.5, was added, and the tube was vortexed briefly. After centrifugation for 60 sec, 300 μL of supernatant was transferred to a new tube, mixed with 300 μL of isopropanol, and left at room temperature for 120 sec. DNA was sedimented by centrifugation for 5 min, the supernatant was removed, and the pellet was allowed to dry. DNA was redissolved in 100 μL of 10 mM Tris-Cl and 1 mM EDTA, pH 8.0.

Mapping

The following markers were used in initial mapping of *sfr6*: nga63 (I:9), M235 (I:32), nga280 (1:81), nga111 (1:111), nga1145 (2:1), *PHYB* (2:36), nga168 (2:73), nga172 (3:7), nga162 (3:21), *GL1* (3:48), *BGL1* (3:75), nga6 (3:85), *GA1* (4:17), nga8 (4:24), G3883 (4:61), *PRHA* (4:76), nga1107 (4:102), nga225 (5:12), nga106 (5:33), nga139 (5:55), *DFR* (5:91), and *LFY3* (5:118). The markers prefixed "nga" are microsatellites and were analyzed according to Bell and Ecker (1994); the remainder were cleaved amplified polymorphic sequences that were analyzed according to Konieczny and Ausubel (1993). Numbers within parentheses refer to map positions in the coordinates of the August 1998 recombinant inbred map from the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk). They are given as chromosome number: position to the nearest centimorgan.

Arabidopsis sequences were found in the GenBank database by using the BLAST2 program (Altschul et al., 1997) at Stanford University (Stanford, CA; http://genome-www2.stanford.edu/cgi-bin/AtDB/nph-blast2atdb) and retrieved from GenBank through the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/).

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