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Research paper

Excess fertilizer responsive miRNAs revealed in *Linum usitatissimum* L

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ABSTRACT

Effective fertilizer application is necessary to increase crop yields and reduce risk of plant overdosing. It is known that expression level of microRNAs (miRNAs) alters in plants under different nutrient concentrations in soil. The aim of our study was to identify and characterize miRNAs with expression alterations under excessive fertilizer in agriculturally important crop – flax (*Linum usitatissimum* L.). We have sequenced small RNAs in flax grown under normal and excessive fertilizer using Illumina GAIIx. Over 14 million raw reads was obtained for two small RNA libraries. 84 conserved miRNAs from 20 families were identified. Differential expression was revealed for several flax miRNAs under excessive fertilizer according to high-throughput sequencing data. For 6 miRNA families (miR395, miR169, miR408, miR399, miR398 and miR168) expression level alterations were evaluated on the extended sampling using qPCR. Statistically significant up-regulation was revealed for miR395 under excessive fertilizer. It is known that target genes of miR395 are involved in sulfate uptake and assimilation. However, according to our data alterations of the expression level of miR395 could be associated not only with excess sulfur application, but also with redundancy of other macro- and micronutrients. Furthermore expression level was evaluated for miRNAs and their predicted targets. The negative correlation between miR399 expression and expression of its predicted target ubiquitin-conjugating enzyme E2 gene was shown in flax for the first time. So we suggested miR399 involvement in phosphate regulation in *L. usitatissimum*. Revealed in our study expression alterations contribute to miRNA role in flax response to excessive fertilizer.

1. Introduction

Nutrients are needed for plant growth and development, and an effective system of fertilizer application is necessary to increase crop yields, fertilizer's efficiency and reduce risk of plant overdosing [1]. It has been reported previously that expression level of microRNAs (miRNAs) alters in plants under different nutrient concentrations in soil or nutrient solutions [2–4]. MiRNA is a class of small non-coding RNA that has been found in diverse organisms including plants. These consisting of approximately 20–24 nucleotides RNAs could control numerous biological processes through negative regulation of gene expression by specific binding and cleavage or translation inhibition of target mRNAs [5]. Certain miRNAs were up- or down-regulated under environmental stress such as drought, hypoxia, cold, salinity, heavy metal and high or low level of nutrients. Their target mRNAs regulate plant growth and development, stress response and nutrient homeostasis [2,6,7].

There are a lot of investigations of miRNA expression under nutrients deficiency [3,8,9], but there are only a few studies dedicated to the influence of excess nutrients application on miRNA

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regulation. In the present work, we estimated expression level alterations of flax miRNAs under the excess fertilizer application.

Flax (*Linum usitatissimum* L.) is agriculturally important crop that is used as a source of stem fiber and seed oil. Flax seed oil is utilized for the production of oils, paints, varnishes, linoleum, plastics, human and animal food. Flax fiber also has broad industrial applications [10]. In addition, *L. usitatissimum* is of research interest because some flax lines ('Stormont cirrus' and other) undergo phenotype and genome changes in response to the specific nutrient conditions [11–13]. We sequenced *L. usitatissimum* small RNAs and identified miRNAs that were expressed in flax under excess fertilizer for the first time. Also we validated high-throughput sequencing data by quantitative real-time PCR (qPCR) and characterized differentially expressed miRNAs and their targets.

2. Materials and methods

2.1. Plant materials

Flax (*L. usitatissimum* L.) line 'Stormont Cirrus' and cultivar 'TOST' were planted in a climate chamber under a 16-h light/8-h dark cycle at 22 °C in 5″ pots with pearlite for six weeks. Plants were grown under normal (*N*) nutrition (700 ml of 0.5× Hoagland's Solution [14], pH5.5, was applied weekly) and the excess nutrition (NPK): 700 ml of 0.5× Hoagland's Solution, pH5.5, with 2.56 g/l MASTER (Valagro, Italy; NPK 13:40:13 and micronutrients: Mg, S, B, Fe, Mn, Zn, Cu, Mo) was applied every seven days. Thus under the excess fertilizer, concentrations of applied nutrients were increased up to 12-fold (see Table 1).

2.2. RNA isolation

Leaf samples were collected from individual plants of line 'Stormont Cirrus' and cultivar 'TOST' after 6 weeks of growth and frozen in liquid nitrogen. Plant samples were stored at −70 °C. Total RNA was extracted using RNA MicroPrep reagents (Zymo Research, USA). RNA quality and quantity were evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and Qubit 2.0 (Life Technologies, USA). 'Stormont cirrus' RNA was used for sequencing (one biological replicate for each library) and qPCR while 'TOST' RNA was used for qPCR analysis (five and six biological replicates for NPK and N conditions respectively).

2.3. Small RNA high-throughput sequencing and bioinformatics analysis

High-quality RNA samples were used for small RNA (sRNA) library preparation using the Illumina TruSeq small RNA preparation kit (Illumina, USA) according to manufacturer's sample preparation guide. Two miRNA libraries were performed for high-throughput sequencing: 1) 'Stormont cirrus' grown under NPK conditions; 2) 'Stormont cirrus' grown under N conditions. Purified sRNA libraries were used for cluster generation on Illumina's c-Bot and sequenced on Illumina GAIIx.

Raw sequence reads were converted into full-length reads by removing low-quality reads and adapter reads. For the next processing, we used cleaned reads with abundance six or more.

Table 1
The concentration increase of applied nutrients in excess fertilizer compared to normal (n-fold).

N	P	K	Mg	S	B	Mn	Zn	Cu	Mo	Fe
4.1	8.2	2.1	2.0	1.6	3.1	4.1	12.1	11.0	4.9	3.3

In the present work we have studied only conserved miRNAs. To identify conserved known miRNAs in flax, sRNAs were aligned with known matured miRNA sequences from miRBase 20.0 [15].

2.4. Expression analysis of flax miRNAs

Expression analysis of miRNAs was performed as described in Ref. [16] for sequence reads generated from the N and NPK libraries. Expression levels of miRNAs in flax libraries were normalized to obtain reads number per million genome-matched reads (RPM).

The comparison of the expression level of each miRNA in NPK and N libraries was performed by the following formula:

$$fold\ \ change = log_2(RPM\ \ in\ \ NPK/RPM\ \ in\ \ N)$$

P-values were obtained according to the calculations of [17].

2.5. Quantitative real-time PCR

QPCR was performed for lus-miR395, lus-miR169, lus-miR408, lus-miR399, lus-miR398, lus-miR168, and sulfate adenylyltransferase (*ATP*), low-affinity sulfate transporter 2.1 (*SULTR2;1*), ubiquitin-conjugating enzyme E2 (*UBE2*) genes. Stem-loop RT primers were designed as described [18]. The 20 μl RT reaction mix contained 50 ng of total RNA, 50 nM of stem-loop RT primer, 250 nM each of dNTPs, 1× RT buffer, 3.33 U/μl M-MuLV reverse transcriptase (Fermentas, Lithuania). The reactions were incubated in 2720 Thermocycler (Applied Biosystems, USA) for 30 min, 16 °C; 30 min, 42 °C; 5 min, 85 °C and then held at 4 °C. All reverse transcriptase reactions, including no-template controls and RT minus controls, were run in duplicate.

QPCR was performed using a 7500 Real-Time PCR System (Applied Biosystems) by the following program: 95 °C, 15 min; 40 cycles of 95 °C, 15 s; 62 °C, 60 s. Each reaction was repeated three times with Eva Green dye and primers listed in Table 2. Sizes of

Table 2
Primer sequences.

Primer name	Primer sequence (5′ → 3′)
miR-R	CCAGTGCAGGGTCCGAGGTA
lus-miR395-SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACGAGTTC
lus-miR395-F	CACGCACTGAAGTGTTTGGGG
lus-miR169-SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACAGGCAA
lus-miR169-F	CACGCATAGCCAAGGATGAC
ETIF3E-F	TTACTGTCGCATCCATCAGC
ETIF3E-R	GGAGTTGCGGATGAGGTTTA
ETIF3H-F	CAGCGTGCTTGAAGTAACCA
ETIF3H-R	AACCTCCCTCAAGCATCTCA
lus-miR398-SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACCAGGGG
lus-miR398-F	CACGCATGTGTTCTCAGGT
lus-miR399-SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACCAGGGC
lus-miR399-F	CACGCATGCCAAAGGAGAGTT
lus-miR408-SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGCCAGG
lus-miR408-F	CACGCAATGCACTGCCTCTTC
lus-miR168-SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACTTCCCG
lus-miR168-F	CACGCATCGCTTGGTGCAGGT
ATP-F	CCTACGCGGGTTCATGAGAG
ATP-R	TCGATTGCCAGTACGATGGG
SULTR2; 1-F	CATATTCCAGGAGGTGGCGG
SULTR2; 1-R	ACCTCGAGAAAGCTGCAACA
UBE2-F	ACTTGGACTCCCAGATTGCC
UBE2-R	CAGAAGCTCCGTGTAACAAACA

SL – steam-loop; R – reverse; F – forward.