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Transcriptional down-regulation of various genes in alfalfa enhances tolerance to abiotic stresses

Udaya Subedi[1,2], Gaganpreet Kaur Dhariwal[1], Kimberley Burton Hughes[1], Guanqun Chen[2], Surya Acharya[1], Stacy D. Singer[1]*

[1]Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, Lethbridge, AB, T1J 4B1; [2]University of Alberta, Department of Agricultural, Food and Nutritional Science, Edmonton, AB, T6G 2P5

Introduction

• Alfalfa (*Medicago sativa* L.) is the most widely grown forage crop with an estimated global cropping area of approximately 30 million hectares.

• Despite the many benefits of this species, adverse environmental conditions such as drought and/or waterlogging often have a severe negative impact on alfalfa production.

• These factors are expected to become increasingly problematic in coming years due to climate change, as well as the use of intensive agricultural practices.

• The aim of this study is to assess the effect of down-regulating several alfalfa homologs of genes shown previously in other species to elicit improvements in abiotic stress tolerance when down-regulated/mutated.

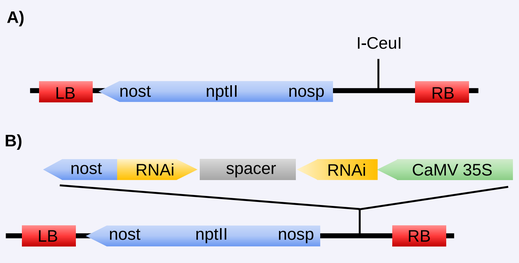
• Such targets have the potential to be utilized downstream for the development of novel alfalfa cultivars with superior climate resiliency using a CRISPR/Cas9 platform.

Methods and Results

• Putative homologs of *ACYL-COA BINDING PROTEIN3* (*ACBP3*), *C-REPEAT BINDING FACTOR2* (*CBF2*), *FATTY OXIDASE3* (*FAO3*), *TELOMERASE ACTIVATOR1* (*TAC1*), and *HOMEOBOX LEUCINE ZIPPER2* (*HB2*) were retrieved from publicly-available databases.

• RNAi vectors were generated to target putative *MsHB2*, *MsACBP3*, *MsTAC1*, *MsFAO3* and *MsCBF2* (two separate vectors targeting genes in clades I and III, respectively) genes (**Figure 1**).

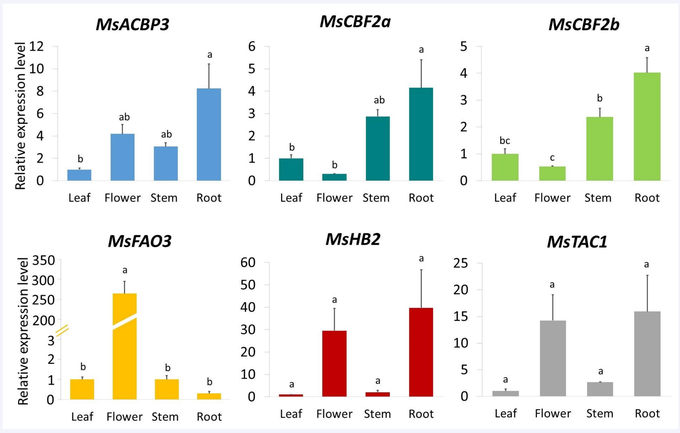
Figure 1. Schematic diagram (not to scale) of plant binary vectors



Empty vector is shown in (A), while a generalized RNAi vector is displayed in (B). RNAi fragments include those targeting *MsACBP3*, *MsCBF2-a*, *MsCBF2-b*, *MsHB2*, *MsFAO3* and *MsTAC1*, respectively. LB, left border; nosp, nos promoter; nost, nos terminator; nptII; neomycin phosphotransferase II; RB, right border.

• Quantitative real-time RT-PCR was carried out to determine whether 6 target genes exhibited preferential expression in particular tissue types.

Figure 2. Relative transcript levels of six alfalfa genes in distinct tissue types



Expression levels of *MsACBP3*, *MsCBF2a*, *MsCBF2b*, *MsFAO3*, *MsHB2* and *MsTAC1* were assessed using qRT-PCR and cDNA derived from leaf, flower, stem and root tissues of the untransformed N.4.4.2 genotype. All values represent the means of three technical and biological replicates, and are relative to the control *UBL-2a* gene. Bars denote standard errors and lowercase letters indicate statistically significant differences at $p \leq 0.05$.

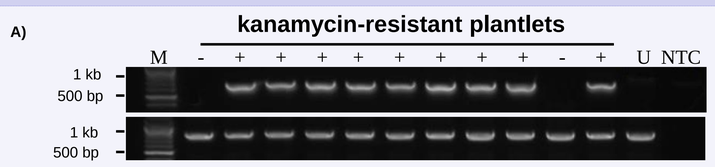
***MsACBP3, MsCBF2* variants and *MsFAO3* transcript levels vary according to the tissue type**

• RNAi vectors, along with the empty vector control, were transformed into alfalfa genotype N.4.4.2 (Badhan et al., 2014) using *Agrobacterium*-mediated transformation of leaf explants (Aung et al., 2015).

• Kanamycin-resistant alfalfa regenerants were assessed via PCR to confirm introduction of the transgenic cassette in each case (**Figure 3**).

Figure 3. PCR confirmation of alfalfa regenerants

A)



B)

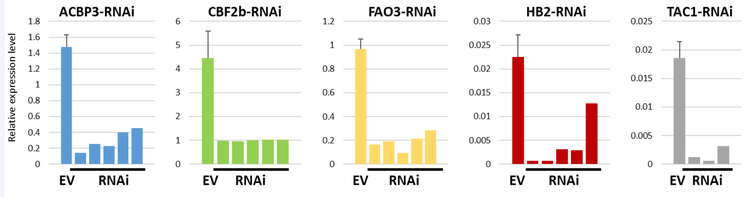
RNAi vector	Empty vector	*ACBP3*	*CBF2-a*	*CBF2-b*	*FAO3*	*HB2*	*TAC1*
Number of PCR positive genotypes	6	49	19	9	9	10	11

(A) Representative PCR results with top row displaying 675 bp transgene-specific fragment and bottom row displaying positive control 883 bp fragment. (B) Number of RNAi genotypes confirmed in each case. M, molecular weight marker; NTC, no template control; U, untransformed control.

Between 6 and 49 independent transgenic alfalfa genotypes were obtained bearing each construct, respectively

• Quantitative real-time RT-PCR was carried out with individual RNAi genotypes to confirm down-regulation of the targeted genes.

Figure 4. qRT-PCR validation of alfalfa RNAi genotypes



Expression levels of *MsACBP3*, *MsCBF2b*, *MsFAO3*, *MsHB2* and *MsTAC1* were assessed in individual RNAi genotypes, as well as empty vector controls, using qRT-PCR and cDNA derived from leaf tissues. No reduction in *MsCBF2a* expression was noted in any of the associated RNAi genotypes. All values represent the means of three technical replicates, except for empty vector (EV) blocks, which represent the mean values of six independent EV genotypes. Values are relative to the control *UBL-2a* gene.

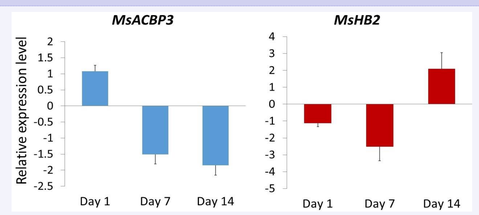
At least 5 *MsACBP3, MsCBF2-b, MsFAO3* and *MsHB2* RNAi genotypes exhibited 1.1- to 34.6- fold reductions in expression in leaf tissue compared to EV genotypes.

Three *MsTAC1*-RNAi genotypes exhibited 6- to 35-2- fold reductions in expression compared to EV genotypes.

No target gene down-regulation was observed in any of the *MsCBF2-a* RNAi genotypes

• Quantitative real-time RT-PCR was carried out to examine the response of *MsACBP3* and *MsHB2* after flooding.

Figure 5. Assessment of *MsACBP3* and *MsHB2* expression under flooding stress conditions

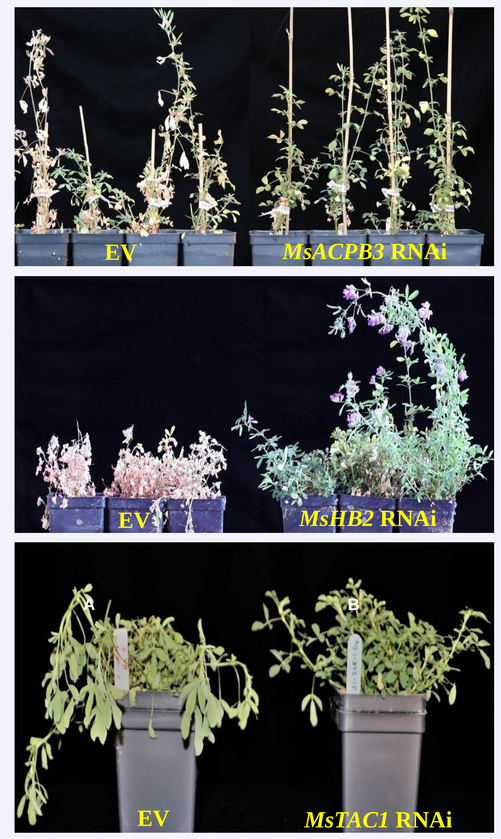


Expression levels of *MsACBP3* and *MsHB2* were assessed in the untransformed N.4.4.2 alfalfa genotype under flooding and control conditions at various time points using qRT-PCR and cDNA derived from leaf tissues. All values represent the means of three technical and biological replicates. Expression levels were normalized to the control *ADF* gene, and blocks represent levels of expression under flooding treatment relative to control conditions .

***MsACBP3* and *MsHB2* genes are differentially expressed post-flooding compared to control conditions**

• In the greenhouse, RNAi-downregulated genotypes and empty vector controls (EV) were subjected to flooding (pots were held in water up to ¾ of pot height) and drought (water was withheld) to assess stress tolerance.

Figure 6. Comparison of abiotic stress response in RNAi and empty vector control genotypes



Independent RNAi and empty vector (EV) genotypes were propagated by stem cuttings, and flooding and drought experiments were carried out approximately 2 months following the second cut. EV plants typically began exhibiting yellowing and senescence after approximately 8 days of flooding, while *MsACBP3* or *MsHB2*-RNAi lines were still green even 14 days post-flooding **(6A and 6B)**. For the drought trial, while EV genotypes began to wilt at a soil moisture level of around 7-10% and died below a soil moisture level of approximately 3%, *MsTAC1*-RNAi lines remained green even below 3%, and wilting did not commence until soil moisture levels were below this point **(6C)**.

Alfalfa plants with reduced levels of *MsACBP3* and *MsHB2* expression exhibit improved flooding tolerance

Down-regulation of *MsTAC1* in alfalfa leads to enhanced drought tolerance

Conclusions and Future Directions

• Putative alfalfa homologs of *HB2*, *ACBP3*, *TAC1*, *CBF2* and *FAO3* genes were identified and alfalfa RNAi genotypes were generated.

• Down-regulation of genes has been achieved in the case of *MsACBP3*-RNAi, *MsCBF2b*-RNAi, *MsFAO3*-RNAi, *MsHB2*-RNAi and *MsTAC1*-RNAi genotypes.

• The down-regulation of *ACBP3* and *HB2* in alfalfa led to enhanced tolerance to flooding. Similarly, alfalfa genotypes with reduced expression of *TAC1* exhibited increased tolerance to drought.

• Further experiments are underway to unravel the mechanisms driving increased abiotic stress tolerance in these genotypes.

Acknowledgements

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