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The *Arabidopsis* Mediator Complex Subunits MED16, MED14, and MED2 Regulate Mediator and RNA Polymerase II Recruitment to CBF-Responsive Cold-Regulated Genes[C][W][OPEN]

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The Mediator16 (MED16; formerly termed SENSITIVE TO FREEZING6 [SFR6]) subunit of the plant Mediator transcriptional coactivator complex regulates cold-responsive gene expression in *Arabidopsis thaliana*, acting downstream of the C-repeat binding factor (CBF) transcription factors to recruit the core Mediator complex to cold-regulated genes. Here, we use loss-of-function mutants to show that RNA polymerase II recruitment to CBF-responsive cold-regulated genes requires MED16, MED2, and MED14 subunits. Transcription of genes known to be regulated via CBFs binding to the C-repeat motif/drought-responsive element promoter motif requires all three Mediator subunits, as does cold acclimation–induced freezing tolerance. In addition, these three subunits are required for low temperature–induced expression of some other, but not all, cold-responsive genes, including genes that are not known targets of CBFs. Genes inducible by darkness also required MED16 but required a different combination of Mediator subunits for their expression than the genes induced by cold. Together, our data illustrate that plants control transcription of specific genes through the action of subsets of Mediator subunits; the specific combination defined by the nature of the stimulus but also by the identity of the gene induced.

INTRODUCTION

Cold acclimation is the process by which some temperate plant species increase their freezing tolerance in response to a prolonged period of low nonfreezing temperatures (Thomashow, 1999). During cold acclimation, extensive transcriptional and biochemical changes occur in plants, allowing them to withstand subsequent freezing conditions. The *sensitive-to-freezing6* (*sfr6-1*) mutant was first identified in a screen for *Arabidopsis thaliana* mutants that failed to cold acclimate to survive subsequent freezing temperatures (Warren et al., 1996). We have shown that *sfr6* mutants are unable to cold acclimate due to an inability to express *COLD ON-REGULATED* (*COR*) genes to sufficient levels in response to low, nonfreezing temperatures (Knight et al., 1999). In wild-type plants, expression of *COR* genes whose promoters contain the C-repeat motif (CRT; or drought-responsive element [DRE]) (Yamaguchi-Shinozaki and Shinozaki, 1994) is upregulated in response to cold by the action of the AP2 transcription factors C-repeat binding factor1 (CBF1), CBF2, and CBF3 (Stockinger et al., 1997; Gilmour et al., 2004), also known as DREB1B, DREB1C and DREB1A, respectively (Liu et al., 1998). The CBFs are essential regulators of freezing tolerance across a range of monocotyledonous as well as dicotyledonous species (Jaglo et al., 2001; Badawi et al., 2007; Pearce et al., 2013).

COR genes are also inducible by dehydration stress; in this case, CBF4 (Haake et al., 2002) and the DREB2 family of transcription factors activate expression via the same motif (Liu et al., 1998). Dehydration-induced *COR* gene expression is also defective in *sfr6* mutants (Knight et al., 1999; Boyce et al., 2003). We have shown previously that SFR6 acts downstream of CBF transcription factors to control expression of *COR* genes via the CRT motif (Boyce et al., 2003; Knight et al., 2009). For target genes to be successfully expressed, transcription factors must bind to promoters (Lee and Young, 2000) and may recruit chromatin remodeling complexes (Clapier and Cairns, 2009) and subsequently activate the transcription of coding regions by RNA polymerase II (Pol II). After our previous study, all of these remained as possible mechanisms that might be regulated by SFR6.

Recently, we cloned *SFR6* (Knight et al., 2009) and identified it as At4g04920, which encodes a protein identified as the MED16 subunit of the Mediator complex (Bäckström et al., 2007). Mediator is a eukaryotic transcriptional coactivator complex consisting of between 25 and 35 subunits (Björklund and Gustafsson, 2005). Mediator links transcriptional regulator binding at gene promoters with changes in activation of Pol II, thus effecting positive and negative control of transcription (Conaway and Conaway, 2011). Much of the work on Mediator to date has been performed in

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yeast (*Saccharomyces cerevisiae*), where Mediator has been shown to play a role in constitutive as well as inducible expression of most protein coding genes (Ansari et al., 2009). An interaction map based on yeast two-hybrid and coimmunoprecipitation experiments has been constructed for the protein subunits that make up yeast Mediator, indicating their positions relative to other subunits (Guglielmi et al., 2004). The positions of other subunits have been inferred by genetic studies. Yeast Mediator has been described as consisting of head, middle, and tail domains, with the head interacting with Pol II (Kang et al., 2001). The tail module is considered to be the site through which direct interaction between transcriptional regulators and Mediator occurs; however, many questions still remain as to how activator-specific responses are achieved.

Our current understanding of yeast and human Mediator is the result of almost 20 years of research; however, very low sequence similarity (Bourbon, 2008) delayed the bioinformatic identification of Mediator subunit homologs from plants (Kidd et al., 2011), an issue that was only resolved by the biochemical identification of the plant Mediator complex (Bäckström et al., 2007). The plant Mediator complex is estimated to be composed of in the region of 34 protein subunits (Mathur et al., 2011). Developmental aberrations have been reported previously for *Arabidopsis* mutants in a number of genes subsequently identified as encoding Mediator subunits. These include SETH10 (MED8), STRUWWELPETER (SWP; MED14), REF4-RELATED1 (RFR1; MED5a), REDUCED EPIDERMAL FLUORESCENCE4 (REF4; MED5b), and PHYTOCHROME FLOWERING TIME1 (PFT1; MED25) (Autran et al., 2002; Cerdán and Chory, 2003; Lalanne et al., 2004; Stout et al., 2008). More recently, MED25 has been shown to regulate jasmonic acid (JA)–responsive and abscisic acid–responsive signaling, affecting susceptibility to the necrotrophs *Alternaria brassicicola* and *Botrytis cinerea* (Kidd et al., 2009) and sensitivity to abscisic acid (Chen et al., 2012).

The identification of SFR6 as part of the Mediator complex offers an explanation for the wide variety of aberrations seen in *sfr6* mutants. Loss of SFR6 disrupts transcriptional outputs beyond low-temperature gene regulation, also affecting expression of flowering time pathway and circadian clock genes (Knight et al., 2008) and the expression of pathogen-associated genes activated by both salicylic acid and JA pathways (Wathugala et al., 2012; Zhang et al., 2012). In the case of low-temperature-regulated genes, the identity of the transcription factors that operate via SFR6 is known (Knight et al., 2009); therefore, in this study, we focused on the role of SFR6/MED16 in cold-responsive gene expression and sought to gain mechanistic information to explain how SFR6/MED16 regulates the activation of CBF-controlled transcription.

RESULTS

MED16 Acts Downstream of CBFs in *COR* Gene Activation but Is Not Required for CBF1 Recruitment or CBF-Mediated Chromatin Remodeling

We have shown that SFR6/MED16 is required for low temperature-inducible expression of *COR* genes in *Arabidopsis* and that a failure to express *COR* genes results in freezing sensitivity in *sfr6* mutants (Knight et al., 1999). *COR* gene expression is activated by the CBF family of transcription factors via the CRT promoter *cis*-element (Stockinger et al., 1997). The *CBF* genes are themselves inducible by low temperature, and CBF proteins are expressed to wild-type levels in cold-treated *sfr6* mutants, suggesting that failure to express their *COR* gene targets occurs downstream of CBFs in *sfr6* (Knight et al., 2009). Furthermore, while overexpression of CBFs in wild-type *Arabidopsis* leads to constitutive activation of *COR* genes and increased freezing tolerance in the absence of low-temperature treatment (Jaglo-Ottosen et al., 1998), it fails to do so in *sfr6* mutants (Knight et al., 2009). Together, these observations indicated to us that SFR6 may be required for either CBF recruitment to the CRT motif of *COR* gene promoters or to facilitate the action of CBFs after their recruitment.

To investigate the first possibility, we overexpressed epitope-tagged versions of CBF1 in *sfr6-1* and Columbia-0 (Col-0) backgrounds to be able to monitor the presence of CBF1 at *COR* gene promoters using chromatin immunoprecipitation (ChIP). CBF1-YFP (for yellow fluorescent protein) fusions were expressed via the cauliflower mosaic virus (CaMV) 35S promoter in both genetic backgrounds, and lines with equivalent levels of *CBF1-YFP* expression were chosen for further analysis (Supplemental Figure 1A; Col-0 lines 35 and 40; *sfr6-1* lines 12 and 20). Overexpression of CBF1-YFP in Col-0 resulted in constitutive expression of the known CBF targets *KIN2* and *GOLS3* (Knight et al., 1999; Fowler and Thomashow, 2002; Taji et al., 2002), consistent with previous reports (Jaglo-Ottosen et al., 1998) and indicating that the CBF1-YFP fusion is functional. As expected, overexpression of the construct failed to increase expression of *KIN2* and *GOLS3* in *sfr6-1* (Supplemental Figures 1B and 1C). Using polyGFP-Trap antibody beads to isolate chromatin-bound CBF1-YFP, we performed ChIP assays on the four CBF1-YFP–expressing transgenic lines. Our results demonstrate that CBF1 binds directly to the CRT-containing region of the promoters of *KIN2* and *GOLS3* in vivo and does so with equal efficiency in *sfr6-1* and the wild type (Figures 1A and 1B). No significant enrichment above background occurred at the promoter of the non-CRT-containing *PEX4* gene (Figure 1C) that is not regulated by low temperature (www.genevestigator.com). These data show that SFR6/MED16 is not required for CBF recruitment to *COR* gene promoters and indicate that SFR6/MED16 acts either downstream or independently of this event.

Mediator has been shown in other systems to play a part in the control of chromatin remodeling (Ding et al., 2008; Malik and Roeder, 2010), and yeast strains in which Sin4 (MED16) was deleted were originally described as having similar phenotypes to histone mutants, causing some workers to suggest that in yeast MED16 was involved in remodeling (Jiang et al., 1995). Therefore, while CBF binds normally to CRT-containing cold gene promoters in *sfr6* mutants (Figures 1A and 1B), it seemed possible that its failure to activate *COR* gene expression could be due to a requirement for SFR6/MED16 in the action of histone remodeling complexes or in their recruitment by CBFs or other transcriptional regulators. Covalent modification of histones by acetylation, methylation, and phosphorylation affects their affinity for DNA, resulting in altered nucleosome occupancy and chromatin remodeling. Transcriptional activators recruit histone remodeling complexes to promoters where their effects on nucleosome

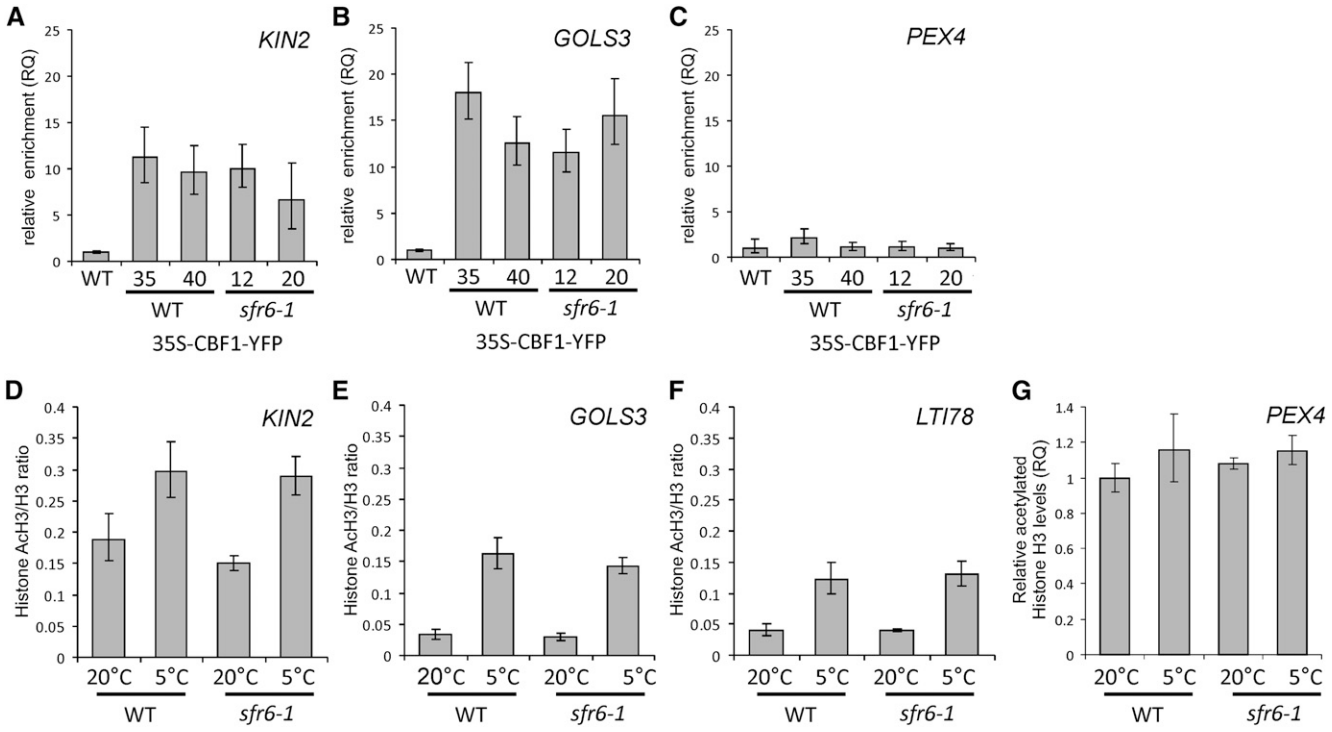

Figure 1. CBF1 Recruitment and Cold-Induced Changes in AcH3/H3 Ratio at *COR* Gene Promoters Are Unimpaired in the *sfr6-1* Mutant.

(A) to **(C)** ChIP qRT-PCR analysis of CBF1-YFP binding to the promoters of *KIN2* **(A)**, *GOLS3* **(B)**, and *PEX4* **(C)** in 14-d-old wild-type (lines 35 and 40) and *sfr6-1* (lines 12 and 20) seedlings expressing 35S-CBF1-YFP. Nontransgenic wild-type plants serve as a negative control, and values represent nonspecific binding. Data represent relative enrichment compared with input and show fold enrichment change relative to the wild type.

(D) to **(F)** ChIP qRT-PCR analysis of relative levels of histone H3 acetylation at promoters of *KIN2* **(D)**, *GOLS3* **(E)**, and *LTI78* **(F)** in 3-week-old wild-type and *sfr6-1* seedlings subjected to 5°C for 4 h or maintained at 20°C.

(G) Relative *PEX4* histone H3 acetylation was referenced against input and shows fold enrichment change compared with the wild type at 20°C.

Data for *KIN2*, *GOLS3*, and *LTI78* represent relative histone H3 acetylation at a given promoter standardized against *PEX4* histone H3 acetylation from the same ChIP sample. Data for histone acetylation are representative of two biological replicates. Data from the second biological replicate experiment are shown in Supplemental Data Set 4. Values were calculated using the $\Delta\Delta C_T$ method, and the error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Error bars indicate the level of variation between technical replicates within one biological replicate experiment.

occupancy result in changes in transcriptional activation (Lee and Young, 2000).

Histone acetylation and nucleosomal depletion at promoters have been shown in many systems to be synonymous with transcriptional activation (Struhl, 1998), and in *Arabidopsis* responding to low temperature an increase in histone H3 acetylation and a reduction in nucleosome occupancy precede *COR* gene transcription (Pavangadkar et al., 2010). CBFs and other transcriptional activators have been proposed to recruit histone acetyl transferases to cold-responsive gene promoters to achieve this. Such changes can be mimicked by CBF overexpression in the absence of cold, suggesting that CBFs facilitate this response (Pavangadkar et al., 2010). Therefore, we tested whether the defect in *COR* gene activation in *sfr6* results from a failure to acetylate histone H3 at gene promoters leading to a consequent lack of change in nucleosome occupancy. We performed ChIP using antibodies specific to nonacetylated and acetylated Histone H3 (AcH3) to compare AcH3/H3 ratios in Col-0 and *sfr6-1* plants before and after 4 h of cold treatment at 5°C. Col-0 and *sfr6-1* plants showed identical increases in AcH3/H3 ratios at the promoters of the *COR* genes *KIN2*, *GOLS3*, and *LTI78* in response to cold treatment (Figures 1D to 1F). No remodeling was observed at the promoter of *PEX4* in response to cold (Figure 1G).

As expected given these results, nucleosome occupancy at *COR* gene promoters decreased relative to occupancy at *PEX4* in response to low temperature to a similar degree in wild-type and *sfr6-1* plants (Supplemental Figures 1D to 1F). This also means that other forms of histone modification leading to changes in nucleosome occupancy can be ruled out as targets of SFR6/MED16 action. These data indicate that the defect in CBF-mediated *COR* gene expression observed in *sfr6* plants occurs downstream, or independently, of CBF recruitment and nucleosome remodeling.

RNA Pol II Recruitment to Cold-Inducible CBF-Controlled Genes Increases under Low Temperature and Requires MED16

Having established that SFR6/MED16 influences cold-regulated gene expression via a mechanism other than CBF recruitment and chromatin remodeling, we tested whether it is required for

the recruitment of RNA Pol II and its progression along two typical CBF-regulated *COR* genes: *KIN2* and *GOLS3* (Jaglo-Ottosen et al., 1998; Fowler and Thomashow, 2002). We performed ChIP with an antibody to Pol II C-terminal domain repeats to monitor Pol II occupancy using primers designed to amplify genomic DNA at specific sites along the genes. The regions chosen were at ~500 bp upstream (referred to hereafter as −500 bp) of the transcriptional start site (TSS), at the TSS itself (including the TATA box if present), at ~50 bp after the start codon (the site of Pol II stalling, if it occurs), and then at ~500-bp intervals thereafter. This resulted in the use of two regions in *GOLS3* (termed "Mid" and "End") but only one in *KIN2*, which has a shorter open reading frame (Figure 2A).

There was a large increase in Pol II recruitment to both genes in response to cold (4 h at 5°C), but this increase was markedly reduced in *sfr6-1* mutants when compared with wild-type controls (Figures 2B and 2C). This correlated with the reduced *COR* gene transcript levels that are observed in response to low temperature in *sfr6* mutants (Knight et al., 1999, 2009) and indicated that a failure to recruit Pol II is the reason for this defect in gene expression. As expected, relatively little signal was detected in either the wild type or *sfr6-1* at −500 bp for either of the genes tested, corresponding to little or no Pol II occupancy. In wild-type plants, Pol II occupancy was higher at the TSS of *KIN2* than at −500 bp and increased further at the two downstream sites assessed, even under ambient conditions (Figure 2B). This suggests that *KIN2* is transcribed at a low but detectable level even under ambient conditions and that cold-inducible increases in transcription do not rely entirely upon de novo recruitment of Pol II in response to low temperature. Recruitment of Pol II to *KIN2* was impaired in *sfr6-1* even under ambient conditions (Figure 2B), offering an explanation for the reduced levels of *KIN2* transcripts we observed previously at ambient temperatures in *sfr6* mutants when compared with the wild type (Knight et al., 2009).

In contrast with *KIN2*, Pol II occupancy at the TSS of *GOLS3* was similar to that observed at −500 bp under ambient conditions, but levels increased significantly after cold treatment. This suggests that, unlike *KIN2*, little or no *GOLS3* transcription occurs under ambient conditions and Pol II is recruited de novo to *GOLS3* in response to low temperature. Similar to *KIN2*, Pol II recruitment to the *GOLS3* TSS was impaired in *sfr6-1* (Figure 2C). These results are consistent with the possibility that *GOLS3* expression requires de novo formation of transcriptional preinitiation complexes in response to low temperature, while genes such as *KIN2* are transcribed, to a greater or lesser extent, under all conditions from a preexisting preinitiation complex.

While Pol II recruitment to the TSS of *GOLS3* and *KIN2* is clearly lower in *sfr6-1* than in the wild type, Pol II occupancy increases downstream of the TSS are proportionally equal in *sfr6-1* and the wild type, indicating the same degree of Pol II activation following recruitment. Pol II occupancy was ~2 times higher at the *GOLS3* stall site than at the TSS in both the wild type and *sfr6-1*. At *KIN2*, the relative increases in occupancy between the TSS and stall site were ~3-fold for both the wild

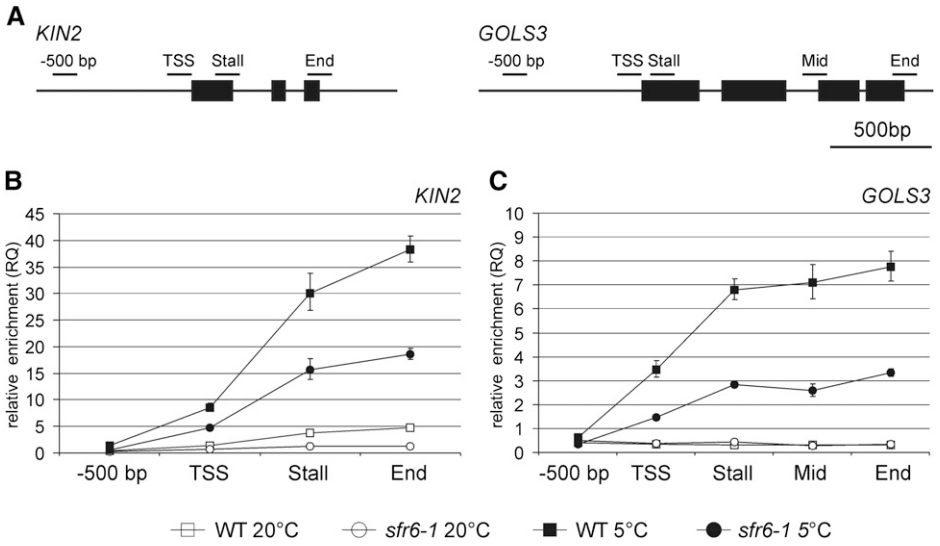

Figure 2. RNA Pol II Recruitment and Progression along CBF-Controlled Genes Is Impaired in *sfr6-1* Mutants.

Pol II abundance at specific locations along cold-responsive genes was assayed by ChIP.

(A) Map of primer pair locations along two cold-responsive genes. Thick black bars indicate the exons of the gene, and thin black lines above show the regions amplified by the primers in each ChIP reaction.

(B) and **(C)** ChIP qRT-PCR analysis of relative levels Pol II abundance along *KIN2* **(B)** and *GOLS3* **(C)** in 14-d-old seedlings of Col-0 (WT) and *sfr6-1* subjected to 5°C for 4 h or maintained at 20°C. ChIP enrichment at each location is referenced to input and enrichment at each location was standardized to Pol II abundance at *PEX4* in the same ChIP sample. Values were calculated using the $\Delta\Delta C_T$ method, and the error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Error bars indicate the level of variation between technical replicates within one biological replicate experiment. Data are representative of three biological replicate experiments. Data from the second biological replicate experiment are shown in Supplemental Data Set 4.

type and *sfr6-1* (Figures 2B and 2C; ratios are shown in Supplemental Figure 2). This indicates that while initial recruitment is reduced in *sfr6-1*, lack of SFR6/MED16 does not impair Pol II progression beyond the TSS and the reduced occupancy seen at the transcribed regions in *sfr6-1* is consistent with the failure in initial recruitment. This is comparable to reports in yeast that while the Sin4 complex (comprising MED16 and other tail subunits) was required for the stable association of Pol II with Mediator and their efficient recruitment into preinitiation complexes, the complexes that were formed did not require further involvement of the Sin4 complex to initiate transcription normally (Reeves and Hahn, 2003).

Transcriptomic Analysis of the Influence of SFR6/MED16 on Cold-Inducible Gene Expression

Having shown that MED16 is needed for recruitment of Pol II to the well-studied CBF-controlled *COR* genes *KIN2* and *GOLS3* and for their expression, we asked whether SFR6/MED16 plays a role in Pol II recruitment to, and expression of, other cold-regulated genes and whether it requires the cooperation of other Mediator subunits. We began by assessing the extent and diversity of cold-inducible gene expression that is controlled by SFR6/MED16, interrogating microarray data to identify for further study cold upregulated genes that are misregulated in *sfr6* mutants. First we used our microarray data to generate a list of genes upregulated by a 3-h 5°C cold treatment in wild-type Col-0 *Arabidopsis* (Supplemental Data Set 1). Consistent with many previous studies, this list of 1177 genes contained a number of genes well known to be upregulated in *Arabidopsis* responding to low temperature, such as *DREB1A*, *GOLS3*, *KIN1*, *COR15a*, *COR15b*, *LTI78*, and *LTI30* (Fowler and Thomashow, 2002; Vogel et al., 2005). (The well studied *COR* gene *KIN2* is not represented on this particular Affymetrix array.)

Using this list, we interrogated data from three separate microarray experiments in which *sfr6-1*, *sfr6-2*, or *sfr6-3* were each compared with a Col-0 wild-type sample under cold conditions. We identified 81 cold-upregulated genes that showed reduced expression in all three *sfr6* mutant alleles in the cold (Supplemental Data Set 2). This list included genes we have shown previously to be misregulated in *sfr6-1* mutants: *KIN1*, *COR15a*, *LTI78*, and *COR15b* (Knight et al., 1999, 2009). For comparison, we generated a second list of 81 cold-upregulated genes, the expression of which was close to equivalent in *sfr6* and the wild type for all three *sfr6* mutant alleles (Supplemental Data Set 3).

We identified heptamer nucleotide sequences that were significantly enriched in the promoters of either list of 81 genes described above relative to the whole genome (Supplemental Tables 1 and 2). For the genes misregulated in *sfr6* mutants, the consensus sequences of the most significant assembly showed similarity to the CRT motif (Sakuma et al., 2002) (Figure 3A); the second most significant assembly showed similarity to the evening element (EE) motif (Harmer et al., 2000) (Figure 3B). For the CRT-like motif this assembly was created from nine different elements (superscript "a" in Supplemental Table 1), representing 24 different promoter sites. For the EE-like motif, this assembly was created from only one element (superscript "b" in Supplemental Table 1), representing 60 different promoter sites. None of the 10 heptamer motifs annotated with a superscript "a" or "b" in Supplemental Table 1 was significantly enriched in the list of 81 genes not misregulated in *sfr6* (Supplemental Data Set 3). This suggested that both the CRT and EE are specifically overrepresented in the promoters of cold upregulated genes misregulated in *sfr6* mutants. This finding was independently corroborated by identifying known transcription factor binding sites using the Athena program (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl): Both the CRT and EE were identified specifically as enriched in the genes misregulated in *sfr6*, whereas they were not enriched in the nonmisregulated gene list (Supplemental Figure 3). Indeed, for nonmisregulated genes, Athena did not identify any known transcription factor binding site enrichment.

We therefore focused our further studies of gene expression and Pol II recruitment in Mediator subunit mutants upon genes containing CRT or EE motifs in the 500 bp upstream of their TSS. To select genes containing the CRT, we used the core consensus "RCCGAC" that had been defined in previous reports (Sakuma et al., 2002) and was the consensus obtained from our own analysis of 81 genes misregulated in *sfr6* (Figure 3A). In total, 48 promoters out of 81 contained this CRT motif (70 copies in total), representing 59% of promoters, compared with 12% in the total genome, thus, an almost 5-fold enrichment. To select genes with the EE, we used the consensus "AAAATATCT" established in previous reports (Harmer et al., 2000), which was also the most prevalent consensus from our analysis of 81 genes misregulated in *sfr6* (Figure 3B). In total, 16 promoters out of 81 contained this EE motif (18 copies in total), representing 19% of promoters, compared with 3% in the total genome, thus, a >6-fold enrichment.

MED16 Shares the Control of *COR* Gene Expression with Other Subunits of the Mediator Complex

A number of other plant Mediator subunits have emerged as key controllers in development (Kidd et al., 2011; Xu and Li, 2011, 2012) and in the response to abiotic (Elfving et al., 2011) and biotic stresses (Kidd et al., 2009; Wathugala et al., 2012; Zhang

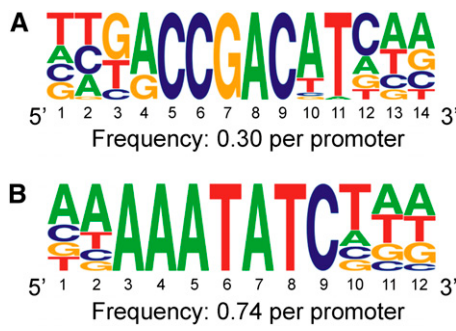

Figure 3. The CRT/DRE and EE Motifs Are the Two Most Significantly Enriched Sequences within Promoters of Cold-Upregulated Genes Showing Reduced Expression in *sfr6* Mutants.

Consensus sequences for the two most significantly enriched sequences within promoters of the 81 cold-upregulated genes showing reduced expression in *sfr6* mutants (Supplemental Data Set 2).
(A) The most significant consensus resembles CRT/DRE.
(B) The second most significant consensus resembles EE.
[See online article for color version of this figure.]

et al., 2012). To date, however, only SFR6/MED16 has been implicated in the transcriptional response to cold; therefore, we investigated whether other Mediator subunits might share this role. Mediator structure is well conserved across eukaryotes and is predicted to be as similar in plants as in those organisms previously studied (Bourbon, 2008). Based on physical models of Mediator from yeast (Guglielmi et al., 2004), we chose the MED2, MED5, and MED14 subunits for our study as they are predicted to be physically close to SFR6/MED16 in the Mediator complex and, therefore, likely candidates for cooperating with SFR6/MED16 in the control of gene expression. In yeast Mediator, each of these subunits, including MED16, occupy positions within or adjacent to the tail submodule (Guglielmi et al., 2004). The tail has been described in yeast as particularly important in regulating environmental responses (Ansari et al., 2012) and is considered to be the main site of transcriptional activator and repressor binding.

We identified homozygous insertion lines for MED2 (MED32), MED14 (SWP), and both MED5 (MED5a/MED33a/RFR1 and MED5b/MED33b/REF4) subunits showing levels of full-length transcript so low that they are likely to be close to null (Supplemental Figure 4). Under all conditions investigated, MED5a and MED5b were functionally redundant; we therefore created a double mutant (referred to as *med5 dbl*) that was used in all the experiments presented. We also included in our study the MED8 and MED25/PFT1 subunits, which have in common with SFR6/MED16 a role in the transcription of biotic stress–related genes (Kidd et al., 2009; Wathugala et al., 2012).

Seedlings of wild-type and Mediator subunit mutant plants were subjected to 5°C treatment for 6 h and *COR* gene expression measured in response to cold using quantitative real-time RT-PCR (qRT-PCR). As expected, *sfr6* mutants showed reduced transcript levels of the CBF-controlled *COR* genes *KIN2* and *GOLS3*, the promoters of which contain the CRT motif (Figures 4A and 4B). Reduced levels of *KIN2* transcript were also observed in *med2* and *med14* mutants, while *med5 dbl*, *med8*, and *med25*/*pft1* mutants were unaffected (Figure 4A; data for additional mutant alleles are shown in Supplemental Figures 5A and 5E). *GOLS3* was expressed to lower than wild-type levels in all of the mutants tested but was most severely affected in *sfr6*, *med2* and *med14* mutants (Figure 4B; Supplemental Figures 5B and 5F). A possible reason for the sensitivity of *GOLS3* expression to a wider range of Mediator mutations may be that an imperfect Mediator complex is more detrimental in situations where rapid assembly of transcriptional complexes is required. In the case of *KIN2*, where Pol II is always present, transcription is perhaps more resistant to minor conformational changes caused by loss of MED25, MED8, or MED5 within Mediator. Alternatively, *GOLS3* transcription may require Mediator to interact with a wider range of transcriptional and remodeling components than are required for the expression of *KIN2*, rendering expression of *GOLS3* more sensitive to any change in Mediator structure.

As our microarray analysis indicated that *sfr6*-misregulated cold genes were enriched for genes containing the EE as well as the CRT, we measured the expression of a gene, At1g20030 (Supplemental Data Set 2), the promoter of which contained the EE but not the CRT. We confirmed that as At1g20030 expression in the cold was reduced in *sfr6* mutants and like the two CRT-containing genes, its expression was also reduced in *med2* and *med14* mutants although as seen for *GOLS3*, some reduction in expression was observed for all of the mutants (Figure 4C; Supplemental Figures 5C and 5G).

Not All Cold-Regulated Expression Uses MED2, MED14, and MED16

Our microarray experiment indicated that some genes are cold-inducible but do not require SFR6/MED16 for their expression (Supplemental Data Set 3). We examined the expression in the Mediator subunit mutants of one of these, At3g52740, the promoter of which contains neither EE nor CRT motifs. As indicated by the microarray data, levels of expression of this gene in *sfr6-1* under cold conditions were very similar to those of wild-type plants. Furthermore, expression reached wild-type levels in all of the other mutants tested, indicating that neither SFR6/MED16 nor the other Mediator subunits in this study play a role in the expression of this gene (Figure 4D; Supplemental Figures 5D and 5H).

These data show that expression of cold-inducible genes requires specific Mediator subunits in addition to SFR6/MED16 but that not all Mediator subunits are required in all cases. In addition, some genes are cold-upregulated independently of either SFR6/MED16 or of several other Mediator subunits. As our results suggested that MED2 and MED14 in particular are required for expression of the same cold-upregulated genes that use SFR6/MED16, we tested the expression of further genes selected from our microarray in *med2* and *med14* mutants to strengthen this conclusion. Our data confirmed that *LTI78*, *COR15a* (both containing the CRT), *ABF1* (At1g49720), At5g46710 (both containing the EE), At1g68500, and At5g62360 (containing both motifs) were misregulated in *sfr6* and demonstrated they were expressed to reduced levels in *med2* and *med14* mutants (Figures 5A to 5F). The microarray data indicated that *SZF2* (At2g40140; a gene containing neither EE nor CRT motifs), like At3g52740, was expressed normally in *sfr6*; we confirmed its expression in *sfr6* was normal and showed this to be the case also in *med2* and *med14* (Figure 5G).

Effect of Loss of MED2 or MED14 on RNA Pol II Recruitment

Having shown that expression of CRT-containing CBF-controlled genes was reduced in *med2* and *med14* mutants as well as in *sfr6*, we measured Pol II occupancy at the CBF-controlled genes *KIN2* and *GOLS3* in *med2* and *med14* mutants (Figure 4). The data shown in Figures 6B and 6C are from the same experiment as shown in Figure 2, allowing direct comparisons between the two mutants and *sfr6-1*. As was the case for *sfr6* mutants, the reduced levels of *COR* gene transcripts seen in *med2* and *med14* mutants responding to cold was mirrored by poor recruitment of Pol II to these genes, offering an explanation for the expression defect seen in *med2* and *med14* mutants. Recruitment of Pol II to the TSS in *KIN2* was impaired in both mutants under cold conditions, and occupancy was reduced at both downstream loci (Figure 6B). Interestingly, as seen for *sfr6-1*, recruitment was noticeably lower in the mutants compared with the wild type even at ambient temperature, matching the reduced levels of *KIN2* transcripts observed in all three mutants under these conditions (Figure 4A). Similarly, Pol II recruitment to *GOLS3* in response to cold was

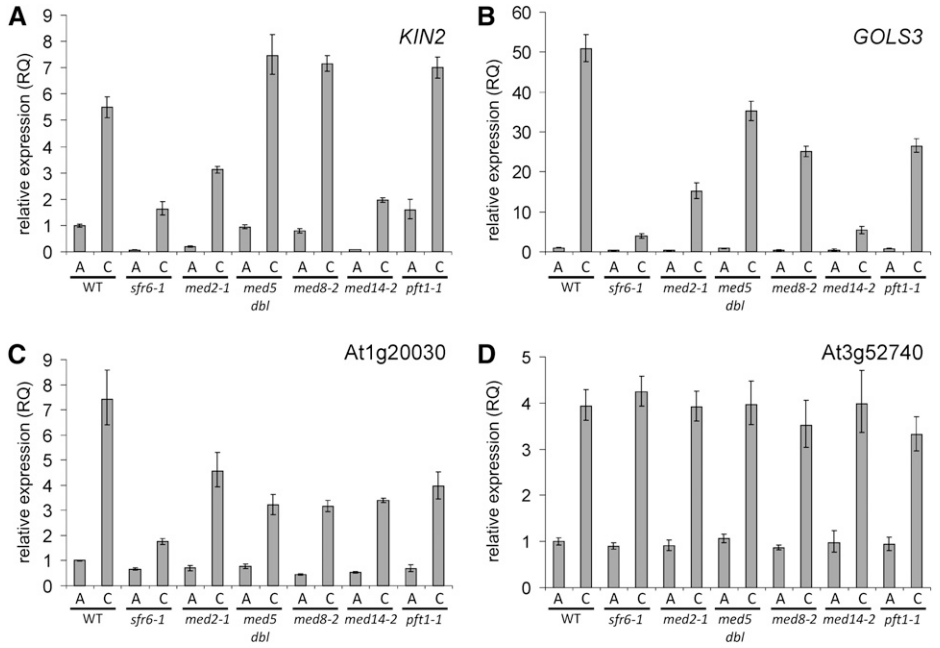

Figure 4. Cold-Responsive Gene Expression Is Altered in Specific Mediator Subunit Mutants.

qRT-PCR analysis of relative expression levels of *KIN2* **(A)**, *GOLS3* **(B)**, At1g20030 **(C)**, and At3g52740 **(D)** in 8-d-old seedlings of Col-0 (WT) and Mediator subunit mutants exposed to cold (C) temperatures (5°C) for 6 h or maintained at ambient (A) temperature (20°C). Expression is shown after normalization to *PEX4*. Values were calculated using the $\Delta\Delta C_T$ method, and the error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Error bars indicate the level of variation between technical replicates within one biological replicate experiment. Data are representative of three biological replicate experiments. Data from the second biological replicate experiment are shown in Supplemental Data Set 4.

impaired in both mutants (Figure 6C). For both genes, loss of MED2 or MED14, like loss of SFR6/MED16, appears to affect initial recruitment but have no additional consequences on the progression of Pol II. Pol II occupancy ratios between the stall site and TSS of *GOLS3* and *KIN2* after cold treatment were similar in the two mutants and in Col-0 (Supplemental Figure 2).

In contrast with the above, little change in Pol II occupancy occurred at At1g20030 in response to cold: Occupancy along the gene remained at a low level relative to the −500-bp position and was similar in the wild type and mutants (Figure 6D). At1g20030 is not a CBF-responsive gene and its promoter contains the EE but not the CRT. Two other cold-inducible genes, *ABF1* and At5g46710, that were misregulated in the three mutants (Figure 5) also contained the EE but not CRT in their promoters and showed no discernible changes in recruitment in response to cold (Supplemental Figures 6A and 6B). Interestingly, At3g52740, a gene that was not misregulated in the mutants tested, did show modest increases in recruitment levels in response to cold and these increases were virtually identical in the wild type and the three mutants (Figure 6E), consistent with its expression levels (Figure 4D). This result serves as an important control, illustrating that SFR6/MED16 is not required ubiquitously for the recruitment of Pol II to transcribed genes but that its role is specific to a subset of genes under its control. As we observed wild-type levels of Pol II recruitment to this gene in the cold, and transcript levels were unaffected by loss of any of the subunits we studied, we elected not to investigate the control of At3g52740 further.

As we had observed cold-responsive Pol II recruitment that was reduced by Mediator subunit mutation only for CBF-controlled genes, we tested whether the effects we observed with *KIN2* and *GOLS3* were likely to be widespread and possibly common to all CBF-controlled genes. Pol II occupancy was measured at the transcribed region of two additional CBF-controlled CRT-containing genes (Figure 7A). As for *GOLS3* and *KIN2*, Pol II occupancy at *LTI78* and *COR15a* increased in the cold but was reduced in all three mutants (Figures 7B and 7C).

Mediator Tail Subunits MED16, MED2, and MED14 Are Necessary for Cold Acclimation

We have shown previously that in *sfr6* mutants, reduced expression of the cold-inducible CBF-controlled regulon results in a failure to cold acclimate to freezing temperatures. We investigated whether loss of MED2 or MED14 caused similar effects. Loss of either subunit resulted in a reduction in the proportion of plants that survived freezing after cold acclimation; *med2* mutants were the least severely affected, with 40% survival compared with 100% of wild-type plants surviving. *med14* mutant plants showed lower survival rates although not so low as *sfr6* (Figures 8A and 8B). Consistent with the appearance and survival of whole plants after freezing, analysis of electrolyte leakage indicated that leaves of *med2-1*, *med14-2*, and *sfr6-1* mutants were more sensitive to electrolyte loss than wild-type plants subjected to sub-zero temperatures. At the least severe freezing temperatures, *med2-1*

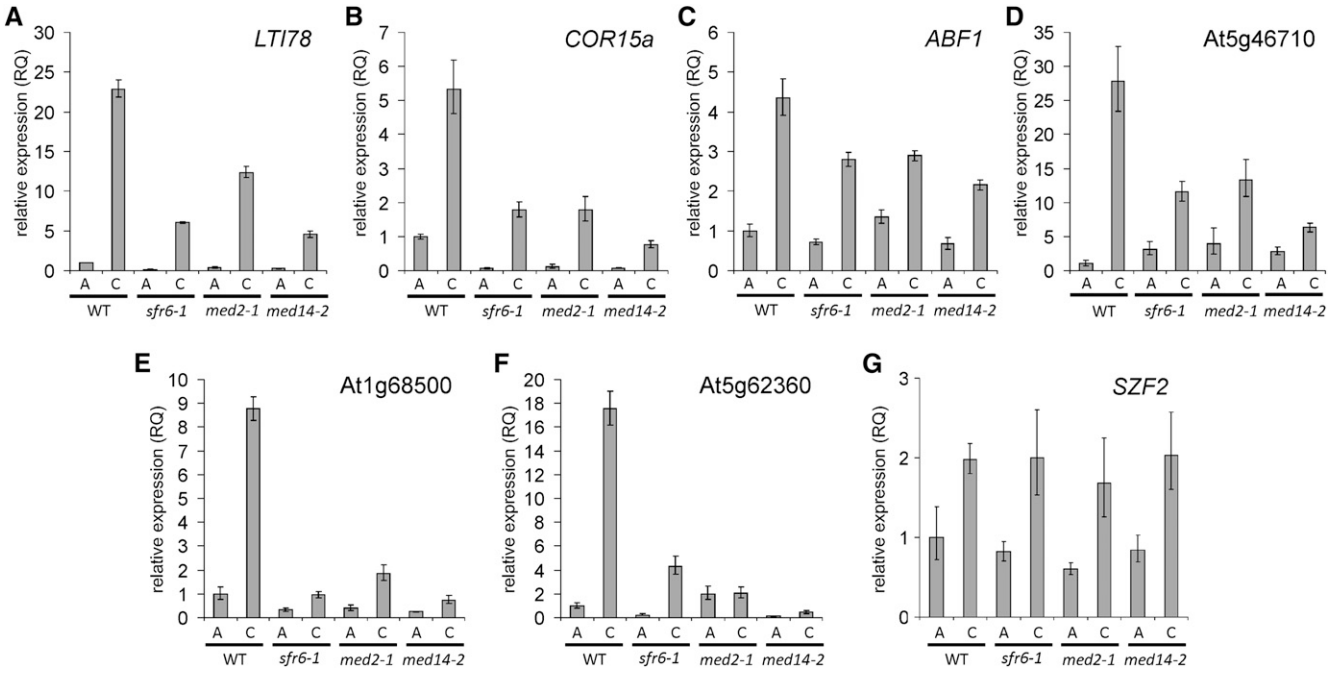

Figure 5. Additional Cold-Responsive Genes Selected from Our Microarray Experiment Show Similar Patterns of Expression in *sfr6-1*, *med2-1*, and *med14-2* Mutants.

qRT-PCR analysis of relative expression levels of *LTI78* **(A)**, *COR15a* **(B)**, *ABF1* **(C)**, At5g46710 **(D)**, At1g68500 **(E)**, At5g62360 **(F)**, and *SZF2* **(G)** in 8-d-old seedlings of Col-0 (WT), *sfr6-1*, *med2-1*, and *med14-2* mutants exposed to cold (C) temperatures (5°C) for 6 h or maintained at ambient (A) temperature (20°C). Expression is shown after normalization to *PEX4*. Values were calculated using the $\Delta\Delta C_T$ method, and the error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Error bars indicate the level of variation between technical replicates within one biological replicate experiment. Data are representative of two biological replicate experiments. Data from the second biological replicate experiment are shown in Supplemental Data Set 4.

was relatively unaffected compared with the wild type, but at lower temperatures, both *med2-1* and *med14-2* showed levels of sensitivity comparable to *sfr6-1* (Figure 8C). All three mutants were significantly more sensitive to low freezing temperatures than the wild type ($P < 0.001$) and differences between mutants reflected the differences in survival rate and gene expression observed. These data demonstrate that like SFR6/MED16, MED2 and MED14 are required for cold acclimation to freezing tolerance.

Mediator Recruitment to CRT-Containing Cold-Regulated Genes

To discover whether failure to recruit Pol II to cold-inducible genes in Mediator mutants was likely to be a consequence of reduced Mediator recruitment, we performed ChIP using an antibody to MED6, a Mediator subunit from the head submodule that has been used before for the isolation of plant Mediator (Bäckström et al., 2007). While Pol II has been reported to be associated only with active loci, Mediator has been reported to be constitutively associated with intergenic regions, coding sequences, and promoters of both transcriptionally active and transcriptionally inactive DNA (Andrau et al., 2006). We therefore measured relative Mediator occupancy after 4 h at 5°C or ambient treatment at the −500-bp position, TSS, middle, and, for the longer open reading frames, the end of the transcribed region and 3′ intergenic region (3IGR; Figure 9A).

Mediator recruitment to all positions on *KIN2* increased dramatically in wild-type plants after exposure to low temperatures and was highest at the end of the transcribed region. Recruitment was severely reduced in *sfr6-1*, *med2-1*, and *med14-2* mutants (Figure 9B). While changes in recruitment to *GOLS3* in response to cold were more modest, the pattern observed was comparable to that of *KIN2* (Figure 9C). In preliminary experiments, very similar observations were made at the transcribed region of two other CRT-controlled genes (*LTI78* and *COR15a*; Supplemental Figures 7B and 7C). Our results suggest that recruitment of Mediator to these CBF-controlled genes requires either intact Mediator or specific functions that are associated with the tail subunits. While some studies in yeast have shown that Mediator is recruited to upstream activator sequences (Kuras et al., 2003; Fan et al., 2006) with occupancy levels much higher at promoters than in open reading frames (Kim and Gross, 2013), other studies, like ours, have revealed significant levels of Mediator occupancy at the coding regions of genes (Andrau et al., 2006; Zhu et al., 2006). Interestingly, small increases in Mediator occupancy in response to cold were seen at the middle of the transcribed region of At1g20030 (Figure 9D), a gene at which little change in Pol II occupancy had been observed in response to low temperature; in a preliminary experiment, a similar observation was made with another of the EE-containing genes, *ABF1* (Supplemental Figure 7D). However, recruitment was not impaired in any of the mutants. This suggests that recruitment of

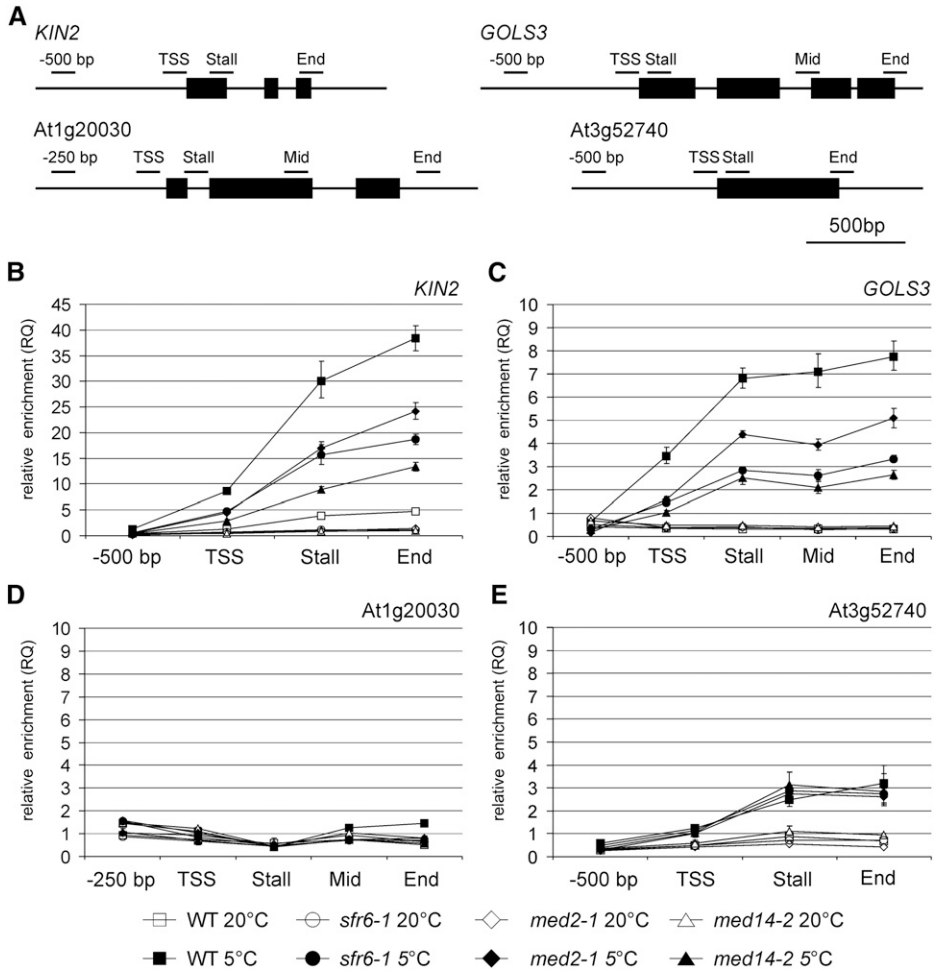

Figure 6. RNA Pol II Recruitment and Progression along Cold-Responsive Genes in *med2-1*, *med14-2*, and *sfr6-1* Mutants Varies between Genes Containing or Lacking the CRT Motif.

ChIP qRT-PCR analysis of relative abundance of Pol II along cold-responsive genes in 14-d-old seedlings subjected to 5°C for 4 h or maintained at 20°C.
(A) Map of primer pair locations along four cold-responsive genes. Thick black bars indicate the exons of the gene and thin black lines above show the regions amplified by the primers in each ChIP reaction.
(B) and **(C)** Pol II abundance along two CRT-containing genes: *KIN2* **(B)** and *GOLS3* **(C)** in Col-0 (WT), *sfr6-1*, *med2-1*, and *med14-2*. The data shown are from the same experiment shown in Figure 2.
(D) and **(E)** Pol II abundance along two genes that do not contain the CRT: At1g20030 **(D)** and At3g52740 **(E)** in Col-0 (WT), *sfr6-1*, *med2-1*, and *med14-2.*
ChIP enrichment at each location is referenced to input and enrichment at each location was standardized to Pol II abundance at *PEX4* in the same ChIP sample. Values were calculated using the $\Delta\Delta C_T$ method, and the error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Error bars indicate the level of variation between technical replicates within one biological replicate experiment. Data are representative of two biological replicate experiments. Data from the second biological replicate experiment are shown in Supplemental Data Set 4.

Mediator to such genes is not dependent upon activators that bind any of these three tail subunits.

Dark-Induced Gene Expression Requires MED16, MED14, and MED5

Having discovered that MED2 and MED14 act in conjunction with SFR6/MED16 to control expression of some, but not all cold-responsive genes, we sought to discover whether these three same subunits always share the same functions. For this purpose, we chose to study a MED16-dependent abiotic stress response unrelated to cold. In a previous study, we revealed that a number of genes were differentially expressed in *sfr6-1* but not known to be regulated by cold (Boyce et al., 2003) and we showed using a further microarray experiment that many of these *sfr6*-misregulated genes were upregulated by darkness (http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=194). A number of these genes have been reported previously to be upregulated by darkness or starvation conditions (Fujiki et al., 2001; Kim and von Arnim, 2006).

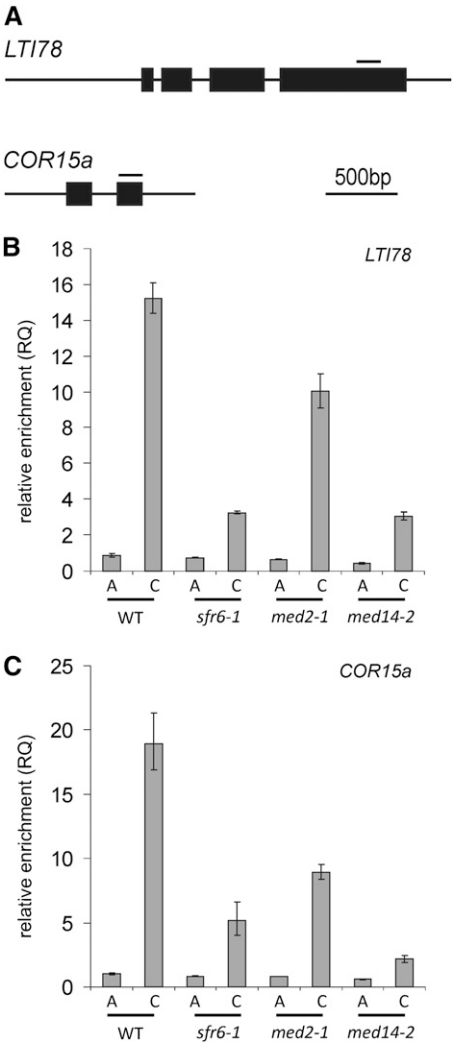

Figure 7. Pol II Recruitment to *LTI78* and *COR15a* Increases in the Cold and Is Impaired in *sfr6-1*, *med2-1*, and *med14-2* Mutants, Similarly to Other CBF-Controlled CRT-Containing Genes.

ChIP qRT-PCR analysis of relative abundance of Pol II along two additional cold-responsive genes in 14-d-old seedlings subjected to cold (C) temperatures (5°C) for 4 h or maintained at ambient (A) temperature (20°C) monitored at the position shown in **(A)**. Thick black bars indicate the exons of the gene and thin black lines above show the regions amplified by the primers in each ChIP reaction. Pol II abundance at the end of the transcribed region of *LTI78* **(B)** and *COR15a* **(C)** in Col-0 (WT), *sfr6-1*, *med2-1*, and *med14-2*. ChIP enrichment is referenced to input and standardized to Pol II abundance at *PEX4* in the same ChIP sample. Values were calculated using the $\Delta\Delta C_T$ method, and the error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Error bars indicate the level of variation between technical replicates within one biological replicate experiment. Data are representative of two biological replicate experiments. Data from the second biological replicate experiment are shown in Supplemental Data Set 4.

We focused on the expression of *DIN6* (At3g47340) and *BCAT2* (At1g10070), two genes that encode enzymes involved in amino acid catabolism (Fujiki et al., 2001; Diebold et al., 2002). We first confirmed that reduced expression is linked to the *SFR6*/*MED16* locus by measuring dark-inducible expression in three *sfr6* mutant alleles. *DIN6* and *BCAT2* expression increased in response to 6 h of darkness and was significantly reduced in *sfr6-1*, *sfr6-2*, and *sfr6-3* (Figures 10A and 10B). We then measured dark-induced expression in Mediator subunit mutants and found that like *sfr6* mutants, *med5 dbl* and *med14* mutants showed reduced expression of *DIN6* and *BCAT2* but relatively little reduction in expression was seen in mutants in MED2, MED8, or MED25 (Figures 11A and 11B; Supplemental Figures 8A and 8B). *DIN6* expression was unimpaired in dark-treated *med5a* and *med5b* single mutants, indicating that MED5a and MED5b act redundantly to control dark-inducible expression (Supplemental Figure 9). Our results indicate that while MED14 and SFR6/MED16 are required for both cold-regulated and dark-regulated transcription, there are other Mediator subunits required specifically for the response to one or other of these stresses but not for both.

DISCUSSION

MED16 Is Required for Cold-Inducible *COR* Gene Expression in *Arabidopsis* but Is Not Necessary for CBF Recruitment or Histone Remodeling

We have shown previously that *sfr6* mutants lack the ability to respond to cold with an increase in CBF-mediated *COR* gene expression (Knight et al., 1999, 2009); our current study demonstrates a mechanism for this.

After we cloned *SFR6*, we found that it encodes the MED16 subunit of the transcriptional coactivator complex Mediator (Bäckström et al., 2007). It has been well documented that transcriptional activators recruit Mediator and are not recruited by Mediator (Kuras et al., 2003). However, yeast *med16*Δ deletion mutants show reduced Hsf1 transcription factor occupancy at target promoters in response to heat shock, suggesting Mediator tail subunits enhance either binding or retention of activated Hsf1 to promoters (Kim and Gross, 2013). The presence of Mediator at a gene can also affect the occupancy of transcriptional activators at promoters by causing their displacement or degradation (Chi et al., 2001). However, loss of SFR6/MED16 did not lead to depletion of CBF1 at target gene promoters. Our data show that CBF1 binds to the promoters of the *COR* genes *KIN2* and *GOLS3* in vivo (Figure 1), providing direct in planta confirmation of previous hypotheses proposed through overexpression and yeast one-hybrid studies (Stockinger et al., 1997; Liu et al., 1998; Gilmour et al., 2004; Vogel et al., 2005).

Mediator can recruit chromatin remodeling complexes (Ding et al., 2008) and some Mediator subunits have intrinsic histone remodeling activity (Lorch et al., 2000), but there is uncertainty as to whether intact Mediator is a prerequisite for all remodeling. Mediator is necessary for remodeling at specific gene promoters (Sharma et al., 2003; Lemieux and Gaudreau, 2004), but other studies have shown remodeling occurs independently of Mediator and prior to recruitment of the transcriptional machinery (He et al.,

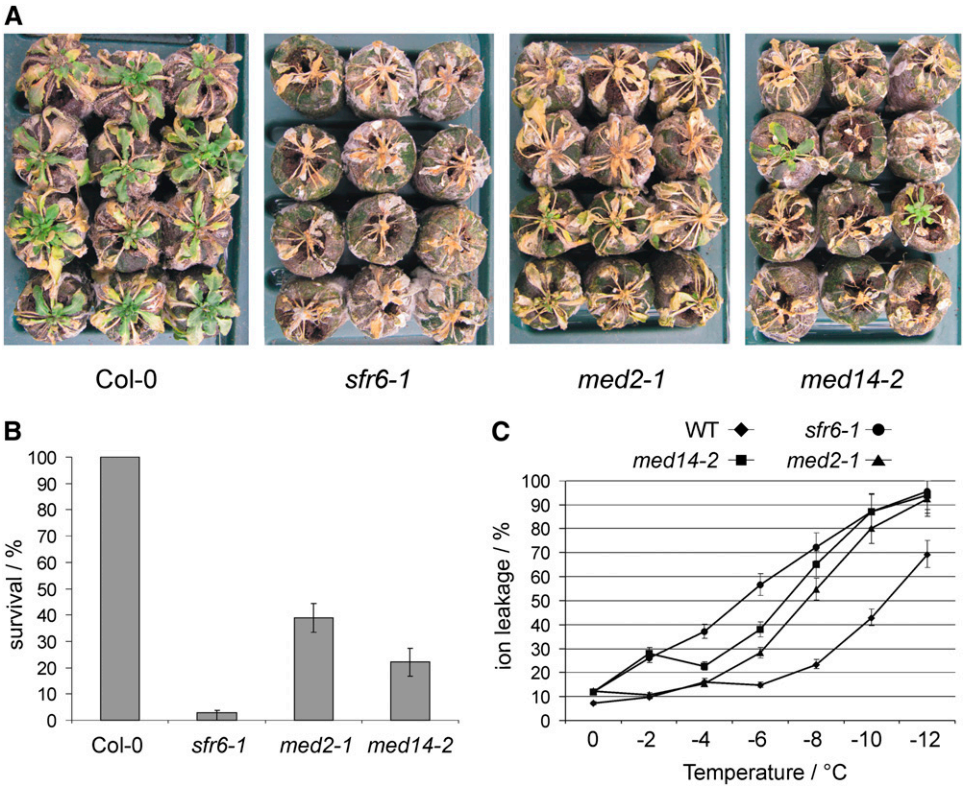

Figure 8. *med2-1* and *med14-2* Mutants, Like *sfr6* Mutants, Exhibit Reduced Freezing Tolerance.

Freezing tolerance of in Col-0 (WT), *sfr6-1*, *med2-1* and *med14-2* plants.
(A) and **(B)** Five-week-old plants grown under short days (12:12 LD cycle) were cold acclimated for 2 weeks at 5°C before subjecting to freezing temperatures (−7.5°C) for 24 h. Plants were returned to ambient conditions for 1 week and then photographed **(A)** and scored for survival based on whether regrowth had occurred **(B)**. Three biological replicate experiments were performed; the photograph is of one representative biological replicate experiment, and the histogram represents the average of three percentage survival rates from three separate biological replicate experiments with 12 plants per experiment. Error bars shown are SE calculated from arcsine transformed values as appropriate for proportional data.
(C) Electrolyte leakage in Col-0 (WT), *sfr6-1*, *med2-1*, and *med14-2* plants grown for 32 d before cold acclimating at 5°C for 2 weeks. Values represent the percentage loss of electrolytes after leaves were subjected to temperatures of between 0 and −12°C. Each data point represents the average of three separate biological replicate experiments; each experiment used three leaves of each genotype per replicate tube, with three tubes per genotype/temperature in each experiment. Natural logarithm transformed percentage of leakage data were analyzed using a one-way ANOVA at each temperature point and error bars represent 1 SE. Data points with nonoverlapping error bars are significantly different ($P < 0.001$).

2008). We showed that cold-induced alterations in histone occupancy and acetylation can occur at cold-inducible genes in the absence of SFR6/MED16, making it unlikely that this Mediator subunit plays any role in chromatin remodeling at these genes (Figure 1).

Control of CBF-Responsive Cold Gene Expression and Cold Acclimation Is Shared between SFR6/MED16 and Other Mediator Subunits

Our transcriptomic analysis showed *sfr6* mutants were deficient in the expression of a large number of cold-inducible genes, many of which are known to be inducible by the CBF transcriptional activators (Fowler and Thomashow, 2002). However, SFR6/MED16 did not exert sole control over these genes; loss of either MED2 or MED14 reduced the expression of genes that required MED16. Clearly, other Mediator subunits that we have not tested may also play a role in the expression of this regulon. Not all of the Mediator mutants we tested showed altered expression of CBF-responsive genes, indicating that individual subunits of the plant Mediator complex have specific roles and that a complete, intact Mediator complex is not required for Mediator to fulfill all of its roles.

CBF-controlled *COR* gene expression is linked to the ability to cold acclimate and overexpression of the CBF regulon results in constitutive freezing tolerance (Jaglo-Ottosen et al., 1998; Fowler and Thomashow, 2002). We showed that MED2 and MED14, as well as SFR6/MED16, were necessary for cold acclimation (Figure 8). Cold acclimation requires the coordinate upregulation of a whole suite of genes and is unlikely to be achieved to any significant level by upregulation of any of these individually (Thomashow, 2001). The failure of *med2* and *med14* mutants to cold acclimate therefore suggests that the expression defects we reported in these mutants are likely to be representative of much more

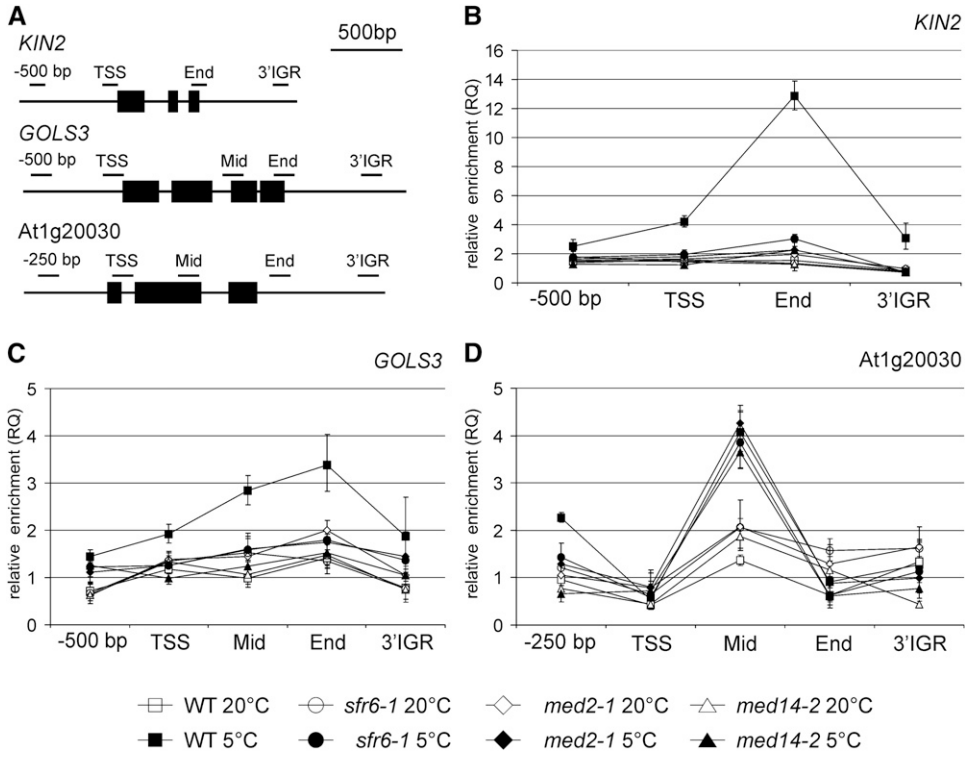

Figure 9. Mediator Recruitment to CBF-Controlled CRT-Containing Genes Increases in the Cold and Is Impaired in *sfr6-1*, *med2-1*, and *med14-2* Mutants.

ChIP qRT-PCR analysis of relative abundance of MED6 (a core Mediator subunit) along cold-responsive genes in 14-d-old seedlings subjected to 5°C for 4 h or maintained at 20°C.

(A) Map of primer pair locations along three cold-responsive genes. Thick black bars indicate the exons of the gene, and thin black lines above show the regions amplified by the primers in each ChIP reaction.

(B) to **(D)** Mediator recruitment along the CBF-responsive CRT-containing genes *KIN2* **(B)** and *GOLS3* **(C)** and in the non-CRT, EE-containing gene At1g20030 **(D)** in Col-0 (WT), *sfr6-1*, *med2-1*, and *med14-2*. ChIP enrichment is referenced to input and standardized to MED6 abundance at *PEX4* in the same ChIP sample. Values were calculated using the $\Delta\Delta C_T$ method, and the error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Error bars indicate the level of variation between technical replicates within one biological replicate experiment. Data are representative of two biological replicate experiments. Data from the second biological replicate experiment are shown in Supplemental Data Set 4.

widespread failure in cold-inducible gene expression, possibly as widespread as those we have shown by microarray analysis for *sfr6* mutants. Our data demonstrate that the Mediator tail submodule is an essential requirement for cold acclimation in *Arabidopsis*.

SFR6/MED16, MED2, and MED14 Are Required for Mediator Recruitment and Pol II Recruitment to CBF-Responsive Genes

We demonstrated that low temperature increased recruitment of both Pol II and Mediator to CBF-responsive CRT-containing genes and that both were dependent upon MED2, MED14, and MED16. This indicates that efficient recruitment of Pol II to CBF-responsive genes requires successful Mediator recruitment. Similarly, deletion of either the MED16 or MED15 tail subunit in yeast reduces Mediator recruitment and Pol II occupancy (Kim and Gross, 2013). Our data suggest that Mediator recruitment to CBF-responsive genes may depend upon activators that bind specifically to MED16, MED2, or MED14; the CBF transcription factors would be obvious candidates for this role.

Control of Cold-Inducible Genes That Do Not Contain a CRT

Our transcriptomic analysis confirmed our earlier findings (Boyce et al., 2003) that *sfr6* mutants show impaired expression of CRT-containing genes (targets of the CBFs) as well as other cold-inducible genes that do not contain CRT motifs. These genes could be indirect targets of the CBFs or they may require interaction of SFR6/MED16 with an alternative transcriptional activator that recognizes another motif. These genes generally showed lower levels of cold induction than the well-characterized CBF-responsive genes (Figures 4 and 5; Supplemental Data Set 2), consistent with previous reports that the majority of the most strongly cold-inducible genes are those controlled by CBFs (Vogel et al., 2005). The second most common motif in *sfr6*-misregulated

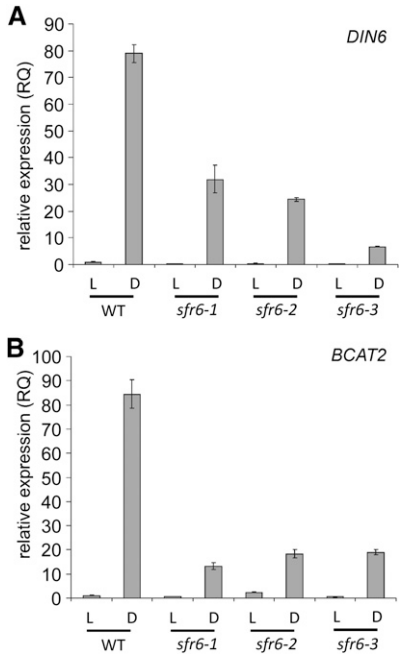

Figure 10. Loss of Dark-Responsive Gene Expression Is Linked to Loss of SFR6/MED16 Function.

qRT-PCR analysis of relative expression levels of *DIN6* **(A)** and *BCAT2* **(B)** in 8-d-old seedlings of Col-0 (WT) and *sfr6-1*, *sfr6-2*, and *sfr6-3* mutant alleles exposed to darkness (D) or maintained in the light (L) for 6 h. Expression is shown after normalization to *PEX4*. Values were calculated using the $\Delta\Delta C_T$ method, and the error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Error bars indicate the level of variation between technical replicates within one biological replicate experiment. Data are representative of three biological replicate experiments. Data from the second biological replicate experiment are shown in Supplemental Data Set 4.

genes showed similarity to the EE. While cold-inducible expression of several genes containing this motif but no CRT was deficient in *med2*, *med14*, and *sfr6* mutants, we found no evidence that the defect in their expression was due to failure to recruit Pol II or Mediator. While Mediator was recruited to these genes in the cold, little corresponding change in Pol II occupancy was observed, suggesting that for these genes Mediator may play a postrecruitment role in regulating transcription (Wang et al., 2005)

In yeast, it has been proposed that that transcript stability may explain the apparent discrepancies between levels of stress-inducible transcripts and Pol II recruitment (Kim et al., 2010; McKinlay et al., 2011) and low temperatures are known to stabilize some plant transcripts (Zarka et al., 2003). Alternative transcript splicing is often temperature sensitive (Sablowski and Meyerowitz, 1998; James et al., 2012), another potential explanation for the disparity between transcript levels and Pol II recruitment. Genes like At1g20030 may require Mediator tail subunits for temperature-dependent recruitment of splicing factors or spliceosome assembly (Seo et al., 2013).

Levels of Some Transcripts Increase in Response to Cold Independently of SFR6/MED16

Our microarray data indicated a number of genes were cold upregulated and not misregulated in *sfr6* mutants and were enriched in neither the CRT nor EE motifs. At3g52740 and *SZF2* were modestly upregulated by cold, requiring none of the Mediator subunits we tested and did not depend upon MED16, MED2, or MED14 for Pol II recruitment. Our observations emphasize the role of specificity within the control of cold gene expression; recruitment of Pol II to these genes may require Mediator subunits that we did not test or might be regulated independently of Mediator.

Starvation-Induced Gene Expression Is Controlled by SFR6/MED16 and Involves an Overlapping but Distinct Subset of Mediator Subunits

In addition to a role in cold-responsive expression, we showed that SFR6/MED16 is involved in the regulation of dark-inducible genes (Figure 10), a role it shares with MED14. There is no major role for MED2, but, interestingly, MED5 is required (Figure 11), with MED5a and 5b acting redundantly (Supplemental Figure 9). These

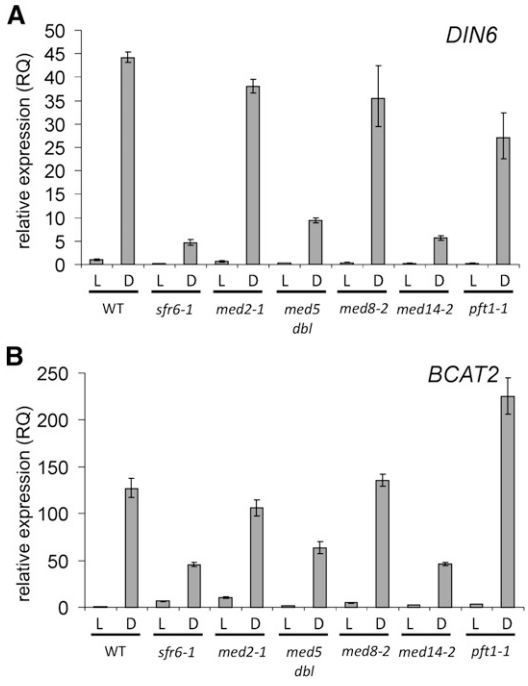

Figure 11. Dark-Responsive Gene Expression Is Reduced in Specific Mediator Subunit Mutants.

qRT-PCR analysis of relative expression levels of *DIN6* **(A)** and *BCAT2* **(B)** in 8-d-old seedlings of Col-0 (WT) and Mediator subunit mutants exposed to darkness (D) for 6 h or maintained in the light (L). Expression is shown after normalization to *PEX4*. Values were calculated using the $\Delta\Delta C_T$ method, and the error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Error bars indicate the level of variation between technical replicates within one biological replicate experiment. Data are representative of three biological replicate experiments. Data from the second biological replicate experiment are shown in Supplemental Data Set 4.

data demonstrate that SFR6/MED16 works in conjunction with select Mediator subunits to respond to different stresses and indicates that specificity of transcriptional responses may be achieved via the joint action of unique combinations of Mediator subunits.

A Structural Perspective on Mediator Subunit Roles in *Arabidopsis*

Our study demonstrates in an intact multicellular organism that particular Mediator subunits facilitate Mediator and Pol II recruitment to specific genes to control their transcription. Three of the Mediator subunits used in this study (MED2, MED5a/b, and MED14) would be predicted from yeast Mediator interaction maps to reside close to *Arabidopsis* MED16 in the plant complex. While empirical data showing intersubunit interactions for plant Mediator is not yet available, it is likely that subunit positions are similar in yeast and plants. Despite low sequence homology, Mediator is surprisingly well conserved between humans and *S. cerevisiae* and exhibits very similar structural topology (Cai et al., 2009), with sequence similarity motifs conserved between Mediator subunit proteins from diverse eukaryotic species (Bourbon, 2008). We made the following observations as to how our data may further support the hypothesis that yeast and plant Mediator structures share similarities.

Our observation that not all SFR6/MED16-dependent functions require MED2 is similar to the case in yeast (Myers et al., 1999; Zhang et al., 2004; Ansari et al., 2012). This is consistent with two alternative possibilities: that some transcriptional activators need to bind both subunits to achieve transcriptional activation or that SFR6/MED16 acts as a "tethering protein" for MED2 and its elimination results in loss of MED2 from the complex, causing failure of any functions associated specifically with MED2. In either eventuality, common roles for MED2 and SFR6/MED16 may reflect a close physical association in *Arabidopsis* Mediator as in yeast. Med2p, Med3p, and Med15p in yeast make up the so-called "triad" of subunits that are attached to the remainder of Mediator via the MED16 subunit Sin4p (Li et al., 1995; Kang et al., 2001). The triad can function independently, though less effectively, when released from the Mediator complex by loss of MED16 (Galdieri et al., 2012). Responses that depend specifically on triad function might therefore be expected to be reduced by loss of MED16/SFR6 but abolished in *sfr6 med2* double mutants, similar to that seen in yeast *med15*Δ *med16*Δ deletions (Kim and Gross, 2013). Alternatively, genes requiring activation by two or more transcription factors may rely on different subunits to bind them. Future analysis of the responses of *sfr6 med2* double mutants may distinguish between these possibilities.

We showed that an *Arabidopsis med14* mutant shared a greater number of phenotypes with *sfr6* mutants than did *med2* mutants. This is consistent with MED14 and SFR6/MED16 occupying adjacent positions in *Arabidopsis* Mediator and possibly both tethering to Mediator other subunits essential for the responses we studied. *Arabidopsis* MED14 and MED16 play overlapping roles in defense gene expression (Zhang et al., 2013), also suggesting close physical proximity between the subunits. A physical association between Sin4p and Rgr1p (the yeast MED14 subunit) has been demonstrated in vivo (Jiang et al., 1995) and deletion of the C-terminal domain of Rgr1p causes loss of both Sin4p and the triad, indicating that Rgr1p anchors the tail to the remainder of Mediator (Li et al., 1995).

The subunit requirement in *Arabidopsis* differed for the transcriptional responses to cold and starvation. The requirement for SFR6/MED16 and MED5 in the response to starvation suggests that these subunits may be closely associated physically and act together to control responses to carbon availability. In yeast, Sin4p is situated close to Nut1p (the yeast MED5 subunit) (Guglielmi et al., 2004; Béve et al., 2005) and *nut1*Δ deletions share many characteristics with *sin4*Δ deletions, including improved growth on nonfermentable carbon sources (Béve et al., 2005). Our data indicated that *Arabidopsis* MED14 also plays a role in the response to carbon availability; similarly, an *rgr1* truncation strain exhibits enhanced growth on nonfermentable carbon sources and reduced repression of the starvation response during glucose availability (Wang and Michels, 2004). Together, these data might suggest that a conserved mechanism controls the transcriptional response to the switch between starvation conditions and glucose availability across eukaryotes.

In a number of experiments, we saw reduced stress-responsive expression in mutants of MED8. This subunit is not part of the tail submodule and would not be expected to bind transcriptional activators. However, as part of the head submodule, MED8 has been shown to play a nonspecific role in gene expression in yeast, where it is considered to be one of 10 essential scaffold subunits that are necessary for the assembly of the Mediator submodules (Kang et al., 2001; Takagi et al., 2006). MED8 in yeast binds the TATA box binding protein (Larivière et al., 2006); therefore, it is not surprising that in *Arabidopsis*, loss of MED8 affects diverse transcriptional responses.

We found no evidence that MED25 acts in conjunction with SFR6/MED16 to control low temperature and starvation-responsive transcription, despite sharing roles in biotic stress tolerance and JA-responsive expression (Kidd et al., 2009; Wathugala et al., 2012; Zhang et al., 2013). Importantly, this supports the hypothesis that individual Mediator subunits or select combinations thereof are responsible for specifying different transcriptional outputs. While *Arabidopsis* MED25 has been shown to bind numerous transcription factors (Elfving et al., 2011; Ou et al., 2011; Çevik et al., 2012; Chen et al., 2012), it clearly plays a role in specific transcriptional responses only.

Our study illustrates that specificity in transcriptional control in plants may be achieved through the cooperation of a group of Mediator subunits whose membership depends upon both the identity of the stimulus and the target gene. Where Mediator subunits prove necessary for a particular transcriptional response this could be due either to a requirement for direct binding to transcriptional regulators or as an indirect result of their contribution to Mediator composition and/or Mediator–Pol II interactions. Future biochemical analyses will help to distinguish between these possibilities.

METHODS

Plant Materials

Col-0 lines overexpressing a 35S-CBF1-YFP construct were created as follows. CBF1 was PCR amplified from cDNA with *Xba*I and *Kpn*I sites

engineered at the 5′ end and a *Xho*I site at the 3′ end (primers are shown in Supplemental Table 3). *CBF1* was cloned as an *Xba*I-*Xho*I fragment into a pBluescript SK− plasmid harboring YFP-cmyc with a NOS terminator (Petersen et al., 2009). A CBF1-YFP fusion was excised using *Kpn*I and cloned into pDH51, which contains the CaMV promoter (Pietrzak et al., 1986). An *Eco*RI-*Kpn*I fragment (consisting of the CaMV promoter, CBF1-YFP fusion, and NOS terminator) was excised from this vector and inserted into the Binary vector pBin19 (Bevan, 1984). Col-0 wild-type *Arabidopsis* was transformed using the floral dip method (Clough and Bent, 1998) with *Agrobacterium tumefaciens* (strain C58C1) harboring the construct and transformants selected by kanamycin resistance before crossing with *sfr6-1* mutant lines.

sfr6-1, *sfr6-2*, and *sfr6-3* alleles were as published previously (Knight et al., 2009). T-DNA insertion lines in Mediator subunits were obtained from the Nottingham Arabidopsis Stock Centre. Homozygotes were identified by PCR using primers designed using the T-DNA express PrimerL program (http://signal.salk.edu/cgi-bin/tdnaexpress). Expression levels of individual subunits were determined by real-time PCR using primers spanning or downstream of the proposed insertion site (Supplemental Table 3). Insertion lines with almost complete reduction in full-length transcript (Supplemental Figure 4) are as follows: *med2-1* (SALK_023845), *med2-2* (SALK_028490), *med5a-2/rfr1* (SALK_011621), *med5b-2/ref4-3* (SALK_037472) both published by Bonawitz et al. (2012), *med8-2* (Kidd et al., 2009) (SALK_092406), *med14-2* (SAIL_373_C07) (Zhang et al., 2013), *med25-1/pft1-1* (Cerdán and Chory, 2003) (SALK_060450), and *med25-2/pft1-2* (Kidd et al., 2009) (SALK_129555).

Plant Growth and Stress Treatments

Plants were grown on 1× Murashige and Skoog and 0.8% agar in 9-cm diameter Petri dishes placed in 16:8 long-day (LD) cycle at 20°C in a Percival CU-36L5 growth chamber (CLF PlantClimatics) unless stated otherwise. Stress treatments were administered to 8-d agar-grown seedlings for all transcriptional experiments. Cold treatment was performed as described previously (Knight et al., 2009), and light conditions were matched in cold (5°C) and ambient (20°C) temperature MLR-350 growth chambers (Panasonic). Plants were harvested after 6 h for RNA extraction or after 4 h for ChIP analysis. ChIP analyses were performed on plants grown for 14 d (or 3 weeks in the case of the histone acetylation experiments) in 14-cm diameter Petri dishes on 1× Murashige and Skoog and 1.5% agar. Dark (starvation) treatment was performed by wrapping plates in three layers of foil 3 h after commencement of the light period. Plants were harvested and RNA extracted after 6 h dark treatment.

Freezing tolerance assays were conducted on rosette stage plants grown at 20°C under short days (12:12 LD cycle) for 5 weeks with 150 to 200 $\mu E/m^2/s$ light. Plants were transferred to cold acclimating conditions (150 $\mu E/m^2/s$ light, 5°C, 10:14 LD cycle) for 14 d prior to freezing at −7.5°C for 24 h. Plants were returned to the original growth conditions and monitored for 7 d after freezing, photographed, and survival scored.

Electrolyte Leakage Assay

Quantitative assessment of damage to plants after freezing conditions was made using a modification of the electrolyte leakage method previously described (Gilmour et al., 1988). Plants were grown for 7 d on agar plates and transferred to soil for growth in 12:12 LD cycles, 150 to 200 $\mu E/m^2/s$ at 20°C for 27 d before transfer to cold acclimating conditions, 5°C, 10:14 LD cycles, 150 $\mu E/m^2/s$ for 14 d. Rosette leaves of a comparable size in all mutants and wild-type plants were excised and washed in deionized water. Leaves were blotted gently on tissue paper to remove excess water, and three replicate test tubes were prepared for each measurement, with each tube containing three leaves. Test tubes were held on ice until all had been prepared. One set of tubes was retained on ice, while the others were transferred in a randomized order to a Clifton stirred circulating bath with immersion dip cooler (Nickel-Electro) set at −2°C. Tubes were allowed to equilibrate for 1 h before the addition of two to four ice chips (made with deionized water) to each tube. Tubes were plugged with a foam bung and retained at −2°C for a further 2 h. One set of tubes (three replicate tubes for each plant type) were removed and placed on ice before decreasing the bath temperature to −4°C. After 30 min at this temperature, the next set of tubes was removed and this procedure continued until the final set were removed after incubation for 30 min at −12°C. All tubes were retained on ice in a cold room (5°C) where samples were allowed to thaw overnight.

The following day 5 mL of deionized water was added to each tube and tubes shaken gently for 3 h at room temperature. The liquid from each tube was decanted and electrical conductivity measured using a hand-held conductivity meter (Hannah Instruments). The test tubes containing leaves were then transferred to a −80°C freezer for 1 h, allowing the complete release of remaining solutes from the plant tissue. The contents of the tubes were allowed to thaw on ice for 30 min, after which the decanted fluid was returned to each tube and the tubes shaken again for 3 h before remeasurement of conductivity. Percentage electrolyte leakage was calculated by expressing the first reading as a percentage of the second.

Microarray Analysis

Col-0 wild-type *Arabidopsis* and *sfr6-1* were subjected to 5°C or maintained at 20°C for 3 h before harvesting and subsequent microarray analysis on Affymetrix gene chips (Gene Expression Omnibus [GEO] reference GSE6167). Subsequent data analysis was performed using the Windows version of dChip (Li and Wong, 2003). Raw CEL files were imported, along with the corresponding MAS data. The data were then modeled (to produce expression index and detect outliers) and normalized using an invariant set of genes as described by Li and Wong (2001). Gene lists presented here were generated using the "compare samples" function within the dChip program, using selected parameters detailed below. Comparison of expression levels between the two wild-type slides allowed us to generate a list of 1177 potentially cold-upregulated genes that had a cold/ambient expression ratio of at least 1.5 and that had a 100% present (P) value for the cold sample (Supplemental Data Set 1). Present (P) values were not made a requirement in the ambient sample as many cold-upregulated genes are expressed to almost undetectable levels under ambient conditions. It was necessary to include genes exhibiting low levels of cold inducibility (a minimum of 1.5× upregulation) to ensure inclusion of mildly cold-upregulated genes that were unlikely to be targets of the CBF transcription factors. In this way, we hoped to gain as comprehensive information as possible regarding the possible role of SFR6/MED16 in the regulation of all cold gene regulons.

Expression values for these genes were extracted for cold-treated wild-type and *sfr6-1* samples from this microarray experiment and from two further separate experiments in which cold-treated *sfr6-2* or *sfr6-3* were each compared with the wild type (GEO reference GSE46084). Making a requirement for a present call (P) in all three wild-type cold samples reduced to 1111 the number of genes that were used as the input data set for analysis of the *sfr6* mutant microarray data. This approach identified 81 genes that showed reduced expression in *sfr6* mutants in the cold (Supplemental Data Set 2). We also generated a second list of 81 genes showing the least difference in expression between *sfr6* mutants and the wild type (i.e., centered on an *sfr6*/wild type ratio of 1) using the criterion that expression of these genes in the cold showed a *sfr6*/wild type ratio of between 0.85 and 1.164 for all three *sfr6* mutant alleles (Supplemental Data Set 3). Analysis of overrepresented heptamer sequences in 500 bp of upstream sequences (downloaded from the TAIR website: http://www.arabidopsis.org/tools/bulk/index.jsp) was performed using the "oligo analysis (words)" program available online at the Regulatory Sequence Analysis Tools site (http://rsat.ulb.ac.be/rsat) according to the developers' instructions (Thomas-Chollier et al., 2011). Default settings were used, with the exception of setting "oligomer length" to 7

and "organism" to *Arabidopsis thaliana*. This program provides output in the form of assemblies and consensuses of related heptamer motifs and matrices corresponding to frequency of nucleotides at specific positions for these assemblies. These matrices were then used manually to produce sequences to enter into the Weblogo program (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al., 2004) to provide a graphical representation of the nucleic acid multiple sequence alignments. All settings were default except for checking the box marked "frequency plot." For mapping previously characterized transcription binding sites, the Athena program (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/analysis_select.pl) (O'Connor et al., 2005) was employed, using default settings, apart from choosing 500 bp of upstream sequence to analyze.

Measurement of Gene Expression

Gene expression levels were analyzed by qRT-PCR as described previously (Wathugala et al., 2012). RNA was extracted using an RNeasy mini kit with on-column DNase treatment (Qiagen). All transcripts studied were detected using validated gene-specific primers (Supplemental Table 3) using Go-Taq quantitative PCR mastermix with SYBR green and ROX (Promega). Expression levels were normalized to the expression of *PEX4* (At5g25760) (Wathugala et al., 2011). For each cDNA sample tested, three technical replicate reactions with gene specific primers and three technical replicate reactions with *PEX4*-specific primers were performed in a 96-well optical plate. Relative quantification (RQ) was performed by the $\Delta\Delta C_T$ (comparative cycle threshold) method (Livak and Schmittgen, 2001), and RQ values and estimates of statistical variation for each sample calculated as previously (Knight et al., 2009). Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test. Separate biological replicate experiments were performed on different occasions; the data presented in each figure are from one of these biological replicate experiments using separately sown seed and are representative of all those performed. Error bars represent the technical error within the data set shown.

ChIP Analyses

ChIP was performed as described in detail below. ChIP grade antibodies against Pol II C-terminal domain repeats (Ab5408; Abcam), Histone H3 (Ab1791; Abcam), AcH3 (06-599, Millipore), and affinity-purified MED6 antibody (a kind gift from Stefan Björklund, Umeå University) were used in conjunction with ChIP grade preblocked protein A/G plus agarose beads (Thermo Fisher). CBF1-YFP was immunoprecipitated using polyGFP-Trap beads (gift from ChromoTek).

Two to three grams of 3-week-old seedlings were harvested into 30 mL of cross-linking buffer (10 mM Tris HCl, pH 8, 10 mM $MgCl_2$, 0.4 M Suc, and 1% [v/v] formaldehyde) and incubated at room temperature, −95 kPa for 20 min. Then, 2 M Gly was added to a final concentration of 200 mM and samples held under vacuum for a further 5 min. Samples were rinsed with 50 mL of cross-linking buffer (without formaldehyde), briefly dried on filter paper, flash frozen, and stored at −80°C until required.

Chromatin was prepared as follows with all steps performed at 4°C or on ice. Tissue was ground in liquid nitrogen to a fine powder and mixed with 25 mL of extraction buffer 1 (10 mM Tris HCl, pH 8, 10 mM $MgCl_2$, 0.4 M Suc, 1% [v/v] Triton X-100, 5 mM β-mercaptoethanol, 0.35% protease inhibitor cocktail; Sigma-Aldrich P-9599]). Samples were incubated on ice for 5 min with occasional gentle mixing then filtered through two layers of Miracloth and the filtrate centrifuged at 4°C, 3000*g*, for 15 min. The supernatant was discarded and crude nuclear pellets were gently resuspended in 20 mL of extraction buffer 1 and incubated on ice for 5 min with occasional gentle swirling followed by centrifugation at 4°C, 3000*g*, for 15 min. Each pellet was resuspended in 1 mL of extraction buffer 2 (10 mM Tris HCl, pH 8, 10 mM $MgCl_2$, 0.25 M Suc, 1% [v/v] Triton X-100, 5 mM β-mercaptoethanol, and 0.35% protease inhibitor cocktail), transferred to a 1.5-mL microfuge tube, and pelleted by centrifugation at 4°C, 3000*g*, for 15 min. Each sample was resuspended in 500 μL of nuclear lysis buffer (10 mM Tris HCl, pH 9, 150 mM NaCl, 1 mM EDTA, 0.1% Na-deoxycholate [w/v], 0.5% sarkosyl, and 0.35% protease inhibitor cocktail) and incubated on ice with gentle mixing for 5 min. Chromatin was sheared to an average size of 400 bp using a Diagenode Bioruptor (20 cycles, high power, 15 s on and 30 s off). After sonication, one-tenth volume of 10% Triton X-100 was added and gently mixed before centrifugation at 16,000*g* for 30 min. The supernatant was retained and used for further steps.

The following immunoprecipitation steps were performed at 4°C or on ice. Samples were diluted to 50 ng/μL in ChIP dilution buffer (10 mM Tris HCl, pH 8, 1% [v/v] Triton X-100, 160 mM NaCl, 5 mM EDTA, and 0.35% protease inhibitor cocktail) to provide 500 μL of 50 ng/μL DNA per ChIP reaction. Each sample was precleared for 4 h using 20 μL of protein A/G plus ChIP beads (Thermo Fisher) for conventional antibody immunoprecipitation or 20 μL sepharose CL-4B (blocked with 100 mg/mL BSA and 500 mg/mL sheared salmon sperm DNA in ChIP dilution buffer for 1 h). Two micrograms of antibody was bound to 20 μL protein A/G plus ChIP beads per ChIP reaction for 4 h in ChIP dilution buffer. PolyGFP-trap beads (for CBF1-YFP ChIP) were blocked for 1 h with 100 mg/mL BSA and 500 mg/mL sheared salmon sperm DNA in ChIP dilution buffer. Mock immunoprecipitation reactions were set up identically but used whole mouse or rabbit preimmune serum as appropriate. Mock reactions for polyGFP-Trap immunoprecipitation used blocked sepharose CL-4B. Aliquots of precleared ChIP DNA were incubated for 12 h with blocked antibody bound resin or polyGFPtrap beads. One hundred microliters of precleared chromatin was retained to act as input control. ChIP reactions were washed twice each with low-salt wash buffer (20 mM Tris HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS), high-salt wash buffer (20 mM Tris HCl, pH 8, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS), once with lithium chloride wash buffer (10 mM Tris HCl, pH 8, 1mM EDTA, 0.25 mM LiCl, 1% Igepal CA-630, and 1% Na-deoxycholate), and twice with TE and then resuspended to a final volume of 100 μL in TE (10 mM Tris-HCl, pH 8, and 1 mM EDTA, pH 8).

To each ChIP sample and input control, 20% Chelex solution in water was added and mixed at room temperature for 5 min and then boiled for 10 min. Once samples returned to room temperature, 20 μg of proteinase K was added, mixed, and incubated at 50°C for 30 min followed by boiling for 10 min. The supernatant was removed and retained, beads washed with 100 μL TE, and the washings added to the initial supernatant. Five micrograms of RNase A was added to each sample and incubated at 37°C for 30 min. Samples were phenol:chloroform:isoamylalcohol (25:24:1) extracted followed by chloroform extraction. DNA was precipitated using 2 μL of linear polyacrylamide, 0.1 volume Na acetate, and 2.5 volumes ethanol incubated at −80°C for 1 h, recovered by centrifugation, and washed. DNA was resuspended in 200 to 400 μL TE.

Recovered DNA was quantified by real-time PCR using the protocol described above, with validated locus-specific primers (Supplemental Table 3), Go-Taq quantitative PCR master mix with SYBR green and ROX (Promega), and relative levels of DNA calculated using the $\Delta\Delta C_T$ method described above. Signal from mock reactions was undetectable or multiple orders of magnitude lower than for experimental reactions. ChIP DNA levels were standardized to the *PEX4* promoter (using PEX4 pro F and R primers; Supplemental Table 3) for Histone and CBF1-YFP ChIP experiments or to the middle of the *PEX4* open reading frame (PEX4 mid F and R) for Pol II and MED6. All values shown represent the mean of three technical replicates performed on each ChIP sample normalized to the mean of three technical replicates performed on the relevant input sample. Pol II and MED6 occupancy at a transcriptionally silent locus, TSI-A (Steimer et al., 2000), showed no change across genotypes or conditions demonstrating that observed enrichments are not stochastic events.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: *SFR6/MED16* (At4g04920),

PFT1/MED25 (At1g25540), *MED2* (At1g11760), *RFR1/MED5a* (At3g23590), *REF4/MED5b* (At2g48110), *MED8* (At2g03070), *MED14* (At3g04740), *KIN2* (At5g15970), *GOLS3* (At1g09350), *CBF1* (At4g25490), *DIN3* (At3g06850), *DIN6* (At3g47340), *COR15a* (At2g42540), *ABF1* (At1g49720), *SZF2* (At2g40140), *LTI78* (At5g52310), *BCAT2* (At1g10070), *PEX4* (At5g25760), At1g20030, At1g68500, At5g62360, and At5g46710. Microarray data can be accessed from GEO (GSE46084 and GSE6167).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Overexpression of CBF1-YFP Elicits *COR* Gene Expression in the Wild Type but Not *sfr6-1*.

Supplemental Figure 2. The Ratio of RNA Polymerase II Occupancy at Stall Sites to Occupancy at TSS Sites in Wild-Type Col-0 and *sfr6-1*, *med2-1*, and *med14-2* Mutant Plants after 4 h at 5°C Is Similar.

Supplemental Figure 3. CRT/DRE and EE Are Enriched in Cold Upregulated Genes That Are Downregulated in *sfr6* Mutants.

Supplemental Figure 4. Mediator Subunit Transcript Levels in Mediator Subunit Mutants.

Supplemental Figure 5. Cold-Responsive Gene Expression in *med2-2*, *pft1-2*, and *sfr6-3* Compared with the Wild Type Is Similar to That Seen in *med2-1*, *pft1-1*, and *sfr6-1*, Respectively.

Supplemental Figure 6. RNA Polymerase II Recruitment and Progression along Cold-Responsive Genes in Wild-Type Col-0, *sfr6-1*, *med2-1*, and *med14-2* Mutants.

Supplemental Figure 7. Mediator Recruitment to Further CRT- and EE-Containing Genes.

Supplemental Figure 8. Dark-Responsive Gene Expression Is Not Reduced in *med2-2* and *pft1-2*, Similar to *med2-1* and *pft1-1*, Respectively.

Supplemental Figure 9. Expression of Dark-Induced *DIN6* Is Reduced in *med5 amed5b* Double Mutants but Not in *med5a* or *med5b* Single Mutants.

Supplemental Table 1. Frequencies of Occurrence of Heptamer Sequences in the Promoters of the 81 Genes Misregulated in *sfr6* Mutants and Probabilities of Occurrence Relative to the Whole Genome.

Supplemental Table 2. Frequencies of Occurrence of Heptamer Sequences in the Promoters of the 81 Genes Not Misregulated in *sfr6* Mutants and Probabilities of Occurrence Relative to the Whole Genome.

Supplemental Table 3. Oligonucleotides Used in This Study.

Supplemental Data Set 1. Genes That Are Upregulated at Least 1.5-Fold in Col-0 Wild-Type Plants.

Supplemental Data Set 2. Cold-Upregulated Genes That Are Misregulated in All Three *sfr6* Alleles.

Supplemental Data Set 3. Cold-Upregulated Genes That Are Not Misregulated in All Three *sfr6* Alleles.

Supplemental Data Set 4. Biological Replicate Data for Experiments Shown in the Main Figures.

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AUTHOR CONTRIBUTIONS

P.A.H. and H.K. designed and performed the experiments, analyzed the data, and wrote the article. M.R.K. analyzed data and wrote the article. R.L., E.A.D., E.K., C.H.H., and J.F.S. performed the research.

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