Analysis of miRNA polymorphism during the selected developmental processes of flax

Analýza polymorfizmu miRNA vo vybraných vývojových štádiách ľanu siateho

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Abstract

MicroRNAs represent small non-coding RNAs that play important role in regulating gene expression under various biotic and abiotic stresses as well as in different developmental stages of plants. They are involved in wide variety of biological and metabolic pathways of plants. The research was focused on the potential of selected miRNA-based molecular markers (miR156 and miR168) to map the polymorphism level of flax genome in the selected developmental stages (flower bud - flowering – boll development) as a reflection of the activity of specific miRNAs in these flax organs and tissues. The miRNA polymorphism was evaluated on 8 flax genotypes by miRNA-based molecular marker assays and data were supported by the morphology measurements on buds, flowers, petals and developing bolls. Extent of PCR amplification of miRNA fragments ranged from 40 bp to 200 bp (miR168a-F/miR-R primer pair) or 35 bp to 120 bp (miR156b-F/miR-R primer pair), respectively. MiRNA-based primers amplified in total 196, respectively 158 miRNA loci. Based on the results can be concluded that the representation of miR168 loci have increased according to developmental stages ascending (stage of bud, flower and boll). In overall miRNA156b loci profile in all three analyzed developmental stages is possible to observe the increase of miRNA loci amplification almost in all genotypes. Taking into account the genetic background of genotypes, based on the peak analysis profile of amplified miRNA loci, implemented by GeneTools software, was possible to identified unique loci in linseed and intermediate genotypes in individual developmental stages. Have been found out that only the genotype Bolley Golden differ from other genotypes in both analysis (molecular and morphological) if the

variability of miR168 loci number in the flower bud growing stage was compared to the variability in trait petals colour in bud.

Keywords: developmental processes, flax, microRNA, miRNA, morphology, polymorphism

Abstrakt

MikroRNA sú malé nekódujúce molekuly RNA, ktoré majú dôležitú úlohu pri regulácii génovej expresie v podmienkach vplyvu rôznych biotických a abiotických faktorov stresu, ako aj v rámci rôznych vývojových štádii rastlín. Sú súčasťou mnohých biologických a metabolických procesov rastlín. Výskum bol zameraný na potenciál vybraných typov mikroRNA (miR156 a miR168), ako molekulárnych markérov pre účely mapovania polymorfizmu genómu ľanu vo vzťahu k určitým vývojovým štádiám (púčik – kvitnutie – tvorba tobolky), ako odraz ich aktivity v daných pletivách a orgánoch ľanu siateho. Polymorfizmus miRNA bol hodnotený na 8 genotypoch ľanu pomocou molekulárnych markérov na báze mikroRNA a údaje boli podporené hodnotením morfologických znakov na púčikoch, okvetných lístkoch a v tvoriacich sa tobolkách. Rozsah PCR amplifikácie fragmentov miRNA bol zaznamenaný v rozmedzí od 40 bp do 200 bp (prajmer miR168a-F/miR-R), alebo 35 bp do 120 bp (prajmer miR156b-F/miR-R). Prajmery na báze miRNA amplifikovali celkovo 196, respektíve 158 miRNA lokusov. Je možné konštatovať, že zastúpenie miR168 lokusov sa zvyšovalo v závislosti na vývojovom štádiu (kvetný púčik, kvitnutie a tvorba tobolky). Celkový profil mir156b lokusov mal stúpajúcu tendenciu od fázy tvorby kvetného púčika až po fázu tvorby tobolky a to takmer vo všetkých genotypoch. V závislosti na genetickom pozadí genotypov, bolo možné identifikovať jedinečné miRNA lokusy v olejných a olejno-priadnych genotypoch v rámci vývojových fáz. Identifikácia jedinečných lokusov bola realizovaná pomocou softvéru GeneTool (Syngene). Zistilo sa, že iba genotyp Bolley Golden sa líši od iných genotypov v oboch typoch analýz (molekulárnych aj morfologických), porovnávaním variability počtu lokusov miR168 v štádiu tvorby púčika s variabilitou znaku, farba lupienkov v púčiku.

Kľúčové slová: ľan, mikroRNA, miRNA, morfológia, polymorfizmus, vývojové štádiá

Introduction

Flax (*Linum usitatissimum* L.) represents a multipurpose usable plant of major importance. It is a one of the key resources of alpha-linolenic acid which is in the recent period of intense research center in terms of improving the quality of human life. Besides, flax genome specificity is the source of many research topics.

MicroRNAs are hairpin-derived short non-coding RNAs that bind to the target mRNAs, leading to either translation delay or mRNA degradation (Erson-Bensan,

2014). miRNAs control gene expression of plants exposed to various environmental stresses as well as in different developmental stages (Ganie and Mondal, 2015). Transcription factors represent the main target sequences of microRNA molecules (Barvkar et al., 2013; Neutelings et al., 2012). Recent advances suggest that miRNA-guided translational inhibition is a major component of miRNA activity (Reis et al., 2015). Molecules miRNA are produced from non-coding mRNAs that undergo various processing steps to form mature miRNA (Ganie and Mondal, 2015).

One of the best understood and extensively reviewed miRNA network is one that regulates developmental timing and involves the antagonistic miR156 and miR172 (Rubio-Somoza and Weigel, 2011). The family miR156 targets squamosa promoter binding protein (SBP), transcription factor in monocot and dicots which is involved in controlling flowering time and controls the transition from the juvenile to the adult vegetative phase (Barvkar et al., 2013). Increased levels of miR156, restrain development transitions, prolong juvenile features and delay flowering. In turn, release of SBP from miR156 regulation leads to early flowering (Rubio-Somoza and Weigel, 2011).The highest expression of SBP target is in the flower and ovary of flax.

One of the target sequences of miR168 family are sequences of cytochrome P450 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). The highest expression of flax miR168 is exhibited in anthers and flowers (Barvkar et al., 2013).

DNA-based molecular markers are an integral part of the evaluation and assessment of genomic significance of plant genetic resources. MicroDNA sequences are common in the genomes of plants where most annotated miRNAs are located in intergenic regions (Fu et al., 2013; Zhang et al., 2009). Evolutionary, they are highly conserved and hence can be useful for studying genetic diversity (Ganie and Mondal, 2015). The high conservation of miRNA sequences provides an opportunity to develop a novel type of molecular markers. miRNA-based markers were applied in two species, so far, *Brassica sp.* (Fu et al., 2013) and *Setaria italica* including some related grass species (Yadav et al., 2014). miRNA-based microsatellite marker system was applied for rice genotyping applications (Ganie and Mondal, 2015) and for identification salt responsive miRNA-SSR markers in rice (Mondal and Ganie, 2014).

Plant genetic resources should be evaluated by an integrated approach of several methods, including the most advanced molecular methods but also classic methods (morphological analysis) (FAO, 1996; Hammer et al., 2003). Further research of morphological and molecular markers and their application will be beneficial to quantify the genetic diversity and genetic erosion (Hammer et al., 2003; Rao and Hodkin, 2002).

The aim of the study was to apply and analyze the miRNA-based markers polymorphism during the developmental stages of flax (flower bud - flowering – boll development) as a reflection of the activity of selected miRNAs (miR156 and miR168) in these organs and tissues.

Material and methods

A total of 8 genotypes of different type and origin of *Linum usitatissimum* L. species were used in the present study (Table 1.).

Table 1. List of samples of genotypes of *Linum usitatissimum* L. species in miRNA marker assay

Tabuľka 1. Zoznam vzoriek genotypov druhu *Linum usitatissimum* L. pre analýzy pomocou miRNA markérov

Code	Name	Type	Origin
1	Amon	Intermediate	Czech Republic
2	Libra	Linseed	Czech Republic
3	Raciol	Linseed	Czech Republic
4	Lin 225	Intermediate	The Netherlands
5	Bolley Golden	Intermediate	USA
6	Currong	Intermediate	Australia
7	Mermilloid	Intermediate	Czech Republic
8	Eyre	Intermediate	Australia

The seed material of genotypes Amon, Libra and Raciol has been provided by company AGRITEC, Research, Breeding and Services, Ltd. from Šumperk in Czech Republic. These genotypes of flax are characterized by low (less than 3%) content of alpha-linolenic acid (genotype Amon), high (more than 57,5%) content (genotype Libra) and middle (30%) content (genotype Raciol) of alpha-linolenic acid. Genotypes marked with codes 1, 2, 3 were grown on experimental fields of Department of Genetics and Plant Breeding (Slovak University of Agriculture in Nitra) and rest of genotypes were grown on experimental fields of N. I. Vavilov Research Institute of Plant Industry at the Department of Oil and Fiber Crops at Russia-Pushkin. Samples for analyzing polymorphism by newly developed type of molecular markers based on microRNA molecules were taken from buds, flowers petals and bolls in the following phenological stages:

- buds- BBCH 55 (first individual flowers visible- still closed),
- flowers petals- BBCH 65 (full flowering: 50% of flowers opened),
- bolls- BBCH 71 (10% of bolls have reached final size).

Total genomic DNA from 8 genotypes of buds, flowers petals and bolls of mentioned above stages was extracted using the modified method according Padmalatha and Prasad (2006). Extracted DNA was quantified by the Implen NanoPhotometer®, and diluted into 70 ng*μl^{-1} with nuclease-free water for PCR amplification.

The primers for the miRNA-based markers were designed according to the mature miRNAs sequences, which are part of the miRNA precursors (pre-miRNA) originated from the miRNA database (http://www.mirbase.org/). Mature miRNAs have shown to be more conserved in plants rather than their precursor sequences (Bartel, 2004). The single forward primers were combined with universal miRNA reverse primer (Chen et al., 2005; Kulcheski et al., 2010) to perform a marker assay (Table 2.).

Table 2. Primers combinations used for miRNA-based marker assay

Tabuľka 2. Kombinácie prajmerov použité pre analýzy pomocou miRNA markérov

Primer name	Sequences
mir168a-F	5´-CACGCATCGCTTGGTGCAGGT-3´
miR-R	5´-CCAGTGCAGGGTCCGAGGTA-3´
miR156b-F	5´-TGACAGAAGAGAGAGAGCACA-3´
miR-R	5´-CCAGTGCAGGGTCCGAGGTA-3´

miR- microRNA; F- forward primer; R- reverse primer

The following types of miRNA were selected for the assay: gm-miR156b and lus-miR168a. The gm-miR156b of soybean genome was selected based on the study by Kulcheski et al. (2010). They provide evidence that the expression stability of gm-mir156b was the highest across the different soybean tissues and genotypes as well as after the abiotic or biotic stress treatment. Lus-miR168a is flax genome own miRNA.

The primers applied in this study have been designed and used in genotyping of flax and medicinal plant *Sylibum marianum* (Hlavačková et al., 2015a, 2015b; Ražná et al., 2015a, 2015b) and in soybean (unpublished). For the purpose of this research, the suitability of the primer sequences of gm-miR156b have been verified by the bioinformatic approach described below.

The sequences stem-loop structure sequences of gm-miR156b (accession number MI0001790) were compared to the stem-loop structure sequences of lus-miR156b (accession number MI0021181) by the BLASTn algorithm of the NCBI database (http://blast.ncbi.nlm.nih.gov/) by program selection optimized for somewhat similar sequences. Consequently were analyzed sequences of mature miRNAs by BLASTn program selection optimized for highly similar sequences (Table 3.). Based on the E-values and the percentage of identities, the primer designed based on mature gm-miR156b was considered as suitable for the purposes.

Table 3. Statistics of evaluation of BLASTn alignment of stem-loop structures sequences and mature miRNA sequences of gm-miR156b and lus-miR156b

Tabuľka 3. Štatistické vyhodnotenie BLASTn prirovnania sekvencií vlásenkovitých štruktúr a sekvencií zrelých molekúl miRNA gm-miR156b a lus-miR156b

Description of BLASTn statistics	Max Score	Total Score	Query cover	E-value	Identity
Stem-loop structures sequences	41.0	67.5	35%	8e-09	86%
Sequences of mature miRNAs	32.2	32.2	95%	6e-08	95%

E – expectancy value

The miRNA-based markers were PCR amplified in a 20-µl reaction mixture that contained 70 ng of genomic DNA, 10 pmol*dm^{-3} of each primer, 2 units of DreamTaq 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 a final extension at 72 °C for 10 min. The samples were then stored at 8 °C. The PCR products were separated using 15% TBE-Urea gels (Invitrogen), running in 1 × TBE Running Buffer at a constant power 200 V, 35 mA for 60 min. The polyacrylamide gels were stained with GelRed^{TM} Nucleic Acid Gel stain and were visualized on G-Box Syngene electrophoresis documentation system. For the recording of loci number and unique fragment identification were the gels analyzed by GeneTools software (Syngene).

Results and discussion

Polymorphism analysis by miR168a-based molecular marker

miRNA-based primers combining with different places of the same stem-loop structure of miRNA can produce fragments of useful size for marker genotyping. It is also likely that miRNA-based primers amplify regions between neighboring miRNAs, resulting in additional variation (Fu et al., 2013). Extent of PCR amplification of miRNA fragments ranged from 40 bp to 200 bp (miR168a-F/miR-R primer pair) or 35 bp to 120 bp (miR156b-F/miR-R primer pair), respectively (Table 4.) miRNA-based primers amplified in total 196, respectively 158 miRNA loci.

The number of amplified loci by miR168a-based marker varied depending on selected developmental stages of flax (Table 5.). Overall could be observed an increasing number of miRNA loci amplified sequentially from phase to phase (Figure 1.).

Table 4. Description of PCR amplification parameters

Tabuľka 4. Charakteristika parametrov PCR amplifikácie

Developmental stage of flax	miR168a-F/miR-R primer pair		miR156b-F/miR-R primer pair	
	Extent of amplification of miRNA fragments	Number of amplified miRNA fragments	Extent of amplification of miRNA fragments	Number of amplified miRNA fragments
Flower bud	40–170 bp	62	35–100 bp	50
Flower petals	40–200 bp	67	35–120 bp	64
Bolls	40–200 bp	69	35–100 bp	70
Total	-	198	-	184

F – forward primer; R – reverse primer; bp – base pair

Table 5. Number of amplified loci by miR168a-based marker assay

Tabuľka 5. Počet amplifikovaných lokusov pomocou markéra miR168a

Plant organ/code of genotype	Number of amplified miRNA loci by primer miR168a_F/R							
	1	2	3	4	5	6	7	8
Flower bud	7	9	11	7	5	9	8	6
Flower petals	7	9	9	8	12	9	4	9
Bolls	8	10	8	5	11	11	8	8

F – forward primer; R – reverse primer

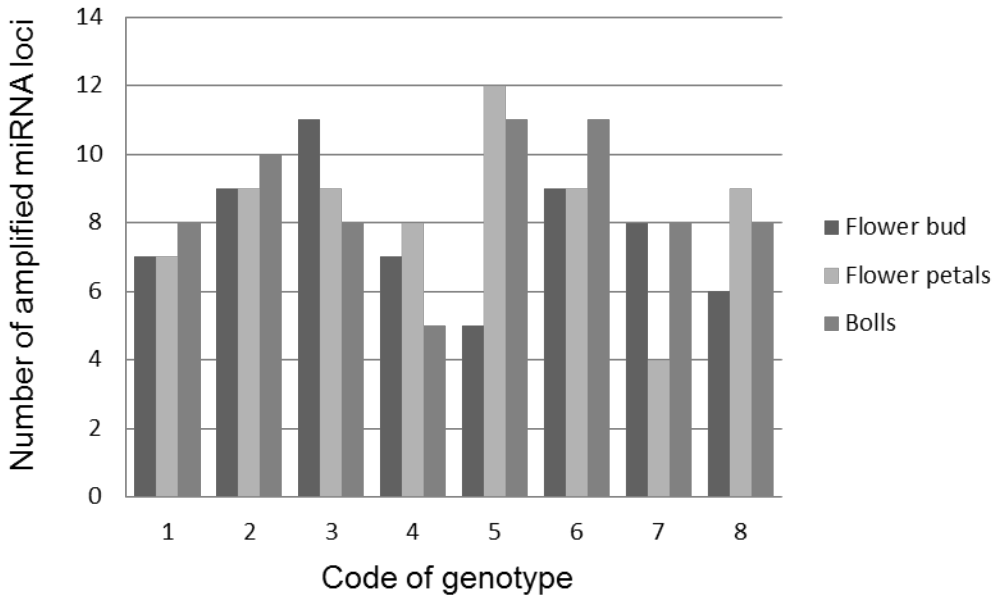


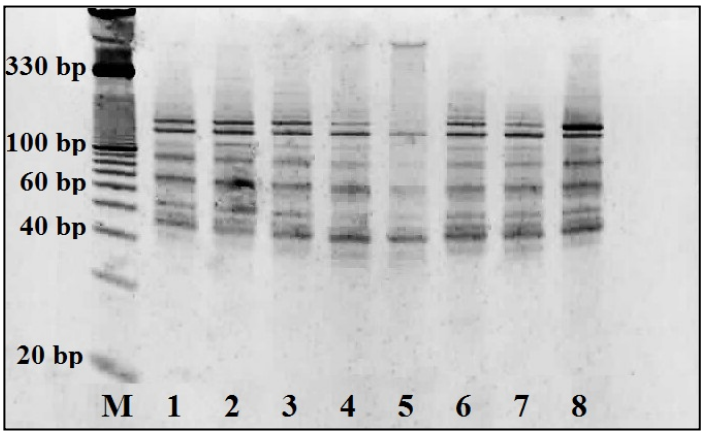
Figure 1. Number of amplified miR168 loci of eight genotypes depending on developmental stage of flax

Obrázok 1. Počet amplifikovaných lokusov miR168 pri ôsmych genotypoch v závislosti na vývojovej fáze ľanu

For genotypes Amon (code 1), Libra (code 2), Bolley Golden (code 5) and Currong (code 6) was observed an increasing number of amplified miRNA loci depending on the development phase. The genotype Raciol (code 3) is characterized by proportional reducing of loci frequency with respect to the stages. The lowest number of miRNA loci was observed in the stage of flower bud formation in intermediate genotype Bolley Golde (code 5; 5 loci) and the highest number in linseed genotype Raciol (code 3; Figure 2.). On the other hand, the same genotype Bolley Golden (code 5) was characterized by the most intensive amplification in the following phase from flowering (Figure 3.), in contrast to genotype Mermilloid (code 7; 4 loci). In the phase of boll formation (Figure 4.) was the highest number (11) of miRNA loci identified for genotypes bolley Golden (code 5) and Currong (code 6) and the lowest one for genotype Lin 225 (code 4).

Taking into account the genetic background of genotypes, based on the peak analysis profile of amplified miRNA loci, implemented by GeneTools software (pictures not shown), was possible to identified unique loci in the stage of flower buds (linseed genotype Raciol, code 3) and intermediate genotype Currong (code 6), in the stage of flowering (linseed genotype Libra, code 2) and intermediate genotype Bolley Golden (code 5) and in the stage of boll formation (genotypes Lin 225, code 4, Bolley Golden, code 5 and Currong, code 6).

Polymorphism amplified by miRNA-based molecular marker primer pair indicate sequence changes in the miRNA loci, which may result in a change in the regulation pattern of targeted genes. Therefore, miRNA-based molecular markers comprise a novel functional molecular marker (Fu et al., 2013; Htwe et al., 2015).



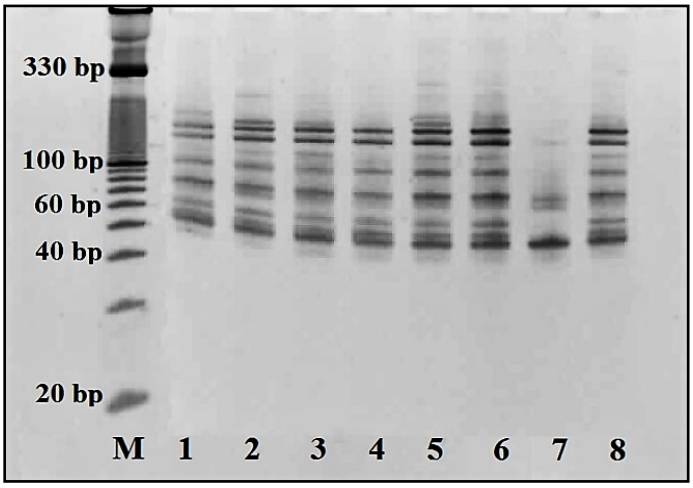
Legend: M - 10 bp DNA Ladder , 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Poznámka: M - 10 bp DNA veľkostný markér, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Figure 2. PCR amplification profile generated with miR168a-F/miR-R primer pair across buds tissues of 8 flax genotypes

Obrázok 2. Profil PCR amplifikácie vytvorený pomocou kombinácie prajmerov miR168a-F/miR-R v pletivách kvetných púčikov 8 genotypov ľanu

Overall, it can be concluded that the representation miR168 loci have increased according to developmental stages ascending (stage of bud, flower and boll). Considering the indirect correlation between the abundancy of miRNAs and the level of expression of their target sequences (Barvkar et al., 2013; Neutelings et al., 2012) is possible to assume that some of the metabolic mechanisms associated with the synthesis of fatty acids take place already in the process of formation of flower bud. Cytochrome P450, which is one of the studied target sequences of miR168 is involved in the formation of fatty acid precursors. Different situation could be observed in the case of linseed genotype with code 3 (Raciol, characterized by high content of alpha-linolenic acid). The miRNA loci amplification depending on the development stage gradually decreased, indicating the shift of fatty acids metabolism towards seed development stages.

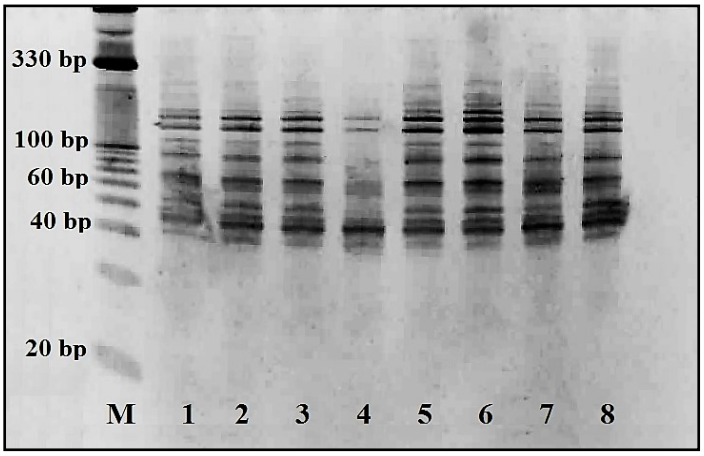


Legend: M - 10 bp DNA Ladder Invitrogen, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Poznámka: M - 10 bp DNA veľkostný markér, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Figure 3. PCR amplification profiles generated with miR168a-F/miR-R primer pair across flowers petals tissues of 8 flax genotypes

Obrázok 3. Profil PCR amplifikácie vytvorený pomocou kombinácie prajmerov miR168a-F/miR-R v pletivách kvetných lupienkov 8 genotypov ľanu



Legend: M - 10 bp DNA Ladder Invitrogen, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Poznámka: M - 10 bp DNA veľkostný markér, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Figure 4. PCR amplification profiles generated with miR168a-F/miR-R primer pair across bolls tissues of 8 flax genotypes

Obrázok 4. Profil PCR amplifikácie vytvorený pomocou kombinácie prajmerov miR168a-F/miR-R v pletivách toboliek 8 genotypov ľanu

However, flax miRNAs may target multiple genes involved in a wide variety of biological and metabolic pathways (Barozai 2012; Bej and Basak, 2014). The synthesis of fatty acids is regulated by multigene families and depends on developmental stage of the plant. By using *in silico* gene mining and comparative analysis You et al. (2014) implemented differential expression analysis of gene families related to fatty acid biosynthesis and they found out that some of them reflected expression during mature flower stage and boll development 12 day after flowing.

Polymorphims analysis by miR156b-based molecular marker

The number of amplified miRNA loci as well as the extent of PCR amplification reached lower values in comparison to miRNA168 based molecular marker. In overall miRNA loci profile in all three analyzed developmental stages is possible to observe the increase of miRNA loci amplification almost in all genotypes (Table 6., Figure 5.).

Table 6. Number of amplified loci by miR156b-based marker assay

Tabuľka 6. Počet amplifikovaných lokusov pomocou markéra miR156b

Plant organ/code of genotype	Number of amplified miRNA loci by primer miR156b_F/R							
	1	2	3	4	5	6	7	8
Flower bud	6	5	7	7	5	7	7	6
Flower petals	6	7	8	8	9	10	8	8
Bolls	8	9	10	7	10	9	9	8

F – forward primer; R – reverse primer

The lower representation of miR156 loci, in the phases of flower buds formation and flowering, comparing to miR168 loci profile is supporting the results of Barvkar et al. (2013) concerning an inverse relation between the expression of the miRNAs and their putative target gene, squamosa promoter-binding protein (SBP protein). Low miRNA profile in these stages is pointing toward higher expression of targeted sequences in flowers and flower organs which role is to control flowering time and the transition from the juvenile to the adult vegetative phase.

In the stage of flower bud was unique miRNA loci recorded in linseed genotype Libra (code 2) and intermediate genotype Eyre (code 8) (Figure 6.). Linseed genotype Raciol (code 3) and intermediate genotypes Bolley Golden (code 5) and Currong (code 6) were characterized by individual loci profile in the stage of flowering (Figure 7.) and in the stage of boll formation (Figure 8.).

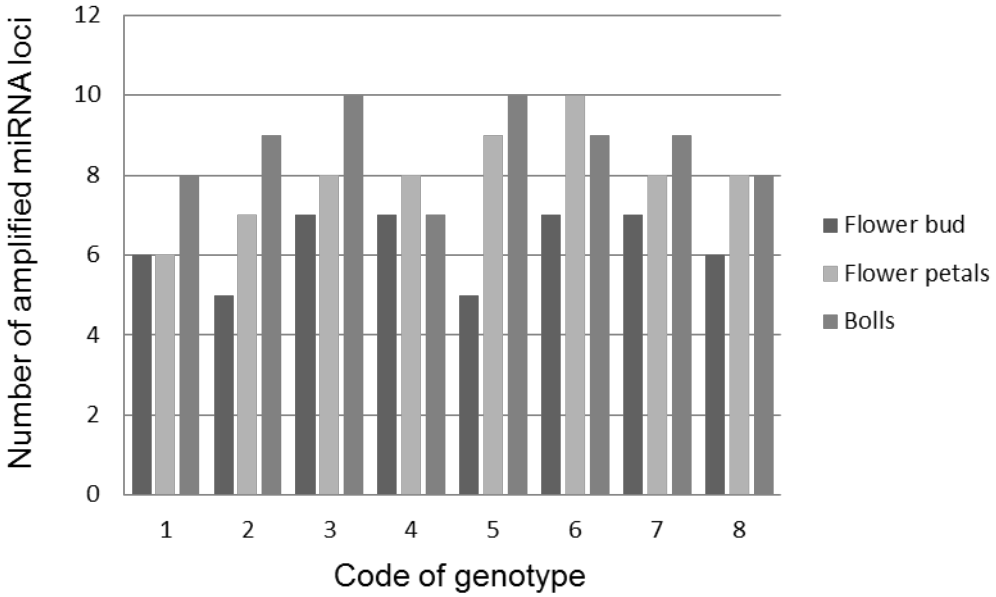
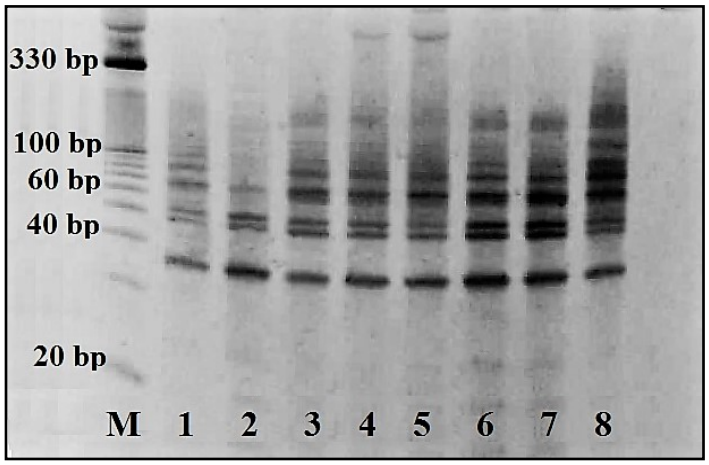


Figure 5. Number of amplified miR156 loci of eight genotypes depending on developmental stage of flax

Obrázok 5. Počet amplifikovaných lokusov miR156 ôsmych genotypov v závislosti na vývojovej fáze ľanu

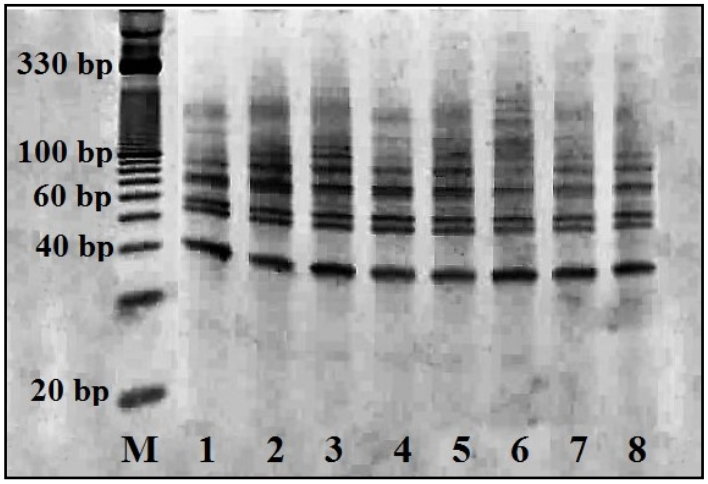


Legend: M - 10 bp DNA Ladder Invitrogen, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Poznámka: M - 10 bp DNA veľkostný markér, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Figure 6. PCR amplification profiles generated with miR156b-F/miR-R primer pair across buds tissues of 8 flax genotypes

Obrázok 6. Profil PCR amplifikácie vytvorený pomocou kombinácie prajmerov miR156b-F/miR-R v pletivách kvetných púčikov 8 genotypov ľanu

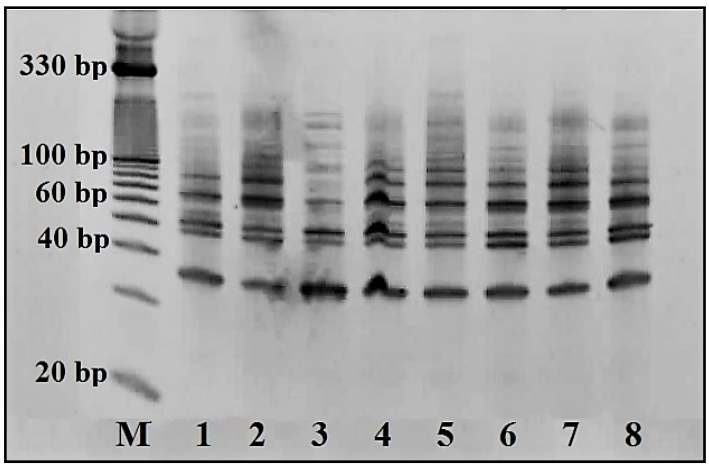


Legend: M - 10 bp DNA Ladder Invitrogen, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Poznámka: M - 10 bp DNA veľkostný markér, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Figure 7. PCR amplification profiles generated with miR156b-F/miR-R primer pair across flowers petals tissues of 8 flax genotypes

Obrázok 7. Profil PCR amplifikácie vytvorený pomocou kombinácie prajmerov miR156b-F/miR-R v pletivách kvetných lupienkov 8 genotypov ľanu



Legend: M - 10 bp DNA Ladder Invitrogen, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Poznámka: M - 10 bp DNA veľkostný markér, 1- Amon, 2- Libra, 3- Raciol, 4- Lin 225, 5- Bolley Golden, 6- Currong, 7- Mermilloid, 8- Eyre

Figure 8. PCR amplification profiles generated with miR156b-F/miR-R primer pair across bolls tissues of 8 flax genotypes

Obrázok 8. Profil PCR amplifikácie vytvorený pomocou kombinácie prajmerov miR156b-F/miR-R v pletivách toboliek 8 genotypov ľanu

Besides analysis of polymorphism of miR168a and miR156b loci the morphological traits (Table 7.) were followed on selected genotypes.

Table 7. List of morphological traits and growing stages

Tabuľka 7. Zoznam morfologických znakov a rastových fáz

Growing stage of trait evaluation	Name of trait
Just before flowering	colour of petals in bud
Flowering	colour of petals on fully opened flower
Flowering	colour of nerves on flower petals
Flowering	presence of longitudinal folding on flower petals
Flowering	margin folding on flower petals
Flowering	flower size
Full maturity	boll shape

Results of evaluation of genotypes phenotype expression on selected traits were processed by multilevel data sorting of qualitative traits. Statistical units (morphological traits) were situated in Pivot Table were the absolute count of follow up objects (genotypes) was determined by classes frequency.

The Table 8. is the final table of multilevel data sorting. In the table are genotypes distinguished according phenotype expressions of individual traits by different hues of grey colour and by “x”. An intermediate genotype Bolley Golden (code 5) has been found out that it shares unique expression in traits colour of flower petals in bud, colour of flower petals, colour of nerves on flower petals compared to other genotypes. The unique expression in traits colour of flower petals in bud, colour of flower petals has also flaxseed genotype Libra (code 2) having unique miRNA loci in both marker assays. Genotypes Amon (code 1) and Raciol (code 3) have same phenotype expression in these traits. Genotypes Lin 225 (code 4), Currong (code 6), Mermilloid (code 7) and Eyre (code 8) have same expression in traits for colour. The groups of genotypes Amon (code 1), Libra (code 2), Raciol (code 3), and Lin 225 (code 4), then Lin 225 (code 4) and Mermilloid (code 7), and Currong (code 6), Eyre (code 8) have the same phenotype expression in the trait longitudinal folding of flower petals. The same groups were formed for trait flower petals margin folding, except genotype Raciol (code 3) which has expression like genotypes Currong (code 6) and Eyre (code 8). The genotypes were divided into two groups, according the size of flower, on genotypes with big flowers (codes 1, 6 and 8) and genotypes with medium flower (codes 2, 3, 5, 4 and 7). All evaluated genotypes have globular shape of capsule.

It was found out that only the genotype with Bolley Golden (code 5) differ from other genotypes in both analysis if comparing the variability of miR168 loci number in the flower bud growing stage with variability in trait petals colour in bud. Genotypes with Lin 225 (code 4) Currong (code 6), Mermilloid (code 7), and Eyre (code 8) have identically phenotype expression in trait petals colour in bud. Following the variability connection, in traits for opened flower (colour, shape, size) and for boll with miRNA

168 loci in the growing stages – flowering and green boll, any connection was not determined in evaluated genotypes.

Following the variability connection, in traits for opened flower (colour, shape, size) and for boll with miR156 loci in all followed growing stages, was not determined any connection in evaluated genotypes. Only one exception was flaxseed genotype Raciol (code 3) in flowering stage and boll formation stage with unique miRNA loci and trait petals colour in bud.

Also in the field of flax genetic resources it is still inadequate seek appropriate genetic material for breeding and improving varieties of flax for food use. The source of new traits and features could be especially *ex situ* flax collections. In the world are institutions and scientific teams dealing with characterization and evaluation of genetic resources of flax (Brutch and Porokhovinova, 2011a, 2011b; Diederichsen, 2001; Diederichsen and Raney, 2006). There are used molecular methods (Everaert et al., 2001; Žiarovská et al., 2012), image analysis (Smykalova et al., 2013; Wiesnerová and Wiesner, 2008) or classical morphometric analysis (Brutch and Porokhovinova, 2011a, 2011b; Diederichsen, 2001; Porokhovinova, 2011).

Despite the fact that miRNA-based molecular markers have been so far established for genotyping applications in related grass species (Yadav et al., 2014) or *Brassica* species (Fu et al., 2013), in combination with microsatellite markers they have been applied for intraspecific genotyping of rice (Ganie and Mondal, 2015; Mondal and Ganie, 2014). Authors of this paper have published primary results of intraspecific miRNA-based markers application in flax (Hlavačková and Ražná, 2015) and *Sylibum marianum* (Ražná et al., 2015a, 2015b). The results have confirmed the high efficiency, stability and good transferability of miRNA-based molecular markers for intraspecific genotyping. Success of transferability of these markers relies on the fact that since these markers are derived from the sequences of mature miRNAs, the high degree of cross-transferability across the genera is expected (Yadav et al., 2014).

However, the use of miRNA-based markers in terms of characterization and differentiation of individual developmental phases of the plant will require the application of several different types of miRNA and also the inclusion of several developmental phases into experimental evaluation.

Table 8. Results of multilevel data sorting genotypes in qualitative traits on bud, petals and boll

Tabuľka 8. Výsledky viacstupňového triedenia údajov kvalitatívnych znakov púčika, korunných lupienkov a tobolky

	Trait	Phenotype expression	GENOTYPES							
			1	2	3	5	4	7	6	8
COLOUR	petal colour in bud	dark blue	x	x						
	petal colour	pale blue with small violet hue	x		x					
	petal nerves colour	dark blue with small violet hue	x		x					
	petal colour in bud	very pale pink				x				
	petal colour	white with pink hue				x				
	petal nerves colour	blue-pink				x				
	petal colour in bud	white					x	x	x	x
	petal colour	white					x	x	x	x
	petal nerves colour	white					x	x	x	x
	petal colour in bud	blue-violet			x					
	petal colour	blue		x						
	petal nerves colour	dark blue		x						
SHAPE	petals longitudinal folding	absent	x	x	x	x				
	petal margin folding	absent	x	x		x				
	petals longitudinal folding	present - weak							x	x
	petal margin folding	present - weak crimp			x				x	x
	petals longitudinal folding	present - strong					x	x		
	petal margin folding	present - folded inwards					x	x		
SIZE	flower size	medium		x	x	x	x	x		
		big	x						x	x
BOLL	shape	globular	x	x	x	x	x	x	x	x

1 – Amon, 2 – Libra, 3 – Raciol, 4 - Lin 225, 5 - Bolley Golden, 6 - Currong, 7 - Mermilloid, 8 – Eyre

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