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FINGERPRINT CHARACTERISTICS AND EXPRESSION VARIABILITY OF miRNA BASED MARKERS IN WHEAT VARIETIES WITH DIFFERENT SUSCEPTIBILITY TO DROUGHT

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Abstract

Drought susceptible (Aladin, Dagmar) and drought tolerant (Seladon, Venturero) varieties of summer wheat were examined in experiment. Genotypes were tested under *In vitro* conditions on Murashige-Skoog culture medium with different concentrations (0, 5, 10, 15 and 20 %) of polyethylene glycol to induce dehydration stress conditions. Genomic response of seedlings was tested by stress-sensitive miRNA markers, tae-miR156, tae-miR408 and tae-miR827. In general, the fingerprints generate activity of tae-miR156 was higher in resistant genotypes in comparison to susceptible ones and lower in tae-miR408. The genome response to induced dehydration stress was genotype-specific, what has been statistically proven. The phosphate-induced tae-miR827 marker activity was balanced, since the optimal nutritional composition of the medium, create appropriate conditions to cope with the stress factor. The expression levels of analysed miRNA correspond to the differences in obtained fingerprints what confirmed the DNA based analysis of miRNAs markers as a very good screening tool for their analysis in plat genomes.

Key words: Wheat, Drought tolerance, miRNAs-based markers, Expression.

Introduction

Triticum aestivum L., is one of the basic crops used in human nutrition that is widely grown in a range of environments all over the world (Shewry & Hey, 2015). Wheat is very variable for the response of its genome in different agroclimatic zones (Ashraf *et al*., 2003). Currently there is reported a limited water supply for irrigation in 70% of the land in areas for wheat cultivation and this may increase in future and would thus become the major cause of limiting global wheat production (Wheeler & von Braun, 2013). Therefore, development of drought-resilient and water-use efficient cultivars is a thrust area of research for wheat breeders to meet the future demands of wheat production.

Drought is a very common stress that plants face and therefore it is one of the research interest of actual agriculture programmes. Drought stresses occur in plants at any growth stage and depends on the local environment and different varieties of one specie should be tested for their tolerating drought. The adverse effects of drought are also recognizable at the level of phytohormonal regulation of growth processes (Aschari & Nadia, 2003; Aschari & Samina, 2010). Drought tolerant plants have less reduction in water content, membrane stability, and photosynthetic activity. The tolerant group tries to accumulate soluble sugars, proline content, amino acids, chlorophyll content and enzymatic and non-enzymatic antioxidant activities (Abid *et al*., 2016). Many different types of markers were analysed and identified yet in wheat genome that are related to drought tolerance from classical up to the very modern ones. Length polymorphism-based markers such as RAPD, SSR or SCoT were applied to discriminate different wheat germplasm or for their response to drought stress (Huseynova & Rustamova, 2010; Bousba *et al*., 2012; Kuťka-Hlozáková *et al*., 2016). Multilocus genome wide association mapping in wheat under different water regimes were performed by Gahlaut *et al*., (2019).

Some of the new approach-based marker mapping in plants is connected to the biological characteristics of microRNAs molecules. The miRNAs are short endogenous non-coding RNAs that are derived from single-stranded RNA precursors (Barvkar *et al*., 2013). The miRNA molecules are produced from the non-coding mRNAs, which undergo different processing steps to form a mature miRNA form a precursor. They possess an ability to bind to the target mRNAs that results either in a translation delay or mRNA degradation (Erson-Bensan, 2014). miRNAs were reported previously to control gene expression of plants under various biotic and abiotic stress as well as in different developmental stages (Mondal & Ganie, 2014). The plant miRNAs are embedded in regulatory networks that coordinate different gene expression programmes in support of developmental plasticity (Rubio-Somoza & Weigel, 2011).

DNA-based molecular markers are an integral part of the research of genomes of plant genetic resources. MicroDNA sequences are common and abundant in the plant genomes where most annotated miRNAs are in intergenic regions (Fu *et al*., 2013; Cuperus *et al*., 2011). These sequences are highly conserved and hence belong to the useful markers for studying genetic diversity (Ganie & Mondal, 2015). The high conservation of miRNA sequences allows their use as molecular markers, as the miRNA-based primers might amplify not only its own

sequences but the regions between the neighbouring miRNAs, too. A good knowledge exists for the mechanisms of expression of various genes associated with drought, but the research on the roles of miRNAs involved in drought tolerance is still ongoing (Zhao *et al*., 2010; Figueroa *et al*., 2011; Sunkar *et al*., 2012)

Here, a miRNA fingerprints and expression levels were analysed for wheat varieties with different physiological response to drought. Drought susceptible (Aladin, Dagmar) and drought tolerant (Seladon, Venturero) varieties of summer type of *Triticum aestivum* L. were examined in experiment.

Material and Methods

Plant material and drought treatment: Biological material of the winter wheat summer form was provided by the research institute of the VÚRV, Piešťany, Slovak Republic. According to the susceptibility index testing results (Žofajová *et al*., 2018), the drought tolerant varieties Seladon and Venturero were selected together with the drought susceptible varieties Aladin and Dagmara. The conditions of dehydration stress were induced by treatment with polyethylene glycol at various concentrations (PEG 6000; 0; 5; 10; 15 and 20%) under *in vitro* conditions on Murashige & Skoog medium (1962) according to the protocol of Nawaz *et al*., (2013).

Nucleic acids extraction and analysis: The leaves of 6-week-old plants from *In vitro* conditions were used to extract genomic DNA according to the Rogers & Bendich (1994). Total RNA from the non-treated wheat varieties planted under the *In vitro* conditions was extracted by GeneJET™ Plant RNA Purification Mini Kit (Thermo Scientific) according to the manufacturer´s recommendations. Quality and concentrations of extracted nucleic acids were quantified by Nanodrop (Implen).

Methodological procedure of application of miRNA-based DNA markers was realized according to the Ražná *et al*., (2015). cDNA transcription was performed by RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific) using 1000 ng of total RNA and random hexamer primers. Primers used for the analysis are listed in Table 1.

Elongation factor alpha was used as housekeeping gene Lopato *et al*., (2006) to normalize the expression of analysed miRNAs against the Aladin variety, that was used as control genotype to all the analysed ones. qRT-PCR reactions were performed in CFX thermocycler (Biorad). The reactions were performed in triplicates in Maxima SYBR Green qPCR Master Mix (Thermo Scientific) with 10-fold diluted cDNA. The temperature and time conditions were as follows: 95°C 3 minutes, 40 cycles of 95°C 15 seconds and 60°C 25 seconds with fluorescence reading. Melting curves of generated amplicons were determined to check the specifity.

Data processing: miRNA based DNA amplicons were separated in 15% PAGE and scored for their length and gel migration characteristics in GeneTools gel analysis software (Syngene). Multi-factorial ANOVA was performed for the number of amplified miRNA loci for comparing the effect of treatment, wheat variety and miRNA markers in fingerprint characteristics of individual concentration of PEG for analysed genotypes. Standard curves were prepared by 5 serial dilutions of Aladin wheat variety cDNA on 10-fold diluted cDNA. MiRNA quantification was performed according to the Pfaffl, (2001).

Table 1. miRNA based primers and housekeeping gene primers used for the analysis.

Primer name	Primer sequence
tae-miR156	F:GCGGCGGTGACAGAAGAGAGAGT R:GTGCAGGGTCCGAGGT
tae-miR408	F:GCGGCGGATGCACTGCCTCTTC R: GTGCAGGGTCCGAGGT
tae-miR827	F:GCTACCCATGAACCTGTTTTG R:ACAAGTTCGTGAGACGCATGC
TaE EF1a	F: CAGATTGGCAACGGCTACG R: CGGACAGCAAAACGACCAAG

Results and Discussion

The genomic response of wheat varieties to the induced drought conditions was tested through the activity of three types of stress-sensitive miRNA markers, tae-miR156, tae-miR408, and tae-827miRNA. The Seladon and Venturero varieties are characterized by drought tolerance and the varieties Aladin and Dagmar are prone to this stress factor. Genotypes were tested under *In vitro* conditions on Murashige-Skoog culture medium with varying percentages (0, 5, 10, 15 and 20%) of polyethylene glycol (PEG 6000) to induce dehydration conditions. MiR156 and miR408 are identified as stress-responsive miRNAs mediating the response of the plant genome to dehydration stress (Akdogan *et al*., 2016). Dehydration stress activates these molecules. The conserved class of miR-156 molecules is involved in the regulation of growth processes and plant development (Xie *et al*., 2012; Barvkar *et al*., 2013). MiR156 is highly active in embryos and seedlings, and with increasing plant age its expression decreases (Spanudakis & Jackson, 2014). The tae-miR827 marker plays an important role in regulatory mechanisms related to nutrient homeostasis. It is characterized by increased activity in conditions of phosphorus deficiency (Bej & Basak, 2014; Melnikova *et al*., 2016; Pacak-Barciszewska *et al*., 2016). Target genes that are regulated by miR827 molecules encode proteins involved in phosphorus transport.

The genome response to dehydration stress was genotype specific, and the fingerprint relationships were confirmed to be statistically highly relevant ($p \leq 0.01$) (Table 2). At the same time, statistical analysis confirmed the functionality of individual types of applied markers, as each of these, statistically highly demonstrable effect of wheat genome response to dehydration stress due to specific regulatory function of applied miRNA markers in various biological processes. The concentration of polyethylene glycol (5-20%) applied for the purpose of inducing dehydration stress was not a statistically significant factor affecting the activation of stress-sensitive miRNA molecules. The activity of the miRNA, due to the plant's genome's dry response, was found here to be varietal dependent. Similar was reported in (Barrera-Figueroa *et al*., 2012). For susceptible genotypes, the activity of the tae-

miR156 marker was higher than for sensitive genotypes. The most noticeable difference in generated fingerprints was observed by PAGE between the tolerant Venturero variety and the susceptible variety Aladin. Very similar profile was obtained for the expression levels of tae-miR156. The difference in its expression was about 20% (Fig. 1). The efficiency of the qRT-PCR was 0.94 for tae-miR156 and 0.99 for housekeeping elongation factor gene.

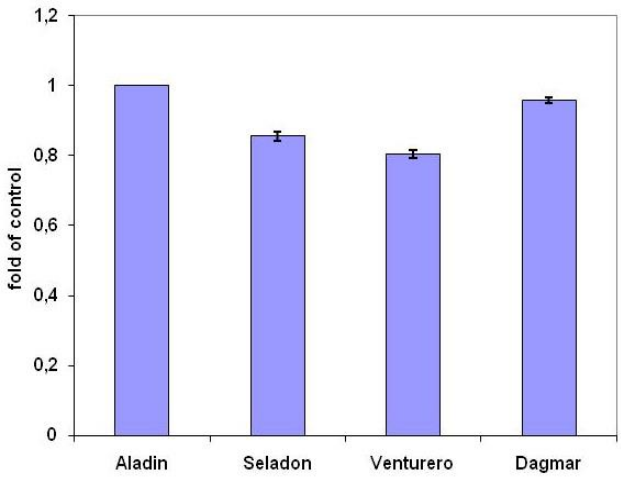

Fig. 1. Comparison of expression ratios of miR156 in drought tolerant and drought susceptible wheat varieties.

By increasing the activity of transcription factors, which are one of the target sequences of microRNA molecules, a better adaptation of wheat to dryness may occur. Achakzai *et al.*, (2019) confirmed 25% representation of transcription factors as targeted sequences of wheat miRNA clusters and 5% of total miRNA targets were stress related. Several studies have reported findings of increased synthesis of primary transcripts in several miRNA cases (including miR156) (Kruszka *et al.*, 2012; Bej & Basak, 2014; Pacak-Barciszewska *et al.*, 2016). MiR156 has agricultural significance for plant development and stress tolerance (Sunkar & Zhu 2004). In *Triticum aestivum*, miR156 is responsive to heat stress with upregulation (Xin *et al.* 2010). Moreover, overexpression of miR156 elevates level of anthocyanin biosynthesis in *Arabidopsis* (Gou *et al.* 2011), which was reported to minimize plant sensitivity to salt and drought stress (Cui *et al.* 2014). Role of miR156 in drought stress was revealed in many studies by high throughput data covering microarray and microRNA sequencing (Sunkar and Zhu 2004, Liu *et al.* 2008, Lee *et al.* 2010). Kantar *et al.* (2011) reported that miR156 is responsive to drought stress in *Triticum dicoccoides* root tissue.

The activity of the tae-miR408 marker was more than 58% higher in generating the DNA based miRNA amplicons for susceptible varieties than for tolerant varieties, demonstrating the important role of miR408 in plant dry tolerance (Fig. 2).

The expression levels of tae-miR408 confirmed the higher activity of this type of miRNA in drought tolerant varieties Seladon and Venturero. The difference in expression was approximately about 40-50 % when comparing the varieties *per se* without any drought stress induction (Fig. 3).

Table 2. Multi-factorial analysis of variance for the number of amplified miRNA loci of summer wheat genome relative to individual sources of variability. Tukey method, 99%.

Variability source		Sum of squares	df	Mean square	F-value	Level of confidence	
	Treatment	6,17	4	1,54	0,21	0,93	-
	Variety	93,65	3	31,22	4,22	0,01	***
Main effects	Marker	510,63	2	255,32	34,49	0,00	***
	Error	370,58	50	7,40			
	Total	980,58	59				

Notes: - No significant difference, *** Statistically highly significant difference, df – Degree of freedom

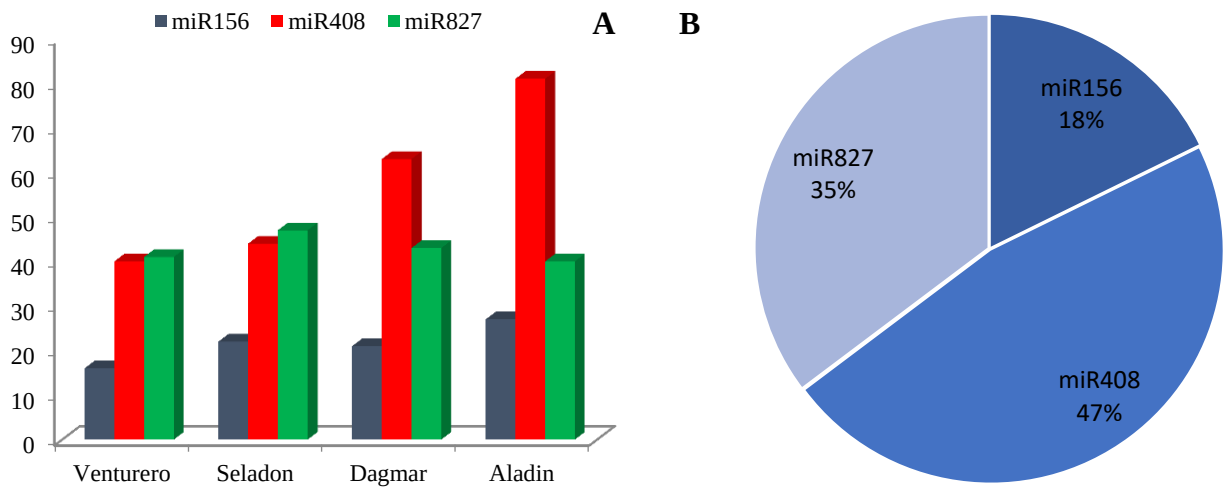

Fig. 2. Representation of miRNA loci amplified by stress-sensitive markers in the winter wheat genome exposed to dehydration stress (A). Activity of individual markers under stress conditions (B).

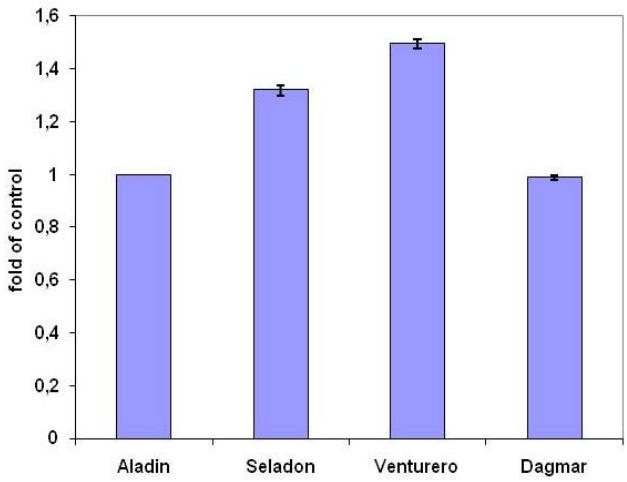

Fig. 3. Comparison of expression ratios of miR408 in drought tolerant and drought susceptible wheat varieties.

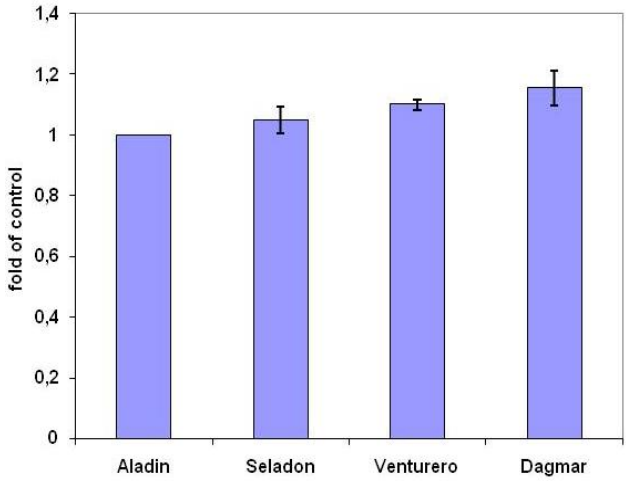

Fig. 4. Comparison of expression ratios of miR827 in drought tolerant and drought susceptible wheat varieties.

MiR408 is one of the most conserved classes of miRNAs and has been reported to date in more than 30 plant species, indicating that its role is essential for the development and existence of plants (Kozomara & Griffiths-Jone, 2014). Studies have shown that miR408 is involved in the development, light signalling pathway and biotic stress reactions as well as biomass production in *Arabidopsis* seedlings (Jones-Rhoades *et al*., 2006; Bej & Basak, 2014; Liang *et al*., 2015; Pacak-Barciszewska *et al*., 2015). It has been reported in Zhao *et al*., (2017) that constitutive expression of miR408 affects various stages of development and promotes intense plantg rowth and seed yield by increasing the efficiency of photosynthesis. Therefore, miR408 is likely to have a pleiotropic effect on plant growth and development.

MicroRNAs biomarker response is not only tissue-specific but also species-specific (Kruszka *et al*., 2014) moreover in the case of miR408 marker its response might be cultivar-specific (Melnikova *et al*., 2016). The expression pattern of miR408 in roots of *Medicago truncatula* was strong up-regulated while its target gene was down-regulated. In Arabidopsis leaves under drought stress was observed a minor decrease of miR408 level. According to Kantar *et al*. (2010), miR408 expression in barley root tissue was not changed upon dehydration stress. This statement is supported by findings of (Liu *et al*., 2008) where the miR408 targeted genes respond to several stresses. However, it has been proved (Hajyzadeh *et al*., 2015) that miR408 is involved in drought stress regulation and its increased level is important to drought tolerance. There is evidence that many of the miRNAs (including miR156 and miR408) are involved in the stress tolerance and these miRNAs might be co-regulated by both environmental factors and developmental stimulus (Liu *et al*., 2008; Zhao *et al*., 2010; Ma *et al*., 2015; Zhang, 2015). The induction of hvu-miR156a-and hvu-miR408- based markers has been detected in leaves and stems, respectively in stems and root tissues of barley samples under dehydratation stress (Ražná *et al*., 2020a). Our results indicate that markers based on miRNA sequences are a suitable tool for genomic screening of plant genetic resources under environmental stress conditions (Ražná *et al*., 2020b).

In terms of miRNA expression pattern should be considered the specification of miRNA family. MiRNAs encoded by different genes of the same MIR gene family might differ in their expression regulation pattern (Kruszka *et al*., 2014). For example, significant down regulation of miR408e of sugar cane was observed in response to drought stress (Zhao *et al*., 2010). The differential response of miR408 could also be due to the different developmental stage of testes plant, stress intensity, growth conditions and methods employed to monitor miRNAs activity (Zhang, 2015).

The activity of the tae-miR827 in the genome of resistant and susceptible genotypes of wheat was nearly balanced. This was obtained for DNA based generated fingerprints as well as for expression analyse (Fig. 4). MiR827 is part of the regulatory processes involved in nutrient homeostasis, especially phosphorus (Liang *et al*., 2015). From this perspective, the result can be interpreted in such a way that under ideal conditions of *In vitro* cultivation, and microelements, as well as lack of moisture, do not affect the genome of wheat as negative as it would probably be under natural environmental conditions. It has also been shown that a low nitrogen content affects the expression of transcription factors or cofactors that drive the transcription of miR827 genes, thereby reducing the expression of mature miRNAs. In the absence of water, nutrient intake is reduced, and the plant reacts to these changes by activating defence mechanisms and by increasing the expression of stress-sensitive genes Chiou (2007).

Abiotic stress conditions induced marked activation of the tae-mir408 biomarker, regulating the adaptation mechanisms of the plant genome to dehydration. In susceptible genotypes, its activity was 42% higher than that of the tae-miR156 marker. In the case of tolerant genotypes, this difference was 45% for these markers. This means that both resistant and susceptible wheat genotypes try to cope with a given stress factor by significantly activating microRNA molecules that are involved in the necessary regulatory and adaptation mechanisms, but at the expense of reducing growth processes. The results indicate that the Aladin genotype has a higher drought tolerance adaptation capability than

the Dagmar variety. In the case of Venturero variety, lack of moisture will be a significant limiting factor that the genome can deal with, within defense mechanisms, to the detriment of developmental and growth indicators. The mechanism of plant adaptation to environmental conditions involves minimizing their growth and reorganizing their resources (Rajwanshi, 2014). The activity of miRNA molecules of the same type may vary depending on the type of tissue or developmental phase, suggesting that the MIR gene expression pattern is spatially and temporally regulated (Bartel, 2004). Despite their size, microRNA molecules have a broad-spectrum effect due to their regulatory potential and have a vital function in almost all biological and metabolic processes.

Conclusion

We focused on the application of stress-sensitive microRNA markers in terms of mapping the response of summer wheat genome (*Triticum aestivum* L.) for the purpose of selection for drought resistance. The genome of resistant genotypes of summer wheat (Aladin and Dagmar) responded compared to susceptible genotypes (Seladon and Venturero), marked activation of the tae-miR408 biomarker due to dehydration stress. This reaction points to a more efficient ability to adapt the genome of resistant genotypes to abiotic stress. Multi-factor analysis of variance confirmed a statistically highly significant relationship between the type of biomarker applied and its potential to map the genome response to induced stress. At the same time, this genome response to abiotic stress was genotype specific.

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