Pak. J. Bot., 55(1): 379-385, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-1(6)

ANALYSIS OF miRNA EXPRESSION ASSOCIATED WITH GENE *Lr34* RESPONSIBLE FOR RESISTANCE MECHANISMS TO WHEAT LEAF RUST

AGNIESZKA TOMKOWIAK[1], ROKSANA BOBROWSKA[1], MICHAŁ KWIATEK[1], JULIA SPYCHAŁA[1*]
JAKUB KUCZYŃSKI[2], AGATA TYCZEWSKA[2], PRZEMYSŁAW ŁUKASZ KOWALCZEWSKI[3], DOROTA WEIGT[1] AND TOMASZ KOSIADA[4]

[1]*Department of Genetics and Plant Breeding, Poznań University of Life Sciences, 11 Dojazd St., 60-632 Poznań, Poland*
[2]*Institute of Bioorganic Chemistry, Polish Academy of Sciences, 12/14 Noskowskiego St., 61-704 Poznań, Poland*
[3]*Department of Food Technology of Plant Origin, Poznań University of Life Sciences, 31 Wojska Polskiego St., 60-624 Poznań, Poland*
[4]*Department of Phytopathology, Seed Science and Technology, Poznań University of Life Sciences, 159 Dąbrowskiego St., 60-594 Poznań, Poland*
[*]*Corresponding author's email: julia.spychala@up.poznan.pl*

Abstract

Diseases caused by pathogenic fungi significantly limit the yield and quality of common wheat. The most dangerous fungal diseases of wheat include leaf rust, stripe rust and powdery mildew. Cultivating resistant wheat varieties is the most effective way to minimize the development of fungal diseases. Genetically determined resistance of the horizontal type (racially non-specific, adult plant resistance genes) is preferred due to its more persistent expression compared to the major (R) genes determining race-specific resistance, which is often overcome by pathogens. Currently, hexaploid wheat resistance gene *Lr34/Yr18/Sr57/Pm38* (hereafter referred to as *Lr34*) is the best characterized gene determining horizontal-type resistance. The aim of this study was to analyze miRNA expression in selected common wheat varieties, showing the presence of resistance gene *Lr34* in response to infection with the fungus *Puccinia triticina* responsible for leaf rust. The reference varieties (Pavon'S', Myna'S', Frontana'S' and Sparrow'S') contained slow rust resistance genes, including *Lr34*, whereas the control variant HN ROD did not have these genes. The presence of *Lr34* in four reference varieties was confirmed using polymerase chain reaction (PCR). Biotic stress in adult plants was induced by inoculating fungal spores under monitored conditions in a growth chamber. Differences in the expression of various microRNAs (miR9653b, miR9657b, miR9773, miR9677b) associated with gene *Lr34* were tested using emulsion PCR (ddPCR). Plant material for analysis was collected before inoculation and 6, 12, 24 and 48 hours after inoculation. The results showed an increase in miR9653b expression in varieties carrying gene *Lr34* as a result of plant infection with *P. triticina*. The ddPCR analysis of miR9657b, miR9773 and miR9677b yielded too low a copy number for correct inference. The expression level of miR9653b in a control variety (HN ROD) lacking this resistance gene remained stable. This demonstrated that miR9653b could be involved in plant resistance mechanisms in response to leaf rust.

Key words: miR9653b; *Triticum aestivum*; *P. triticina*; APR; plant disease.

Introduction

Currently, wheat breeders focus on increasing resistance to stress and pathogens while maintaining high yield and its quality (Małecka *et al*., 2018). In addition, due to environmental protection and human health safety, the principle of integrated pest management (IPM) by all agricultural producers in the European Union applies from January 1, 2014. IPM mainly involves the use of non-chemical methods of plant protection in pathogen control, thereby minimizing the risk to humans, animals and environmental health (Sawinska *et al*., 2020, Vida *et al*., 2009). One of the general principles of IPM is to prevent the occurrence of harmful organisms or limit their negative impact, which should be achieved, among others, by using resistant or tolerant varieties. To date, over 100 alleles of genes determining leaf rust resistance, about 130 alleles of genes conditioning stripe rust resistance and over 140 alleles of genes responsible for powdery mildew resistance have been identified (McIntosh *et al*., 2019, Goriewa-Duba *et al*., 2020, Tomkowiak *et al*., 2019). In recent years, special attention has been paid to genetic diversity already at the stage of starting materials for plant breeding. Resistance genes have been divided in two groups and the first one includes the so-called “major resistance genes”, responsible for the induction of vertical-type resistance (“gene-for-gene”), also known as “race-specific resistance” (Flor, 1971). These genes provide resistance throughout the plant’s growth cycle. Therefore, this type of resistance is often referred to as “seedling resistance”. Vertical immunity is only effective against specific physiological races of pathogenic fungi (McCallum *et al*., 2007). Expression of resistance is only possible when the resistance gene in the plant and the avirulence effector gene (*Avr*) in the pathogen genotype are present simultaneously. Major genes are very popular among growers due to their strong expression and high efficiency in reducing plant diseases. The plant’s hypersensitivity response to pathogen infection is a characteristic feature of the expression of these genes (Bolton *et al*., 2008, Kolmer *et al*., 2008). Unfortunately, the basic disadvantage of this resistance is that it is subject to a “breakdown” phenomenon resulting from adaptation processes in pathogen populations. It involves a mutation in the *Avr* gene sequence of the pathogen, resulting in the lack of recognition by the major gene of the host plant (Dodds & Rathjen, 2010). Another type of resistance is adult plant resistance (APR). It is a type of quantitative resistance, usually racially-nonspecific, characterized by slow pathogen development and reduced epidemic risk (Krattinger *et al*., 2011, Ellis *et al*., 2014). The action of genes slows down the process of infection,

which is directly related to slow rusting of plants (Tiwari *et al.*, 2009, Singh & Huerta-Espino, 1997). Plants with APR genes are more stable under production conditions.

To date, gene *Lr34/Yr18/Sr57/Pm38* (hereafter referred to as *Lr34*) is the best-known gene conditioning horizontal resistance. It is located on wheat chromosome 7DS and the presence of this gene determines adult plant resistance (APR), which effectively inhibits the development of rust diseases at the adult plant stage. Gene *Lr34* is believed to be the source of permanent and non-specific resistance to leaf, streak and stem rust, as well as to powdery mildew (Lagudah *et al.*, 2009). In addition, gene *Lr34* is also used as a model to study the molecular basis of durable resistance (Kumar *et al.*, 2018).

Disease resistance and susceptibility are regulated by a mechanism for rapid recognition of invading pathogens and efficient activation of host plant defense mechanisms. The hypersensitive response (HR) has an essential function in limiting the spread of the pathogen and activating defense mechanisms in the uninfected part of the plant. Plant functions in response to infection are altered at the cellular level through the action of endogenous small non-coding miRNAs (Dutta *et al.*, 2019, Gupta *et al.*, 2014, Kumar *et al.*, 2016). In recent years, scientists have turned their attention to small non-coding RNAs (miRNAs, siRNAs) that play a significant role in the control of biological processes (Sanz-Carbonell *et al.*, 2019, Tyczewska *et al.*, 2017, Yao *et al.*, 2010). They regulate not only the processes of plant growth and development, but also actively participate in plant responses to biotic stress. Studies in plants have indicated the key importance of miRNAs in various regulatory pathways involving almost all biological processes, including plant growth and defense response development to biotic and abiotic stresses (Guleria *et al.*, 2011, Chu *et al.*, 2016). The miRNA molecules bind to complementary gene sequence fragments, thereby inhibiting the translation process (Li *et al.*, 2017). Another regulatory molecules, siRNAs, arise from longer RNA fragments and can affect DNA methylation of target sequences.

This work is a report of miRNA expression analyses in selected varieties of common wheat (*Triticum aestivum* L.) exhibiting the presence of resistance gene *Lr34* in response to infection with the fungus *Puccinia triticina* responsible for leaf rust.

Materials and Methods

Experimental material: The plant material used in the study consisted of 5 common wheat varieties, and the seeds for the study were obtained from the National Small Grains Collection (Agricultural Research Station in Aberdeen, WA, USA). Four of the wheat varieties tested were considered very valuable sources of leaf rust resistance genes and they also carried gene *Lr34* (Pavon'S', Myna'S', Frontana'S' and Sparrow'S'). In addition to gene *Lr34*, they also had gene *Lr46*. The negative control was the variety HN ROD, which did not carry gene *Lr34*.

Experiment under controlled conditions in a growth chamber: The present experiment was conducted in a growth chamber under controlled conditions. The chamber temperature was initially set to 18°C during the day and 16°C during the night. The emission spectrum of the light source with a photon flux of 572 µE was evaluated. After one month, the temperature was raised to 20°C and 17°C during the day and night, respectively. Induction of biotic stress at the adult plant stage was performed by infecting the plants with fungal spores. For each variety, 5 plants were potted in two variants (inoculated and non-inoculated), in triplicate, for a total of 30 pots (5 varieties x 3 replicates x 2 variants). Leaf tissue samples were taken from each pot for molecular analysis before inoculation and 6, 12, 24 and 48 hours after infection. In total, 150 samples [5 varieties x 3 biological replicates x 5 time points (0 h, 6 h, 12 h, 24 h and 48 h) x 2 variants (inoculated and non-inoculated)] were collected to examine the expression of miRNAs associated with gene *Lr34*. The material used to inoculate the tested plants was a mixture of 4 isolates of *Puccinia triticina*. Fungus spores were collected from infected field experiments located in various parts of Poland, including Smolice village (52.1000°N 19.0500°E), Strzelce (53.0167°N 16.9667°E), Kobierzyce (50.9667°N 16.9167°E) and Nagradowice (52.4167°N 16.9667°E), where branches of Polish breeding companies are located. Fungi were propagated on a medium consisting of agar, distilled water, growth factors (glucose, peptone) and penicillin (to inhibit the growth of bacteria). Before inoculating the microorganisms, the medium was sterilized in an autoclave and the pH was adjusted to 5.5. The culture was carried out at the temperature of 25°C. The study wheat varieties were artificially inoculated at the adult plant stage by spraying a spore suspension at a concentration of approximately $5x10^5$ spores/mL.

Identification of the *csLV34* marker of gene *Lr34*: Genomic DNA was extracted from the leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands). The quality and concentration of isolated DNA was determined using a DeNovix spectrophotometer. To identify gene *Lr34*, the *csLV34* STS (sequence tagged site) marker was used, which was mapped 0.4 cM from *Lr34*. The *csLV34* marker was used by coworkers in an earlier study (Skowrońska *et al.*, 2019), which confirmed its effectiveness in identifying the presence of this gene. The primer sequences (Merck KGaA, Darmstadt, Germany) were as follows: *csLV34*F – 5′-GTTGGTTAAGACTGGTGATGG-3′, *csLV34*R – 5′-TGCTTGCTATTGCTGAATAGT-3′ (Lagudah *et al.*, 2006). A 150 bp band indicated the presence of gene *Lr34*, and a 229 bp product was amplified in susceptible genotypes that did not carry this gene, which was consistent with literature data.

PCR was conducted using Labcycler thermal cyclers (SensoQuest GmbH, Göttingen, Germany) and the previously described reaction mixture and PCR profile parameters (Tomkowiak *et al.*, 2020). After amplification, PCR products were separated on a 2% agarose gel (Sigma-Aldrich, Saint Louis, MO, USA) at 100 V for 2.5 h in 1 × TBE buffer (BioShop Canada Inc., Burlington, Canada) with 0.5 µL of Midori Green Direct (NIPPON Genetics EUROPE GmbH, Düren, Germany). A DNA size marker (100 bp ladder) was used to evaluate the size of amplification products. The Molecular Imager Gel Doc™ XR UV system with Bio-Rad Bio Image™ software was used to visualize PCR reaction products.

Expression analysis of individual miRNAs linked to gene *Lr34*: Gene *Lr34* expression analysis was performed according to the method described in detail by Tomkowiak *et al*., (2020). Briefly, gene *Lr34* sequence was initially searched and compared between Ensembl Plants and NCBI databases, and the following 4 complementary microRNAs were selected based on the sequence of this gene in the psRNAtarget database: miR9653b, miR9657b, miR9773 and miR9677b. The miRNA sequences were found in the miRBase database and reverse transcription and ddPCR primers were designed using the retrieved sequences and IDT software (Integrated DNA Technologies). The mirVana™ miRNA Isolation Kit with phenol (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract RNA fraction that contained miRNAs, the concentration and quality of which were measured spectrophotometrically. Subsequently, extracted RNA was reverse-transcribed using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). A T100 PCR thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to amplify cDNA. The products were quantified in a QX100 droplet reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Data were acquired and analyzed using QuantaSoft™ Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Results

The *csLV34* STS molecular marker was mapped 0.4 cM from gene *Lr34* (Lagudah *et al*., 2006). According to the literature, a 150 bp product indicated the presence of this gene, while a 229 bp band should be amplified in susceptible common wheat genotypes. The product indicating the presence of the *csLV34* marker of gene *Lr34* was detected in the varieties Pavon'S', Myna'S', Frontana'S' and Sparrow'S'. A 229 bp product was amplified in the control variety HN ROD, which indicated the absence of the analyzed gene (Fig. 1).

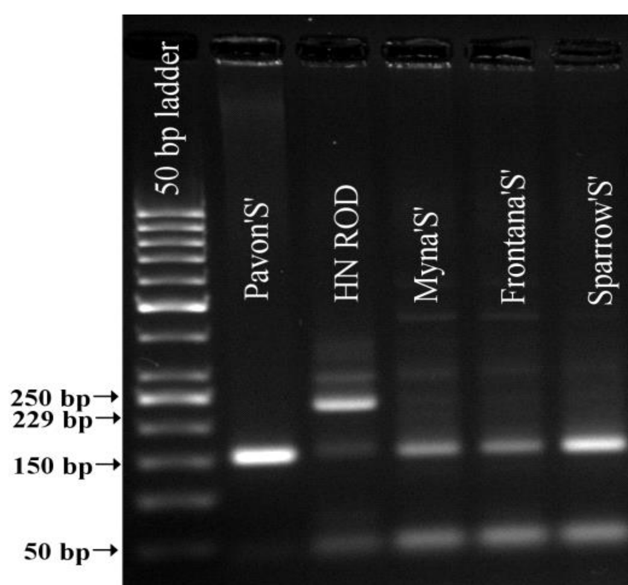

Fig. 1. Electropherogram showing the occurrence of the *csLV34* marker in the varieties of wheat (*Triticum aestivum* ssp. *vulgare* L).

The study analyzed the expression of four miRNAs associated with gene *Lr34*. Complementary miRNAs (miR9653b, miR9657b, miR9773 and miR9677b) were selected based on nucleotide sequence analysis of *Lr34* gene using the psRNAtarget tool (http://plantgrn.noble.org/psRNATarget/). The quantity of all analyzed miRNAs was assessed using ddPCR. Ultimately, a sufficient number of copies for correct inference and further statistical analysis were obtained only for miR9653b. In the experimental variant without inoculation, miR9653b concentrations in the analyzed varieties in all replications (biological and technical) and time point variants (0 h, 6 h, 12 h, 24 h, 48 h) did not show significant differences, i.e. miR9653b (complementary to gene *Lr34*) expression was not observed. However, expression of this miRNA was observed in the inoculated variant. The results were averaged and presented in Table 1 as no significant differences in miR9653b concentration were observed between biological and technical replicates. The lack of significant differences between replicates is the result of well-designed reaction conditions. An instance of ddPCR analysis is shown in (Fig. 2).

All the tested varieties, except the control variety HN ROD, responded to *Puccinia triticina* infection (Table 1). The number of miR9653b copies/μL before inoculation varied among varieties from 126 (Myna'S') to 216 (Sparrow'S'). A slight increase in miR9653b expression could be observed in all varieties, except Frontana'S', six hours after infestation (Table 1), while a simultaneous reduction in miR9653b copy number from 215 to 201 was recorded. This difference could be due to an increase in gene *Lr34* activity caused by infection. Over time, increased miR9653b expression was observed in all varieties 12, 24 and 48 hours after inoculation, except the HN ROD control. The observed growth rate depended on the variety. Sparrow'S' showed the slowest response to infection, as miR9653b concentration 12 h after inoculation was only 17 copies/μL higher than before inoculation. For this cultivar, the breakthrough occurred after 24 h, when a doubling of miR9653b copies/μL was recorded (Table 1). This could mean that plants of this variety were able to defend themselves against the pathogen for the longest time. The fastest plant response to infection was recorded for the variety Myna'S', in which miR9653b quantity increased by 61 copies/μL 6 h after inoculation, 229 copies/μL after 12 h, and 157 copies/μL after 24 h. In this case, the genetic resistance mechanism was rapidly blocked by the pathogen. The expression of miR9653b in varieties with gene *Lr34* in comparison to the control variety HN ROD is presented in separate charts (Fig. 3). The graphs clearly indicate that, unlike other varieties carrying gene *Lr34*, we did not observe clear differences in miRNA expression in the control variety HN ROD after plant infection with *Puccinia triticina*.

Table 1. Concentration of miR9653b in the analyzed varieties (tested before infection and 6, 12, 24 and 48 hours after infection in biological and technical replicates) – replicate means.

Analyzed genotypes	miR9653b concentration (copy number/µL) – replicate means				
	before infection	6 hours post infection	12 hours post infection	24 hours post infection	48 hours post infection
HN ROD	197	198	200	170	221
Pavon ‘S’	165	234	289	393	607
Myna ‘S’	126	187	416	573	600
Frontana ‘S’	215	201	366	489	719
Sparrow ‘S’	216	227	233	515	650

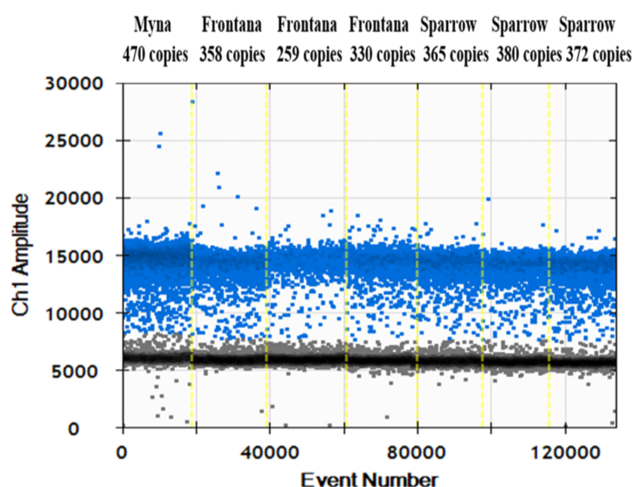

Fig. 2. Example of ddPCR reaction showing miR9653b concentrations (copy number/µL) for selected cultivars and replicates.

Discussion

Common wheat (*Triticum aestivum* L.) is one of the most important crop species of great civilization importance. The economic value of this species is determined by many traits, although the most important of them are the size and quality of the yield. Currently, breeders strive to create highyielding varieties that are also resistant to biotic stresses. In recent years, horizontal adult plant resistance (APR) has been preferred in individual varieties because it is mostly racially non-specific, more persistent and reduces the risk of epidemics (Lagudah *et al*., 2009).

Currently, gene *Lr34/Yr18/Sr57/Pm38* (hereafter referred to as *Lr34*) is the best-known gene conditioning horizontal resistance of wheat varieties. Thus far, gene *Lr34* has been recognized as a source of persistent and racially non-specific resistance to leaf, stripe and stem rust and powdery mildew. Gene *Lr34* has been cloned and its sequence is known (Krattinger *et al*., 2009). It encodes an ATP-binding transporter (so-called ABC transporter – ATP-binding cassette transporter), a multi-domain membrane protein that uses energy from ATP hydrolysis to translocate substances across the cell membrane in all living organisms. Therefore, gene *Lr34* is present in all hexaploid wheat varieties in several allelic variants. The resistance-conferring allele (*Lr34res*) differs from the commonly present *Lr34sus* allele in three SNP (single nucleotide polymorphism) mutations in intron 4 and exons 11 and 12 of the gene *Lr34* sequence (Krattinger *et al*., 2009). The specific action of this allele is to increase resistance to infection regardless of race and type of pathogen. Gene *Lr34* is widely applied in breeding due to its stability and universality (non-specific immunity).

This work confirmed the association of the *csLV34* marker with gene *Lr34* in four reference varieties derived from the GeneBank “National Small Grains Collection” located at the Agricultural Research Station in Aberdeen USA. The varieties have been described as resistant to leaf rust. A 150 bp product indicating the presence of the *csLV34* marker was found in the reference varieties Pavon'S', Myna'S', Frontana'S 'and Sparrow'S', while a 229 bp product, indicating the absence of the analyzed gene, was amplified in the control variety HN ROD.

In addition, this gene (*Lr34*) has been shown to provide not only increased resistance to leaf rust, but also to stripe rust (*Yr18*), powdery mildew (*Pm38*) and in certain forms to leaf rust (*Sr57*). The morphological characteristic of the *Lr34res* allele is leaf necrosis conditioned by gene *Ltn1* (leaf-tip necrosis). It probably originated from local Chinese varieties (Kolmer *et al*., 2008, Krattinger *et al*., 2009). Gene *Lr34* is present in European varieties, which have varieties Mentana and Ardito in their pedigree, registered at the beginning of the 20th century (Lagudah *et al*., 2009).

Gene *Lr34* is effective in the adult plant stage and under favorable conditions, i.e., at 4-10°C, at the flag leaf and seedling stage (Risk *et al*., 2012). Unfortunately, plant resistance conditioned by *Lr34* is less effective at high environmental temperatures (Wellings 2011). Inoculation assays conducted under field conditions on inbred wheat lines (F6) with the *Lr34res* allele indicated 50% infestation with 15% yield reduction compared to control forms, where 100% infestation and 84% yield reduction were observed (Singh & Huerta-Espino 2003).

This study investigated the expression of various miRNAs associated with gene *Lr34* in plant response to *Puccinia triticina* infection. Four miRNAs associated with *Lr34* have been identified: miR9653b, miR9657b, miR9773 and miR9677b. As a result of ddPCR analyses, the highest copy number necessary for correct inference was obtained only for miR9653b. Various authors have previously reported that miRNAs analyzed in this study are involved in the immune response to both biotic and abiotic stress. According to a study of Li *et al*., (2017), miR9773 demonstrated an important role in wheat response to biotic stress. Although miRNAs are small in size, they play key regulatory roles in many abiotic stress reactions such as soil salinity (Gao *et al*., 2011), nutrient deficiency (Liang *et al*., 2015), UV-B radiation (Casadevall *et al*., 2013), heat (Goswami *et al*., 2014) or metal stress (Gupta *et al*., 2014).

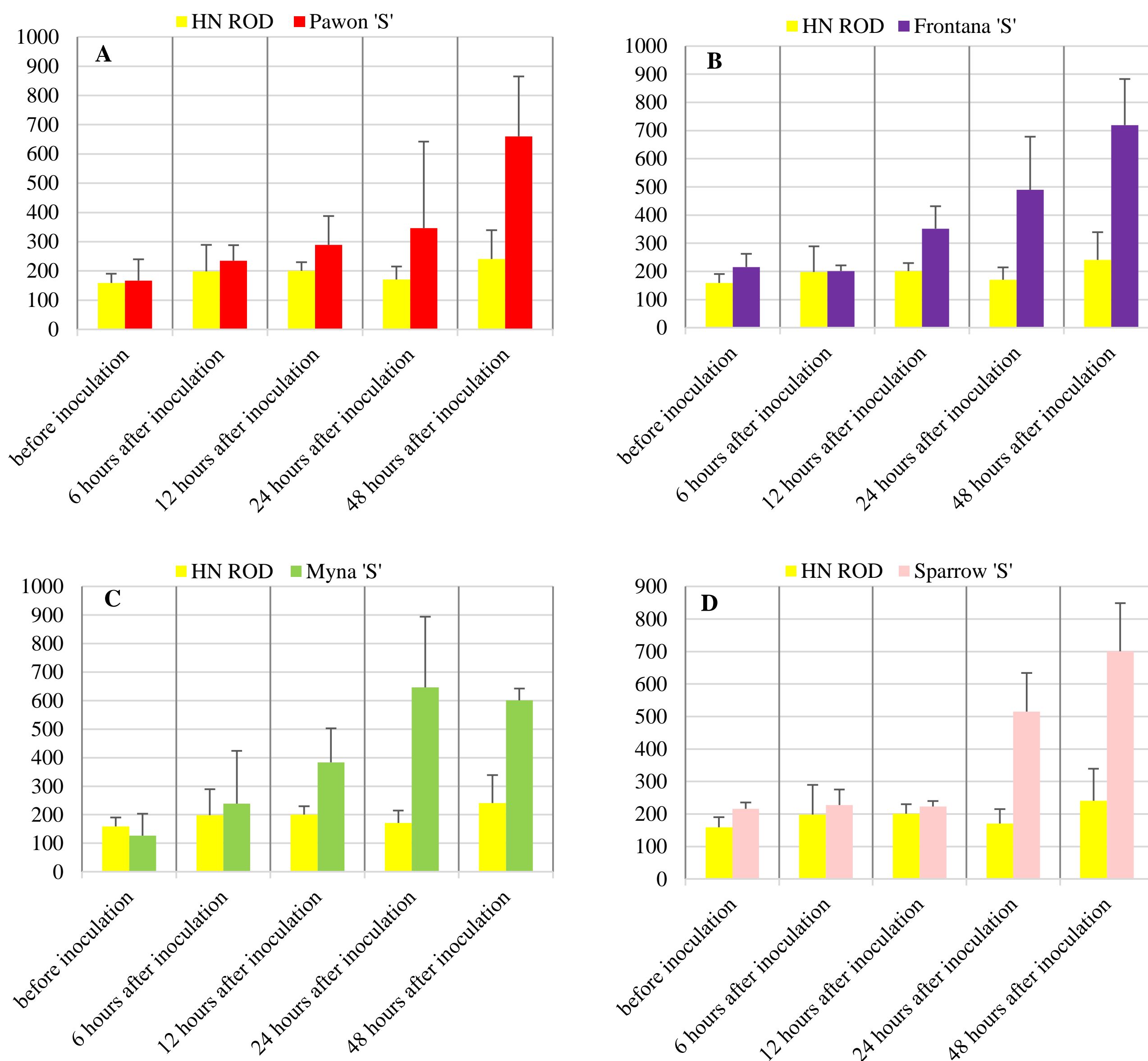

Fig. 3. miR9653b concentration (copy number/µL) in the varieties Pavon'S', Myna'S', Frontana'S' and Sparrow'S' compared to HN ROD (control genotype) before and 6, 12, 24 and 48 hours post-infection.

Chu *et al.*, (2016) claimed that miR9657b was associated with a protein kinase belonging to the CDPK family, which was also involved in signal transduction pathways in response to injury and the presence of a pathogen. Many authors (Cheng *et al.*, 2002, Rudd & Franklin-Tong, 2001) have shown in their works that Ca^{2+} acts as an intracellular regulator in many aspects of plant growth and development, as well as in stress response. It is a key messenger that plays an important role in cell signaling. Under the influence of stimuli, spatio-temporal calcium waves are generated with a frequency and amplitude characteristic of the acting stimulus. Such specific increases in the concentration of Ca^{2+} ions in the cytosol, called 'Ca^{2+} signature', are received, interpreted and transferred to effector elements by special types of sensor proteins, such as calmodulin (CaM), calcineurin B-like proteins (CBL) or calcium-dependent kinases (CDPK) containing Ca^{2+} ion-binding structural motifs called EF hand motifs. Recently, *In vivo* activation of CDPK after Cf-9/Avr9 interaction has been demonstrated in Cf-9 transgenic tobacco plants, suggesting that CDPKs are important calcium-induced sensors in defense responses (Romeis *et al*., 2000, Romeis *et al.*, 2001).

Kumar *et al.*, (2016) analyzed ten miRNAs responding to leaf rust infection using wheat varieties carrying gene *Lr24* (resistant genotypes) and varieties lacking this gene (susceptible genotypes). The latter authors applied stem-loop qPCR to identify and analyze the expression of individual miRNAs. The expression patterns of individual miRNAs differed, which according to these authors could have indicated their role at different time points after pathogen infection. Maximum expression of two miRNAs, miR119 and miR256, occurred at 48 hpi (hours post-infection) while four miRNAs: miR148, miR181, miR383 and miR441 demonstrated maximum expression at 12 hpi, which decreased with time. On the other hand, three other miRNAs: miR218, miR337 and miR358 showed

maximum expression at 168 hpi, suggesting their late response to leaf rust. As indicated by the researchers, a significant correlation was observed for all the analyzed miRNAs, whose pathogen-induced expression levels were higher in susceptible plants compared to resistant ones, revealing an important role of gene *Lr24*.

Bulut (2016) showed in her work that miR9677 was associated with plant response to drought stress. The latter author tested the expression of various miRNAs in response to drought using qPCR. Her study demonstrated average expression levels of miR395, miR2275 and miR9677, but increased miR166 and miR397 expression during water scarcity. The latter author showed that the expression level of different miRNAs fluctuated depending on the type of tissue exposed to drought stress and stress duration. In the case of leaf tissues, Bulut (2016) observed that the expression of miR156, miR164, miR9673, miR9772 and miR9778 increased due to water deficiency. Similar fluctuations in miRNA expression under different biotic and abiotic stress conditions were also observed by other authors (Pandey *et al*., 2014, Wu *et al.*, 2014).

Li *et al*., (2017) examined various miRNAs after wheat exposure to phenanthrene and found that miR9653b expression was down-regulated. Sibisi (2020) analyzed the expression of eleven miRNAs, including miR9653b, using the qPCR method. Expression of miR9653b was up-regulated in a susceptible wheat cultivar after RWASA1 infection (Russian wheat aphid). Unfortunately, knowledge about the role of miR9653b in plant pathogen stress responses is still limited. The present study showed that inoculation of wheat varieties with *P. triticina* spores resulted in altered miR9653b expression in the varieties carrying gene *Lr34*. The concentration of this miRNA varied depending on the variety and time since inoculation. A slight increase in miR9653b expression could be observed in all varieties, except the HN ROD control, 6 hours after infection. These varieties showed an enhanced expression of this miRNA 12, 24 and 48 hours post-infection. The rate of an increase in miR9653b expression depended on the variety. The slowest reaction to infection was observed in the variety 'Sparrow'S', which could indicate that plants of this variety defended themselves the longest against the pathogen. The fastest plant response to infection was recorded in the variety Myna'S', where the genetic mechanism of resistance was quickly blocked by the pathogen.

Conclusions

The discovery of miRNAs as particles that specifically modulate proteome composition has unveiled a new level of control over cellular processes. Since miRNAs are endogenous molecules, their expression is regulated by protein transcription factors and epigenetic mechanisms. Transcription factors, involved in cellular signaling pathways responsible for response to environmental influences, can also modify the miRNA pool, and thus the proteome. The current study has indicated that miR9653b may be involved in plant resistance mechanisms to leaf rust caused by *P. triticina*. According to the psRNATarget database, miR9653b is associated with leaf rust resistance conferred by gene *Lr34*, and expression differences induced by fungus infection seem to confirm this information. These small miRNAs are a key component of the cellular response to stress, in this case biotic stress. They enable the cell to adapt to the external environment or survive its adverse effects.

References

Bolton, M.D., J.A. Kolmer and D.F. Garvin. 2008. Wheat leaf rust caused by *Puccinia triticina*. *Mol. Plant Pathol.*, 9 (5): 563-75.

Bulut, R.F. 2016. Genome-wide microRNA identification in drought tolerant wild emmer wheat: assessment of antimicrobial activity of surface active antimic agent on raw fruit and vegetable packaging. (Doctoral dissertation).

Casadevall, R., R.E. Rodriguez, J.M. Debernardi, J.F. Palatnik and P. Casati. 2013. Repression of growth regulating factors by the microRNA396 inhibits cell proliferation by UV-B radiation in *Arabidopsis* leaves. *Plant Cell*, 25: 3570-3583.

Cheng, S.H., R.M. Willmann., H. Chen and J. Sheen. 2002. Calcium signaling through protein kinases. The Arabidopsis calcium–dependent protein kinase gene family. *Plant Physiol*., 129: 469-485.

Chu, Z., J. Chen, H. Xu, Z. Dong, F. Chen and D. Cui. 2016. Identification comparative analysis of micro RNA in wheat (*Triticum aestivum* L.) callus derived from mature and immature embryos during in vitro culture. *Front. Plant Sci*., 7: 1302.

Dodds, P.N. and J.P. Rathjen. 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.*, 11(8): 53948.

Dutta, S., S.K. Jha, K.V. Prabhu, M. Kumar and K. Mukhopadhyay. 2019. Leaf rust (*Puccinia triticina*) mediated RNAi in wheat (*Triticum aestivum* L.) prompting host susceptibility. *Fun. & Integ. Genom*., 19(3): 437-452.

Ellis, J.G., E.S. Lagudah, W. Spielmeyer and P.N. Dodds. 2014. The past, present and future of breeding rust resistant wheat. *Front. Plant Sci.,* 5: 641. doi:10.3389/fpls.2014. 00641.eCollection 2014.

Flor, H.H. 1971. Current status of the gene–for–gene concept. *Ann. Rev. Phytopathol*., 9: 275-296.

Gao, P., X. Bai, L. Yang, D. Lv, X. Pan, Y. Li, H. Cai, W. Ji, Q. Chen and Y. Zhu. 2011. Osa-MIR393: a salinity-and alkaline stress-related microRNA gene. *Mol. Biol. Rep*., 38: 237-242.

Goriewa-Duba, K., A. Duba, E. Suchowilska and M. Wiwart. 2020. An Analysis of the genetic diversity of bread wheat x spelt breeding lines in terms of their resistance to powdery mildew and leaf rust. *Agronomy*, 10(5): 658.

Goswami, S., R.R. Kumar and R.D. Rai. 2014. Heat-responsive microRNAs regulate the transcription factors and heat shock proteins in modulating thermo-stability of starch biosynthesis enzymes in wheat (*Triticum aestivum* L.) under the heat stress *Aust. J. Crop. Sci*., 8: 697.

Guleria, P., M. Mahajan, J. Bhardwaj and S.K. Yadav. 2011. Plant small RNAs: biogenesis, mode of action and their roles in abiotic stresses. *Genom. Proteom. & Bioinform.*, 9(6): 183-199.

Gupta, O.P., P. Sharma, R.K. Gupta and I. Sharma. 2014. MicroRNA mediated regulation of metal toxicity in plants: Present status and future perspectives. *Plant Mol. Biol*., 84: 1-18.

Kolmer, J.A., R.P. Singh, D.F. Garvin, L. Viccars, H.M. William, J. Huerta-Espino, F.C. Ogbonnaya, H. Raman, S. Orford, H.S. Bariana and E.S. Lagudah. 2008. Analysis of the *Lr34/Yr18* rust resistance region in wheat germplasm. *Crop Sci.*, 48: 1841-1852.

Krattinger, S.G., E.S. Lagudah, T. Wicker, J.M. Risk, A.R. Ashton, L.L. Selter, T. Matsumoto and B. Keller. 2011. *Lr34* multi-pathogen resistance ABC transporter: Molecular analysis of homoeologous and orthologous genes in hexaploid wheat and other grass species. *Plant J.*, 65: 392-403.

Krattinger, S.G., E.S. Lagudah, W. Spielmeyer, R.P. Singh, J. Huerta-Espino, H. McFadden, E. Bossolini, L.L. Selter and B. Keller. 2009. A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science,* 323(5919): 1360-1363.

Kumar, D., S. Dutta, D. Singh, K.V. Prabhu, M. Kumar and K. Mukhopadhyay. 2016. Uncovering leaf rust responsive miRNAs in wheat (*Triticum aestivum* L.) using high-throughput sequencing and prediction of their targets through degradome analysis. *Planta,* 245(1): 161-182.

Kumar, S., R.P. Singh, A.K. Joshi, M.S. Roder, P. Chhuneja, G.S. Mavi and U. Kumar. 2018. Association of *Lr34* gene complex with spot blotch disease resistance at molecular level in wheat (*Triticum aestivum* L.). *Ind. J. Genet*., 78(3): 302-308.

Lagudah, E.S, S.G. Krattinger, S. Herrera-Foessel, R.P. Singh, J. Huerta-Espino, W. Spielmeyer, G. Brown-Guedira, L.L. Selter and B. Keller. 2009. Gene-specific markers for the wheat gene *Lr34/Yr18/Pm38* which confers resistance to multiple fungal pathogens, *Theor. & Appl. Genetics,* 119(5): 889-898.

Lagudah, E.S., H. McFadden, R.P. Singh, J. Huerta-Espino, H.S. Bariana and W. Spielmeyer. 2006. Molecular genetic characterization of the *Lr34/Yr18* slow rusting resistance gene region in wheat. *Theor. & Appl. Genetics*, 114(1): 21-30.

Li, J., L. Yoe, Y. Shen, Y. Sheng, X. Zhan, G. Xu and B. Xing. 2017. Phenanthrene-responsive microRNAs and their targets in wheat roots. *Chemosphere,* 186: 588-598.

Liang, G., Q. Ai and D. Yu. 2015. Uncovering miRNAs involved in crosstalk between nutrient deficiencies in *Arabidopsis. Sci. Reports*, 5: 11813.

Małecka, I., A. Blecharczyk, Z. Sawinska, D. Swędrzyńska and T. Piechota. 2018. Winter wheat yield and soil properties response to long-term non-inversion tillage. *J. Agric. Sci. Technol*., 17: 1571-1584.

McCallum, B.D., T. Fetch and J. Chong. 2007. Cereal rust control in Canada. *Aust. J. Agric. Res.*, 58: 639-647.

McIntosh, R.A., J. Dubcovsky, W.J. Rogers, C. Morris and X.C. Xia. 2019. Catalogue of gene symbols for wheat: 2009 supplement. Annu. *Wheat Newslett*., vol. 55, p. 256-278.

Pandey, R., G. Joshi, A.R. Bhardwaj, M. Agarwal and S. Katiyar-Agarwal. 2014. A comprehensive genome-wide study on tissue-specific and abiotic stress-specific miRNAs in *Triticum aestivum*. *PLoS One*, 9(4): e95800.

Risk, J.M., L.L. Selter, S.G. Krattinger, L.A. Viccars, T.M. Richardson, G. Buesing, G. Herren, E.S. Lagudah and B. Keller. 2012. Functional variability of the *Lr34* durable resistance gene in transgenic wheat. *Plant Biotech. J.*, 10(4): 477-487.

Romeis, T., A.A. Ludwig, R. Martin and J.D. Jones. 2001. Calcium-dependent protein kinases play an essential role in a plant defence response. *E.M.B.O. J.,* 20: 5556-5567.

Romeis, T., P. Piedras and J.D. Jones. 2000. Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. *The Plant Cell*, 12(5): 803-815.

Rudd, J.J. and V.E. Franklin-Tong. 2001. Unravelling response-specific in Ca2+ signalling in plants cell. *New Phytol.*, 151: 7-33.

Sanz-Carbonell, A., M.C. Marques, A. Bustamante, M.A. Fares, G. Rodrigo and G. Gomez. 2019. Inferring the regulatory network of the miRNA-mediated response to biotic and abiotic stress in melon. *B.M.C. Plant Biol.*, 19(1): 1-17.

Sawinska, Z., S. Świtek, R. Głowicka-Wołoszyn and P.Ł. Kowalczewski. 2020. Agricultural Practice in Poland Before and After Mandatory IPM Implementation by the European Union, *Sustainability*, 12(3): 1107.

Sibisi, P.P. 2020. Characterisation of microRNA and selected targets in the wheat defence response against *Diuraphis noxia*. (Doctoral dissertation).

Singh, R.P and J. Huerta-Espino. 1997. Effect of leaf rust resistance gene *Lr34* on grain yield and agronomic traits of spring wheat. *Crop Sci.,* 37(2): 390-395.

Singh, R.P. and J. Huerta-Espino. 2003. Effect of leaf rust resistance gene Lr34 on components of slow rusting at seven growth stages in wheat. *Euphytica,* 129(3): 371-76.

Skowrońska, R., M. Kwiatek, A. Tomkowiak and J. Nawracała. 2019. Development of multiplex PCR to detect slow rust resistance genes *Lr34* and *Lr46* in wheat. *J. Appl. Genet.*, 60(3): 301-304.

Tiwari, P.R., M.S. Saharan, R. Chatrath, P. Siwach and B. Mishra. 2009. STS marker based tracking of slow rusting *Lr34* gene in Indian wheat genotypes, *Ind. J. Biotechnol*., 8: 07-213.

Tomkowiak, A., R. Skowrońska, A. Buda, D. Kurasiak-Popowska, J. Nawracała, P.Ł. Kowalczewski, M. Pluta and D. Radzikowska. 2019. Identification of leaf rust resistance genes in selected wheat cultivars and development of multiplex PCR, *Open Life Sci*., 14: 327-334.

Tomkowiak, A., T. Jędrzejewski, J. Spychała, J. Kuczyński, M.T. Kwiatek, A. Tyczewska, R. Skowrońska and T. Twardowski. 2020. Analysis of miRNA expression associated with the *Lr46* gene responsible for APR resistance in wheat (*Triticum aestivum* L.). *J. Appl. Genet.,* 61: 503-511.

Tyczewska, A., J. Gracz, J. Kuczyński and T. Twardowski. 2017. Deciphering soybean molecular stress response via high-throughput approach, *Acta Biochim. Pol*., 63(4): 631-643.

Vida, G., M. Gál, A. Uhrin, O. Veisz, N.H. Syed, A.J. Flavell, Z. Wang and Z. Bedo. 2009. Molecular markers for the identification of resistance genes and marker-assisted selection in breeding wheat for leaf rust resistance. *Euphytica,* 170: 67-74.

Wellings, C.R. 2011. Global status of stripe rust: a review of historical and current threats. *Euphytica,* 179(1): 129-41.

Wu, F., J. Shu and W. Jin. 2014. Identification and validation of miRNAs associated with the resistance of maize (*Zea mays* L.) to *Exserohilum turcicum*. *PloS One*, 9(1): e87251.

Yao, Y., Z. Ni, H. Peng, F. Sun, M. Xin, R. Sunkar, J.K. Zhu and Q. Sun. 2010. Non-coding small RNAs responsive to abiotic stress in wheat (*Triticum aestivum* L.). *Fun. Integr. Genomics,* 10: 187-190.

(Received for publication 26 April 2021)