Tropical Agricultural Research & Extension 15(2): 2012

MED16/SFR6 IS NECESSARY BUT NOT SUFFICIENT FOR *COR* GENE EXPRESSION OF CBF PATHWAY

Deepthi Lakmini Wathugala[1*], Heather Knight[2] and Marc R Knight [2]
Department of Crop Science, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri Lanka [2] School of Biological and Biomedical Sciences, Durham University, United Kingdom

Accepted: 5th November 2011

ABSTRACT

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 crop plants by this approach. SFR6 (SENSITIVE TO FREEZING6) is one of plant mediator protein which has identified first with its involvement to tolerance against freezing in *Arabidopsis*. The freezing sensitivity of 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, the over expression of SFR6 in wild type *Arabidopsis* did not increase *COR* (*Cold on regulated*) gene expression under ambient temperature. Therefore, the effect of SFR6 on cold gene expression was further characterized in this study. Results showed that over expression of *SFR6* did not alter *CBF1* expression levels under ambient temperature as well as *COR* gene expression under cold and osmotic stress indicating SFR6 alone cannot use as a molecular tool to improve stress tolerance of crop plants. Further over expression of both SFR6 and *CBF1* also did not have additive effect on *COR* gene expression. Therefore, further studies of the mechanism of SFR6 on *COR* gene regulation need to be conducted to evaluate the potential of use of SFR6 as a molecular tool to increase stress tolerance of crop plants.

***Key words*:** CBF Pathway, COR Gene, MED16/ SFR6, Stress Tolerance

INTRODUCTION

Low temperature is an adverse environmental condition that affects plant growth and development and thus crop yield reduce mainly in temperate countries and higher altitudes in tropical countries. Cold acclimation is important in mediating the freezing tolerance of plants grown in these regions. During the acclimation process numerous molecular and physiological changes take place including alterations in gene expression. The complexity of this process is implied by the number of genes altered during cold acclimation. According to some estimations 25 % of the transcriptome in *Arabidopsis* is involved in cold acclimation (Kreps *et al*. 2002). Many are also inducible by drought (Thomashow 1999). Therefore, a basic understanding of the molecular mechanisms of cold acclimation-related gene expression and the signaling pathways leading to them is important to improve the freezing and drought tolerance of agricultural crops.

A group of transcription factors called C-repeat binding factors (CBF) (Stockinger *et al*. 1997), and also known as dehydration responsive element binding factors (DREB1) (Liu *et al*. 1998), control cold induced *COR* (Cold on regulated) gene expression. CBF/DREB1 transcription factors belong to the EREBP/AP2 family of DNA binding proteins (Stockinger *et al*. 1997; Liu *et al*. 1998). There are 3 closely related cold inducible *CBF/DREB1* genes, named *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A* all located on chromosome 4 of *Arabidopsis* with approximately 88 % identical and 91 % similar amino acid sequences (Gilmour *et al*. 1998; Liu *et al*. 1998). CBF/DREB1 transcripts increase markedly within 15 minutes of transfer to low temperature, and continue to increase over next 1-2 h. These 3 genes encode transcriptional activators which bind CRT/DRE elements (Gilmour *et al*. 1998; Liu *et al*. 1998). After about 2 h, the transcripts of *COR* genes containing CRT/DRE in the promoters accumulate (Gilmour *et al*. 1998). The

*Corresponding author: wgdlakmini@crop.ruh.ac.lk

over-expression of *CBF*/*DREB1* transcription factors in *Arabidopsis* result in a great increase in freezing tolerance in wild type *Arabidopsis* (Jaglo-Ottosen *et al*. 1998; Liu *et al*. 1998). AtSFR6 a plant mediator protein (Backstrom *et al*. 2007) has also been identified as a protein that regulate CBF/DREB dependent *COR* gene expression in *Arabidopsis* (Knight *et al*. 2009). The freezing sensitivity of *sfr6* mutants are due to defective expression of *COR* genes controlled by CBF/DREB1 transcription factor (Knight *et al*. 1999; Knight *et al*. 2009). However, *CBF/DREB1* gene expression is not misregulated at the transcriptional and translational level in *sfr6*, indicating that SFR6 operates downstream of CBF translation (Knight *et al*. 1999; Knight *et al*. 2009). However, over expression of *AtSFR6* did not increase *KIN2* expression in wild type *Arabidopsis* (Wathugala *et al*. 2011). SFR6 also mediates tolerance to osmotic stress induced by drought and salinity (Knight *et al*. 1999; Boyce *et al*. 2003) and gene expression affecting other important developmental processes such as flowering (Knight *et al*. 2008). AtSFR6 homologue has also been identified in rice and over expression of *OSSFR6* in *Arabidopsis sfr6-1* mutant showed functional orthology through genetic complementation (Wathugala *et al*. 2011). Therefore, the effect of SFR6 on cold gene expression was further characterized in this study.

MATERIALS AND ME-THODS

Production of both AtSFR6 and AtCBF1 over expressing lines

Crosses between *35S*::*AtCBF1* plants in Col-0 background (Knight *et al*. 2009) with *35S*::*AtSFR6* in *sfr6-1* mutant background (Wathugala *et al*. 2011) were performed to construct *Arabidopsis* lines over-expressing both *CBF1* and *SFR6* genes. *35S*::*AtSFR6* plants were used as recipient plants when crossing. Col-0 and *sfr6-1* mutant plants were also crossed with *35S*::*AtCBF1* as controls. As *35S*::*AtCBF1* plants harbour a kanamycin (*Kan*) resistant gene F1 plants were selected on *Kan* 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 collected. As *35S*::*AtSFR6* construct harbour a phosphinothricine (*PPT*) resistant gene F2 seeds were germinated on MS agar plates supplemented with *Kan* and *PPT*. The seeds from Col-0 and *sfr6-1* crossed with *35S*::*AtCBF1* were selected on *Kan* supplemented agar plates. Twelve day old plants were harvested for RNA extraction. *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*.

Cold stress

Seven-day-old seedlings grown on horizontal agar plates were transferred to 4^{o}C for the appropriate amount of time in constant light. The whole plants were collected after the indicated time of cold exposure and quickly frozen in liquid nitrogen. One biological replicate contained 15-20 seedlings.

Osmotic stress

Seven-day-old seedlings grown on MS agar plates were floated in 6 ml of sterile water or 350 mM mannitol contained in transparent multi well plates. Before adding mannitol, seedlings were floated in 3 ml of sterile water for 3 h to recover from transfer to water from agar medium. Then, 3 ml of 700 mM mannitol solution was added to each well, except for control plants to which 3 ml of water was added. Fifteen to 20 seedlings were used for each treatment. Samples were collected 6 h post treatment. Immediately before harvesting, seedlings were blotted on tissue paper to remove excess solution. Samples were then quickly placed into microfuge tubes and snap-frozen in liquid nitrogen. Samples were processed so that the minimum time elapsed (less than 1 minute) between harvesting and freezing.

Quantitative real time PCR

A high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe cDNA from 1.5µg total RNA extracted using the Quagen Rneasy plant mini kit in conjunction with RNAse-free DNase (Quagen) to remove any genomic DNA contamination. Quantitative real time PCR (qRT-PCR) was performed on 10µl of 1 : 50 diluted cDNA reaction in a 25µl reaction using an Applied Biosystem 7300 system. Relative transcript abundance of *KIN2*

(At5g15970; At02354775_s1) were measured using gene-specific TaqMan probes from Applied Biosystem.

RESULTS

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 (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) indicating that *SFR6* alone might not influence activation of target *COR* genes in *Arabidopsis*. 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). Therefore, the effect of overabundance of *AtSFR6* on *CBF1* transcript levels was first analysed.

The same cDNA samples prepared to determine *KIN2* and *SFR6* levels (Wathugala *et al*. 2011) were used. Apart from 4 lines all other lines showed comparatively reduced levels of *CBF1* expression (Fig 1A), but there is no significant difference. Although the overabundance of AtSFR6 has no effect on *KIN2* (Wathugala *et al*. 2011) and *CBF1* expression in unstressed seedlings, there is, however, a

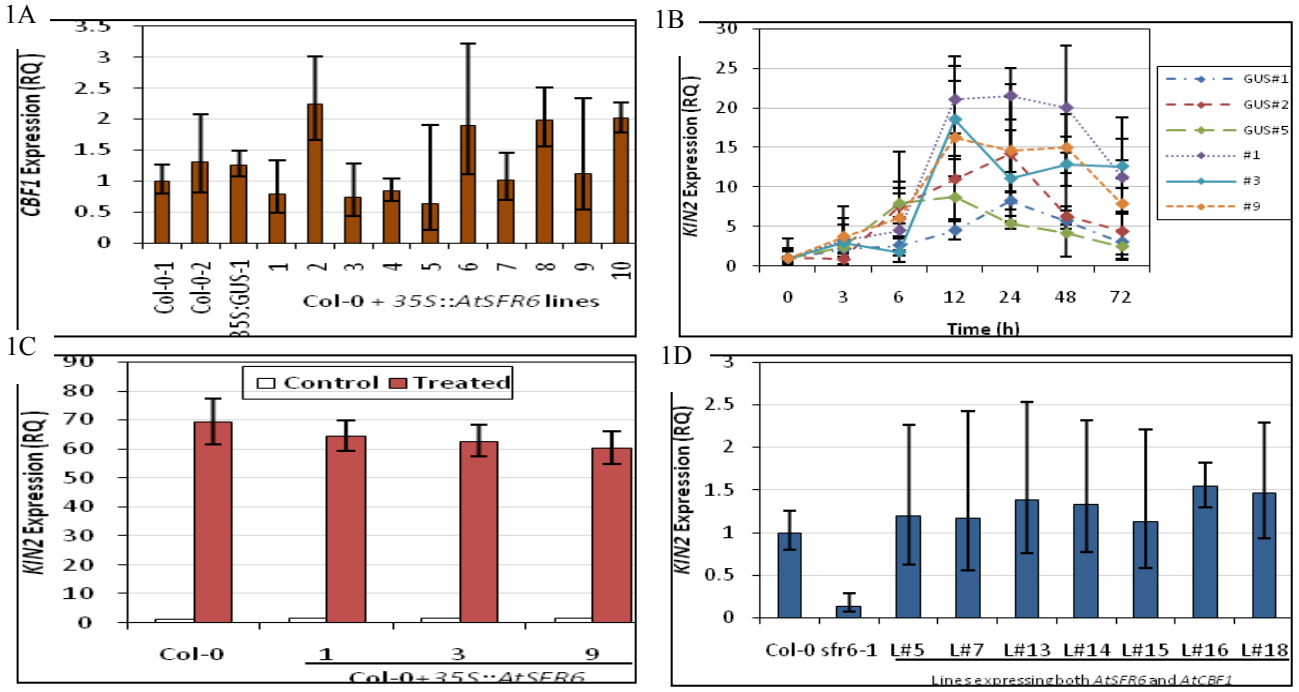

Figure 1: (A)Expression of *CBF1* in unstressed *35S*::*AtSFR6* over-expressing lines. Graph shows the relative quantification (RQ) values for *CBF1* expression relative to the level of *CBF1* expression in non-transgenic Col-0 plants. *ß-TUBULIN4* was used as endogenous control. **(B) *KIN2* expression during a time course of cold exposure in Col+*35S*:: *AtSFR6* lines and Col+*35S*::*GUS* lines.** Graph shows relative quantification (RQ) values of *KIN2* levels relative to the *KIN2* level of each line's unstressed conditions. *ß-TUBULIN4* was used as an endogenous control.
(C) *KIN2* expression after osmotic stress in Col-0+*35S*:: *AtSFR6* lines. Relative quantification (RQ) of *KIN2* expression relative to the *KIN2* level of non-transgenic Col-0 treated with water are shown here. *ß-TUBULIN4* used as endogenous control.
(D) Transcript levels of *KIN2* in transgenic lines expressing both *35S*::*AtSFR6* and *35S*::*AtCBF1*. Data represented here are relative quantification (s) values of *KIN2* expression relative to the *KIN2* levels of Col-0 over-expressing *CBF1*. *PEX4* was used as endogenous control.
Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

possibility that SFR6 has its effect after low temperature stimuli. To test this, the expression of *KIN2* was further characterized in *SFR6* over-expressing transgenic lines during a time course of cold exposure. Three Col-0 + *35S*::*GUS* lines were used as controls and three Col-0 + *35S*::*AtSFR6* transgenic lines (Line #1, #3 and #9) (Wathugala *et al*. 2011) with different levels of *SFR6* expression (strong, moderate and weak respectively) were chosen.

KIN2 transcripts increased up to a maximum in 12h cold treatments and then gradually decreased (fig. 1B). The three *SFR6* over-expressing transgenic lines showed higher *KIN2* transcript level than three *GUS* lines from 12 to 72h (fig.1B). At the end of 3days (72 h) of 4°C exposure transcript levels of *KIN2* in *GUS* lines had returned to ambient levels. Apart from these changes no consistent difference in expression of *KIN2* was observed in any time course between the *AtSFR6* over-expression lines and the *GUS* expression lines. These results suggest that SFR6 may have effect to keep *KIN2* expression at a higher level for a longer period of time.

The expression of *KIN2* was characterized further by subjecting plants to osmotic stress, as the *sfr6-1* mutant displayed reduced expression of *COR* genes after mannitol induced osmotic stress (Boyce *et al*. 2003). However, there was no significant difference between non-transgenic Col-0 and *35S*::*AtSFR6* over-expressers (fig. 1C) further suggesting that the over-expression of *SFR6* alone does not have an effect on *KIN2* expression.

Although we have evidence that *SFR6* is essential for *COR* gene expression of *CBF* cold response pathway, all the above analyses suggested that the over-expression of *SFR6* alone is not sufficient to induce cold gene expression. However, the over expression of *CBF1* alone significantly increases cold tolerance in *Arabidopsis* (Jaglo-Ottosen *et al*. 1998) by increasing levels of *COR* gene expression. As *SFR6* does not have effect on *CBF* gene expression, the additive effect of *CBF1* and *SFR6* over-expression on *KIN2* expression was determined.

Crosses between *35S*::*AtCBF1* plants in Col-0 background (Knight *et al*. 2009) with *35S*::*AtSFR6* in *sfr6-1* mutant plants (Wathugala *et al*. 2011) were performed to construct *Arabidopsis* lines over-expressing both *CBF1* and *SFR6* genes. A slight increase of *KIN2* could be observed in transgenic lines over-expressing both *SFR6* and *CBF1* but these differences were not significant (95% confidence limit according to Student's *t* test) (Fig. 1D).

DISCUSSION

Previous work identified *At4g04920* a plant mediator gene as the locus for *SFR6*; a gene required for *COR* gene expression and cold acclimation in *Arabidopsis* (Knight *et al*. 2009). However the overexpression of AtSFR6 in wild type *Arabidopsis* did not increase *KIN2* expression either in ambient temperatures (Wathugala *et al*. 2011) or after cold exposure indicating that the levels of *SFR6* alone might not influence activation of target *COR* genes in *Arabidopsis*.

Previous studies also showed reduced *COR* gene expression in the *sfr6-1* mutant, not only after cold exposure, but also after osmotic stress induced by mannitol (Boyce *et al*. 2003). However, *KIN2* expression in *AtSFR6* over-expressing lines after osmotic stress did not alter compared to wild type *Arabidopsis*. These results further confirm SFR6 is necessary but not sufficient for *COR* gene expression, not only at low temperature, but also in response to osmotic stress.

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). Therefore, the possible additive effect on *COR* gene expression due to an overabundance of *SFR6* and *CBF* was examined. The data showed that increasing both *SFR6* and *CBF1* had no effect on *COR* gene expression indicating that overabundance of both *SFR6* and *CBF1* has no additive effect on *COR* gene expression.

As At4g04920 is a sub unit of plant mediator

complex (Backstrom *et al*. 2007) one explanation for the lack of effect when *SFR6* is over-expressed in wild type is that the SFR6 protein is required in stoichiometric proportions with one or more other proteins as a part of a complex and that an increase in the amount of any one of these individually cannot influence the amount of the complex as a whole.

Mediator is the evolutionary conserved multi-protein complex that binds RNA polymerase II and controls transcription of genes (Flanagan *et al*. 1991). The possible mechanism suggested by Chadick and Asturias (2005), Bjorklund and Gustafsson (2005), and Haha (2004) for transcriptional activation by mediator was that the gene specific activators (transcription factors) recruits mediator to the transcription initiation site. Then, general transcriptions factors involved in gene transcription interact with the DNA-mediator complex to form a platform to bind RNA polymerase II. According to this mechanism when plants are exposed to low temperature CBF proteins activate and bind to the promoter of *COR* genes and recruits mediator complex to the promoter to build the RNA polymerase assembly platform. It is possible that MED16/SFR6 might directly bind with CBF or with other mediator subunit which links with MED16 (Myers *et al*. 1999; Chadick and Asturias, 2005). Based on these results over-expression of a single gene of the mediator complex does not result in increased activity of the complex as a whole.

CONCLUSION

In conclusion the results reported here showed that there is no possibility to use of AtSFR6 alone as a molecular tool to improve crop tolerance to environmental stress. As well as there is no additive effect on *COR* gene expression by over expressing both *AtSFR6* and *AtCBF1*. Therefore, the mechanism of regulation of stress induced gene expression via SFR6/ MED16 remains to be further investigated. The future research on specific roles of individual subunits and of the whole complex will widen our knowledge of the transcriptional regulation of gene expression in plant and will create new routes to improve crop tolerance to environmental stress.

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