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PROCEEDINGS OF THE LATVIAN ACADEMY OF SCIENCES. Section B,
Vol. 77 (2023), No. 5/6 (746/747), pp. 271–274.

DOI: 10.2478/prolas-2023-0038

Short Communication

miR159c EXPRESSION UNDER NANOPARTICLE STRESS IN FLAX (*LINUM USITATISSIMUM* L.) CALLI

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Communicated by Isaak Rashal

The rapid development of nanotechnologies has raised great interest in the use of engineered nanoparticles (NPs) and their release in the environment. The interaction between nanoparticles and crops is currently a topical problem, as nanoparticles are capable to enter the soil, plant cells and to translocate in plants. MicroRNA (miRNA) is an endogenous small RNA with a significant regulatory role in plants, cleaving or repressing the translation of a particular mRNA in response to multiple biotic and abiotic factors. In this work, the expression of miR159c in Linum usitatissimum *L. calli cultures caused by various concentrations of Fe_3O_4 nanoparticles was studied. The obtained results showed an increase in miR159c expression with a decrease in the concentration of Fe_3O_4 nanoparticles. The results obtained are important in explaining the potential of nanoparticle utilisation to provide plant immunity responses.*

Keywords: *miRNA, Fe_3O_4 nanoparticles, abiotic stress, flaxseed, callus culture.*

The human population size and wealth is increasing continuously and is expected to grow in the future, and therefore, global food needs to be increased, but crop and herbage productivity is decreasing due to constantly changing environment and various abiotic (drought, high salinity, nanoparticles, etc.) and biotic (viral, bacterial, fungal pathogens) stresses (Khraiwesh *et al.,* 2012; Xu *et al.*, 2020a). Plants are a crucial part of human daily diet, ecosystem and environment (Ma *et al.*, 2018). Plants are unable to move away from environmental stresses and are susceptible to growth, development, and productivity changes that are induced by various stress factors (Gehan *et al.*, 2015). Fungi are known to be the most common microbial pathogens of plants around the world (Cools *et al*., 2017). Many scientists worldwide are working with the aim to exploit agents that can provide plants with resistance against the attack of pathogens including fungi. Currently, nanotechnology has been found to be an essential tool in agricultural applications (Fincheira *et al.*, 2020).

Magnetic nanoparticles (NPs) such as iron oxide (Fe_3O_4) NPs are multifaceted NPs that have attracted attention in scientific and agricultural fields. Numerous experiments have been performed to investigate plant response to Fe_3O_4 NPs, and many results show a positive effect on different plant germination, growth, and development. For example, Fe_3O_4 NPs (50 to 200 mg/g in soil) increased growth and biomass of lettuce (Zahra *et al.*, 2015). Furthermore, magnetite NPs showed a positive influence on soybean root elongation (Ghafariyan *et al*., 2013). Also, it was found that Fe_3O_4 NPs treatment increased chlorophyll level in oak leaves in comparison with control plants.

MiRNAs are small sequence-specific regulatory molecules that play a significant role in target gene silencing at the transcriptional level through DNA methylation and post-transcriptional level in eukaryotic organisms. miRNAs are actually regarded as the most important gene regulators, negatively regulating gene expression in plants (Bofill-De Ros *et al.*, 2020). Small RNAs can act as a new breeding tool in plant genetic improvement (Li and Zhang, 2016). Recently, it has been shown that plant miRNAs are highly involved in several processes including abiotic and biotic stress responses (Singh *et al.*, 2020). Numerous investigations confirm that the induction or repression of specific miRNAs contributes to the defence of plants against a broad

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range of non-viral pathogens. Many of them present up- or down-regulation of specific miRNAs in response to infection with fungal pathogens (Zogli and Libault, 2017). For instance, in wheat, miR159c is involved in response to fungal infection (Khraiwesh *et al.*, 2012).

Pectic polysaccharide homogalacturonan is a main component of the plant primary walls. Homogalacturonan is exported to cell wall in highly methylesterified form, but to acquire successful infection of host, pathogens including fungi need degradation of pectin by pectin methylesterases (PMEs) (Xu *et al.*, 2020b). A large family of PMEs catalyse the de-methyl esterification of homogalacturonan, thereby leading to cell wall loosening. This causes targeting of pathogen pectin-degrading enzymes, affecting the texture and rigidity alteration of the cell wall, which leads to easier entry of pathogens, plant damage and death (Raiola *et al.*, 2011).

Flax *Linum usitatissimum* L. is a common oilseed industrial crop that is considered as economically important. Flax is cultivated worldwide for high quality fibre (linen) and seed oil. Unfortunately, flax, as well as other crops, is susceptible to several fungal diseases that lead to serious losses in seed yield and quality and stem fibre quality in flax (He *et al.*, 2019). Therefore, the aim of the present study was to detect the amount of plant resistance-related specific miRNA in flax *in vitro* tissues in different callus development stages under Fe_3O_4 nanoparticle stress conditions.

Fe_3O_4 NPs were synthesised at Daugavpils University in the G. Libert's Centre of Innovative Microscopy, using the electrochemical method of magnetite nanoparticle synthesis (Cabrera *et al.*, 2008). The size of derived nanoparticles was 25 nm. The following concentrations of nanoparticle solutions were prepared for the study: 0.5 mg/l, 1 mg/l, and 1.5 mg/l.

For callus formation, one donor plant of *Linum usitatissimum* 'Vega 2' was used. *In vitro* cultures were achieved as described in Kokina *et al.* (2012). Two experiments with callus culture were conducted according to Kokina *et al.* (2017) as follows: in experiment A, explants (n = 135) were divided into three experimental groups, where the Murashige and Skoog (MS) medium was supplemented by different concentrations of Fe_3O_4 NPs: 0.5 mg/l, 1 mg/l, and 1.5 mg/l. Explants were cultivated for nine weeks in order to obtain callus cultures till analysis with molecular biology methods.

In experiment B, explants (n = 135) were left on MS medium without NPs for five weeks to accumulate callus biomass. Then obtained calluses (n = 135) were divided into control group (medium without NPs) and three experimental groups, where MS medium was supplemented by the same Fe_3O_4 NPs concentrations and were left on medium for four weeks (Kokina *et al.*, 2017).

DNA was extracted from obtained calluses in both experiments, and real-time qPCR analysis was carried out. Total RNA from flax calluses of both experiments was isolated according to Plaksenkova *et al.* (2019). miRNA target-specific primer lus-miR159c with locked nucleic acid was designed. The target miRNA lus-miR159c sequence was: 5'- UUUGGAUUGAAGGGAGCUCUU -3'. In the relative quantification analysis, the flax elongation factor 1-alpha (*EF1i*) gene was used as a reference gene in order to normalise expression values as gene shown expression in a stable manner in flax tissue (Neutelings *et al.*, 2012). Reverse transcription for miRNA was performed according to Plaksenkova *et al.* (2019). The results were analysed using the $\Delta\Delta C_T$ method (Kumar and Lorand, 2021).

RT-qPCR reactions were performed to quantify known miRNA expression level. The results showed that Fe_3O_4 NPs treatment altered miRNA gene expression in a dosage-dependent manner. The miRNA expression level was up-regulated at all tested concentrations. However, NPs exposure insignificantly ($p > 0.05$) affected miRNA expression in experiment A. The expression level of miR159c decreased (1.23, 1.14, and 1.03-fold) with increase in NPs concentration. Nevertheless, 0.5 mg/l concentration of NPs resulted in the largest increase of specific miRNA expression level (1.23-fold), while 1 mg/l and 1.5 mg/l concentrations increased expression only by 1.18 and 1.12-fold, respectively, in callus cultures obtained during cultivation on MS medium with different concentrations of NPs, only at the four-week callus stage (experiment B).

One way to improve plant productivity is through use of miRNAs. Some plant miRNAs can up- or down-regulate the expression of target genes to get through the necessity of growth and environmental stress (Wang *et al.*, 2018). The results indicated that lower concentrations of Fe_3O_4 NPs cause higher expression of miR159c in callus cultures induced from flax explants grown on MS medium with different concentrations of NPs (Experiment A) (Fig 1). This data is consistent with previous results of investigation of miR159c expression in five weeks old rocket seedlings that were affected by 0.5 mg/l, 1 mg/l, and 1.5 mg/l Fe_3O_4 NPs concentrations (Plaksenkova *et al.*, 2019). However, in callus cultures obtained during cultivation on MS medium with different concentrations of NPs, this was shown only at the four-week callus stage. This suggests that miR159c is slightly sensitive to Fe_3O_4 NPs exposure. The results can be beneficial in the field of increasing specific miRNA expression level using NPs. The obtained data are consistent with another independent study, where TiO_2 NPs also slightly increased miR159c expression level in the same way in three-week-old tobacco seedlings (Frazier *et al.*, 2014). Since miRNAs are plant species-dependent they may be evolved in various plant response mechanisms. For instance, was shown that in *Arabidopsis*, miR159 is drought responsive (Kantar *et al.*, 2011), while in maize it is involved in response to hypoxia (Khraiwesh *et al.*, 2012). Moreover, in wheat, miR159 is involved in response to fungal infection (Khraiwesh *et al.*, 2012). Xin *et al.* (2010) showed significant decrease of miR159 both in powdery mildew susceptible and resistant wheat lines. However, this

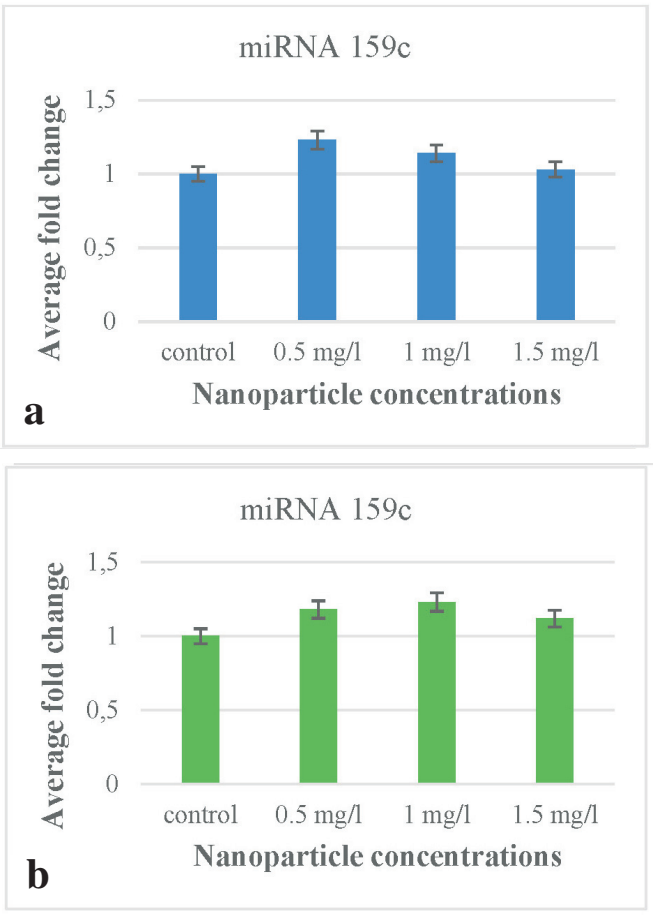


Fig. 1. Expression of miRNA in flax calluses. a – in callus cultures induced from flax explants grown on Murashige and Skoog (MS) medium with different concentrations of nanoparticles (NPs) (experiment A); b – in callus cultures obtained during cultivation on MS medium with different concentrations of NPs, only at the 4-week callus stage (experiment B). Values are means of three replicates with standard deviation.

miRNA was up-regulated under heat stress in wheat seedlings. Furthermore, according to Zhang *et al.* (2016), cotton plants increase production of miR159 in response to infection with fungal pathogen. Unfortunately, data about the role of miR159 in flax is unknown, but the obtained results and data of independent studies allow to suggest that there is a possibility that Fe_3O_4 NPs may increase plant resistance to biotic stress such as a fungal pathogen. Therefore, the next step of investigation will be developed where flax cultures grown with and without NPs are exposed to fungal pathogen, with evaluation of miRNA expression. Nevertheless, further investigations are needed to clarify interaction mechanisms between NPs and miRNA. These results are significant in terms of use of nanoparticles to control resistance level for fungal infections in plants.

In this study, the expression of miR159c in different *in vitro* flax callus cultures in response to Fe_3O_4 NPs stress was investigated. The obtained results allow to conclude that these NPs are capable of affecting miRNA expression in *in vitro* flax cultures. We can assume that small Fe_3O_4 NPs (25 nm) possibly can affect flax resistance against fungal pathogen. To verify this, an experiment will be carried out, where flax seedlings treated with Fe_3O_4 NPs will be used to explore miR159c expression in control and infected flax seedlings.

To the best of our knowledge, this is the first report concerning determination of miRNA expression that is associated with plant response toFe_3O_4 NPs in flax cultures. Mechanisms of interactions between NPs and flax miRNAs related with fungal resistance will be conducted in our following work. We believe that the results will help us to better understand plant nanoparticle stress-related mechanisms and will provide a new tool for regulation of miRNAs expression to withstand fungal pathogen attack.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENTS

This study was supported by Daugavpils University internal grant No. 14-95/13. The authors also acknowledge the scientific staff of Daugavpils University G. Libert's Centre of Innovative Microscopy for kindly provided nanoparticles.

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Received 12 January 2021
Accepted in the final form 12 April 2023

miR159c EKSPRESIJA LINU (*LINUM USITATISSIMUM* L.) KALLUSOS NANODAĻIŅU STRESA IETEKMĒ

Nanotehnoloģiju strauja attīstība izraisa lielu interesi par ražotu nanodaļiņu izmantošanu un nonākšanu apkārtējā vidē. Nanodaļiņu un kultūraugu mijiedarbība pašlaik ir aktuāla problēma, jo nanodaļiņas bieži nonāk augsnē, augu šūnās un transportējas augos. MikroRNS (miRNS) ir endogēnās mazās RNS ar nozīmīgu regulējošu lomu augos, sašķeļot vai represējot konkrētas mRNS translāciju, atbildot uz vairākiem biotiskiem un abiotiskiem faktoriem. Šajā darbā tika pētīta miR159c ekspresija *Linum usitatissimum* L. kallusu kultūrās, ko izraisīja dažādas Fe_3O_4 nanodaļiņu koncentrācijas. Iegūtie rezultāti parādīja, ka miR159c ekspresija palielinās līdz ar Fe_3O_4 nanodaļiņu koncentrācijas samazināšanos. Iegūtie rezultāti ir būtiski, skaidrojot nanodaļiņu izmantošanas potenciālu augu imunitātes reakciju nodrošināšanā.