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SFR6 PROTEIN OF FREEZING TOLERANCE IN ARABIDOPSIS DOES NOT AFFECT LOCALIZATION OF CBF1 PROTEIN

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ABSTRACT

SFR6 (SENSITIVE TO FREEZING6) is one of plant mediator proteins which has been identified first with its involvement to tolerance against freezing in *Arabidopsis*. The freezing sensitive *sfr6* mutant is lack of expression of downstream genes in CBF cold response pathway. SFR6 also mediates tolerance to osmotic stress induced by drought and salinity. However, *sfr6-1* mutant shows normal levels of *CBF* gene expression suggesting that SFR6 acts downstream of *CBF* transcription. The over-expression of *CBF1* and *CBF2* in the *sfr6-1* mutant did not increase downstream cold gene expression. Further, SFR6 does not affect the level of accumulation of CBF protein suggesting that SFR6 may effect on localization of CBF proteins in *Arabidopsis*. Therefore, the localization of CBF1 protein in the *sfr6-1* mutant was examined in this study. *sfr6-1* and Col-0 plants were crossed with wild type plants over-expressing *35S*::*CBF1*::*GFP* and F3 generation was used to visualize CBF1 localization. However, the localization of CBF1 protein was not altered in *sfr6-1* mutant compared to the wild type *Arabidopsis* indicating that the involvement of SFR6 for *COR* gene expression needs to be further studied.

Key words: SFR6, Freezing tolerance, Mediator protein

INTRODUCTION

Developing stress tolerant crops to cope with the rapid environmental degradation that is occurring is an absolute requirement in order to provide enough food for growing population. One of the basic genetic engineering approaches currently being used to improve crop stress tolerance is generation of transgenic plants by introducing novel genes into the genome of agriculturally important crops or altering the expression of existing genes. Understanding stress response signaling pathways is the prime requirement to manipulate stress tolerance of crops by this approach.

The *sfr6* mutant of *Arabidopsis* was isolated on the basis of its failure to cold acclimate (Warren *et al*., 1996). Further studies showed the freezing sensitivity of the *sfr6* mutant is due to greatly reduced levels of the expression of *COR* genes controlled by CBF/DREB1 transcription factors in response to low temperature (Knight *et al*., 1999; Knight *et al*., 2009). However, CBF/DREB1 gene expression itself was not mis-regulated at the transcriptional and translational level in the *sfr6-1* mutant indicating that SFR6 operates downstream of CBF translation (Knight *et al*., 1999; Knight *et al*., 2009). In addition *sfr6* is compromised in its ability to express genes in response to drought, salinity (Knight *et al*., 1999; Boyce *et al*., 2003), UV radiation and pathogen attacks (Wathugala *et al*., 2012) and in other important developmental processes such as flowering (Knight *et al*., 2008). *At4g04920* locus in chromosome 4 near the centromere was identified as *SFR6* locus through mapping and cloning. *SFR6* was identified as a 135kDa protein (Knight *et al*., 2009) of subunit MED16 of plant mediator complex (Backstrom *et al*., 2007). Further the over expression of *AtSFR6* did not increase *KIN2* (downstream gene of CBF pathway) expression in wild type *Arabidopsis* either under ambient temperatures (Wathugala *et al*., 2011) or after cold exposure (Wathugala *et al*., 2012). Thus it is possible that SFR6 controls localization of CBF proteins in *Arabidopsis*. Therefore, the effect of

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SFR6 on localization of CBF protein was analyzed in this study.

METHODOLOGY

Crosses between *35S*::*AtCBF1*::*GFP* (GFP-Green Florescent Protein) plants in Col-0 background (kind gift from Prof. M. Thomashow's lab, MSU-DOE plant research lab, Michigan State University) with Col-0 and *sfr6-1* mutant plants were performed to construct *Arabidopsis* lines over-expressing both *CBF1* and *GFP*. As *35S*::*AtCBF1*::*GFP* plants harbour a *kanamycin* resistant gene F1 plants were selected on *kanamycin* supplemented MS agar plates. Plants were grown to maturity in a growth room maintained at approximately 20 ± 1^{o}C with a long day photoperiod (16 h light/8 h dark) and light level of 100-150 µmol m^{-2} s^{-1} and F2 seeds from individual plants were collected. Seeds were again selected on *kanamycin* supplemented agar plates and F2 plants were grown for maturity. F3 seeds were grown on *kanamycin* supplemented agar plates for 12 days. The seeds from Col-0 and *sfr6-1* crossed with *35S*::*AtCBF1* were also selected on *kanamycin* supplemented agar plates. Twelve day old plants were used for segregation of GFP florescence with the *sfr6* mutant phenotype. *sfr6-1* mutants have pale green colored leaves (Knight *et al*., 2009). Therefore, seedlings with pale coloured leaves on *Kan* supplemented plates were selected as homozygous *sfr6-1* over-expressing *CBF1*::*GFP*. A Confocal laser scanning microscope (Zeiss LSM50) with 40× objectives was used to visualize GFP. The excitation wavelength for GFP visualization was 488 nm, with emission measured using a 505nm long pass filter. All images were taken from seedlings grown under similar conditions, or subjected to the same treatments.

RESULTS AND DISCUSSION

SFR6 gene controls freezing and osmotic stress tolerance in *Arabidopsis* (Knight *et al*., 1999, 2009; Boyce *et al*., 2003). AtSFR6 has been identified as At4g04929 protein (Knight *et al*., 2009) a plant mediator sub-unit (Backstrom *et al*., 2007). Overexpression of *AtSFR6* in *sfr6-1* mutant background complemented all visible phenotypes of *sfr6-1* mutant (Wathugala *et al*., 2011). However, overexpression of *AtSFR6* in wild type *Arabidopsis* did not alter downstream cold gene expression (Wathugala *et al*., 2011**).** Further, over-expression of *CBF* genes in the *sfr6-1* mutant did not alter *COR* gene expression (Knight *et al*., 2009). However, over-expression of *CBF* transcription factors in wild type *Arabidopsis* causes large increase in *COR* gene expression and freezing tolerance at ambient temperatures (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998; Kasuga *et al*., 1999; Gilmour *et al*., 2000). These results indicate that SFR6 is essential but not sufficient for *COR* gene expression. Previously, it was also observed that the expression of CBF proteins (translation and protein stability) was not defective in the *sfr6-1* mutant (Knight *et al*., 2009). Therefore, it was suggested that *SFR6* might affect CBF activity after CBF translation (Knight *et al*., 2009). Therefore, it might be possible that *SFR6* recruits CBF protein to the nucleus. Therefore, the localization of CBF1 was analyzed by producing wild type and SFR6 plants over-expressing *35S:CBF1:GFP*. However, as shown in figure 1 the localization of CBF1 protein was not altered in *sfr6-1* mutant compared to the wild type *Arabidopsis* suggesting that SFR6 is not involved in localization of CBF1 in *Arabidopsis*.

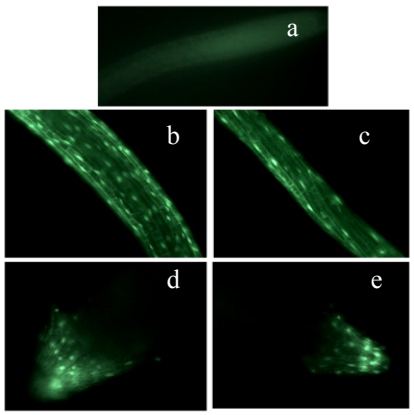

Figure 1: **Localization of 35S::CBF1::GFP in root cells of *Arabidopsis* plants over-expressing *35S::CBF1:GFP*. Images were captured using a 40 × objective. (a) Root of wild type *Arabidopsis* plant over-expressing *35S*::*CBF1*. (b, c) Images of Col-0 and *sfr6-1* over-expressing *CBF1* and *GFP* respectively were taken from root elongation zone and (d, e) root cap. Images were taken with identical parameters to allow comparison.**

Mediator is a transcriptional co-activator complex which acts as a bridge to convey DNA-bound transcriptional regulators and enhancers to the general RNA polymerase II transcription machinery (Chadick and Asturias, 2005; Bourbon, 2008). Components of mediator were first identified thought biochemical and genetic studies in baker's yeast (*Saccharomyces cerevisiae*), as subunits linking to the RNA Polymerase II holoenzyme (Flanagan *et al*, 1991). Mediator complexes were subsequently found in other organisms including higher eukaryotes (Bourbon *et al*., 2004; Bjorklund and Gustafsson, 2005; Bourbon, 2008). Of these, the structure and composition of yeast mediator has been studied in most detail. The yeast complex comprises 26 subunits (Guglielmi *et al*., 2004), and has been grouped through interactor studies into 4 sub-modules known as the head, middle, tail and kinase (CDK) (Asturias *et al*., 1999). In mammalian mediator, 30 subunits have been identified (Bourbon *et al*., 2004). However, prior to 2007 there were no reports of a plant mediator complex, probably due to the low sequence homology between *Arabidopsis* and other non-plant eukaryotic mediators (Backstrom *et al*., 2007).

Physical interactions between mediator subunits were revealed in 1992 by Jiang and Stillman, through research on yeast SIN4 (MED16) subunit mutant strain. A mutation in the SIN4 (MED16) subunit of yeast showed loss of function of other tail subunits and therefore, it was suggested that mediator subunits physically interact with each other (Jiang and Stillman, 1992). Consistent with these results, electron microscopic (EM) studies of holoenzyme complexes purified from mutant yeast lacking MED16, showed that the tail mediator modules (MED15, MED3 and MED2) were absent (Chadick and Asturias, 2005). Therefore they also suggested that the tail portion of mediator formed a subset of physically interacting units. Moreover, the physical association of yeast MED16 and MED14 (Rgr1) proteins were also apparent with the same spectrum of phenotypes observed in *sin4* and *rgr1* mutant strains (Jiang *et al*., 1995). According to these literatures SFR6/MED16 in *Arabidopsis* may play very complex role in stress gene regulation. Therefore, further studies of role of SFR6/MED16 will give us clear picture on the mechanism of plant stress gene regulation.

CONCLUSION

We observed that AtSFR6 has no effect in localization of CBF1 gene. Therefore, the mechanism of regulation of cold induced *COR* gene expression via SFR6/MED16 remains to be further investigated. Not only SFR6/MED16 subunit, the future research on other subunits and of the whole complex of plant mediator will help us to understand the mechanism of stress gene expression.

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