Expression of *CBF*-like Genes in Alfalfa (*Medicago sativa* L.)

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Introduction

Temperature is one of the major environmental cues regulating the growth and development of plants. Chilling or subzero-freezing temperature results in cold stress, which is responsible for damage and yield loss of crop plants. Identification of germplasm with superior freezing tolerance and understanding the molecular biology of the underlying mechanisms would be key to improving freezing tolerance in plants. As an effort to improve freezing tolerance in alfalfa, we recently discovered a germplasm, River side (RS), that showed greater freezing tolerance compared to some of the known freezing tolerant germplasm. To understand the molecular biology of the superior freezing tolerance of the germplasm, we examined the expression of the C-repeat binding factor (*CBF*) genes in cold response in alfalfa. Studies in Arabidopsis and other plants show that *CBF*s, specifically *CBF3*, play a very important role in response to low temperature cues and regulate a number of downstream genes involved in the cold tolerance. The objective of this study was to identify the potential functional homolog of *AtCBF3* in alfalfa and to analyze its association to the freezing tolerance in RS.

Methods

To identify the members of the *CBF-like* gene family in alfalfa, Arabidopsis CBF3 protein sequence was used to search against the *Medicago truncatula* genome database version Mt 4.0V1(https://phytozome.jgi.doe.gov/pz/portal.html#!search show=KEYWORD&method=Org_Mtruncatula). This legume shares a 98% sequence similarity with alfalfa. The phylogenetic tree was build using MEGA 6 (Tamura et al, 2011) based on the protein sequence alignments. For the expression analysis of *CBF*s under cold stress, seedlings of the alfalfa genotype, SD201, exposed to cold temperature were harvested at various time points. Tissues were also harvested for expression at different developmental stages and for the diurnal expression pattern. Transcript levels were quantified using qRT-PCR. For a comparison of gene expression in different germplasm, 20~30-day-old plants of Riverside, Foster ranch, Apica and CUF-101 were subjected to cold temperature and samples were harvested at 0h, 2h, and 24h after the treatment.

Results

1. Phylogenetic analysis of the CBF-like sequences in *Medicago truncatula* grouped them into 5 distinct groups based on their homology to CBF/DREB transcription factors from Arabidopsis and soybean.

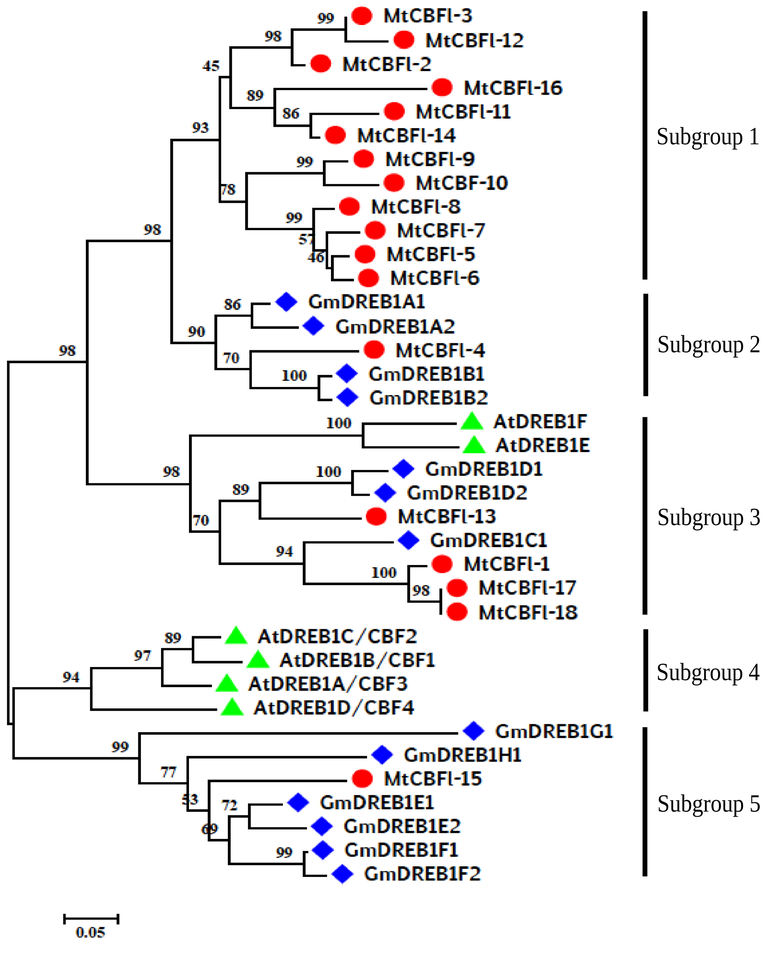

Fig 1.Phylogenetic tree of CBF transcription factors in *Medicago truncatula* (red circles), **Arabidopsis** (green triangles) and ***Glycine max*** (blue diamonds).

2. Thirteen *CBF-like* genes are induced under cold stress in alfalfa

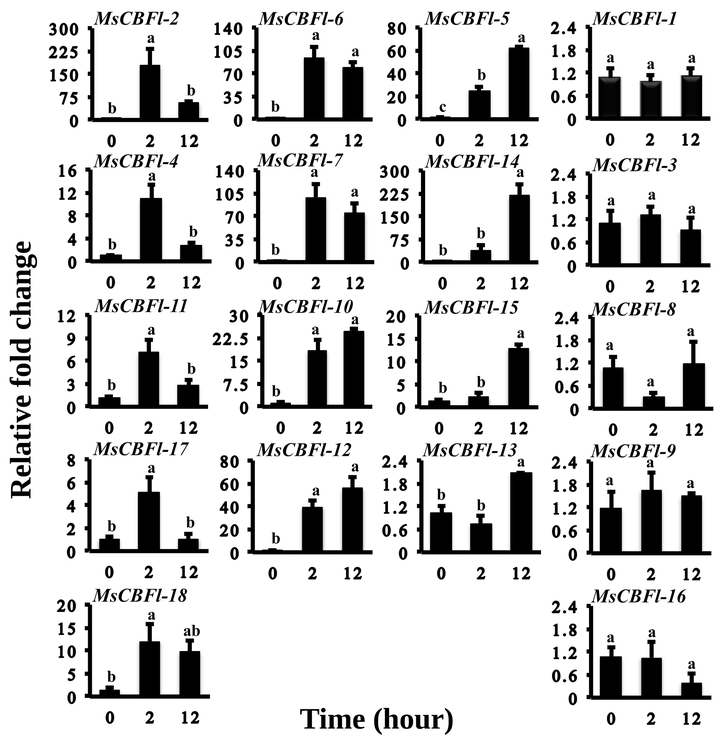

Fig 2. Cold-responsive expression of the *CBF-like* genes in alfalfa. One-week-old SD201 seedlings were exposed to cold (4° C) and samples were collected at 0h, 2h and 12h after the cold treatment. The transcripts were quantified by qRT-PCR. The values represent the mean fold change ±SE (n=3). Bars with different letters are significantly different (p< 0.05).

3 Expression of three early cold-induced *MsCBF-like* genes showed different diurnal pattern (a), and genes had varied expression patterns in different tissues (b) and at different developmental stages (c)

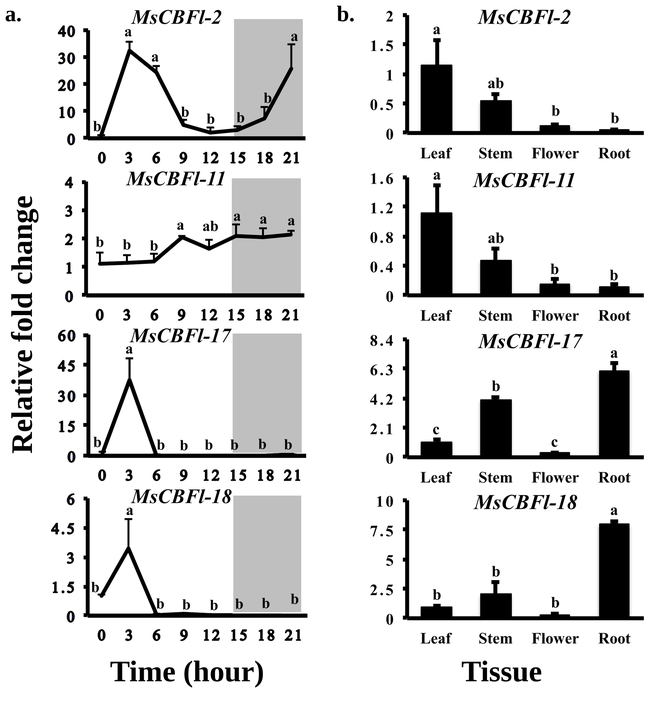

Fig 3a. **Diurnal regulation of four cold-induced genes in alfalfa**. Young shoots were harvested from SD201 plants, of the same age, every 3h after dawn. The shaded area in each graph represents sampling points during night. The values represent the mean fold change ± SE (n=3). Data points with different letters are significantly different (p< 0.05).

Fig 3b. **Expression analysis of *MsCBF-like* genes in different tissues**: Leaf, stem, root and flower tissues were harvested from mature SD201 plants for gene expression analysis. The values represent the mean fold change ± SE (n=3). Bars with different letters are significantly different (p< 0.05).

Acknowledgements

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References

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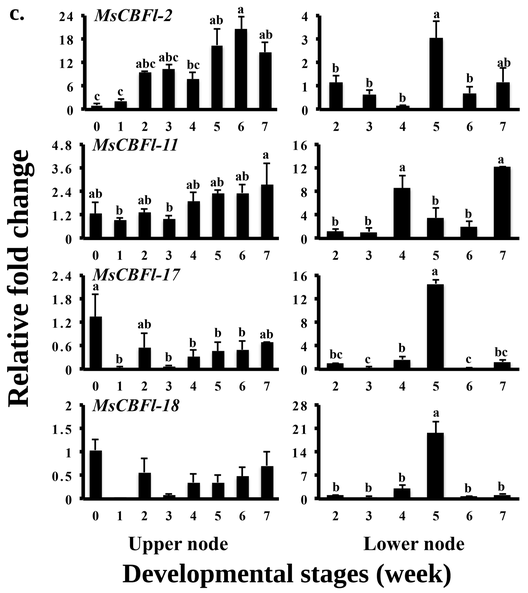

Fig 3c. Expression analysis of the *MsCBF-like* genes at different developmental stages: Samples were harvested from SD201 plants, every week starting from the seventh day after germination. For the first two weeks the whole seedlings were harvested (0&1), and from the third week onwards the young shoots from the upper node (2-7) and the shoots from the lowest node were harvested (2-7). At week 8 the plants were flowering (early flower stage). The transcripts were quantified by qRT-PCR and the values represent the mean fold change ± SE (n=3). Bars with different letters are significantly different (p< 0.05). For the mean fold change values of *MsCBFl-18* in upper node samples the homogeneous group format could not be used because of the pattern of significant differences.

4. *MsCBFl-17* and *MsCBFl-18* are upregulated very early in cold-tolerant but not in cold-susceptible germplasm under cold stress

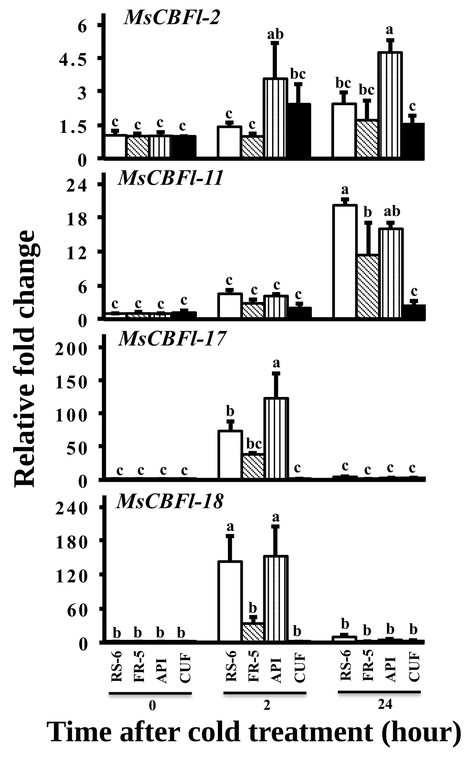

Fig 4. Cold-responsive expression of *CBF-like genes* in four different alfalfa germplasm: Plants (28~30-day-old) of Riverside (RS), Foster ranch (FR), Apica (Api) and CUF-101 (CUF) grown in cone-tainers in a greenhouse were subjected to cold temperature (2°C) treatment and samples were harvested at 0h, 2h, and 24h after the treatment. The transcripts were quantified by qRT-PCR and the values represent the mean fold change ± SE (n=3). Bars with different letters are significantly different (p< 0.05).

Conclusions

in-silico analysis of Medicago genome resulted in the identification of 18 *CBF*-like genes. Their protein sequences were grouped into five distinct clusters in relationship to Arabidopsis and soybean CBFs. Five of the eighteen genes showed an early induction of expression, as that of *CBF3* in Arabidopsis under cold stress. Three of them showed clear diurnal patterns. Expression levels of the *CBF*-like genes also vary due to developmental stages and tissue types. Finally, transcript levels of *MsCBFl-17* and *MsCBFl-18* showed an early and greater induction in RS, FR and Apica when compared to non-freezing tolerant germplasm, suggesting that these two genes are potentially the functional homologs of At*CBF3* and may contribute towards the superior freezing tolerance in alfalfa.