Chapter

MicroRNA-Based Markers in Plant Genome Response to Abiotic Stress and Their Application in Plant Genotyping

Katarína Ražná, Jana Žiarovská and Zdenka Gálová

Abstract

The high conservation of miRNA sequences provided an opportunity to develop an effective type of markers that is useful not only for genetic diversity study but also as potential biomarkers in plant stress responses. The fundamental potential of miRNA-based markers relies on the primer design based on the sequences of mature miRNAs, which are part of the step-loop structures. The advantages of this marker system include high polymorphism, reproducibility, and transferability across species. The abundance of mature miRNAs, which is linked to the expression of *MIRNA* genes, varies greatly among miRNAs, tissue types, or developmental stages, indicating the spatially and temporally regulated expression patterns of plant miRNAs. The results confirm the significance, reliability, and the position of miRNA-based markers as stress-sensitive biomarkers, indicating their potential in a wide range of applications of agricultural research.

Keywords: molecular markers, miRNAs, genotyping, stress-biomarkers

1. Introduction

Systematic documentation and evaluation of plant genome based on molecular markers that capture variability at the level of DNA or its protein products is essential for plant genetic resources management. These markers complement the morphological, agronomic, and other characteristics necessary to classify and identify plant genotypes within the species. Their importance and benefits are significant both for research and breeding, and for practice [1, 2]. The study and comparison of molecular information of individual organisms involves the search for DNA polymorphisms [3]. Polymorphisms are differences in DNA sequence caused by mutations. Molecular techniques in which polymorphisms can be visualized without sequencing are called molecular marker techniques [4]. A molecular marker is a specific fragment of DNA that can be identified within the whole genome and that is transmitted to the next generation following the standard rules of inheritance (Mendel's Laws). Marker may be located in or close to a gene or in noncoding regions.

The presence of polymorphisms between individuals will lead to a different pattern of markers after electrophoresis. These patterns are comparable with a "fingerprint"; therefore, these techniques are referred to as fingerprinting techniques.

These patterns reveal the DNA polymorphisms between the studied individuals. The more the individuals are related, the more their fingerprint will match. The level of polymorphisms in a group of individuals reveals the genetic diversity within this group [5].

There are different types of molecular markers. Molecular markers can be divided into two groups (a) biochemical markers, which detect variation at the gene product level such as changes in proteins and amino acids and (b) molecular markers, which detect variation at the DNA level such as nucleotide changes: deletion, duplication, inversion, and/or insertion. DNA markers are based on hybridization or PCR (amplification of DNA) [3]. The multiplex ratio is defined as the amount of markers generated with one single reaction (e.g., one PCR, one hybridization reaction). The obtained pattern can be simple (one or a few bands, low multiplex ratio) or complex (a high number of bands, high multiplex ratio).

Molecular marker methods are either dominant or co-dominant. Using co-dominant marker techniques, the different genotype combinations can be distinguished from each other at the study locus (or multiple loci). This means that homozygous (two identical alleles at a certain DNA locus) and heterozygous (two different alleles at a certain DNA locus) individuals will be identifiable. In the case of a dominant technique, it is not possible to detect the alleles that are present at a certain locus/loci, so homozygotes are not distinguishable from heterozygotes [6, 7].

The high conservation of microRNA sequences provided an opportunity to develop miRNA-based marker system referred to as stable, polymorphic, functional, and transferable genotyping technique.

2. The role of miRNAs in plant genome response to abiotic stress

The plant organism has to cope with the environmental stress in natural and agricultural conditions. The genetic background of the plant organism allows it to adapt and defend itself through different mechanisms at a molecular level.

RNA interference represents the plant immune and defense system. It is a conserved mechanism induced by double-stranded RNA (dsRNA) or hairpin-structured RNA (hpRNA). One of the modules of RNA interference is provided by the microRNA (miRNA) molecules [8–10], which are capable to form double-stranded hairpin-like structures referred to as pre-miRNA. These small molecules have significant regulatory potential in the genetic and epigenetic control of gene expression. They are one of the key players in plant genome response to abiotic and biotic stress factor(s). Especially, deeply conserved miRNA families are integral components of many regulatory networks in plant organism [11, 12].

In general, the function of miRNAs molecules in plant organisms is defined as regulatory in the following processes:

- plant growth and development
- leaf morphology and plant polarity
- root formation
- processes of transition from embryogenic to vegetative phase
- flowering time, formation of flower organs, and reproduction
- defense mechanisms through transferring of signaling molecules.

Plant adaptation mechanism requires complex modifications of gene expression machinery at the transcriptional and posttranscriptional level. Detailed studies of posttranscriptional gene regulation allow identifying stress-responsive miRNAs, which are differentially regulated under various stress factors. In plants, various abiotic stress-regulated miRNAs have been identified and characterized [13–17].

Certain families of miRNAs are either under or over-expressed, or new types of miRNAs can be synthesized under stress [13, 18]. Regulation of target genes expression by miRNA molecules is mediated by hybridization between miRNA sequences and their nascent reverse complementary sequences of mRNAs, which leads to their degradation or translational repression [19–21]. Because of their mode of action, they are generally referred to as negative regulators of gene expression.

Plant adaptation mechanism to environmental conditions includes minimization of their growth rates and reorganizing their resources. The primary focus of the adjustment is cell cycle, cell division, and cell wall constitution [22]; it means developmental processes regulated by conserved families of microRNAs.

3. MicroRNA-based markers

Genomic conservation of miRNA sequences and especially the stem-loop region of precursor molecules of miRNA (pre-miRNA) provided an opportunity to develop a novel type of molecular markers.

MicroRNA-based genotyping technique as a novel type of marker system was published in 2013 by authors Fu et al. [23]. Since then, this system has been applied to genotyping applications of *Setaria italica* and in related grass species [24]. The structure analysis of miRNA genes revealed that repetitive sequences are part of them, which led to development of miRNA-based microsatellite markers in *Oryza sativa* [25, 26] and *Medicago truncatula* and related legume species [27]. Given the origin of sequences of this type of markers, they can be considered as functional markers at the DNA levels [23, 24, 28].

The attributes of miRNA-based markers [23, 24] are as follows:

- good stability due to a direct PCR-based marker system
- improved reproducibility and sequence specificity due to high annealing temperature (more than 60°C) and the use of "touchdown" PCR approach
- relatively high polymorphism because of possible random combinations of primers
- putative functionality due to their polymorphism nature and the ability to predict phenotypes controlled by miRNAs
- cross-genera transferability potential because of the conservation level of miRNAs between species and the way of deriving markers from the consensus sequences of miRNAs.

3.1 MicroRNA-based marker assay

Following subsection provides the approach of microRNA-based marker assay applied in our laboratory. As referred in **Table 1,** the following procedure has been applied in several plant species for different research purposes.

The genomic DNA isolation protocol is based on the type of plant biological material (in terms of secondary metabolite content or other aspects). The primers for the miRNA-based markers are designed according to the mature or precursors

PCR component	Concentration	Final concentration
PCR buffer KCl, $(NH_4)_2SO_4$, 20 mmol × dm^{-3} $MgCl_2$	10×	1×
dNTP mix	2.5 mmol × dm^{-3}	0.8 mmol × dm^{-3}
Primer forward	100 μmol × dm^{-3}	10 pmol × dm^{-3}
Primer reverse	100 μmol × dm^{-3}	10 pmol × dm^{-3}
Taq polymerase	5 U	2 U

Table 1.
Protocol of miRNA-based marker assay.

Amplification process	Temperature	Time period	Number of cycles
Initial denaturation	94°C	5 min	1 cycle
Denaturation	94°C	30 s	5 cycles
Annealing	64°C (with temperature reduction of 1°C per cycle)	45 s	
Polymerization	72°C	60 s	
Denaturation	94°C	30 s	30 cycles
Annealing	60°C	45 s	
Polymerization	72°C	60 s	
Final polymerization	72°C	10 min	

Table 2.
Amplification protocol of miRNA-based markers.

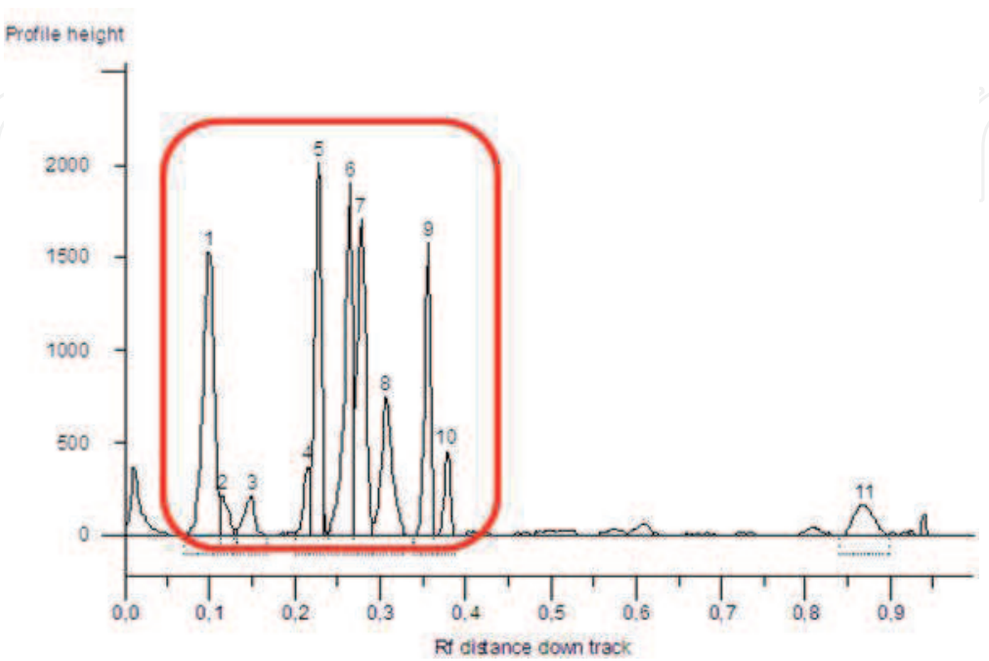

Figure 1.
Representative profile of amplified miRNA loci analyzed by GeneTool software (Syngene).

sequences (pre-miRNAs) available on the miRBase database (http://www.mirbase.org, version 22), taking into account the primer design approach of published methodology [23, 24]. The primers are combined as follows: (a) forward and reverse primers of the same type, (b) forward and reverse primers in random combinations or (c) specific forward primer and universal reverse primer [29].

The amplification protocol has originated from methodologies [23, 24] and was modified [30] (**Tables 1** and **2**). The total volume of PCR was 20 μl and the DNA concentration was 70 ng μl^{-1}. Amplification products are separated on 15% TBE-urea polyacrylamide (PAGE) gels, running in 1× TBE running buffer at constant power 180 V, 30 mA for 90 min. The gels are stained with PAGE GelRed™ Nucleic Acid Gel stain and are visualized on G-Box Syngene electrophoresis documentation system. For the recording of loci number and their position, as well as the identification of unique fragments, the gels are analyzed by GeneTools software (Syngene) (**Figure 1**).

4. Contribution of miRNA-based markers on plant genome response to abiotic stress and for genotyping applications

Genomic polymorphism of plants is the basis of their survival and ability to different climatic conditions. The cognition and mapping of plant genome variability using molecular markers is a prerequisite for extending the genetic base of crops to reduce their susceptibility to adverse environmental conditions [26]. An ideal molecular marker should be polymorphic, stable, reproducible, providing sufficient resolution, fast, and with fairly low cost [31]. The miRNA-based marker system is characterized by relatively high polymorphism, reproducibility, transferability across species, and ease of use with putative functionality [23]. The high level of transferability demonstrates the usability of miRNA-based markers for comparative genome mapping and phylogenetic studies [24].

5. MiRNA-based markers in genotyping applications

Recognizing relationships between species or within species help to focus more closely on a wide range of human interests, from basic description and disaggregation, through efficient resource genetic management to the production of quality and safe food, whether plant or animal origin [32].

Within our research, we focused on the use of miRNA-based markers to highlight their broad spectrum of regulatory impact activities in different plant species of nutritional and pharmaceutical uses (**Table 3**): flax (*Linum usitatissimum* L.), medlar (*Messpilus germanica* L.), milk thistle (*Silybum marianum* (L.) Gaertn.), ginkgo (*Ginkgo biloba* L.), common ivy (*Hedera helix* L.), avocado (*Persea americana* Mill), and ribwort plantain (*Plantago lanceolata* L.). A total of 13 miRNA-based markers were applied of which 28 primer combinations were made.

Useful molecular markers produce fragments between 150 and 500 bp in length, as this size of fragments can easily be distinguished using agarose or PAGE gels [23]. In our experiments, this size of fragments varied predominantly from 40 to 300 bp and could be clearly identified on agarose gels (**Figure 2**).

This range is due to variability of stem-loop structure of which the mature miRNA sequences are part of and the length of stem-loop structure ranges from less than 100 to over 900 nt. The primers' design based on miRNAs sequences can be linked to different places of the same stem-loop structure. Another possibility is that primers amplify regions between neighboring miRNAs [23].

Research purpose	Plant species	Reference
Genome profiling with regard to genotype origin	*Linum usitatissimum*	[30]
Spatial and temporal abundance of individual miRNA markers	*Linum usitatissimum*	[33, 34]
Functional markers of commercial type of the crop	*Linum usitatissimum*	[35, 36]
Genotyping	*Mespilus germanica*	[36]
Genomic authentication of varieties	*Silybum marianum*	[37]
Genotyping	*Ginkgo biloba*	[38, 39]
Genotyping	*Hedera helix*	[40]
Genotyping	*Persea americana*	—
Genotyping	*Plantago lanceolata*	—

Table 3.
The list of realized miRNA-based markers experiments.

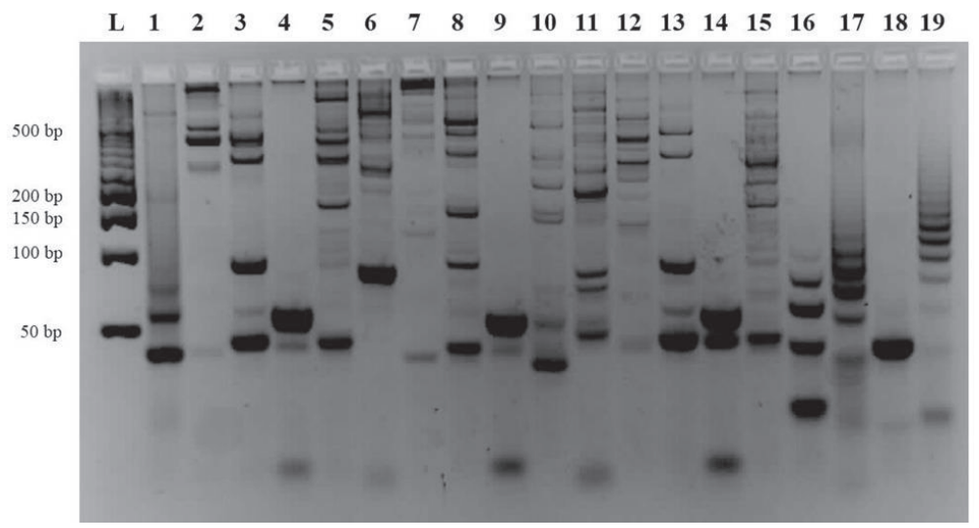

Figure 2.
Representative gel showing amplification profile of miRNA-based markers of Plantago lanceolata (L.). 1–19 combinations of primers. F—forward, R—reverse. 1: lus-miR-R + lus-miR168F, 2: lus-miR-R + gm-miR156bF, 3: lus-miR-R + hyp-miR414F, 4: lus-miR-R + gm-miR171aF, 5: lus-miR-R + lus-miR156aF, 6: gm-miR-R + lus-miR168F, 7: gm-miR-R + gm-miR156bF, 8: gm-miR-R + hyp-miR414F, 9: gm-miR-R + gm-miR171aF, 10: gm-miR-R + lus-miR156aF, 11: miR-R + lus-miR168F, 12: miR-R + gm-miR156bF, 13: miR-R + hyp-miR414F, 14: miR-R + lus-miR171aF, 15: miR-R + lsa-miR156aF, 16: lsa-miR169aF + lsa-miR169aR, 17: lus-miR156aF + lus-miR156aR, 18: hvu-miR827F + hvu-miR827R, 19: lsa-miR396aF + lsa-miR396aR.

The level of polymorphism varied from 70 to 90%. Mature miRNAs are expressed as small 21–24 nt endogenous molecules. As a result of different transcriptional activity among *MIRNA* genes, the miRNAs abundance in the cell varies greatly in dependence of miRNA family [11]. It should be noted that the level of polymorphism depended on the effectiveness of primer combination as well as the level of marker transferability.

The results of the studies have repeatedly confirmed the following:

- MicroRNA-based markers show the cross-genera transferability potential.

- MicroRNA-based markers display sufficient level of polymorphism in analyzed genotypes and are suitable to differentiate within genotypes of one specimen.

- MicroRNA-based markers provide genotype-specific profile of miRNA loci.

- The abundance of selective miRNA-based markers is tissue specific and developmental specific.

5.1 MiRNA-based plant genome response to abiotic stress conditions

Different mechanism of stress response contributes to stress tolerance or resistance at different morphological, biochemical, and molecular level [13]. Many stress-regulated genes are found to be regulated by miRNAs.

In the flax study, we applied nutritional stress factor under *in vitro* conditions [41]. The genome response of the flax genotype CDC Bethune was analyzed under five variants (including control variant) of Murashige-Skoog [42] medium by two miRNA-based markers, lus-miR395 and lus-miR399 [16, 17]. The results show that flax genome responds to the nutritional stress stimulus. Our results have supported the capability of miRNA-based molecules as potential biomarkers of abiotic stress factors.

Another study was conducted in order to test the ultrasound-induced oxidative stress in lettuce (*Lactuca sativa* L.) tissues by miRNA-based markers [43]. We have confirmed that reactive oxygen species (ROS), caused by sonication treatment, induced the polymorphism at the molecular level detected by miRNA-based stress markers. We have observed the statistically significant differences ($p \leq 0.01$) in miRNA markers ability to detect this polymorphism. The response of miR168 marker was statistically more sensitive in comparison with miR156 marker as a result of their specific regulatory nature.

The aim of research into the impact of soil compaction was to identify the barley (*Hordeum vulgare* L.) genome response by stress-responsive miRNA-based markers. A prerequisite for the research was that the plants are exposed to a lack of soil moisture and nutrients due to soil compaction. The effect of soil compaction was analyzed by four different miRNA-based markers (hvu-miR156, hvu-miR399, hvu-miR408, and hvu-miR827), within the leaf, stem, and root tissues of barley plants. We can state that due to soil compaction, the barley plants were exposed to the lack of moisture which subsequently affected the intake and utilization of nutrients from the soil and showed lower plant growth parameters and reduced the yields. Moreover, this genome response was tissue specific. The roots were most affected by dehydration, and the nutrient deficiency was the most pronounced on leaves. The number of amplified miRNA loci was statistically significantly dependent on the stress-sensitive marker applied.

We have conducted experiments in connection with research on the drought resistance of wheat (*Triticum aestivum* L.). Genomes of susceptible and drought-resistant genotypes were screened by stress-sensitive miRNA-based markers (hvu-miR408 and hvu-miR827). Genotypes were tested under *in vitro* conditions on Murashige-Skoog culture medium with different concentrations (0, 5, 10, 15, and 20%) of polyethylene glycol (PEG 6000) to induce dehydration stress. Drought-resistant wheat genotypes responded to dehydration stress, by significantly higher activity of hvu-miR408 biomarker in comparison with susceptible genotypes. This response points to a better genome adaptation ability of the resistant genotypes to abiotic stress. By using the conserved type marker hvu-miR156, which is involved in the regulation of plant growth and development processes, a reduced activity of this type of marker was observed, both in susceptible and resistant genotypes, indicating that the adaptation mechanism of plants to cope with stress conditions is implemented at the expense of growth processes.

6. Conclusions

It can be summarized that the marker system based on microRNA molecules represents (a) flexible marker system based on sequences of regulatory molecules, (b) species-transfer system due to the conserved nature of mature sequences of miRNAs, (c) functionally potential markers, where observed polymorphism points to changes in miRNA loci sequences evoking changes in the target gene regulatory model, (d) tissue-specific and development-specific characters of markers, and (e) screening tool of genome adaptation changes to induced abiotic stress referred to as stress-sensitive biomarkers. It should be noted that in selecting suitable type(s) of miRNA markers for a particular type of study, it is necessary to know the regulatory background, regulation mechanism, and target sequences of particular type of miRNA molecules.

Acknowledgements

This work was supported by the project of Slovak Scientific Agency of Ministry of Education of the Slovak Republic, VEGA, No. 1/0246/18 and Slovak Research and Development Agency APVV-15-0156.

Conflict of interest

The authors declare no conflict of interest.

Author details

Katarína Ražná[1*], Jana Žiarovská[1] and Zdenka Gálová[2]

1 Department of Genetics and Plant Breeding, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Nitra, Slovak Republic

2 Department of Biochemistry and Biotechnology, Faculty of Biotechnology and Food Science, Slovak University of Agriculture in Nitra, Nitra, Slovak Republic

*Address all correspondence to: katarina.razna@uniag.sk

References

[1] Gálová Z, Balážová Ž, Chrenek P, Chňapek M, Libantová J, Matušíková I, et al. Metódy a techniky génových manipulácií [Methods and Techniques of Gene Manipulation]. Nitra: Slovenská poľnohospodárska unvierzita; 2013. 189 p. ISBN 978-80-552-1092-6

[2] Gálová Z, Balážová Ž, Tomka M, Vivodík M, Chňapek M, Oslovičová V. Bielkovinové a DNA markery jačmeňa siateho. [Barley Protein and DNA Markers.]. Nitra: Slovenská poľnohospodárska unvierzita. 2016. 130 p. ISBN 978-80-552-1555-6

[3] Malik AZ, Usha K, Athar KA. Plant Biotechnology: Principles and Applications. Singapure: Springer Nature Singapure Pte Ltd; 2017. 392 p. DOI: 10.1007/978-981-10-2961-5

[4] Clark DP. Molecular Biology. Understanding the Genetic Revolution. USA: Elsevier Academic Press; 2005. 784 p. ISBN-10: 0-12-175551

[5] Batley J. Plant Genotyping. Methods and Protocols. New York: Humana Press. Springer Science + Business Media; 2015. 315 p

[6] Lodish H, Baltimore D, Berk AS, Zipursky L, Matsudaira P, Darnell J. Molecular Cell Biology. 3rd ed. USA: Scientific American Books; 1998. 1344 p

[7] Ricroch A, Chopra S, Fleischer SJ. Plant Biotechnology. Experience and Future Prospects. Switzerland: Springer International Publishing; 2014. 291 p. DOI: 10.1007/978-3-319-06892-3

[8] Baulcombe D. RNA silencing in plants. Nature. 2004;**431**:356-363. DOI: 10.1038/nature02874

[9] Guo Q, Liu Q, Smith NA, Liang G, Wang M-B. RNA silencing in plants. Mechanisms, technologies and applications in horticultural crops. Current Genomics. 2016;**17**:476-489. DOI: 10.2174/1389202917666160520103117

[10] Voinnet O. RNA silencing as a plant immune system against viruses. Trends in Genetics. 2002;**17**:449-459. DOI: 10.1016/S0168-9525/(01)02367-8

[11] Cuperus JT, Fahlgren N, Carrington JC. Evolution and functional diversification of *MIRNA* genes. The Plant Cell. 2011;**23**:431-442. DOI: 10.1105/tpc.110.082784

[12] Xie Z, Khanna K, Ruan S. Expression of microRNAs and its regulation in plants. Seminars in Cell and Developmental Biology. 2010;**21**:790-797. DOI: 10. 1016/j. semedb.2010.03.012

[13] Bej S, Basak J. MicroRNAs: The potential biomarkers in plant stress response. American Journal of Plant Sciences. 2014;**5**:748-759. DOI: 10.4236/ajps.2014.55089

[14] Chiou TJ. The role of microRNAs in sensing nutrient stress. Plant, Cell and Environment. 2007;**30**:323-332. DOI: 10.1111/j.1365-3040.2007.01643.x.

[15] Kruszka K, Pieczynski M, Windels D, Bielewicz D, Jarmolowski A, Szweykowska-Kulinska Z, et al. Role of microRNAs and other sRNAs of plants in their changing environments. Journal of Plant Physiology. 2012;**169**:1664-1672. DOI: 10.1016/j.jplph.2012.03.009

[16] Melnikova NV, Dmitriev AA, Belenikin MS, Speranskava AS, Krinitsina AA, Rachinskaia OA, et al. Excess fertilizer responsive miRNAs revealed in *Linum usitatissimum* L. Biochemie. 2015;**109**:36-41. DOI: 10.1016/j.biochi.2014.11.017

[17] Melnikova NV, Dmitriev AA, Belenikin MS, Koroban NV, Speranskaya AS, Krinitsina AA, et al.

Identification, expression analysis, and target prediction of flax genotroph microRNAs under normal and nutrient stress conditions. Plant Biotechnology. 2016;**7**:1-12. DOI: 10.3389/fpls.2016.00399

[18] Pacak-Barciszewska M, Milanowska K, Knop K, Bielewicz D, Nuc P, Plewka P, et al. Arabidopsis microRNA expression regulation in a wide range of abiotic stress responses. Frontiers in Plant Science. 2015;**6**:410. DOI: 10.3389/fpls.2015.00410

[19] Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell. 2004;**116**:281-297. DOI: 10.1016/S0092-8674(04)00045-5

[20] Erson-Bensan AE. Introduction to microRNAs in biological systems. In: Yousef M, Allmer J, editors. MicroRNAs Biology and Computational Analysis. New York: Springer Science+Business Media; 2014. pp. 1-14. ISBN 978-1-62703-747-1

[21] Jones-Rhoades MW, Bartel DP, Bartel B. MicroRNAs and their tegulatory roles in plants. Annual Review of Plant Biology. 2006;**57**:19-53. DOI: 10.1146/annurev.arplant.57.032905.105218

[22] Rajwanshi R, Chakraborty S, Jayanandi K, Deb B, Lightfoot DA. Orthologous plant microRNAs: Microregulators with great potential for improving stress tolerance in plants. Theoretical and Applied Genetics. 2014;**127**:2525-2543. DOI: 10.1007/s00122-014-2391-y

[23] Fu D, Ma B, Mason AS, Xiao M, Wei L, An Z. MicroRNA-based molecular markers: A novel PCR-based genotyping technique in *Brassica* species. Plant Breeding. 2013;**132**:375-381. DOI: org/10.1111/pbr.12069

[24] Yadav CBY, Muthamilarasan M, Pandey G, Prasad M. Development of novel microRNA-based genetic markers in foxtail millet for genotyping applications in related grass species. Molecular Breeding. 2014;**34**:2219-2224. DOI: 10.1007/s11032-014-0137-9

[25] Mondal TK, Ganie SA. Identification and characterization of salt responsive miRNA-SSR. Gene. 2014;**535**:204-209. DOI: 10.1016/j.gene.2013.11.033

[26] Ganie SA, Mondal TK. Genome-wide development of novel miRNA-based microsatellite markers of rice (*Oryza sativa*) for genotyping applications. Molecular Breeding. 2015;**35**:51. DOI: 10.1007/s11032-015-0207-7

[27] Min X, Zhang Z, Liu Y, Wei X, Liu Z, Wang Y, et al. Genome-wide development of microRNA-based SSR markers in *Medicago truncatula* with their transferability analysis and utilization in related legume species. International Journal of Molecular Sciences. 2017;**18**:2440. DOI: 10.3390/ijms18112440

[28] Htwe NMPS, Luo ZQ, Jin LG, Nadon B, Wang KJ, Qiu LJ. Functional marker development of miR1511-InDel and allelic diversity within the genus glycine. MBC Genomics. 2015;**16**:467. DOI: 10.1186/s12864-015-1665-3

[29] Kulcheski FR, Marcelino-Guimaraes FC, Nepomuceno AL, Abdelnoor RV, Margis R. The use of microRNAs as reference genes for quantitative polymerase chain reaction in soybean. Analytical Biochemistry. 2010;**406**:185-192. DOI: 10.1016/j.ab.2010.07.020

[30] Ražná K, Nôžková J, Hlavačková L, Brutch N, Porokhovinova E, Shelenga T, et al. Genotyping of flax genetic resources by miRNA-based molecular markers and morphology. Agriculture. 2015;**61**:129-138. DOI: 10.1515/agri-2015-0018

[31] Poczai P, Varga I, Laos M, Cseh A, Bell N, Valkonen JPT, et al. Advances in plant gene-targeted and functional markers: A review. Plant Methods. 2013;**9**:6. Available from: http://www.plantmethods.com/content/9/1/6

[32] Žiarovská J, Ražná K, Bežo M. Nestabilné a opakujúce sa prvky genómu ľanu siateho ako molekulárne markéry [Unstable and Repeating Elements of the Flax Genome Genome as Molecular Markers]. Nitra: Slovenská poľnohospodárska unvierzita; 2011. 80 p. ISBN 978-80-552-0633-2

[33] Hlavačková L, Nôžková J, Porokhovinova E, Brutch N, Shelenga T, Bjelková M, et al. Analysis of miRNA polymorphism during the selected developmental processes of flax. Journal of Central European Agriculture Online. 2016;**17**:707-724. DOI: 10.5513/JCEA01/17.3.1767

[34] Ražná K, Hlavačková L. The applicability of genetic markers based on molecules microRNA in agricultural research. Open Access Journal of Agricultural Research. 2017;**2**:000125. DOI: 10.23880/OAJAR-16000125

[35] Ražná K, Hlavačková L. MicroRNA analysis of flax (*Linum usitatissimum* L.) genotypes in regard to alpha-linolenic acid content. In: Plant breeding: The Art of Bringing Science to Life. EUCARPIA. Zürich: Eidgenössische Technische Hochschule; Online; 2016. 426 pp. ISBN 978-3-906804-22-4

[36] Ražná K, Bežo M, Hlavačková L, Žiarovská J, Miko M, Gažo J, et al. MicroRNA (miRNA) in food resources and medicinal plant. Potravinárstvo. 2016;**1**:188-194. DOI: 10.5219/583

[37] Ražná K, Hlavačková L, Bežo M, Žiarovská J, Habán M, Sluková Z, et al. Application of the RAPD and miRNA markers in the genotyping of *Silybum marianum* (L.) Gaertn. Acta Fytotechnica et Zootechnica. 2015;**18**:83-89. DOI: 10.15414/afz.2015.18.04.83-89

[38] Ražná K, Hrubík P. Ginkgo dvojlaločné (*Ginkgo biloba* L.)—genomická štúdia a kultúrne rozšírenie na Slovensku [*Ginkgo biloba* L.—Genomic Study and Cultural Area of Expansion in Slovakia]. Nitra: Slovenská poľnohospodárska unvierzita; 2016. 92 p. ISBN 978-80-552-1594-5

[39] Ražná K, Žiarovská J, Hrubík P, Batyaneková V, Vargaová A. Ecologically conditioned imprinting of miRNA-based profiles of *Ginkgo biloba* L. growing in Slovakia. Folia Oecologica. 2019;**46**:54-62. DOI: 10.2478/foecol-2019-0008

[40] Žiarovská J, Bošeľová D, Zeleňáková L, Bežo M. Utilization of different markers for *Hedera helix* L. germplasm evaluation. Journal of Microbiology, Biotechnology and Food Sciences Online. 2016;**5**:23-26. DOI: 10.15414/jmbfs.2016.5.special1.23-26

[41] Hlavačková L, Ražná K. Polymorphism of specific miRNAs in the context of flax (*Linum usitatissimum* L.) genome adaptability to abiotic stress. In: MendelNet 2015. CD-ROM. Brno: Mendel University; 2015. 615 p. ISBN 978-80-7509-363-9

[42] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiologia Plantarum. 1962;**15**:473-497. DOI: 0.1111/j.1399-3054.1962.tb08052.x

[43] Ražná K, Khasanova N, Ivanišová E, Qahramon D, Habán M. Antioxidant properties of cumin (*Bunium persicum* Boiss.) extract and its protective role against ultrasound-induced oxidative stress tested by microRNA based markers. Potravinárstvo Online. 2018;**12**:11-19. DOI: 10.5219/838