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THE SIX *KNOX* GENES IDENTIFICATION ON TETRAPLOID *Medicago sativa*, BASED ON THE MODEL PLANT RESOURCES

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Abstract

The main goal of this work was to evaluate the genes involved in alfalfa leaf morphology as the first step of the multifoliolate trait deciphering. Because there was no information regarding alfalfa genes, the model plant Medicago truncatula L. *was considered, based on its high degree of genomic similarity. For the model plant six KNOX genes involved in leaf morphology were characterized and their transcription level in different organs was evaluated.*

The present experiments pointed out that the six KNOX genes are also present in the alfalfa tetraploid genome. The sequences are partially conserved between the both species, but some differences appeared, with possible influence on the gene functions. Therefore the length of the KNOX 2 and 3 genes transcripts are higher for alfalfa, compared with the model plant.

The statistical interpretation of the data pointed out a strongly down-regulation in roots for all the studied genes confirming their involvement in leaf morphology. An up-regulation in shoot apex and young leaf for the class I genes and in shoot apex, young leaf and adult leaf for the class II ones were also demonstrated. A different transcription pattern was shown for the genes KNOX 1 and 3, which were strongly down-regulated in all of the studied organs.

Keywords: alfalfa, leaf morphology, KNOX genes

Introduction

Alfalfa, *Medicago sativa* L., called the "Queen of the Forages" is one of the most important fodder plants all over the world due to its high biomass production and high nutritional quality. Alfalfa contains between 15 to 22% crude protein as well as an excellent source of vitamins and minerals. In addition, alfalfa 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, 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 main goal of this work was to evaluate the genes involved in alfalfa leaf morphology as the first step of the multifoliolate trait deciphering. Because there was no information regarding alfalfa genes, the model plant *Medicago truncatula* L. was considered.

Medicago truncatula L. (commonly called barrel medic) has been adopted as a reference legume species for genomic and functional genomic research, because the genetic progress of alfalfa is very slow due to its autotetrapolidy, alogamy and synthetic structure (***1***). As a member of the Papilionoideae subfamily of the legumes, *Medicago truncatula* L. is closely related to the most crop and pasture legumes, it has a relatively small, diploid genome (haploid size less than 500 Mbp), making it useful for both genetics and genomics (***10***), ease of transformation, short lifecycle and high levels of natural diversity. Due to its adoption as

model species by the international community, a number of useful tools and resources have been developed for *Medicago truncatula* L. The studies focused on possible sinteny between *Medicago truncatula* L. and *Medicago sativa* emphasized a high degree of similarity between their genomes, therefore the genomics information in *Medicago truncatula* L. could be successfully transferred to alfalfa (***6, 2***).

It is known that plants morphogenesis is based on the activity of indeterminate cell population termed shoot meristem, where members of the KNOX homeobox genes are expressed. These genes fall into two classes on the basis of aminoacid similarity within consensus domain, intron position and expression patterns. The expression of class I of the KNOX genes in vascular plants leaves is correlated with leaf compounding that arises during primary morphogenesis of the early leaf primordium, although not in cases where compounding is due to later postprimordial morphogenesis (***5***). Therefore, the KNOX1 gene expression in compound primordia is part of the typical pathway that results in the production of a mature compound leaf. Overexpression of class I KNOX genes in different plant species determine an alteration of cell differentiations followed by major changes of the whole plant architecture, leaf morphology and vascular development. Recently, a correlation between class I KNOX gene expression and cytokinins and gibberellins metabolism was establish. Class II of the KNOX genes have more diverse expression patterns (***8, 3*).**

In *Medicago truncatula* L. specie three classes I of KNOX genes where identified: KNOX1, KNOX 2, KNOX 6, and three classes II of genes: KNOX 3, KNOX 4 and KNOX 5. These six KNOX genes were isolated and preliminary characterized (***4***). Gene expression studies showed a wide organ distribution of class II Mt KNOX genes transcripts and a low transcription of the class I genes in the leaves, similar with the corresponding genes from *Arabidopsis*. Mt KNOX 1, 2 and 6 appeared to be down-regulated in cotyledons and leaves and up-regulated in the shoot apex, whereas the class II genes (Mt KNOX 3,4 and 5) were expressed in all tissues. Mt KNOX6 transcript was down-regulated also in roots.

In our work the data emphasized in *Medicago truncatula* L. 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 (*7*).

Materials and Methods

Plant material

Two fully mature wild type alfalfa individual plants, grown in the green-house were used to isolate RNA, from different organs.

RNA isolation and purification

Total RNA was isolated and purified from shoots, young leaves, adult leaves and roots, 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 25 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.

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.

Table 1. The primers sequences for the *Medicago truncatula* L. KNOX homebox genes

Genes	Primer F 5' - 3' sequence	Primer R 3' - 5' sequence
KNOX 1	ATTCATTCAATGGAGGGTAGTT	TTTTGAAAGCATGATAGAAGAGGT
KNOX 2	GAAGCGTTGACACTGGCACATC	TGGAAAATAGAAGCAACAGACACT
KNOX 3	TGCACCAAACTCCCCTAAGAT	TTCCTAAAACAAAAAGACTAA
KNOX 4	AGTGTACTGTAGACCCTCCAATG	ACCACACCTAATAATAACAACAGA
KNOX 5	GGTTCCCAATCCTCCGGTCAATGG	TCGTTATCAAGCATGCCCT
KNOX 6	CATGTGCAAGCCATTCCCTATG	GAAAAATCAACCGTTATGCCCA
control gene (MsGAPDH)	CTBGAGAGGTGGAAGAGC	GGTCAACAACTGAGACATCC

The control gene was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the most commonly housekeeping gene used in comparisons of gene expression data, due to its constitutive and continuous expression in all plant tissues.

Starting from the primers sequences and the KNOX genes identification number, the sequences and the length of the amplified fragments were determined using the National Center of Biotechnology Information (NCBI- http://blast.ncbi.nlm.nih.gov) database, operating with BLAST function.

The amplifications followed the conditions described by Di Giacomo et al., 2008 and were adjusted according to the kit supplier indications, related to the optimum temperature for enzymes proper activity: reverse transcription 45 min/ 45°C; PCR amplification - initial denaturation of cDNA 2 min/ 94° C, (35cycles) 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.

Data analysis

The gels images were analyzed by Image J software (http://rsb.info.nih.gov/ij/idex.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 the gene expression level compared with a control gene.

Results and discussions

One of this research objectives was to investigate the genes involved in alfalfa leaf morphology, starting from the KNOX genes already characterized for the model plant *Medicago truncatula* L. Based on the previous results, which demonstrated a high level of sinteny between *Medicago truncatula.* L. and *Medicago sativa* L. and a high transfer rate of molecular markers it was considered that the KNOX genes sequences could be similar for the both species.

The literature information provided the primer sequences for all of the six *Medicago truncatula* L. KNOX genes but there were no information regarding the fragments length or amplified sequences. Therefore the NCBI (http://blast.ncbi.nlm.nih.gov) database was analyzed. It was possible to determine the NCBI database identification code, liniar mRNA sequence and the length of amplified fragment for each of the six studied *Medicago truncatula* L. genes.

In the following images the sequences corresponding with *Medicago truncatula* L. KNOX genes and the primers anneal positions are presented.

1. *Medicago truncatula* L. class I KNOX homebox transcription factor (KNOX1)
NCBI database identification code AF308454;
Liniar mRNA, sequence length: 1161 bp
Length of amplified fragment: 267 bp

5’ ATTCATTCAA TGGAGGGTAG TT 3’
1 - ATTCATTCAA TGGAGGGTAG TTCTAATGGA AGTTGTTCTT ATGTTATGGG TGCTTTTGGA GAAAACAGTG GTGGGCTATG TCCTCCAATG ATGATGATGC CTTTAGTCAC TTCATCTCAT CATAATGCTC ATCATCCAAT AAATTCCAAT AACAACAACA ACAACAACAA CAACAATACT AATGCAAACA ACACAACCGG TCTTTTCCTT CCTATCCCTA ATTCCACTAA CAATAATAAC AATC
TTTTGA AAGCAT GATAGAAGAG GT - 267
5’ TTTTGA AAGCAT GATAGAAGAG GT 3’

2. *Medicago truncatula* L. class I KNOX homebox transcription factor (KNOX2)
NCBI database identification code EF128057
Liniar mRNA, sequence length: 1267 bp
Length of amplified fragment: 302 bp

5’ GAAGCGTTGA CACTGGCACATC 3’
901 - GAAGCGTTGA CACTGGCACATC TGGCACAT CGACACCAAT TGGTCGGACT TATCTTCAAT ATTATGACCA CTTTATATAT TATATTATAT TATATTATAT TATATATAGG TTAAGTGGAA AGTGAAACAA TCAAAGACTG TTTAAACTGT ATCAGTCAGC ATTATATTTA TATTATAGTT CTAGTTTCAA AGTATTATGA CAAGCATAAC ATGAAACTTG GTTATGCTTG TGCAGTGTGA ATGTTTCAGT TTTTCCCATA TTAATTGTTT TCTTT TGGA AAATAGAAGC AACAGACACT – 1182
5’ TGGA AAATAGAAGC AACAGACACT 3’

3. *Medicago truncatula* L. class II KNOX homebox transcription factor (KNOX3)
NCBI database identification code EF128058
Liniar mRNA, sequence length: 2230 bp
Length of amplified fragment: 468 bp

5’ TGCACCAAAC TCCCCTAAGA T 3’
1571-TGCACCAAAC TCCCCTAAGA TTAGTGGTTA TTACACTAAC GCCCTTCTTG TTAAGGAAAT
CACATTTACC TCTCTTGCTT TGTTATTTTT AGGGAGACTT AGTGAAGGTT ATAGTAAAGG
GGTGTGTACA ATCTATATAG CACAAACACT ACAAATTGAA ACACTACAAA TTGAAGGCTG
TGTCTAGTAG TATCTAACTT GTGTCGGTAT TTGACATAGA CACATATAGT CACATTCAAT CACTTCTATT
TTCGTAAATT ATTTACCAGT GTTTATGTAT CAGCATCAAT ATCGTGTCTG TGTCAATGCT TCATGGTGTG
TTATACTTTA AACAAAGAAG CCTGTTGAGA GGTAAAGAGC AGCATCCAAA GTAAAGGATA
TTGATGTAAT TTCAGTAACA AAAGGGATGT CAGTGTAATA AACATTCATC TTAAGAGGTC TAGTGTCA
TT CCTAAAACAAAAAGACTAA-2039
5’ TT CCTAAAACAAAAAGACTAA 3’

4. *Medicago truncatula* L. class II KNOX homebox transcription factor (KNOX 4)
NCBI database identification code EF128059
Liniar mRNA, sequence length: 1567 bp
Length of amplified fragment: 185 bp

5’ AGTGTACTGT AGACCCTCCA ATG 3’
1536 - AGTGTACTGT AGACCCTCCA ATGAGATGCA CCAAGAGATA TAATAGTAGA TATTATTATG
GTTAATTGGT TTTTACTGAA ATGTAACATA GTTTTATTTC AAAAAAGAGC TGGTTGCTGT
AAATTTCTGG AATTTCATGT ATGCACACTA TTTTTTTTGT TCTCTG
ACCA CACCTAATAA TAACAACAGA – 1536 5’
5’ ACCA CACCTAATAA TAACAACAGA3’

5. *Medicago truncatula* L. class II KNOX homebox transcription factor (KNOX 5)
NCBI database identification code EF128060
Liniar mRNA, sequence length: 1588 bp
Length of amplified fragment: 328 bp

5’ GG TTCCCAATCC TCCGGTCAAT GG 3’
209 - GG TTCCCAATCC TCCGGTCAAT GGCTCTCTCG TCCTATCCTC CACCGAAACC ATAGCGAAGT
CATCGACGAC GTCACCGGCG CCGGCATGAA GGCGGAGGAA AGCGGCGAGG CAACGGCGGA
GGGAGTGATGAACTGGCAGA AAGCGAAACA CAAAGGTGAA ATAATGGCGC ACCCGTTGTA
TGAACAGCTT CTATCAGCAC ACGTGTCGTG TTTAAGAATC GCCACGCCGG TTGACCAGTT
GCCCAGGATC GATGCTCAGC TGGCAGAGTC GCAGAATGTT GTTGCCAAGT ACTCTGCCTT
AGGACAACA TCGTTATCAAG CATGCCCT - 607
5’ T CGTTATCAAG CATGCCCT 3’

6. *Medicago truncatula L.* class I KNOX homebox transcription factor (KNOX6)
NCBI database identification code EF128061
Liniar mRNA, sequence length: 1379 bp
Length of amplified fragment: 321 bp

5’ CAT GTGCAAGCCA TTCCCTATG 3’
1058 - CAT GTGCAAGCCA TTCCCTATGG ATGCCATGCC TATGCTTCTT TAGAACATTA
ATCCTCTCAC CTTATTCAGT AAAACCCTAT CCATTTTAAA ATTACCTAGG TTGTGAAGGT
ACAATATATA TTAATTAATG TATGTTACTGTAATCTTTAA TCTGTAGAAA GGTGAGGATG
AAAAATCCAC ACTATCCCTG AACTACATCT TTATCAGATT GAACTTCAAT CTAATTATAT
ATATAAGCTA
GCTATCCTCT TCACTATTTA TATATGGAGC ATGTACCAAA GTGAAAAAAT ATTTATT
GAA AAATCAACCG TTATGCCCA – 1379
5’ GAA AAATCAACCG TTATGCCCA 3’

In the first step the RNA was extracted from young leaves collected from two alfalfa plants. 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.

Then, the one-step RT-PCR reactions were performed, following the literature recommended amplification conditions. The amplification products were separated by agarose gel electrophoresis and the image was analyzed further on (**Figure 1**).

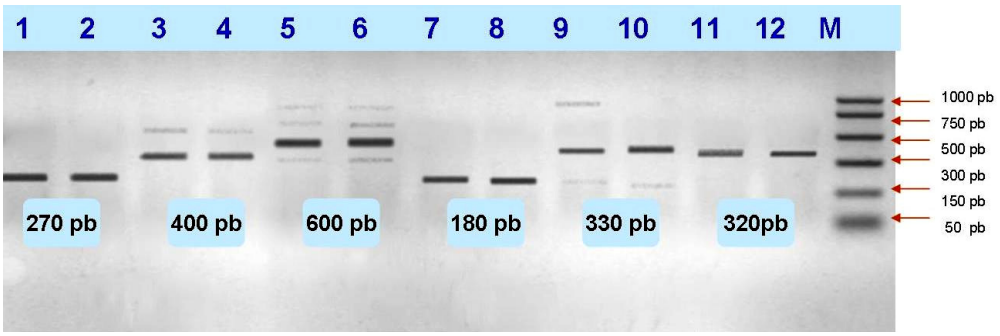

Fig. 1. The amplification products analysis, for two RNA samples, with the primers specific for the six studied genes

***1,2**- KNOX 1 gene; **3,4**- KNOX 2 gene; **5,6**- KNOX 3 gene; **7,8**- KNOX 4 gene;*
***9,10**- KNOX 5 gene; 11,12- KNOX 6 gene, M- PCR marker (Promega)*

The RNA samples extracted and purified from tetraploid alfalfa were successfully amplified for all of the six KNOX genes, using *Medicago truncatula* L. specific primers. Thus, it was demonstrated once again the expression markers transferability between the both species. Separately RT-PCR reactions were performed for each marker in order to optimize the amplification conditions for alfalfa, namely adjusting the number of amplification cycles and more important the annealing temperature.

As it was expected all the six KNOX expression markers were transferable pointing out that the *Medicago truncatula* L. KNOX genes have highly similar orthologues in *Medicago sativa* L. genome. The gel analysis by Image J software showed that there are some differences between the band intensities meaning that the expression level is not identical for all of the KNOX genes.

The fragments sizes for alfalfa were compared with those of the orthologue genes from *Medicago truncatula L.* database (Table 2). The length fragments for *Medicago sativa* L. genome were evaluated from the gel image, comparing with a molecular weight marker (*PCR marker* – Promega) (**Table 2**).

Table 2: The amplified fragment length for tetraploid *Medicago sativa* L. compared with the *Medicago truncatula L.* L. ones, available from the databases

Expression marker	**The amplified fragment length *Medicago sativa* (bp)**	**The amplified fragment length *Medicago truncatula* (according with the NCBI databases) (bp)**
KNOX 1	270	267
KNOX 2	**400**	**302**
KNOX 3	**600**	**468**
KNOX 4	180	185
KNOX 5	330	328
KNOX 6	320	321

The alfalfa transcription products for four genes, two from class I, KNOX 1 and 6 and two from class II, KNOX 4 and 5 had the same length as the *Medicago truncatula* L. ones, emphasizing great similarities between the both genomes. The length of the alfalfa transcript for KNOX 2 and 3 genes were higher compared with the model plant, probably due to some insertions or different splicing processes.

In the second part of the present research, the expression level of the six KNOX genes in different plant organs was studied. The tissues were selected according with their functions, thus, different cells types were analyzed: apical meristematic cells from shoot apex, cells involved in leaf growth after its formation from young leaves and mature and functional cells from adult leaves. Roots samples were also analyzed to confirm the KNOX gene specificity for leaf morphology.

The RNA was extracted from all of the specified tissues, diluted at the same concentration (200ng/µl) and analyzed with the KNOX genes specific primers. 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) (**Figure 2**).

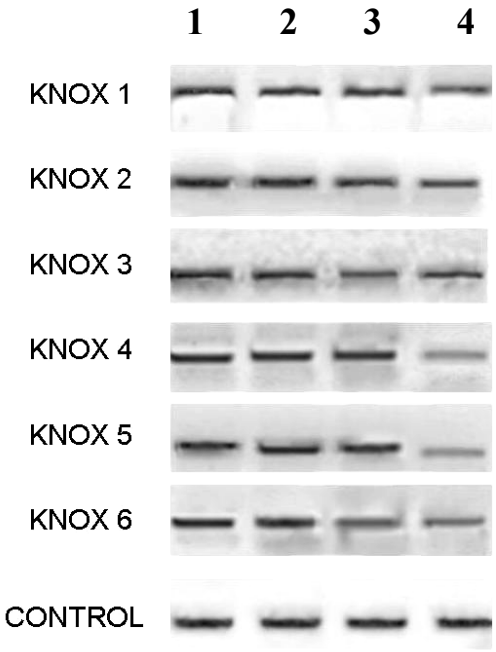

Fig. 2. The agarose gel analysis of the KNOX genes transcription products in *1 – shoot apex*, *2 – young leaf, 3- adult leaf and 4 - root, Control- glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH)*

The intensity of each band was converted into numerical value using the program ImageJ (**Figure 3**). For each experimental variant, the ratio between the KNOX gene values and the control one was determined (R ratio). These ratios were used for the experimental values statistical interpretation.

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

For the KNOX 2 gene the R ratios for shoot apex and young leaf were very closed, with a slightly higher value compared with the control. The KNOX 2 gene was down-regulated in adult leaves and roots.

The KNOX 6 gene was up-regulated in shoot apex and young leaves (R ratios higher than 1) and down-regulated in adult leaf and root.

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.

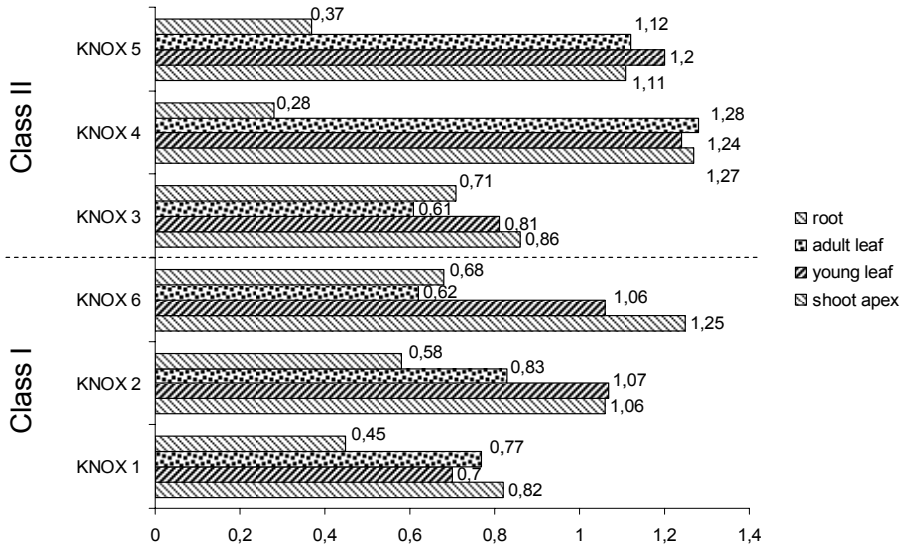

Fig. 3. The ratio between the KNOX gene transcription products and the control one (R),for different plant organs

The statistical interpretation of the data pointed out a distinct significantly negative difference between root and shoot apex and young leaf respectively. The expression level in shoot apex was significantly higher compared to young and adult leaf.

When the model plant, *Medicago truncatula* L. was analyzed it turned out that the class I genes Mt KNOX 1, 2 and 6 are down-regulated in cotyledons and leaves and up-regulated in the shoot apex. In our experiments, when tetraploid alfalfa plants were studied, the KNOX 1 gene had a different pattern, being highly down-regulated in all the organs. The genes KNOX 2 and 6 were up-regulated in shoot apex, but unlike *Medicago truncatula* L. they had also highly transcription in young leaves. The highest value was observed for KNOX 6 gene in shoot apex.

For *Medicago truncatula* L. the class II genes had a wide organ distribution. Our experiments showed a strongly down-regulation of the KNOX 3 gene in all the organs. The other class II genes had a wider distribution compared to class I genes, their transcription products being present in high amounts in all shoot apex, young and adult leaves. These genes were down-regulated only in roots.

Conclusions

From the present experiments turned out that the six KNOX genes described in literature for the model species *Medicago truncatula* L. are also present in the alfalfa tetraploid genome. The sequences are partially conserved between the both species, but some differences appeared, with possible influence on the gene functions. Therefore the length of the KNOX 2 and 3 genes transcripts are higher for alfalfa, compared with the model plant. The transcription pattern of KNOX 2 genes in the both species is similar, but the KNOX 3 gene showed a highly down-regulation in all of the studied alfalfa plant organs. A similar

pattern as the KNOX 3 was emphasized for alfalfa KNOX 1 gene, thus we can not say that the different gene expression is due to the different transcripts length.

The alfalfa studies emphasized similar results with the model plant for the class I KNOX genes (2 and 6). In both species these genes were up-regulated in shoot apex, but unlike model plant, in alfalfa they were down-regulated also in young leaf.

All of the KNOX genes were strongly down-regulated in roots, emphasizing their involvement in leaf morphology.

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