POLYMORPHISM OF SPECIFIC miRNAs IN THE CONTEXT OF FLAX (*LINUM USITATISSIMUM* L.) GENOME ADAPTABILITY TO ABIOTIC STRESS

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Abstract: Polymorphism of flax (*Linum usitatissimum* L.) genome, genotype CDC Bethune, under nutrient stress *in vitro*, was analyzed by newly developed type of molecular markers based on microRNA molecules. Two types of stress-sensitive miRNAs, miR395 and miR399 were evaluated. The miR359 loci profile has shown to be more polymorphic and more specific in comparison to miR399 loci pattern. Our observations have supported the role of miRNA molecules as potential biomarkers of abiotic stress.

Key words: microRNA, *in vitro*, nutrition stress, genome polymorphism

INTRODUCTION

Plants are exposed to a wide variety of environmental stimuli. The different mechanisms of stress response contribute to genome adaptability at different levels of plant organism. MicroRNA as non-coding regulatory molecules are considered as potential biomarkers in plant stress responses (Bej, Basak 2014). Plant miRNA plays a vital role in development, physiological processes and stress responses. Many stress-regulated genes are found to be regulated by miRNAs. Phosphorus is one of the most influential macronutrients in the plant life cycle. It is involved in phosphorylation reactions, energy delivery, synthesis of nucleic acids (Kruszka et al. 2012). It has been documented, that miR399 regulates phosphate equilibrium (Fuji et al. 2005, Kruszka et al. 2012). Under phosphate deficient condition miR399 is up-regulated. In the case of miR395 the crutial role for sulfate homeostasis through regulating the sulfate uptake, transport and assimilation has been demonstrated (Liang, Yu 2010). Furthermore, miR395 also regulates the transport of sulfate into the leaves. Under sulfate starvation conditions miR395 is up-regulated (Jones-Rhoades, Bartel 2004). Plant sulfur in its reduced form is found mainly in amino acids, peptides and proteins (Kruszka et al. 2012). On the other hand, Melnikova et al. (2015) reported statistically significant up-regulation for miR395 under excessive fertilizer. According to their findings the expression level of miR395 could be associated not only with excess sulfur application, but also with redundancy of other macronutrients and micronutrients. There is the possibility that miR395 response varies between different plant species and species like switchgrass that are adapted to unfertile soils have evolved constitutive adaptive mechanisms (Sunkar 2010).

Flax is known by its phytoremediation capabilities (Havel et al. 2010). In addition, flax is of research interest because some lines undergo phenotype and genome changes in response to environmental conditions (Cullis 2004).

In this work we applied nutritional stress under *in vitro* conditions on the genotype CDC Bethune, the genome of which should be stable in the environmental conditions. We were interested whether it is possible, at the level of miRNA-based markers, to record the polymorphism of nutrition stress-sensitive miRNA, miR395 and miR399, in the genome of flax.

MATERIAL AND METHODS

Characterization of biological material, growth conditions and DNA extraction

Seeds of flax genotype CDC Bethune were cultivated on solidified Murashige and Skoog (MS) medium (Murashige, Skoog 1962). The seed material was cultivated on four different nutritional variants of the MS basal medium as follows:

1 – full-strength of microelements and vitamins, half-strength of macroelements,

2 – full-strength of macroelements and vitamins, half-strength of microelements,

3 – full-strength of microelements and macroelements, half-strength of vitamins,

4 – half-strength of microelements, macroelements and vitamins,

C – control variant – basal MS medium.

In vitro cultivation was carried out during the period of six weeks at 22°C under photoperiod 16 h light/8 h dark cycle (Melnikova et al. 2015).

Total genomic DNA was extracted using the modified method according to Padmalantha and Prasad (2006). The DNA concentration was quantified by the Implen NanoPhotometer®, measuring the absorbance at 260 nm. The purity and integrity was assessed by the absorbance 260/280 nm ratio.

Marker assay

The miRNA-based markers were PCR amplified in a 20-µl reaction mixture that contained 70 ng of genomic DNA, 1 × DreamTaq Buffer (KCl, $(NH_4)_2SO_4$, 20 mmol.dm^{-3} $MgCl_2$), 2 units of DreamTaq DNA polymerase, 0.8 mmol.dm^{-3} dNTPs (Bioline), 10 pmol.dm^{-3} of each primer and nuclease-free water for PCR amplification. The PCR amplification program 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 (annealing temperature was decreased with 1°C/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 a final extension at 72°C for 10 min. The primers for the miRNA-based markers were designed according to the mature miRNAs sequences, originated from the miRNA database (http://www.mirbase.org/). A total of 2 miRNA-based forward primers and 1 universal miRNA reverse primer were used and randomly combined together to perform a marker assay. Combination of primer pairs and their sequences used for microRNA-based marker assay are displayed in Table 1.

Table 1 Combination of primer pairs used for miRNA-based marker assay.

Primer combination	Sequences
miR 395_F	5′-CACGCACTGAAGTGTTTGGGG-3′
miR_R	5′-CCAGTGCAGGGTCCGAGGTA-3′
miR 399_F	5′-CACGCATGCCAAAGGAGAGTT-3′
miR_R	5′-CCAGTGCAGGGTCCGAGGTA-3′

Legend: miR- microRNA, F- forward primer, R- reverse primer.

PCR products were separated on 15% TBE-Urea gels (Invitrogen) running in 1 × TBE Running Buffer at a constant power 180 V, 30 mA for 75 min. The polyacrylamide gels were stained with GelRed™ Nucleic Acid Gel stain and were visualized on G-Box Syngene electrophoresis documentation system.

RESULTS AND DISCUSSION

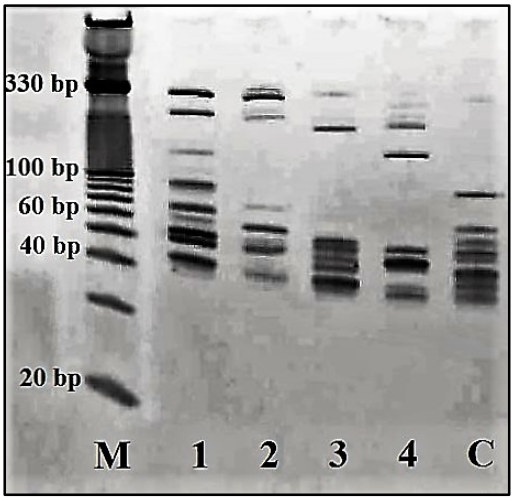
DNA samples were amplified by PCR amplification program using the 'touchdown'method. The maximal annealing temperature was set at 64°C and the minimal at 60°C. The two single forward primers and one universal reverse primer were randomly combined together to perform a marker assay (see Figure 1, 2).

Identification of polymorphism of stress-sensitive miRNA in CDC Bethune

The miR395 polymorphism profile has showen to be very specifically dependent on nutrition stress conditions. In comparison to control variant it is possible to recognize- the variable number of individual miR395 loci per each stress variant. Not only considerable variability in the number

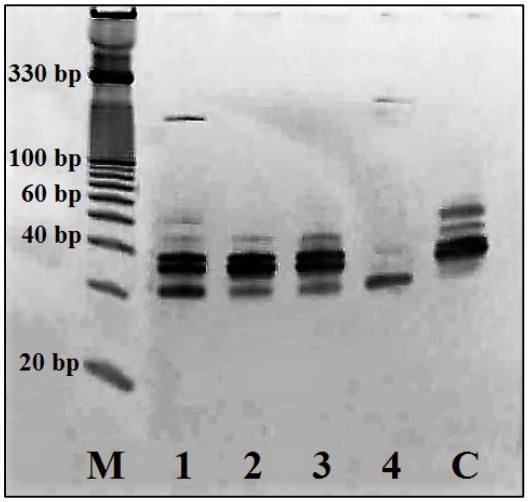
of miRNA loci but also in their size was observed. The highest number of miR395 loci (9) has been recorded at the MS basal medium having half-strength of macroelements. Within the macroelements of MS medium sulfur is present only in one component and that is magnesium sulphate- while, four microelements are in the form of sulphates (copper, iron, manganese and zinc). Our results supports the data of Sunkar (2010) suggesting the possibility that miR395 response varies between different plant species and some plants that are adapted to inferior growing conditions might evolved constitutive adaptive mechanisms. However, it is evident that the flax genome responds to the stimulus caused by this kind of abiotic stress.

Figure 1 Representative gel showing amplification profiles of CDC Bethune generated by primer pair miR 395_F / miR_R.



Legend: M – 10 bp DNA Ladder Invitrogen; 1– modified MS medium with full-strength microelements and vitamins, half-strength of macroelements; 2 – modified MS medium with full-strength of macroelements and vitamins, half-strength of microelements; 3 – modified MS medium with full-strength of microelements and macroelements, half-strength of vitamins; 4 – modified MS medium with half-strength of microelements, macroelements and vitamins; C (Control) – basal MS medium.

Figure2 Representative gel showing amplification profiles of CDC Bethune generated by primer pair miR 399_F / miR_R.



Legend: M – 10 bp DNA Ladder Invitrogen; 1– modified MS medium with full-strength microelements and vitamins, half-strength of macroelements; 2 – modified MS medium with full-strength of macroelements and vitamins, half-strength of microelements; 3 – modified MS medium with full-strength of microelements and macroelements, half-strength of vitamins; 4 – modified MS medium with half-strength of microelements, macroelements and vitamins; C (Control) – basal MS medium.

The pattern of miR399 is less polymorphic than the miR359 pattern. We have observed significantly different miR399 loci pattern in the case of stress variant having half-strength of all components of MS medium. Within the macroelements of MS medium phosphate is present only in one component and that is potassium phosphate- while in the microelements there are no phosphates. That means that the stress variant number 1 represents, in this case, the conditions of low phosphate characterized by miR399 up-regulation (Fuji et al. 2005, Kruszka et al. 2012). In this variant the highest number of miRNA loci in comparison to control has been observed.

CONCLUSION

Obtained results have shown that miRNA-based molecular markers are sufficient for evaluation of flax genome polymorphism under specific conditions of abiotic stress. Our observations have supported the capability of miRNA molecules as potential biomarkers of environmental stress.

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REFERENCES

Bej S., Basak J. 2014. MicroRNAs: The Potential Biomarkers in Plant Stress Response. *American Journal of Plant Sciences*, 5: 748–759.

Cullis C. A. 2004. Mechanisms and control of rapid genomic changes in flax. *Annals of Botany*, 95(1): 201–206.

Fujii H., Chiou T. H., Lin S. I., Aung K., Zhu J. K. 2005. A miRNA involved in phodphate-starvation respomse in *Arabidopsis*. *Current Bioogy*, 15(22): 2038–2043.

Havel L., Trojan V., Krystofova O., Zitka O., Babula P., Zehnalek J. 2010. Phytoremediation potential of flax (*Linum usitatissimum*) to heavy metals with respect to biosynthesis of phytochelatins and activity of phytochelatin synthase. *Journal of Biotechnology*, 150: 223–223.

Jones-Rhoades M. W., Bartel D. P. 2004. Compotational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Molecular Cell*, 14(6): 787–799.

Kruszka K., Pieczynski M., Windels D., Bielewicz D., Jarmolowski A., Szweykowska-Kulinska Z., Vazquez F. 2012. Role of microRNAs and other sRNAs of plants in their changing environments. *Journal of Plant Physiology*, 169(2012): 1664–1672.

Liang G., Yu D. 2010. Reciprocal regulation among miR395, APS and SULTR2;1 in *Arabidopsis thaliana*. *Plant Signal Behay*, 5(10): 1257–1259.

Melnikova N. V, Dmitriev A. A., Belenikin M. S., Speranskava, A. S., Krinitsina A. A., Rachinskaia, O. A., Lakunina, V. A., Krasnov G. S., Snezhkina A. V., Sadriidinova A. F., Uroshlev L. A., Koroban N. V., Samatadze T. E., Amosova A. V., Zelenin A. V., Muaravenko O. V., Bolsheva N. L., Kudryavtseava A. V. 2015. Excess fertilizer responsive miRNAs revealed in *Linum usitatissimum* L. *Biochemie*, 109: 36–41.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tabacco tissue cultures. *Physiologia Plantarum*, 15: 473–497.

Padmalatha K., Prasad M.N.V. 2005. Optimization of DNA isolationand PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India. *African Journal Biotechnology*, 5(3): 230–234.

Sunkar R. 2010. MicroRNAs with macro-effects on plant stress responses. *Semin Cell Dey Biology*, 21(8): 805–811.