International Journal of Innovative Engineering Applications

Journal homepage: https://dergipark.org.tr/ijiea

MOBILE MICRORNAS (MIRNAS) RESPONSIVE TO EXCESS NICKEL IN PUMPKIN (*CUCURBITA MAXIMA* L.)

Guzin Tombuloglu[*1]

[1]*Department of Biophysics, Institute for Research and Medical Consultations (IRMC), Imam Abdulrahman Bin Faisal University, P.O. Box 1982, Dammam 31441, Saudi Arabia*

Abstract

Original scientific paper

Nickel (Ni) is a toxic heavy metal that inhibits plant growth, development, and reproduction. MicroRNAs (miRNAs) travel from cell to cell or organ to carry messages to regulate gene expression. This study aims to find mobile miRNAs that are Ni-responsive and are present in pumpkin (*Cucurbita maxima* L.) phloem sap. For this purpose, pumpkin seedlings were exposed to Ni (100 µM, $NiCl_2$), and root, shoot, and phloem-sap specimens were collected at 0 (control), 24, and 48 hours of the treatment. The stem-loop RT-qPCR and stem-loop semi-quantitative RT-PCR methods were used to determine the abundance of 14 miRNAs in the phloem sap. Compared to the control, the abundance of miR160, miR167, miR393, miR397, and miR398 was suppressed in Ni-treated seedlings. The reduction was verified by grafting experiments, revealing that miR167 and miR393 are Ni-responsive and move/travel from the leaf-to-root direction. Those phloem-residential miRNAs potentially play a role in the Ni-response mechanism. This study can help to understand the early response mechanism of plants against excess Ni and lead to identifying miRNA-mediated long-distance communication of plants.

Keywords: *miRNA, phloem, long-distance communication, nickel, pumpkin.*

BALKABAĞINDA (*CUCURBITA MAXIMA* L.) FAZLA NİKELE YANIT VEREN MOBİL MİKRORNA'LAR (MIRNA'LAR)

Özet

Orijinal bilimsel makale

Nikel (Ni) fazlalığı durumunda, bitki büyümesini, gelişimini ve üremesini engelleyen toksik bir ağır metaldir. MikroRNA'lar (miRNA), gen ekspresyonunu düzenlemek için mesajlar taşımak üzere hücreden hücreye veya organa seyahat eder. Bu çalışma balkabağı (*Cucurbita maxima* L.) floem özsuyunda dolaşan Ni'ye duyarlı mobil miRNA'ları tanımlamayı amaçlamaktadır. Bu amaçla, Ni (100 µM, $NiCl_2$) uygulanan balkabağı fidelerine ait kök, sürgün ve floem özsuyu örnekleri toplanmıştır. Floem özsuyundaki 14 miRNA'nın ekspresyonunu belirlemek için gövde-ilmek RT-qPCR ve gövde-ilmek yarı-kantitatif RT-PCR yöntemleri kullanıldı. Kontrol ile karşılaştırıldığında, Ni ile muamele edilmiş fidelerde miR160, miR167, miR393, miR397 ve miR398'in miktarının azaldığı tespit edildi. Bu azalma, aşılama deneyleriyle de doğrulanarak, miR167 ve miR393'ün Ni'ye duyarlı olduğu ve yapraktan köke doğru hareket ettiğini ortaya çıkardı. Floemde bulunan bu miRNA'lar, Ni-yanıt mekanizmasında potansiyel olarak rol oynamaktadır. Bu çalışma, bitkilerin fazla Ni'ye karşı erken tepki mekanizmasının anlaşılmasına yardımcı olarak, bitkilerin miRNA aracılı kök-yaprak iletişiminin belirlenmesine yol gösterebilir.

Anahtar Kelimeler: *miRNA, floem, uzak organ iletisimi, nikel, balkabağı.*

1 Introduction

An RNA-based communication network through phloem has been discovered between plants' distant organs (root-leaf). So far, different RNA types have been found as mobile RNAs, such as viral RNAs, cellular mRNAs, tRNAs, and microRNAs (miRNAs) [1-4]. The existence of mobile miRNAs has been revealed in different plants, such as gourd, lupine, beans, yucca [5], rapeseed [6], apple [3, 7], and pumpkin [8]. They can move along the phloem and reach distant organs/tissues of plants through the mass flow of water [1-4, 9]. Some mobile RNAs are essential in transcriptional response mechanisms such as nutrient (phosphate, sulfur, and copper) deficiencies. For instance, miR399 was found to be translocated from leaf to root through the phloem upon phosphate deficiency [10-12]. It was also found that phosphate deficiency induces miR169, miR827, and miR2111 in rapeseed (*Brassica napus*) phloem sap [13]. In addition, miR399 has been proven to be a signaling molecule that communicates between leaves and roots via phloem in *Arabidopsis*, pumpkin (*Cucurbita maxima*), and tobacco (*Nicotiana tabacum*) plants [6, 10,

*Corresponding author.

E-mail address: guzinkekec@gmail.com (G. Tombuloğlu)

Received 28 May 2023; Received in revised form 07 February 2024; Accepted 13 February 2024

2587-1943 | © 2024 IJIEA. All rights reserved. Doi: https://doi.org/10.46460/ijiea.1304404

14-15]. miR395 and miR398 abundances in phloem sap have increased due to sulfur and copper deficiency, respectively [6, 12, 16-17]. Besides, the expressions of miR397, miR408, and miR857 were found to be increased against copper deficiency [18]. Moreover, grafting experiments showed that miR172, abundantly found in the phloem sap, is associated with plant growth and affects tuber formation in potatoes [19-20].

Nickel (Ni) is the seventeenth essential element for the development and growth of plants. It is involved in the biosynthesis of some bacterial taxa' hydrogenase, carbon monoxide dehydrogenase, and factor F 430 [21]. Another Ni-containing protein is urease, found in jack beans and several plant species. However, it is a potentially hazardous metal, with an average concentration of 20 to 30 mg/kg in soils and occasionally surpassing 10,000 mg/kg (*e.g*., ultramafic soils) [22-23]. The concentration of Ni in agricultural soils is generally deficient. However, the nickel content of soils composed of ultra-basic igneous rocks such as serpentine varies between 100-5,000 mg Ni/kg [24]. Excess Ni in the plant harms chlorophyll synthesis and lipid metabolism, preventing plant roots from taking other nutrients and causing nutrient deficiencies [25].

This study aims to identify Ni-responsive mobile miRNAs. For this purpose, 14 miRNAs detected in pumpkin's phloem sap were chosen. Their expression at 12 and 24 hours was compared with those of untreated (control) one using stem-loop RT-qPCR and semi-quantitative RT-PCR assays. The mobility of selected miRNAs was verified by grafting assay. This study reveals Ni-responsive mobile miRNAs potentially involved in the long-distance communication of tissues.

2 Material and Methods

2.1 Plant Growth and Nickel Treatment

The seeds of a pumpkin (*Cucurbita maxima* L., Dill's Atlantic Giant) were surface sterilized by submerging them in a 5% sodium hypochlorite (NaClO) solution for 10 minutes before being washed three times with distilled water (dH_2O). The filter paper was adjusted into a Petri dish, adding 5 mL of dH_2O. The seeds were placed between sterile filter papers in the Petri dish (10 cm x 1.5 cm) and kept at room temperature. After seven days, about 80% of the seeds germinated, and root lengths were at least 15 mm long. The seedlings were placed in a hydroponics medium that contained macronutrient solution in mM: 1 $NH_4H_2PO_4$, 4 $Ca(NO_3)_2$; 2 $MgSO_4$, 6 KNO_3, and micronutrient solution in µM: 0.8 $ZnSO_4$, 9 $MnCl_2$, 0.3 $CuSO_4$, 50 H_3BO_3, 25 Fe-EDTA, and 0.02 g MoO_3 (85%) [26]. The plants were grown in a temperature (20-24 °C), humidity (40-60%), and light (6,000 Lux)-controlled growth chamber (WiseCube, Korea). The nutrient solution was kept at a constant pH (5.5) and renewed weekly.

Three-week-old pumpkin seedlings were exposed to 100 µM Nickel ($NiCl_2$) solution at two different time intervals, 24 h and 48 h. Leaf, root, and phloem sap (PS) samples were harvested from the seedlings (n = 3) exposed to Ni (control (0), 24 h, and 48 h). The tissue samples (root and leaf) were flash-frozen by liquid nitrogen and stored at -80 °C. The PS samples collected from each plant were kept in ice until RNA isolation.

2.2 RNA Lsolation from Phloem Sap (PS) and Tissues

The PS exudates were collected using Buhtz et al. [6]'s outlined procedure. For this purpose, the stem of each seedling (n = 3) was cut with a sterile razor blade. To avoid tissue contamination, the first droplets of PS were removed with sterile filter paper from the cut surface. The exudates are then gathered in a pre-chilled tube with 10 µl pipette tips every 10 minutes until the PS exudates reach about 25 µl.

The RNA was extracted according to Zhang et al. [30] and Chomczynski and Sacchi [27]. Accordingly, the PS and Trizol® LS reagent (Invitrogen) were mixed (1:3) and vortexed thoroughly. The mixture was incubated for five minutes at room temperature. After adding 25 µl of chloroform (Merck, Germany), the mixture underwent a 15-sec handshake, incubated for 15 min at room temperature, and cold centrifuged (4 °C) at 12,000 x g for 15 min. The supernatant was mixed with absolute ethanol (Millipore) (1:2) and incubated at -20 °C overnight. After 30 min centrifugation (16,000 x g, 4 °C), the pellet was rinsed with DEPC-treated 70% ethanol (Millipore), dried, and re-suspended with ddH_2O. The RNA samples were quantified by NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific), and kept at -80 °C.

2.3 Stem-Loop 1st-Strand cDNA Synthesis

The stem-loop 1st-strand cDNA synthesis method was conducted to determine the miRNAs in the PS. For this purpose, 1 µl of RT primer (1 µM) (Table S1), 30 ng of RNA, 0.5 µl of dNTP (10 mM) (Bio-Basic) and DEPC-treated water was mixed to make a total volume of 14 µl. The mixture was kept at 65 °C for 5 min and immediately placed on ice. Afterward, 4 µl of Buffer (5x), 2 µl RiboLock RNase Inhibitor (10 U/µl), 1 µl of RevertAid M-MuLV Reverse Transcriptase (200 U/µl), and DEPC-treated water (up to 20 µl) were added. The mixture was kept at 16 °C for 30 minutes, and the following reaction conditions were applied: 30 °C 30 sec, 42 °C 45 sec, and 48 °C 1 sec (60 cycles). The enzyme inactivation was performed at 70 °C (5 min), and then the tubes were kept at -20 °C.

2.4 Stem-loop Quantitative Reverse Transcription PCR

The stem-loop quantitative RT-PCR method was used to determine the expression level of miRNAs under control and Ni-treated specimens [28-29]. miRNA amplification was performed using SYBR®Premix Ex Taq™ (2×) (Tli RNaseH Plus) kit (Takara, Japan). Accordingly, 1 µl (10 µM) of miRNA-specific forward and universal primers (Table S1), 2 µl of cDNA, 8 µl of sterile water, and 10 µl of SYBR®Premix Ex Taq™ (2×) mixture were collected in a 0.2 ml PCR tube. A real-time PCR device (Rotor-Gene® PCR, Corbett Research-Qiagen) was used for the reaction settled at 95 °C (2 min), 40 cycles of 95 °C (5 sec), 56 °C (15 sec), and 72 °C (20 sec). Subsequently, the primer specificity was tested by performing a melting

curve analysis (MCA) between 52 and 95 °C (0.5 °C increments/sec).

2.5 Semi-quantitative RT-PCR

The expression levels of miRNAs in control and Ni-applied specimens were determined using the semi-quantitative RT-PCR technique. For this purpose, 2 µl cDNA, 2.5 µl 10X buffer, 0.5 µl 10mM dNTP, 1.5 µl 2 mM $MgCl_2$, 0.75 µl (10 µM) miRNA specific "forward" and "universal" primers (Table S1), 0.25 µl Taq Polymerase (Fermentas, Thermo Scientific), and 17.75 µl sterile water were mixed. PCR conditions were adjusted as follows: 95 °C (2 min), 30 cycles of 95 °C (5 sec), 56 °C (15 sec), and 72 °C (20 sec). Finally, the polymerization temperature was set at 72°C (7 min) (TECHNE, TC-512 thermal cycler). After the reaction, the PCR products were loaded in a 3% agarose gel using 1x TBE buffer. The gel was visualized under ultraviolet light.

2.6 Contamination Analysis

To check for any contamination that might have occurred during the isolation and/or exudation of phloem sap, the existence of *CmPP16* (AF079170) and *RuBisCo* were tested. The *CmPP16* gene, a phloem-specific transcript found in the PS of *Cucurbitaceae* species [30-31], is used as a positive control. In addition, *RuBisCo*, a transcript exclusive to green tissues, was chosen as a negative control to examine any contamination that might have occurred during PS sampling. The leaf and PS RNAs from all tested plants were used for the analyses. First-strand cDNA synthesis was carried out according to Tombuloglu et al. [26]. The transcripts were amplified in a thermal cycler (Techne TC-512, UK) using standard protocols detailed in the previous section. The PCR products were loaded in 2% agarose gel by labeling SafeView® stain and visualized under UV light.

2.7 Grafting Experiments

Grafting experiments were conducted to demonstrate and verify the translocation of mobile miRNAs between root and leaf in pumpkin seedlings. First, the unstressed (control) root and the unstressed stem are grafted (self-grafting) to detect possible miRNA differences that may occur due to tissue injury. In another set, the unstressed root (rootstock) was grafted with the stressed stem (scion) treated with Ni for 48 h. After grafting, the seedlings were placed in dark and humid conditions (>90%) for overnight. The PS, root, and leaf tissues were collected for RNA isolation. The protocols for RNA isolation, stem-loop cDNA synthesis, and stem-loop RT-PCR were carried out to determine the expression level of selected miRNAs.

3 Results

3.1 Contamination Test

RNA contamination from the neighboring tissues during the collection of phloem sap (PS) is one of the challenges. *CmPP16*, a transcript specific to PS, was used as a positive control to elucidate possible RNA contamination. In addition, *RuBisCo*, a tissue-specific transcript that does not exist in the PS, was used as a negative control to test any possible RNA contamination during PS collection. Results showed that *RuBisCo* transcript was absent in the PS of the control and Ni-treated samples (24h and 48h) (Figure 1a). Conversely, the *CmPP16* transcript was evident in the PS specimens of the same seedlings (Figure 1a). Moreover, *RuBisCo* transcripts were detected in all tested leaves, including control, 24h, and 48h specimens (Figure 1b). These results showed that PS specimens collected from control and Ni-treated seedlings are exempt from RNA contamination that may be derived from neighboring tissues.

3.2 Stem-loop Quantitative RT-PCR Analysis of miRNAs

Expressions of 14 miRNAs in the PS of control, 24h, and 48h Ni-applied seedlings were determined. Among tested miRNAs, the abundance of five miRNAs (miR160, miR167, miR393, miR397, and miR398) was found to be altered at 24h and 48h of Ni treatment (Figure 2). Compared to the control, those miRNAs were down-regulated in Ni-treated seedlings.

3.3 Confirmation of Results with Semi-quantitative RT-PCR

To validate the RT-qPCR findings, a semi-quantitative RT-PCR technique was used to determine the expression level of five down-regulated miRNAs. As depicted in Figure 3, the intensity of miR167 and miR393 bands gradually decreased in 24h and 48h of Ni-treated samples compared to that of the control. These results aligned with the quantitative RT-qPCR results depicted in Figure 2. However, no significant change in band intensities was observed in miR160, miR397, and miR398 samples. These results confirm the existence and down-regulation of miR167 and miR393 in the phloem sap of pumpkin seedlings as responsive to nickel application.

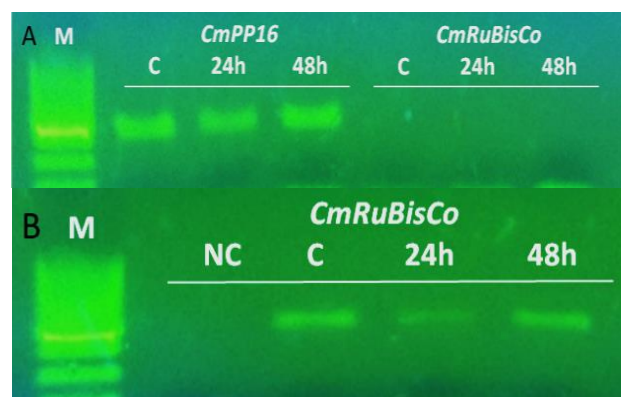

Figure 1. Agarose gel image showing contamination test results upon nickel (Ni) application. M = Marker (100 bp). **(a)** *CmPP16* transcripts were determined in the phloem sap of control (C), and Ni-treated seedlings (24h and 48h). However, *CmRuBisCo* transcripts were not detected in the identical specimens **(b)** The tissue-specific transcript, *CmRuBisCo*, was determined in the leaf tissues of control (C) and Ni-treated seedlings (24h and 48h). NC = negative (no template) control.

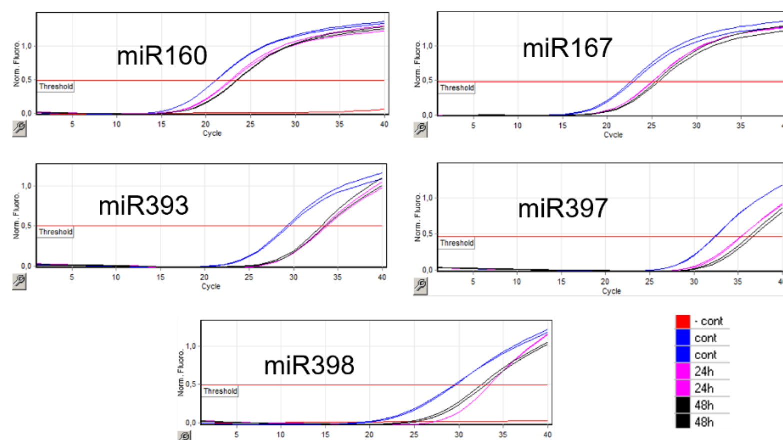

Figure 2. Stem-loop RT-qPCR analysis of miRNAs (miR160, miR167, miR393, miR397, and miR398) in the phloem sap (PS) of Ni-treated and untreated (control) seedlings. The color legend was shown on the right-hand side.

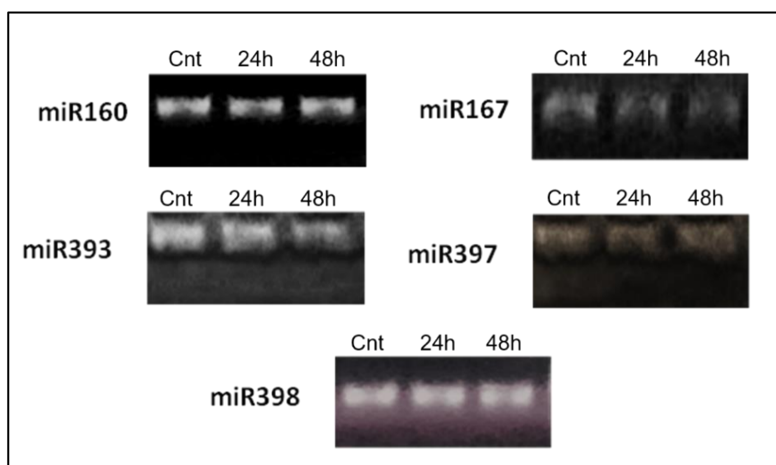

Figure 3. Semi-quantitative RT-PCR analysis of Ni-responsive miRNAs in control (Cnt) and Ni-treated (24h and 48h) plants.

3.4 Expression of miR167 and miR393 in Grafted Seedlings

Grafting experiments were carried out to verify the expression of Ni-responsive miRNAs found in the quantitative RT-qPCR and semi-quantitative RT-PCR analyses. For this purpose, the excess Ni-responsive miRNAs were determined in the PS of control (untreated), self-grafted (control rootstock + control scion), rootstock-grafted (control rootstock + Ni-treated scion), and scion-grafted (Ni-treated rootstock + control scion) seedlings (Figure 4a, b). Self-grafting was applied to elucidate a possible miRNA expression change that could be caused by tissue injury during the grafting process. Besides, Ni-treated scion was grafted on the control rootstock to determine the direction of miRNA movement (*i.e.,* from scion to rootstock or *vice versa*). Self-grafting results showed no difference in miR167 expression. However, the self-grafting process increased miR393 expression, which can be attributed to tissue injury/repair during the grafting (Figure 4c). This result revealed that miR393 could be responsive to tissue injury as well. Once Ni-treated scion is grafted on the control rootstock, the expression of miR167 and miR393 were suppressed, as expected. On the other hand, the grafting of Ni-treated rootstock with control scion increased the abundance of miR167 and miR393 compared to those of untreated (control) and self-grafting seedlings. These results show that miR167 and miR393 are Ni-responsive and move/travel from leaves to the roots.

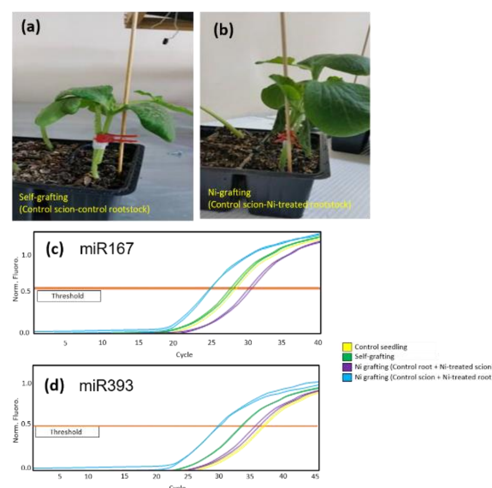

Figure 4. Grafting experiments. (a) The control seedlings were self-grafted. (b) The control scion has grafted on the Ni-treated (48h) rootstock—the stem-loop quantitative RT-PCR analysis of (c) miR167 and (d) miR393.

4 Discussion

Nickel (Ni) is an essential micronutrient for the growth and development of plants. It involves biochemical reactions and takes place in integral components of various biomolecules such as metalloenzymes and enzymes [32]. Besides, it is a heavy metal that limits crop growth and development and eventually yields when its concentration in the soil reaches an excess level [33]. Due to increasing

anthropogenic, technogenic, and geogenic activities, Ni pollution in agricultural soils has become widespread worldwide [34]. Burning fossil fuels, industries using Ni, and mining activities release it into the atmosphere. These releasing substances readily dissolve in water and then build up in the soil and sediments [35-38]. According to the World Health Organization (WHO), Ni concentration in soils is 15-30 mg/kg. Its concentration in most plant parts is between 0.05–10 mg/kg dry weight and is considered toxic above this amount [39]. Although plants' resistance mechanism and homeostasis against Ni stress have been studied [40-43], there is still a lack of information about Ni-induced miRNA mobilization. The information on this mechanism is crucial to understanding tissue or organ-level communication of plants to improve heavy metal resistance.

Small regulatory RNAs known as miRNAs are a diverse class that controls post-transcriptional gene expression. They also play a critical role in gene regulatory networks [44]. "Mobile" RNAs have been found in the phloem fluid/sap, which is functional in root-to-leaf communication [6, 45]. Moreover, they are known as naturally occurring species-to-species signaling molecules, such as between the parasitic plant *Cuscuta* and its hosts like *Arabidopsis* and tobacco [46]. Recent studies documented the first case of plant-to-plant miRNA communication. They provided experimental support for the idea that plants might uptake miRNAs produced by their neighbors, causing post-transcriptional gene silencing (PTGS) in the cells receiving them [47-48].

In this study, pumpkin seedlings were exposed to excess Ni, and stem-loop RT-PCR analysis was conducted for 14 miRNAs (miR156, miR168, miR398, miR166, miR397, miR167, miR164, miR169, miR390, miR172, miR393, miR159, and miR162), which were verified as PS residential. Among those miRNAs, miR160, miR167, miR393, miR397, and miR398 were responsive to Ni treatment, evidenced by stem-loop RT-qPCR (Figure 2). Besides, semi-quantitative RT-PCR analyses confirmed that those of two miRNAs (miR167 and miR393) are Ni-responsive (Figure 3), which were further tested by grafting experiments to find their transport direction (Figure 4). The grafting experiments revealed that miR167 and miR393 migrate from leaf to root. On the other hand, the abundance of other miRNAs (miR156, miR168, miR398, miR166, miR397, miR164, miR169, miR390, miR172, miR159, and miR162) in phloem sap did not change against Ni stress. Instead, they may play a role in systemic long-distance signaling during development or different types of stress.

miR398 is known to be a master regulator of plant development and stress responses [49]. A recent study found that miR398 is downregulated upon $NiCl_2$ treatments (50, 100, and 200 μM) over control seedlings of castor bean (*R. communis* L.) [50]. The same study showed up-regulation of a miR398-target gene, *cupper-zinc/superoxide dismutase* (*Cu-Zn/SOD or CSD*), in both root and leaf tissues upon Ni treatments. Cu-Zn/SOD is an antioxidant protein involved in scavenging superoxide anions. Our findings indicate that miR398 is involved in the long-distance communication of pumpkin seedlings against Ni-stress. Reduced miR398 expression in the PS could release *Cu-Zn/SOD* transcripts in the target tissue. Thus, the antioxidant defense system is promoted as an early stress response. In addition to miR398, miR171, miR395, miR396, and miR838 have been determined as Ni-responsive in the leaves of castor beans [50]. This study focused on identifying miRNAs in the PS of pumpkin. However, except for miR398, those miRNAs were not determined to be Ni-responsive. This can be attributed to the difference in plant type and the tissue studied, which is the PS of pumpkin in this study.

miR167 and miR393 were other Ni-responsive miRNAs found in the PS of pumpkin. Previous studies revealed their functions in different plants under nutrient deficiency. For instance, miR167 and miR160 were nitrate (N)–starvation responsive in *Arabidopsis* [51]. The potential functionality of miR160 in nitrogen (N) deficiency has been demonstrated in *Arabidopsis*. Its expression is upregulated in roots during transient low nitrate treatment [51]. In addition, several studies have revealed the functionality of miR167 in the developmental stages of tissues/organs (*i.e.,* root, leaf, flower, seed, embryo) and biotic and abiotic stress adaptation (reviewed by [52]). For instance, miR167 expression is induced in *Arabidopsis* under salinity, drought, and cold stresses [53-54]. Although miR167 is stress-responsive, its function against Ni-stress is yet to be identified. This study, for the first time, revealed its possible function during the regulation of Ni stress. In addition, the grafting experiments showed the direction of long-distance movement, which was from the leaf to the root.

Additionally, miR393 was found to be phloem-resided and responsive to Ni. The grafting studies revealed its leaf-to-root movement. The roots are the primary tissue exposed to Ni stress. In line with these findings, miR393 was found in the phloem sap of *Brassica napus* under nutrient deficiency [12]. Transgenic rice and *Arabidopsis* are more sensitive to salt stress when OsmiR393 is overexpressed [55]. This study verified miR167 and miR393 as responsive to excess Ni. Their leaf-to-root movement in the plant body through the phloem may regulate the Ni homeostasis in plants. The transcriptional regulation of these miRNAs in the target tissues (*i.e*., roots or leaves) upon the miRNA translocation should be studied.

5 Conclusion

Several phloem-mobile miRNAs have been identified as messenger molecules, which play a molecular switch in target cells/organs and regulate early stress responses. For the first time, this study showed the phloem sap resident miRNAs in pumpkins and their response against excess nickel. Grafting experiments also showed the direction of miRNA movements. Among 14 selected miRNAs, miR167 and miR393 were nickel-responsive, moving from leaves to roots. Functional studies are necessary to clarify their role in the transcriptional regulation of nickel stress at their target tissue.

Acknowledgments

The author states that the study does not require an ethics committee's approval. The author has no conflict of interest.

Declaration

Ethics committee approval is not required.

References

[1] Kehr, J., & Buhtz, A. (2008). Long distance transport and movement of RNA through the phloem. *Journal of Experimental Botany,* 59, 85-92.
[2] Kragler, F. (2010). RNA in the phloem: A crisis or a return on investment. *Plant Science,* 178, 99-104
[3] Kehr, J. (2013). Systemic regulation of mineral homeostasis by micro RNAs. *Frontiers in Plant Science*, 4, 145.
[4] Ruf, A., Oberkofler, L., Robatze, S., & Weiberg, A. (2022). Spotlight on plant RNA-containing extracellular vesicles. *Current Opinion in Plant Biology*, 69, 102272.
[5] Yoo, B. C., Kragler, F., Varkonyi-Gasic, E., Haywood, V., Evans, S. A., Lee, Y. M., Lough, T. J., & Lucas, W. J. (2004). A systemic small RNA signaling system in plants. *Plant Cell*, 16, 1979-2000.
[6] Buhtz, A., Springer, F., Chappell, L., Baulcombe, D. C., & Kehr, J. (2008). Identification and characterization of small RNAs from the phloem of Brassica napus. *Plant Journal*, 53, 739-749.
[7] Varkonyi-Gasic, E., Gould, N., Sandanayaka, M., Sutherland, P., & MacDiarmid, R. M. (2010). Characterisation of microRNAs from apple (Malus domestica 'Royal Gala') vascular tissue and phloem sap. *BMC Plant Biology*, 10, 159.
[8] Tolstyko, E., Lezzhov, A., & Solovyev, A. (2019). Identification of miRNA precursors in the phloem of Cucurbita maxima. *PeerJ,* 7, e8269.
[9] Kehr, J., & Kragler, F. (2018). Long distance RNA movement. *New Phytologist,* 218(1), 29-40.
[10] Pant, B. D., Buhtz, A., Kehr, J., & Scheible, W. R. (2008). MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *Plant Journal*, 53, 731-738.
[11] Lin, S. I., Chiang, S. F., Lin, W. Y., Chen, J. W., Tseng, C. Y., Wu, P. C., & Chiou, T. J. (2008). Regulatory network of microRNA399 and PHO_2 by systemic signaling. *Plant Physiology*, 147, 732-746.
[12] Buhtz, A., Pieritz, J., Springer, F., & Kehr, J. (2010). Phloem small RNAs, nutrient stress responses, and systemic mobility. *BMC Plant Biology*, 10(64).
[13] Pant, B. D., Musialak-Lange, M., Nuc, P., May, P., Buhtz, A., Kehr, J., ... & Scheible, W. R. (2009). Identification of nutrient-responsive Arabidopsis and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. *Plant Physiology*, 150(3), 1541-1555.
[14] Kuo, H. F., & Chiou, T. J. (2011). The role of microRNAs in phosphorus deficiency signaling. *Plant Physiology*, 156, 1016-1024.
[15] Calderwood, A., Kopriva, S., & Morris, R. J. (2016). Transcript abundance explains mRNA mobility data in Arabidopsis thaliana. *Plant Cell*, 28, 610-615.
[16] Yamasaki, H., Abdel-Ghany, S. E., Cohu, C. M., Kobayashi, Y., Shikanai, T., & Pilon, M. (2007). Regulation of copper homeostasis by micro-RNA in Arabidopsis. *Journal of Biological Chemistry*, 282(22), 16369-16378.
[17] Ham, B. K., & Lucas, W. J. (2017). Phloem-mobile RNAs as systemic signaling agents. *Annual Review of Plant Biology*, 68, 173-195.
[18] Fahlgren, N., Howell, M. D., Kasschau, K. D., Chapman, E. J., Sullivan, C. M., Cumbie, J. S., Givan, S. A., Law, T. F., Grant, S. R., Dangl, J. L., & Carrington, J. C. (2007). High-throughput sequencing of Arabidopsis microRNAs: *Evidence for frequent birth and death of MIRNA genes. PLoS ONE*, 2.
[19] Martin, A., Adam, H., Diaz-Mendoza, M., Zurczak, M., Gonzalez-Schain, N. D., & Suarez-Lopez, P. (2009). Graft-transmissible induction of potato tuberization by the microRNA miR172. *Development*, 136, 2873-2881.
[20] Kasai, A., Kanehira, A., & Harada, T. (2010). miR172 can move long distance in Nicotiana benthamiana. *The Open Plant Science Journal,* 4, 1–6.
[21] Alfano, M., & Cavazza, C. (2020). Structure, function, and biosynthesis of nickel-dependent enzymes. *Protein Science*, 29(5), 1071-1089.
[22] Iyaka, Y. A. (2011). Nickel in soils: A review of its distribution and impacts. *Scientific Research and Essays*, 6(33), 6774-6777.
[23] El-Naggar, A., Ahmed, N., Mosa, A., Niazi, N. K., Yousaf, B., Sharma, A., ... & Chang, S. X. (2021). Nickel in soil and water: Sources, biogeochemistry, and remediation using biochar. *Journal of Hazardous Materials*, 419, 126421.
[24] Kacar, B., Katkat, V., & Öztürk, Ş. (2009). *Bitki Fizyolojisi (3. Baskı)*. Nobel Yayınları No: 848.
[25] Zengin, F. K., & Munzuroglu, O. (2005). Effects of some heavy metals on content of chlorophyll, proline and some antioxidant chemicals in bean (Phaseolus vulgaris L.) seedlings. *Acta Biologica Cracoviensia Series Botanica*, 47(2), 157-164.
[26] Tombuloglu, G., Tombuloglu, H., Sakcali, M. S., & Unver, T. (2015). High-throughput transcriptome analysis of barley (Hordeum vulgare) exposed to excessive boron. *Gene*, 557(1), 71-81.
[27] Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, 162, 156-159.
[28] Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E. F., & Hellens, R. P. (2007). Protocol: A highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods*, 3, 12.
[29] Yanik, H., Turktas, M., Dundar, E., Hernandez, P., Dorado, G., & Unver, T. (2013). Genome-wide identification of alternate bearing-associated microRNAs (miRNAs) in olive (Olea europaea L.). *BMC Plant Biology*, 13, 10-31.
[30] Ruiz-Medrano, R., Xocosnostle-Cazares, B., & Lucas, W. J. (1999). Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development*, 126, 4405-4419.
[31] Zhang, S., Sun, L., & Karger, F. (2009). The phloem-delivered RNA pool contains small noncoding RNAs and interferes with translation. *Plant Physiology*, 150(1), 378-387.
[32] Saleh, S. R., Kandeel, M. M., Ghareeb, D., Ghoneim, T. M., Talha, N. I., Alaoui-Sossé, B., & Abdel-Daim, M. M. (2020). Wheat biological responses to stress caused by cadmium, nickel and lead. *Science of The Total Environment,* 706, 136013.
[33] Bhalerao, S. A., Sharma, A. S., & Poojari, A. C. (2015). Toxicity of nickel in plants. *International Journal of Pure and Applied Bioscience*, 3(2), 345-355.
[34] Rizwan, M., Imtiaz, M., Dai, Z., Mehmood, S., Adeel, M., Liu, J., & Tu, S. (2017). Nickel stressed responses of rice in Ni subcellular distribution, antioxidant production, and osmolyte accumulation. *Environmental Science and Pollution Research*, 24(20), 20587-20598.
[35] Mousavi, H. Z., & Seyedi, S. R. (2011). Nettle ash as a low cost adsorbent for the removal of nickel and cadmium from wastewater. *International Journal of Environmental Science & Technology,* 8(1), 195-202.
[36] Harasim, P., & Filipek, T. (2015). Nickel in the environment. *Journal of Elementology,* 20(2).

[37] Shahbaz, A. K., Iqbal, M., Jabbar, A., Hussain, S., & Ibrahim, M. (2018). Assessment of nickel bioavailability through chemical extractants and red clover (Trifolium pratense L.) in an amended soil: Related changes in various parameters of red clover. *Ecotoxicology and Environmental Safety*, 149, 116-127.

[38] AlMisned, G., Al-Abdullah, T., Liadi, F. A., & Hawsawi, A. (2022). Comparison between NIS and TNC channels for the detection of nickel in soil samples. *Applied Radiation and Isotopes,* 186, 110303.

[39] Kozlov, M. V. (2005). Sources of variation in concentrations of nickel and copper in mountain birch foliage near a nickel-copper smelter at Monchegorsk, north-western Russia: results of long-term monitoring. *Environmental Pollution,* 135(1), 91-99.

[40] He, S., He, Z., Yang, X., & Baligar, V. C. (2012). Mechanisms of nickel uptake and hyperaccumulation by plants and implications for soil remediation. *Advances in Agronomy,* 117, 117-189.

[41] Theriault, G., Michael, P., & Nkongolo, K. (2016). Comprehensive transcriptome analysis of response to nickel stress in white birch (Betula papyrifera). PLOS ONE, 11(4). Hassan, M. U., Chattha, M. U., Khan, I., Chattha, M. B., Aamer, M., Nawaz, M., & Khan, T. A. (2019). Nickel toxicity in plants: reasons, toxic effects, tolerance mechanisms, and remediation possibilities—a review. *Environmental Science and Pollution Research,* 26(12), 12673-12688.

[42] Warren, A., Michael, P., & Nkongolo, K. (2022). Nickel translocation, toxicity, and its effect on the expression of genes associated with nickel resistance in red pine (Pinus resinosa). *Ecological Genetics and Genomics*, 25, 100148.

[43] Tombuloglu, G. (2022). Drought-responsive miRNAs in plants: A review. *International Journal of Innovative Engineering Applications*, 6(1), 150-157.

[44] Loreti, E., & Perata, P. (2022). Mobile plant microRNAs allow communication within and between organisms. *New Phytologist*, 235(6), 2176-2182.

[45] Shahid, S., Kim, G., Johnson, N. R., Wafula, E., Wang, F., Coruh, C., Bernal-Galeano, V., Phifer, T., Depamphilis, C. W., & Westwood, J. H. (2018). MicroRNAs from the parasitic plant Cuscuta campestris target host messenger RNAs. *Nature*, 553(7686), 82-85.

[46] Betti, F., Ladera-Carmona, M. J., Weits, D. A., Ferri, G., Iacopino, S., Novi, G., Svezia, B., Kunkowska, A. B., Santaniello, A., & Piaggesi, A. (2021). Exogenous miRNAs induce post-transcriptional gene silencing in plants. *Nature Plants*, 7(12), 1379–1388.

[47] Marzec, M. (2022). MicroRNA: a new signal in plant-to-plant communication. *Trends in Plant Science*, 27(5), 418-419.

[48] Li, J., Song, Q., Zuo, Z. F., & Liu, L. (2022). MicroRNA398: A master regulator of plant development and stress responses. *International Journal of Molecular Sciences,* 23(18), 10803.

[49] Çelik, Ö., & Akdaş, E. Y. (2019). Tissue-specific transcriptional regulation of seven heavy metal stress-responsive miRNAs and their putative targets in nickel indicator castor bean (R. communis L.) plants. *Ecotoxicology and Environmental Safety*, 170, 682-690.

[50] Gifford, M. L., Dean, A., Gutierrez, R. A., Coruzzi, G. M., & Birnbaum, K. D. (2008). Cell-specific nitrogen responses mediate developmental plasticity. *Proceedings of the National Academy of Sciences*, 105(2), 803-808.

[51] Liu, X., Huang, S., & Xie, H. (2021). Advances in the regulation of plant development and stress response by miR167. *Frontiers in Bioscience-Landmark*, 26(9), 655-665.

[52] Liu, H. H., Tian, X., Li, Y. J., Wu, C. A., & Zheng, C. C. (2008). Microarray-based analysis of stress-regulated microRNAs in Arabidopsis thaliana. *RNA*, 14(5), 836-843.

[53] Wang, L. L., Zhao, T. L., Ge, J. T., et al. (2017). Application prospects of miRNAs in response to plant cold stress in the research of plant cold resistance. *Shanghai Journal of Agricultural Sciences,* 33(2), 129-134.

[54] Gao, P., Bai, X., Yang, L., Lv, D., Pan, X., Li, Y., Cai, H., Ji, W., Chen, Q., & Zhu, Y. (2011). Osa-MIR393: A salinity- and alkaline stress-related microRNA gene. *Molecular Biology Reports*, 38(4), 237–242.