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MicroRNA (miRNA) IN FOOD RESOURCES AND MEDICINAL PLANT

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ABSTRACT

MicroRNAs (miRNAs) are a class of 19 – 24 nucleotide long non-coding RNAs derived from hairpin precursors, regulating various biological, metabolic and developmental processes at the post-transcriptional level. Many of the known miRNAs are evolutionary conserved across diverse plant species and function in the regulatory control of fundamentally important biological processes. It is known that exogenous plant miRNAs specifically target approximately 30% of protein-coding genes in mammals. The research was focused to analyze the occurrence of selected families of miRNAs (miR156, miR168 and miR171) in less used species but nutritionally important plant food resources (flax and medlar) and medicinal plant (milk thistle). The analyses were done by two individual approaches, by (a) miRNA-based molecular markers - as a novel type of functional markers and (b) qualitative Real-Time PCR. The expression pattern of selected miRNAs was analyzed depending on various plant tissues and developmental stages. Results have confirmed the significance and reliability of novel type of markers based on miRNA molecules as well as the species-specific and tissues-specific expression patterns of plants miRNAs. Significant polymorphism profile of miR156b was detected in various flax tissues of genotypes varying in the content of alpha-linolenic acid. Conclusions indicate that the variable behavior of the miRNA molecules, depending on various factors, may reflect the variability of the gene expression regulation of the human genome. The exploitation of the background of miRNA functioning within different species and plant tissues will help us to understand the molecular machinery as well as the regulatory mechanisms involved in the expression of miRNAs in plants and consequently in human genome.

Keywords: miRNA; human nutrition; functional food; medicinal plant

INTRODUCTION

Recent findings show that genetic material in plant foods may survive digestion, circulate through our bodies and modulate our gene expression **(Hirschi, 2012)**. Exogenous plant microRNAs that are primarily acquired orally, through food intake, are present in the sera and tissue of various animals **(Zhang et al., 2012)**. Microvesicles (MVs) may encapsulate these miRNAs, because these small vesicles are shed from almost all cell types. Stable microRNAs in mammalian serum and plasma are actively secreted from tissues and cells and can serve as a novel class of biomarkers for diseases, and act as signaling molecules in intercellular communication **(Zhang et al., 2012)**. MicroRNAs (miRNAs) are small RNAs that can regulate target mRNAs by binding to their 3′-UTRs **(Singh et al., 2008)**, leading to either translation delay or mRNA degradation **(Erson-Bensan, 2014)**. A single miRNA can regulate many mRNA targets, and several miRNAs can regulate a single mRNA. All miRNAs have similar secondary hairpin structures, many of these are evolutionary conserved **(Zhang et al., 2006)**. The high conservation of miRNA sequences provides an opportunity to develop a novel type of molecular markers **(Fu et al., 2013; Yadav et al., 2014; Mondal and Ganie 2014; Ganie, Mondal, 2015)**.

miRNAs have been implicated in a number of diseases, and both miRNA inhibition and activation show great promise in the treatment of various types of cancer, and viral and metabolic diseases **(Singh et al., 2008)**.

Plants miRNAs play important roles in plant development and physiology, as well as tolerance to abiotic and biotic stresses **(Taylor et al., 2014)**. Expression of miRNAs in plants involves transcription from *MIRNA* loci by RNA polymerase II, multi-step processing of the primary transcripts, pri-miRNAs by the Dicer-like complex in plants and Drosha and Dicer in animals into precursors, pre-miRNAs with a characteristic hairpin structure **(Xie et al., 2010; Zhang et al., 2006)**. Then, pre-miRNA is further cleaved to a miRNA duplex (miRNA: miRNA*), a short double-stranded RNA (dsRNA) and a mature miRNA. Finally, mature miRNAs are predominantly incorporated in the in the RNA-induced silencing complex (RICS) **(Bartel, 2004)**.

The findings of **Zhang et al., (2012)** have demonstrated that exogenous plant miRNAs in food can regulate the expression of target genes in mammals. miR156a and miR168a are abundant in rice and the miR168a is one of the most highly enriched exogenous plant miRNAs in the sera of Chinese subjects. Functional studies demonstrated that MIR168a could decrease low-density lipoprotein (LDL) removal from mouse plasma.

Lukasik and Zielenkiewicz (2014) by *in silico* approach identified in mammalian breast milk exosomes the highest abundance levels yielded the ath-miR166a (ath, *Arabidopsis thaliana*), while in the porcine breast milk exosomes, the zma-miR168a, zma-miR156a (zma, *Zea mays*) and ath- miR166a.

Several miRNA families have multiple members within the same plant species. For instance, miR395 has 18 members in rice. Although they are conserved as mature miRNA sequences, the other parts of miRNA precursor differ widely, suggesting that the different members of the same miRNA family may evolve at different rates within the same plant species **(Zhang et al., 2006)**.

As the link between metabolism and major disease processes becomes more well-defined, the identification of key molecular targets is leading to new therapeutic strategies **(Palmer et al., 2014)**. Dietary interventions have been used to change metabolism and to potentially alter disease progression. Since microRNAs may fine tune many molecular processes, it is reasonable to assume that dietary alterations that induce miRNA changes will modulate these pathways. Many microRNA families have already been associated with various nutrient interventions. MiRNA represent a link between nutrient intake, obesity and insulin resistance, and disease **(Ali et al., 2011)**.

Within our research we are focused on the exploitation of microRNA as molecular markers of plant genome characterization and mapping their activity in different plant species of nutritional and pharmaceutical importance (*Linum usitatissimum, Messpilus germanica, Silybum marianum, Hedera helix* and *Ginkgo biloba,*), plant organs and tissues (flower buds, flowers, bolls, leaves, seeds) and developmental stages (flowers development, flowering, seed development). The abundance of mature miRNAs, which is linked to the expression of *MIRNA* genes, varies greatly among different miRNAs, tissue types or developmental stages, indicating the spatially and temporally regulated expression patterns of plant miRNAs **(Xie et al., 2010)**.

Understanding the function of miRNAs in the complex molecular network regulating the development and function of various cells and tissues will increases our knowledge about the potential role of miRNAs and their involvement in gene regulation **(Singh et al., 2008)**.

MATERIAL AND METHODOLOGY

Based on the type of plant biological material was the total genomic DNA extracted either commercial isolation kit or different isolation protocols **(Saghai-Maroof et al., 1984; Padmalantha and Prasad, 2006)**. The extracted DNA was quantified by the Implen NanoPhotometer®, and diluted to 70 ng.µl^{-1}. The primers for the miRNA-based markers were designed according to the mature miRNAs sequences, which are part of the miRNA precursors (pre-miRNA), originating from the miRNA database (http://www.mirbase.org/). The single forward primers and the universal miRNA reverse primer **(Kulcheski et al., 2010; Chen et al., 2005)** were combined to perform a marker assays. The effectiveness and species transferability of used primers was confirmed in previous studies **(Hlavačková et al., 2015; Ražná et al., 2015)**.

miRNA-marker assay

Polymorphism analyzes were applied for three flax genotypes of different alpha-linolenic acid content, genotype Amon (less than 3%), Raciol (30%) and Libra (more than 57%), 5 genotypes of milk thistle of various origins, 6 genotypes of medlar and one genotype of ivy.

The modification of miRNA-based markers assay was perfomed based on methodologies **Fu et al., (2013)** and **Yadav et al., (2014)**. PCR amplifications were perfomed in a 20-µl reaction mixture containing 70 ng of genomic DNA, 10 pmol.dm^{-3} of each primer, 2 units of DreamTaq

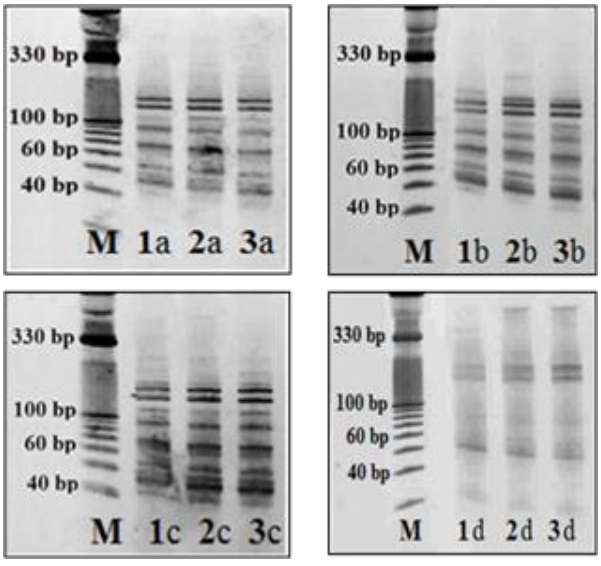

Figure 1 PCR amplification profiles generated with lus-miR168-F/miR-R markers across tissues of buds (a), flower petals (b), bolls (c), leaves (d) of flax genotypes. Legend: M- 10 bp DNA Ladder, genotypes: 1- Amon, 2- Libra, 3- Raciol.

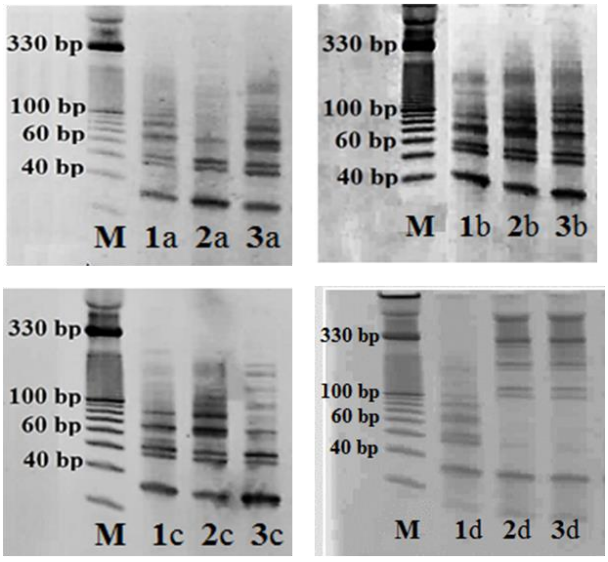

Figure 2 PCR amplification profiles generated with gm-miR156b-F/miR-R markers across tissues of buds (a), flower petals (b), bolls (c), leaves (d) of flax genotypes. Legend: M- 10 bp DNA Ladder, genotypes: 1- Amon, 2- Libra, 3- Raciol.

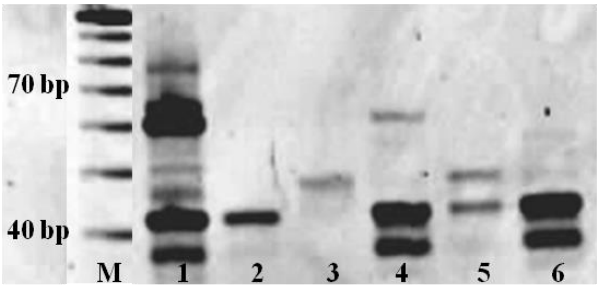

Figure 3 PCR amplification profiles generated with markers gm-miR156b/gm-miR171a of *Mespilus germanica* genotypes. Legend: M - 10 bp DNA Ladder, genotypes: 1 - Sz. Rozsa, 2 - Holandská Veľkoplodá, 3 - GR1, 4 - GR2, 5 - GR3, 6 - GR4.

DNA polymerase, 0.8 mmol.dm^{-3} dNTPs (Bioline) and 1 × DreamTaq Buffer (KCl, $(NH_4)_2SO_4$, 20 mmol.dm^{-3} $MgCl_2$). The PCR amplification programme used the 'touchdown' method as follows: initial denaturation at 94 °C for 5 min; 5 cycles of 30 s at 94 °C, 45 s at 64 °C (with a 1 °C decrease in annealing temperature per cycle), and 60 s at 72 °C; 30 cycles of 30 s at 94 °C, 45 s at 60 °C, and 60 s at 72 °C; and the final extension at 72 °C for 10 min. The samples were subsequently stored at 8 °C.

The PCR products were separated using 15% TBE-PAGE gels, running in 1 × TBE Running Buffer at a constant power 90 V, 25 mA for 120 min. The polyacrylamide gels were stained with the GelRed™ Nucleic Acid Gel stain and were visualized in the G-Box Syngene electrophoresis documentation system. For the recording of loci number and unique identification of fragments, the gels were analyzed by the GeneTools software (Syngene).

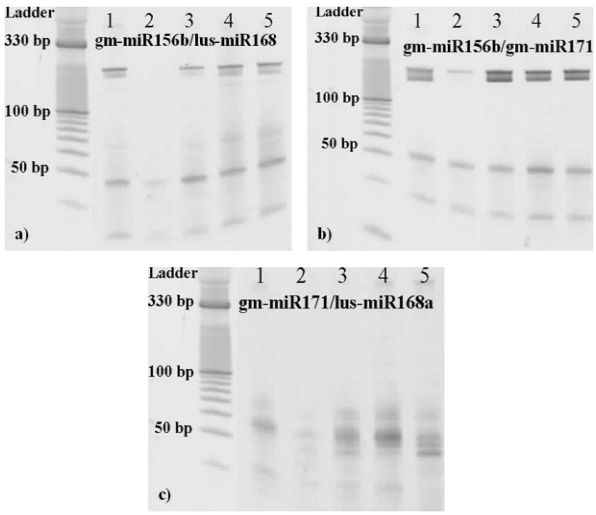

Figure 4 PCR amplification profiles generated with combination of markers: a) gm-miR156b/lus-miR168, b) gm-miR156b/gm-miR171a and c) gm-miR171a/lus-miR168 of *Silybum marianum* samples.
Legend: M - 10 bp DNA Ladder, genotypes: 1 - Silyb 1, 2 - Silyb 2, 3 - Mirel, 4 - Silma, 5 - sample of unknown origin.

miRNA expression analysis by qRT-PCR

The methodology of qRT-PCR analysis of miRNA was done based on **Barvkar et al., (2013)** and **Neutelings et al., (2012)** approach. For qRT-PCR analysis were used three genotypes of flax differing content of alpha-linolenic acid (Amon, raciol, Libra). From the 10-days old *in vitro* seedlings was isolated miRNA by PureLink miRNA Isolation Kit (Life Technologies). Consequently was miRNA diluted in 10 mM Tris-HCl, pH 7.0 in ratio 1:1 and quantified by NanoPhotometr (Implen). By means of the kit NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR (Invitrogen) was done miRNA polyadenylation and cDNA synthesis. qRT-PCR reactions were performed by SYBR® GreenER qPCR SuperMix Universal (Invitrogen) based on manufacturer instructions. cDNA was diluted in ratio 1:10. Two types of miRNA, gm-miR156b and lus-miR168 were analyzed. As a reference gene *UBE2* (Ubiquitin-conjugating enzymes E2) was selected **(Barvkar et al., 2013)**.

The conditions of qRT-PCR were as followed: 2 min incubation at 50 °C, 95 °C 10 min, 40 cycles of 95 °C 15 sec, 57 °C 60 sec and 95 °C 10 sec. Fluorescence reading of the PCR product took place after the analysis phase of the amplification and melting points were read for 30 seconds and the temperature rise of 0.5 °C. Analyzes were performed by CFX96 Real-Time detection system. Reactions were done in triplicates.

On the basis of the average value of threshold cycle of miRNA and reference gene *UBE2* value $2^{-\delta CT}$ **(Shi and Chiang, 2005)** was calculated.

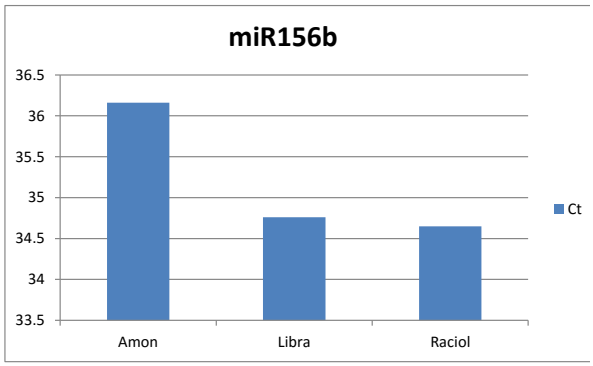

Figure 5 Comparison of gm-miR156b expression based on values of threshold cycle (C_T) in flax genotypes of different alpha-linolenic acid content.

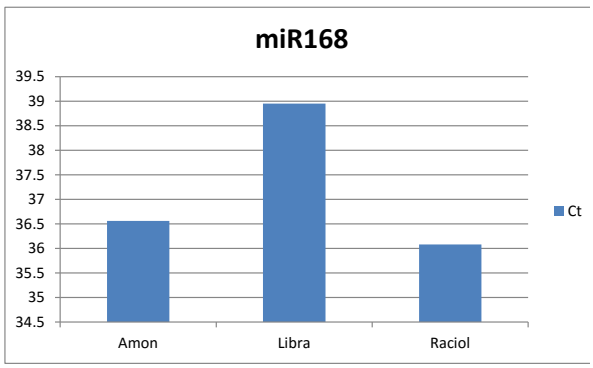

Figure 6 Comparison of lus-miR168 expression based on values of threshold cycle (C_T) in flax genotypes of different alpha-linolenic acid content.

RESULTS AND DISCUSSION

The aim of our research was mapping the abundance, polymorphism and activity of several conservative (miR156, miR168 and miR171) miRNAs in plants genome.

One of the extensively reviewed miRNA networks in plants includes the conservative miR156 family, which consists of 10 miRNAs (miR156a-j), and miRNA156a-f have identical nucleotide sequences (miRBase) **(Bari et al., 2013)**. miR156 family members are predicted to be associated with the mRNAs of genes encoding the DNa-binding proteins - squamosa promoter binding protein (SBP), transcription factor in monocot and dicots and F-box protein sequences **(Barvkar et al., 2013; Xie et al., 2010)**. SBS transcription factors regulated many developmental processes of plants. miR156 regulates processes at post-germinative stages, which is important for the transition to autotrophic growth, it regulates transition phase from the juvenile to adult stage **(Nonogaki, 2010)** and also play a critical role in reproductive phases such as shoot maturation **(Shikata et al., 2009)**. The study of **Kulcheski et al., (2010)** provided evidence that the expression stability of miR156b was the highest across the soybean tissue and applied stress conditions.

One of the target sequences of miR168 family are sequences of cytochromeP450 which is involved in a wide range of biosynthetic reactions, including fatty acid biosynthesis. The miR168 is also considered as the biomarker of plant stress response **(Bej and Basak 2014)**.

Target sequences of the miR171a are represented by HAMs genes **(Bari et al., 2013)** which belong to the GRAS transcription factor family. These genes play crucial roles in diverse fundamental processes of plant growth and development **(Huang et al., 2015)**.

Of the following figures (Figure 1, 2, 3 and 4) it is observed that molecular markers based on microRNA represent polymorphic and significant type of molecular markers. It is more apparent that the expression profile of miR156b, miR168 and miR171a is species specific and even tissue specific as confirmed by several studies (**Barvkar et al., 2013; Neutelings et al., 2012**). Tissue-specific expression of miRNA (Figure 1 and 2) also points to the different levels of miRNA activity in various stages of development of the plant organism. The same miRNA can be found in different abundance among tissue types or developmental stages, indicating the spatially and temporally regulated expression patterns of plants miRNAs **(Jones-Rhoades et al., 2006; Xie et al., 2010)**. From this point of view is quite significant polymorphism profile of miR156b in various flax tissues of genotypes varying in the content of alpha-linolenic acid (ALA). It can be observed visible difference among individual genotypes in regard to miRNA profile. Interesting is distinguished pattern of intermediate type of flax genotype Amon (less than 3% content of ALA) and other two oily genotypes with higher ALA content (Raciol 30%, Libra more than 57%). Detected polymorphism by miRNA-based molecular markers may indicate sequence changes in the miRNA loci, which consequently may change the regulation pattern of targeted genes **(Htwe et al., 2015; Fu et al., 2013)**.

Considering the indirect correlation between the abundance of miRNAs and the expression level of their target sequences **(Barvkar et al., 2013; Neutelings et al., 2012)** we can assume the spatially and temporally machinery of metabolic processes regulation as well as the expression patterns of plant miRNAs.

The effectiveness and reliability of miRNA molecular markers has been confirmed for medlar genotypes (Figure 3). Medlar as a source of new valuable compounds and their pharmacological properties, has gained a value in human consumption and commercial importance in recent years **(Rop et al., 2011)**. By the combination of miRNAs markers, miR156b and miR171a, was possible to distinguish almost all six genetic resources collected on the territory of Slovak Republic. This confirms the status that miRNA-based molecular markers comprise a novel functional molecular marker **(Yadav et al., 2014; Fu et al., 2013)**. miR171 potentially targets a beta-1,3 glycanase-like transcript. The corresponding enzyme is implicated in developmental as well as biotic and abiotic stress processes **(Roy Choudhury et al., 2010)**.

Silybum marianum (L.) Gaertn. or milk thistle is a medicinal plant of unique pharmaceutical properties. It is the most cultivated medicinal plant in Slovakia. In the years 2014-2015 it exceeded the growing area of 1000 hectares **(Habán et al., 2015)**.

Although monomorphic but miRNA-type specific microRNA profile can be observed in milk thistle genotypes of different origine (Silyb 1- Malanta, Slovak Republic, Silyb 2 - Šumperk, Czech Republic, Mirel - Brno, Czech Republic, Silma – Poland and sample of unknown origin) in two miRNA markers combination (Figure 4, a and b). The fingerprint profile amplified by primer pairs combinations ranged from 4 (gm-miR156b/gm-miR171a) to 7 (gm-miR156b/lus-miR168) miRNA loci per genotype. The another marker combination (Figure 4, c) has provided polymorphic miRNA loci pattern, even genotype specific. In comparison with previous two species, namely the flax genotypes, it can be stated that the abundance of analyzed types of miRNAs in milk thistle genome is not so significant although the studied miRNAs families represent conservative types of miRNA families. It seems that for the mapping of this genome will be required the application of species-specific miRNAs.

It is apparent that the research of food resources includes various approaches based on application of different types of molecular markers or molecular analyses **(Balážová et al., 2016; Gálová et al., 2015; Žiarovská et al., 2015)**.

Results based on qRT-PCR and evaluation of $2^{-\delta CT}$ value suggest significant difference in miR156b activity of Amon genotype (low content of ALA) in comparison to other two genotypes with medium and high content of alpha-linolenic acid (Figure 5). Within the miR168 expression analysis was the difference recorded between genotype Libra (high content of ALA) and other two genotypes Amon and Raciol (Figure 6).

The most of miRNA families, including miR156 and miR168, are characterized by negative correlation between miRNA expression and expression of their target sequences. It means, that if the expression of a specific miRNA increased, the activity of target sequences

regulated by this miRNA will be suppressed and vice versa.

The miR168 expression profile, from the above point of view, can indicate two possible explanations. As we mentioned before, most of the miRNA families have several target sequences, not excluding these two types of miRNAs. Significantly higher expression of miR168 in Libra genotype (57% ALA) points out downregulation of the target sequences, one of which is the cytochrome P450 involved in a wide range of biosynthetic reactions. It seems that the genome of this genotype mediates the production of miRNA168 increasingly over other genotypes. . It should be recalled that for the analysis were used 10-days old seedling *in vitro*. There is another explanation connected to reaction of the flax genome to stress factor presented by cultivation under *in vitro* conditions. miR168 as a stress biomarker molecule may indicate greater sensitivity of genotypes with high content of fatty acid to abiotic stress.

From the Figures 2d and Figure 5 can be observed similar pattern of miRNA expression in leaves tissues. miRNA loci profile generated by miR156b-F/miR-R markers and expression profile generated by qRT-PCR seems to show different behavior of miR156 in genotype Amon in comparison to oily genotypes Libra and Raciol. The answer might be found in the character of the major group of target sequences of miR156, what does mean the SBS transcription factors. It seems that in oily genotypes (Libra and Raciol) are, due to downregulation of miR156, its target sequences more active than in intermediate genotype (Amon), which may be associated with a plant structure of oily genotypes or indirectly with higher metabolism of fatty acids in those two genotypes. These results are confirmed by the research of **Nonogaki (2010)**. As the consequence of a decrease expression in miRNA levels is the increased accumulation of some of SPS transcripts (and proteins) which are necessary for the juvenile to adult transition in *Arabidopsis* seedlings.

The aim of the research was to highlight the broad spectrum of miRNA molecules behavior in various food resources, functional foods and medicinal plant. As was observed, different plants miRNAs accumulate at different levels depending on developmental stage or the plant tissues. It can be presumed that their regulation pattern of gene expression in human genome may be influenced also by several aspects of human metabolism and health conditions.

CONCLUSION

The aim of the research was to highlight the broad spectrum of regulatory impact activities of miRNA molecules in different plant species of nutritional and pharmaceutical uses. As has been recorded, the polymorphism and expression of analyzed gm-miR156, lus-miR168 and gm-miR171a is not only species- but also tissue- and developmentally-specific. It points out the fact that, depending on the type of food of plant origin (species, state of maturity, bio products or traditional agriculture), miRNA molecules can regulate the expression of genes of the human genome in many ways.

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