The evaluation of six KNOX genes expression evaluated for in vitro grown tetraploid Medicago sativa plants

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Abstract The main goal of this work was to evaluate the expression of genes involved in alfalfa leaf morphology as the plants are grown in in vitro conditions. For the tetraploid Medicago sativa L. six KNOX genes involved in leaf morphology were identified and their transcription level in different organs was evaluated. The expression of these genes is controlled by the genes that codes for transcription factors but other factors also have a conserved role in determining leaves primordia, they are phitohormones: auxines, giberellines and citokinines. Gene expression is expected to undergo changes due to cultivation in vitro, especially when it comes to genes with morphological role. Therefore, preliminary tests were necessary to confirm the existence of KNOX gene expression in tissues harvested from plants grown in vitro. For gene expression evaluation a semi-quantitative analysis RT-PCR was performed. From the present experiments turned out that the expression of six KNOX genes can also be detected in tetraploid alfalfa plants that are subjected to in vitro culture. However, as expected, compared to control gene the levels of expression for KNOX genes seems to be down-regulated.

Key words alfalfa, leaf morphology, KNOX genes, RT-PCR

Alfalfa (Medicago sativa L.) is one of the most important legume species used in agriculture. Its high nutritional quality and vegetative yield makes alfalfa superior to other forage crops due to its high biomass production (9). Alfalfa contains between 15 to 22% crude protein as well as an excellent source of vitamins and minerals. By using artificial selection, breeders have been able to improve many important traits of alfalfa, such as disease resistance, salt tolerance, nitrogen fixation, and high self-fertility. The majority of these selection efforts have been based on phenotypic evaluations, and the genetic basis of most targeted traits remains unknown. In addition, alfalfa’s ability to fix atmospheric nitrogen makes it valuable both for crop rotation and for a more sustainable and environmentally safe agriculture (9).

In the last time, the alfalfa breeding programs have focused on three general areas: increasing forage yield and quality potential and improving persistence. One of the most important objectives is the increasing of biomass production due to the leaves architecture and size or stems dimensions.

The family of “homedomain” transcription factors Class I KNOX (knotted 1) plays a key role in the formation and maintenance of the shoot apical meristem SAM (8), which controls the process of organogenesis, and so the final morphology of the plant aerial organs.

It was demonstrated that KNOX gene expression is correlated with compound leaf primordial. KNOX expression in leaves was correlated with a state of indetermination and compound leaves appear to maintain this state of indeterminacy in order to develop morphological processes leading to leaflets production (4).

The expression of these genes is controlled by the genes that codes for transcription factors but other factors also have a conserved role in determining leaves primordia, they are phitohormones: auxines, gibberellines and citokinines (2). The involvement of KNOX genes in generating compound leaves has become a subject of intense research but it seems that this involvement is not universal applied, for example the species Pisum sativum (6).

In our work the data emphasized in Medicago truncatula L. (3) studies were used to investigate the KNOX gene expression in alfalfa using one step- RT-PCR. Because this technique combines the reverse transcription (cDNA synthesis) with the PCR amplification it has a high accuracy and can be used in determining the abundance of RNA molecules in a cell or tissue (5).

The main goal of this work was to evaluate the genes involved in alfalfa leaf morphology as the
Material and Methods

Plant material
Two alfalfa individual: in vitro grown two alfalfa individuals: Sigma genotype and a multileaflet genotype were used as a biological material to isolate RNA.

RNA isolation and purification
Total RNA was isolated and purified young leaves, using Maxwell 16 Tissue LEV Total RNA Purification kit (Promega), designed to optimize purification and concentration of high quality RNA. The equipment used for extraction was the automated system for extracting DNA and RNA Maxwell 16 (Promega).

For each extraction, fresh tissue (approximately 30 mg) was collected on ice and kept at -80°C until use. The samples were grinded at low temperature and fast homogenized with the kit solutions. Further on, the working methodology has followed the manufacturer instructions. The total RNA was finally re-suspended in 50µl of nuclease free water.

RNA quantification was performed spectrophotometrically at wavelengths of 260 and 280 nm. To confirm the RNA quality, it was analyzed on a 1.1% agarose gel. After quantification all RNA samples were diluted to the same concentration (200ng/µl).

RT-PCR analysis
For gene expression evaluation a semi-quantitative analysis was performed using the Access RT-PCR System (Promega), containing AMV Reverse Transcriptase, Tfl DNA Polymerase, AMV / Tfl 5X Reaction Buffer and dNTP Mix. This system is designed for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific target RNA in one step, providing sensitive, quick, and reproducible analysis of even rare RNAs. The Access RT-PCR System includes an optimized single-buffer system that permits extremely sensitive detection of RNA transcripts, without a requirement for buffer additions between the reverse transcription and PCR amplification steps. This simplifies the procedure and reduces the potential for contaminating the samples (7).

The primers sequences for the six KNOX genes, specific for Medicago truncatula L. genome, according to Di Giacomo et al., 2008 are presented in Table 1. They were developed by comparing the Arabidopsis genome sequences with the DNA sequences available for Medicago truncatula L. denaturing 30s/ 94° C, primers annealing, 1 min/ variable temperature, final extension 7min/ 68° C.Temperatures for primers annealing were different, depending on the primers lengths and sequences, namely 58°C for KNOX 1, KNOX 2 and KNOX 6 markers and 55°C for KNOX 3, KNOX 4 and KNOX 5 markers.

The amplification products were separated in 2% agarose gel in ethidium bromide presence and visualized by UVP BioImaging System.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer F 5' - 3' sequence</th>
<th>Primer R 3' - 5' sequence</th>
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<tbody>
<tr>
<td>KNOX 1</td>
<td>ATTCATTTCAATGGAGGGTAGTT</td>
<td>TTTTGAAAGCATGATAGAAGAGGT</td>
</tr>
<tr>
<td>KNOX 2</td>
<td>GAAGCGTCTGACACTGGCACATC</td>
<td>TGGAAATAAGAAGCAACAGACACT</td>
</tr>
<tr>
<td>KNOX 3</td>
<td>TGCACAAACTCCCCTAAGAT</td>
<td>TTCCTAAACAAAAAGACTAA</td>
</tr>
<tr>
<td>KNOX 4</td>
<td>AGTGTCACTGTAGACCCTCCCTTG</td>
<td>ACCACACCTTAATATAACACAGA</td>
</tr>
<tr>
<td>KNOX 5</td>
<td>GTTTCCCACATCCCTCCGTCAATGG</td>
<td>TCGTTTCAAGACTGCCCT</td>
</tr>
<tr>
<td>KNOX 6</td>
<td>CATGTGCAAGGAGCCATTCCCTATG</td>
<td>GAAAATCAACCCTTATGCACCA</td>
</tr>
<tr>
<td>control gene (MsGAPDH)</td>
<td>CTBGAGAGGGTGGAAGAGC</td>
<td>GTCCAACAACCTGAGACATCC</td>
</tr>
</tbody>
</table>

Data analysis
The gels images were analyzed by Image J software (http://rsb.info.nih.gov/ij/index.html) version 2. This software analyzed the gels images and transformed the band intensity in a numerically value (densitometry test) allowing an accurate estimation of samples' intensities.
the gene expression level compared with a control gene.

**Obtained results**

The main goal of this research was to investigate the genes involved in alfalfa leaf morphology, starting from the KNOX genes preliminarily characterized for in vivo cultivated alfalfa (teza). The necessity to further study the expression mechanism of alfalfa KNOX genes, implies studies of correlation between hormones concentrations and level of genes expression.

The best way to submit plants to known concentration of hormonal treatments, is by cultivate them in vitro. The previous results concerning KNOX genes expression in alfalfa plant (lucrare Pitesti) were obtained by studying in vivo grown plants. Gene expression is expected to undergo changes due to cultivation in vitro, especially when it comes to genes with morphological role. Therefore, preliminary tests were necessary to confirm the existence of KNOX gene expression in tissues harvested from plants grown in vitro.

Seeds from Sigma genotype were sterilized and inoculated for germination in vitro on Murashige-Skoog, culture media without any hormonal addition. True leaves were prelevated in sterile conditions from two months old plants, and immediately transferred to -80°C freezer.

In the first step the RNA was extracted from young leaves collected. The RNA samples were quantified and then diluted to 200 ng/µl concentration. The method used for gene expression evaluation was a semi-quantitative one, therefore the equal RNA quantity in each reaction was of significant importance.

The RNA samples extracted and purified from the two alfalfa plants were successfully amplified for all of the six KNOX genes, using *Medicago truncatula* L. specific primers (Figures 1, 2).

The amplification products were analyzed by agarose gel electrophoresis and the transcription levels were evaluated compared with the control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The intensity of each band was converted into numerical value using the program ImageJ (Table 2). For each experimental variant, the ratio between the KNOX gene values and the control one was determined (R ratio).

![Figure 1](image-url) **Figure 1**: The amplification products analysis, for two RNA samples, with the primers specific for the three class I studied KNOX genes:

- M - PCR marker (Promega)
- 1, 2 - KNOX 1 gene
- 4, 5 - KNOX 2 gene
- 7, 8 - KNOX 6 gene
- 3, 6, 9 – Control gene - glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH)

For the KNOX 1 gene the values were very low compared with the control gene, each R ratio being less than 1. Therefore the gene was down-regulated in all of the analyzed samples.

For the KNOX 2 gene the R ratios were very closed, with a lower value compared with the control. The KNOX 2 gene was down-regulated in the analyzed samples.

The KNOX 6 gene was down-regulated in the samples. (R ratios lower than 1).
The KNOX 3 gene expression, as a member of the class II was strongly repressed in the studied material.

The KNOX 4 showed low expression levels in the two analyzed tissues. The same pattern was observed when the KNOX 5 gene was analyzed.

The data presented above show that KNOX genes expression is present in in vitro grown alfalfa plants tissues, where, although there is an active sub-expression level of gene expression compared with control.

The KNOX 3 gene expression, as a member of the class II was strongly repressed in all of the studied organs.

The KNOX 4 showed high expression levels in shoot apex, young leaf and adult leaf and it was strongly repressed in root. The same pattern was observed when the KNOX 5 gene was analyzed.

**Conclusions**

From the previous experiments turned out that the six KNOX genes described in literature for the model specie *Medicago truncatula* L. are also present in the alfalfa tetraploid genome. However, the mechanisms of regulation of this genes expression are not known. The present experiment shows that KNOX genes are expressed also in alfalfa plants that are cultivated *in vitro*. As it was shown all studied genes are down-regulated in given conditions, having the level close to the end of vegetation stage plant. Further experiments will imply the addition of different types of phitohormones in the culture media, followed by performing correlation between phitohormones concentration and the level of KNOX genes expressions.

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