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 ([Ca2+]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 ([Ca2+]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.

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 (Ler-0). Plants of the F₁ 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 Ler-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 F₂ plants from the cross, we identified individuals homozygous for sfr6 by freeze testing their progeny. Additional progeny of the identified sfr6/sfr6 F₂ individuals then were grown and tested for the cold inducibility of KIN1. The

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 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 Ler-0; lanes 2, wild-type Col; lanes 3, F₁ SFR6/sfr6 heterozygote line from a cross between sfr6 and wild-type Ler; lanes 4 and 5, two F₂ sfr6/sfr6 homozygote lines from a cross between sfr6 and wild-type Col; lanes 6, F₂ sfr6/sfr6 homozygote line from a cross between sfr6 and transgenic Col expressing the aequorin (AEQ) transgene; lanes 7 and 8, two F₂ sfr6/sfr6 homozygote lines from a cross between sfr6 and wild-type Ler.

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 Ler-0; lanes 2, wild-type Col; lanes 3, F₁ SFR6/sfr6 heterozygote line from a cross between sfr6 and wild-type Ler; lanes 4 and 5, two F₂ sfr6/sfr6 homozygote lines from a cross between sfr6 and wild-type Col; lanes 6, F₂ sfr6/sfr6 homozygote line from a cross between sfr6 and transgenic Col expressing the aequorin (AEQ) transgene; lanes 7 and 8, two F₂ sfr6/sfr6 homozygote lines from a cross between sfr6 and wild-type Ler.
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 F2 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\textsuperscript{2+}]\textsubscript{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).

### Measurement of Cold-Induced [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{cyt} elevation (Knight et al., 1996, 1997), which controls cold- and drought-induced gene expression, including KIN1 and LT778 (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\textsuperscript{2+}]\textsubscript{cyt} elevation in response to cold or drought. Therefore, we measured cold-induced [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{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 \([\text{Ca}^{2+}]_{\text{cyt}}\) 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\(^{\circ}\)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 \(\Delta^1\)-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\(^{\circ}\)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.
Map Location of SFR6

A cross between sfr6 (Col) and wild type (Ler-0) was described above. Eight F2 plants from this cross were genotyped by freeze testing their progeny. DNA representative of each F2 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 F2 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 (Thorby 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 Ca2+ 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).

In this study, we investigated cold-induced gene expression in six sfr6 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, LT178, 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 (I aglo-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 LT178 gene expression in sfr6 mutants during cold acclimation leads to reduced accumulation of KIN1, COR15a, and LT178 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 [Ca2+]cyt Elevation in sfr6

Low-temperature stimulation results in a rapid elevation of [Ca2+]cyt, due mostly to an influx of Ca2+ 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 \([\text{Ca}^{2+}]_{\text{c}}\) elevation has been demonstrated to be necessary for full expression of KIN1 (Knight et al., 1996; Tähtharju 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 \([\text{Ca}^{2+}]_{\text{c}}\) elevation. It has been speculated that the cold-induced \([\text{Ca}^{2+}]_{\text{c}}\) 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 \([\text{Ca}^{2+}]_{\text{c}}\)-dependent luminescent protein aequorin to measure cold-induced \([\text{Ca}^{2+}]_{\text{c}}\) 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 \([\text{Ca}^{2+}]_{\text{c}}\) 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 \([\text{Ca}^{2+}]_{\text{c}}\) 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 \([\text{Ca}^{2+}]_{\text{c}}\) 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 (TGCGCGAC) both contain a core sequence CGCGAC, 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 CBFI was overexpressed in Arabidopsis under the control of a constitutive promoter, induction of COR genes, including LT178, 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 LT178, 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\(^{2+}\)]\(_{\text{cyt}}\) 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 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 KN1 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 (Gnosys, Cambridge, UK): cor15a forward, 5′-CTCCCTCTTTTATTTCCAAA-3′; cor15a reverse, 5′-AGAAATGTGCAGAGTCTG-3′; tubulin forward, 5′-CTGTAT-AACCTGTCTTGGG-3′; and tubulin reverse, 5′-TGAAATCTC-ATCTGCTCAT-3′.

Probes were labeled using [\(^{32}P\)]CTP and DNA labeling beads (Pharmacia Biotech, St. Albans, 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
of supernatant was transferred to a new tube, mixed with 300 μL 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 50% ethanol was added, and the mixture was incubated at −20°C overnight. The precipitated DNA was then collected by centrifugation and air-dried. The DNA was resuspended in 10 μL of sterile water. DNA was quantified spectrophotometrically using a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA).

We used the following primers: cbf1/2/3 forward, 5'-CCTTATTCCAGTTTCTGAAACAGAC-3'; cbf2 reverse, 5'-CGAAATATTAGTAACTCCAAAGCGAC-3'; cbf2 reverse, 5'-CAGTGTTCACGATTACGACCATCACAC-3'; and cbf3 reverse, 5'-GACCATGAGCATCCGTCGTCA-3'.

[Ca2+]i 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 mCi/mL of 32P. 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 mCi/mL of 32P. We used the following primers: cbf1/2/3 forward, 5'-CCTTATTCCAGTTTCTGAAACAGAC-3'; cbf2 reverse, 5'-CGAAATATTAGTAACTCCAAAGCGAC-3'; cbf2 reverse, 5'-CAGTGTTCACGATTACGACCATCACAC-3'; and cbf3 reverse, 5'-GACCATGAGCATCCGTCGTCA-3'.

DNA Preparation

DNA was obtained using the following protocol, which is a modified version of a published method (Knight 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 resuspended 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 (I:81), nga111 (I: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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REFERENCES


Correction


An error was printed in the first full paragraph on page 884. The primer sequences for cbf2 and cbf3 are reversed. The sentence should read, “We used the following primers: cbf1/2/3 forward, 5’-CCTTATCCAGTTTCTTGAAACAGAG-3’; cbf1 reverse, 5’-CGAATATTGTAATCTCCAAAGCAGAC-3’; cbf2 reverse, 5’-GACCATGAGCAGCTCGTATGAC-3’; and cbf3 reverse, 5’-CAGTTTTACAAAC-3’.”
The sfr6 Mutation in Arabidopsis Suppresses Low-Temperature Induction of Genes Dependent on the CRT/DRE Sequence Motif
Heather Knight, Emma L. Veale, Gareth J. Warren and Marc R. Knight

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