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Original Research

Virulence Factors of the Fungal Pathogen *Stagonospora nodorum* Manipulate Hormonal Signaling Pathways in *Triticum aestivum* L. by Regulating Host Plant MicroRNA Expressions

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Abstract

Background: Currently, the role of microRNAs in plant immune responses is being actively studied. Thus, our aim was to research the effect of *Stagonospora nodorum* (Berk.) NEs SnToxA and SnTox3 on the expression of miRNAs involved in the wheat–*S. nodorum* interaction and to determine the role of phytohormones in this process. **Methods**: The expressions of nine conserved microRNAs were studied by quantitative real-time polymerase chain reaction in three different wheat genotypes of bread spring wheat (*Triticum aestivum* L.) infected with *S. nodorum*. Phytohormone treatments (*trans*-zeatin, 2-chloroethylphosphonic acid (etefone is the chemical precursor of ethylene), and salicylic acid) were applied. The results were compared with disease symptoms, the redox status of plants, and the expression of fungal necrotrophic effector (NE) genes of *SnToxA* and *SnTox3* and genes of *SnPf2*, *SnStuA*, alongside *SnCon7* transcription factors (TFs). **Results**: Salicylic acid (SA) and cytokinins (CK) are involved in the development of defense reactions in wheat plants against *S. nodorum*, by regulating the expression of fungal NEs and TFs genes, inducing an oxidative burst in all three wheat genotypes. Moreover, ethylene enhanced the virulence of the pathogen by increasing the expression of fungal NE and TF genes, thereby resulting in a decrease in the generation of reactive oxygen species in all three cultivars. The nine miRNAs played a role in the development of wheat resistance against *S. nodorum*. NE SnTox3 mainly suppressed the expression of three miRNAs: miR159, miR393, and miR408, while NE SnToxA suppressed miR166 expression. Conversely, treatment with CK and SA increased the expression of miR159 and miR408; treatment with CK increased the expression of miR393 and miR166. Ethylene inhibited the expression of miR159, miR408, miR393, and miR166. Suppression of miP159 expression by NE SnTox3 was most likely associated with the activation of the ethylene signaling pathway. NEs SnToxA and SnTox3 suppressed the expression of miR408, whose role most likely consisted of inhibiting the catalase activity, via SA and CK regulation. In addition, NE SnToxA hijacked the SA signaling pathway and manipulated it for fungal growth and development. Fungal TFs SnPf2 and SnStuA could be involved in the regulation of these processes indirectly through the regulation of the expression of NE genes. **Conclusions**: The results of this work show, for the first time, the role of microRNAs in the development of wheat resistance against *S. nodorum* and the effect of *S. nodorum* NEs SnToxA and SnTox3 on the activity of plant microRNAs.

Keywords: *Stagonospora nodorum*; necrotrophic effectors; transcription factors; phytohormones; cytokinins; ethylene; salicylic acid; signaling systems; microRNA

1. Introduction

According to the modern model of the plant immune system, defense mechanisms against biotrophic pathogens, such as bacteria, viruses, nematodes, insects, and fungi, lead a biotrophic lifestyle and consist of several levels of protection, which are separated in space and time [1]. At the first level, plants perceive pathogen-associated molecular patterns (PAMP) by pattern recognition receptors (PRRs), leading to the development of basal immunity, known as pattern-triggered immunity (PTI) [1]. Successful pathogens have evolved with the ability to secrete effectors that disrupt the PTI response and lead to effector-triggered susceptibility (ETS). Accordingly, plants have developed a second level of defense called effector-triggered immunity (ETI), which involves the recognition of pathogen effectors by effector-specific resistance proteins (R) [1]. While PTI is a non-specific reaction, ETI is a specific gene-for-gene response. These abilities develop at different times, however, both are associated with increased generation of reactive oxygen species (ROS) and oxidative bursts [1,2]. PTI activates primary defense reactions that are regulated by classical immune phytohormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene, as well as growth and development regulators—cytokinins (CKs), auxins, abscisic acid (ABA), brassinosteroids (BRs), and gibberellins (GAs) [3]. It should be noted that phytohormones may also play an important role in the development of ETS or ETI since some pathogen effectors target plant hormonal signaling pathways by hijacking and manipulating them [4]. Almost nothing is known about how plants oppose necrotrophic fungal pathogens. However, recent studies have shown that some necrotrophic fungi produce effector proteins designated as

necrotrophic effectors (NEs). NEs suppress PTI and promote the development of a sensitivity called NE-triggered sensitivity (NETS) [5,6].

The pathogenic fungus *Stagonospora nodorum* (Berk.) belongs to the order Pleosporales of the class Dothideomycetes (teleomorph *Phaeosphaeria nodorum*, *Parastagonospora nodorum*) and causes one of the most harmful wheat diseases—Septoria nodorum blotch (SNB) [7]. The main virulence factors of the *S. nodorum* are numerous fungal NEs, which are encoded by the *SnTox* genes. The interaction in the wheat–*S. nodorum* pathosystem mostly follows an inverse gene-for-gene model. When the host gene products, encoded by dominant susceptibility (S) genes, interact with fungal NE, a compatible interaction is established resulting in disease progression and pathogen growth and sporulation [5,7]. Of all NEs, SnToxA, SnTox1, and SnTox3 have been identified at the gene and protein levels and are considered the most common among pathogen strains and isolates [8,9]. The following compatible interactions SnToxA–*Tsn1*, SnTox1–*Snn1*, and SnTox3–*Snn3-B1* play a significant role in the development of SNB [7]. At present, it is believed that the main role of *S. nodorum* NEs is the induction of host cell death by manipulating the plant defense signaling pathways, including the hormonal pathways [7,10]. There are numerous examples in the literature of how pathogen effectors manipulate plant hormonal signaling systems by regulating phytohormone biosynthesis or by interacting with phytohormone signaling participants [4,11]. However, the mechanisms underlying this process are currently only being studied in the *S. nodorum* [10,12]. Concurrently, the question remains as to the role phytohormones perform in the regulation of pathogen-effector processes. In addition to NEs, transcription factors (TFs) can be virulence factors in fungal pathogens. Several recent studies have demonstrated the role of various fungal TFs in the regulation of effector gene expressions in pathogens, including *S. nodorum* [13]. In addition to the direct regulation of NE gene expressions, these TFs act as regulators in many processes in the pathogen organism, such as carbohydrate metabolism, hyphal growth, enzyme biosynthesis, and sporulation, which also affect pathogen virulence [13]. However, there is practically no knowledge about the regulatory mechanisms of *S. nodorum* virulence factors.

Recent studies have shown that small RNAs (sRNAs) play an important role in regulating the expression of genes involved in the defense responses and communication by plants during plant–microbial interactions [14–16]. In plants, non-coding sRNAs can be divided into two categories: microRNA (miRNA) and small interfering RNA (siRNA), both of which are produced by RNase III-like enzymes called DCL—Dicer-like [14]. Most plant miRNAs, after recognition of their targets promote the rapid degradation of the target mRNA. It has also been shown that miRNAs inhibit the translation of their target mRNAs, thereby limiting protein production. miRNAs can also regulate the expression of target genes by histone modifications and DNA methylation [15,16]. Increasing evidence shows that miRNAs participate in PTI and ETI in response to viruses, fungi, and bacteria. In particular, miRNAs are involved in the regulation of a variety of defense signals and pathways, including hormone signals, ROS production, and cross-kingdom gene silencing [15,16]. For example, miR393 limited the production of auxin receptors and repressed auxin signaling during a PTI response to *Pseudomonas syringae* pv DC3000, leading to the resistance of Arabidopsis [17]. It has been reported that miR398b enhances the resistance of rice to the *Magnaporthe oryzae* fungus by elevating superoxide dismutase (SOD) activity and increasing hydrogen peroxide content [18]. The expression of defense-related genes in plants and virulence genes in pathogens are often regulated at the post-transcriptional level by sRNAs. For example, the effector PSR1 (Phytophthora suppressor of RNA silencing 1) leads to the disruption of miRNA processing in plants, while the effector VdSSR1 (secretory silencing repressor 1) from *Verticillium dahliae* interferes with the nuclear export of the AGO1–miRNA complex, which results in a significant reduction in the content of cytoplasmic AGO1 protein and sRNA levels [19,20]. Despite the fact that in recent years a large amount of information has been accumulated on miRNAs involved in plant–pathogen interactions, a deeper understanding of the physiological and molecular roles of miRNAs is required. Moreover, most studies are limited to the computational prediction of sRNA targets, and many of them still need to be experimentally verified [16]. To date, the question of how NEs manipulate hormonal signaling pathways in the defense response of plants and what their targets are remains open. We hypothesize that NEs influence microRNAs and manipulate hormonal signaling pathways.

Our aim was to study the effect of *S. nodorum* NEs SnToxA and SnTox3 on the expression of miRNAs involved in the wheat–*S. nodorum* interaction and to determine the role of phytohormones in this process. To achieve this goal, we studied the expression of nine conserved miRNAs in incompatible and compatible interactions (SnToxA–*Tsn1* and SnTox3–*Snn3-B1*) on various genotypes of bread spring wheat carrying different alleles of susceptibility genes to *S. nodorum* effectors. We compared the data obtained in the course of studying the disease symptoms, such as the generation of hydrogen peroxide, the activity of oxidoreductases, and the expression of NE genes and miRNA, during the treatment of various wheat genotypes with phytohormones. This comparison provided important information on the role of NEs in manipulating hormonal signaling pathways and regulating microRNA expression for the development of the defense response in infected plants.

Overall, our findings indicate that the nine conserved miRNAs studied in this work played a role in the development of wheat resistance against *S. nodorum* through the regulation of hormonal signaling pathways, plant redox metabolism, and interaction with pathogen effectors. NE *S. nodorum* SnToxA and SnTox3 could manipulate hormonal signaling pathways by influencing plant microRNAs to regulate plant susceptibility. The results of this work show, for the first time, the role of microRNAs in the development of wheat resistance against *S. nodorum* and the effect of *S. nodorum* NE SnToxA and SnTox3 on the transcript levels of plant microRNAs. These results will further our understanding of the complex interactions between *S. nodorum* and wheat plants associated with NEs and wheat susceptibility genes.

2. Materials and Methods

2.1 Fungi and Plant Materials

This study used a *S. nodorum* SnB isolate, which has previously been shown to express the NEs *SnTox3* and *SnToxA* genes [21]. This isolate was obtained from a collection at the Institute of Biochemistry and Genetics, Ufa Federal Research Centre, Russian Academy of Sciences, Ufa, Russia. *S. nodorum* SnB isolate was maintained on potato–glucose agar (PGA) at 21 °C for a photoperiod of 12 h.

We used three cultivars of bread spring wheat (*Triticum aestivum* L.), which carried a diverse set of sensitivity genes to *S. nodorum* effectors: Omskaya 35 (Om35), Kazakhstanskaya 10 (Kaz10), and Zhnitsa (Zh). The SnToxA effector interacts with the host gene product *Tsn1*, and the SnTox3 effector interacts with the host gene product *Snn3* [7]. We have previously shown that cultivar Om35 has recessive alleles for both susceptibility genes (*tsn1/snn3*) and is resistant to *S. nodorum* due to an insensitivity to both SnToxA and SnTox3 [22]. Cultivar Kaz10 was only insensitive to NE SnToxA and sensitive to NE SnTox3 (*tsn1/Snn3*), meaning the cv. Kaz10 has one specific compatible interaction of SnTox3–*Snn3* [22]. The cultivar Zhnitsa has two dominant alleles for two susceptibility genes and was susceptible to both NEs SnToxA and SnTox3 (*Tsn1/Snn3*), meaning the susceptibility of Zhnitsa to *S. nodorum* is determined by two compatible specific gene-for-gene interactions (SnToxA–*Tsn1* and SnTox3–*Snn3*) [22]. Wheat seeds were obtained from the Ufa Federal Research Centre, Russian Academy of Sciences, Ufa, Russia.

2.2 Experimental Design

The redox status of the plants was analyzed and the expression of fungal genes and plant–host miRNAs were conducted on intact plants grown in a hydroponic culture (10% Hoagland–Arnon) in a KBW E6 plant growth chamber (Binder GmbH, Tuttlingen, Germany) at 146 W/m^2 PAR (Osram lamps L 36W/77), at temperatures of 24/18 °C (day/night), and a light period of 16 hours [23]. Experiments evaluating genotypic resistances were performed on separated first leaves of 7-day-old seedlings.

Treatment with phytohormones. To study the role of NEs in the manipulation of hormonal signaling pathways and regulation of microRNA expression during defense responses, parts of 6-day-old seedlings were treated with phytohormones: CK, SA, and ethephone (the chemical precursor of ethylene)—since we had previously established the role of these phytohormones in the SnTox3–*Snn3* interaction [10]. For this purpose, a solution of *trans*-zeatin (Merck KGaA, Sigma-Aldrich, Darmstadt, Germany) at a final concentration of 2.5 µM, or SA solution (Merck KGaA, Sigma-Aldrich, Darmstadt, Germany) at a final concentration of 0.05 mM was added to the plant nutrient medium in separate vessels 24 hours before infection. After 24 h, the medium was replaced by Hoagland–Arnon solution without *trans*-zeatin or SA. Other plants were sprayed with a 1.5 mM solution of 2-chloroethylphosphonic acid (ethephone, ET) (Merck KGaA, Sigma-Aldrich, Darmstadt, Germany) in separate vessels 24 hours before infection [24]. The biologically effective concentrations of *trans*-zeatin, SA, and ET were preselected [10].

Pathogen infection. A total of 24 hours after the hormonal treatment, phytohormone-treated and untreated 7-day-old seedlings were placed in separate vessels and sprayed with a spore suspension of *S. nodorum* SnB isolate (1×10^6 spores/mL), as described previously [10].

To study the redox status of plants, the first phytohormone-treated and untreated leaves from intact wheat seedlings (approximately 200 mg from 5 plants) were fixed in liquid nitrogen at either 6, 24, or 72 h after inoculation with the *S. nodorum* SnB isolates. To study the relative gene expressions of *S. nodorum* NEs (*SnToxA* and *SnTox3*) and TFs (*SnPf2*, *SnStuA*, and *SnCon7*), or plant-host miRNAs, the first phytohormone-treated and untreated leaves of wheat seedlings (approximately 100 mg from 5 plants) were fixed in liquid nitrogen 6, 24 and 72 h after inoculation with the *S. nodorum* SnB isolate.

To study the effect of phytohormones on the resistance/susceptibility of the wheat genotypes, the first phytohormone-treated and untreated leaves of 7-day-old seedlings were separated and placed in Petri dishes on wet cotton wool containing 0.004% benzimidazole (10–12 leaves/dish) [10]. Then, the separated leaves were inoculated with a spore suspension (1×10^6 spores/mL), as described previously [10,21]. The development of SNB symptoms on the wheat leaves was registered on the ninth day after infection with *S. nodorum* SnB isolate, as described previously [10]. The damaged area was measured using the ImageJ (version 1.44, LOCI, University of Wisconsin, Madison, WI, USA) computer program (http://rsbweb.nih.gov/ij/download.html, accessed on 7 July 2023) and expressed as a percentage of the total area of the leaf [10,21].

Table 1. Effect of phytohormones on the development of Septoria nodorum blotch in three wheat genotypes nine days after infection with *S. nodorum* SnB isolate.

Variant of treatment	Omskaya 35 (*tsn1/snn3*)	Kazakhstanskaya 10 (*tsn1/Snn3*)	Zhnitsa (*Tsn1/Snn3*)
S. nodorum (SnToxA/SnTox3)*	14.2 ± 0.8^{a}	77.1 ± 7.3^{c}	85.2 ± 7.7^{e}
S. nodorum+*trans*-Zeatin	9.1 ± 0.1^{b}	8.5 ± 0.5^{b}	3.1 ± 0.1^{f}
S. nodorum+Salicylic acid	12.2 ± 0.2^{a}	15.2 ± 0.6^{a}	25.8 ± 2.1^{g}
S. nodorum+Ethephone	75.3 ± 5.6^{c}	94.7 ± 8.4^{d}	95.6 ± 4.2^{d}

* The table shows the leaf damage area in % of the total leaf area, where 100% is the area of the whole sheet. Different letters indicate statistical differences between the values and the control in each column according to the Duncan's test (n = 30, $p \leq 0.05$).

Fig. 1. The effect of phytohormones on the phenotypic manifestation of Septoria nodorum blotch symptoms in three wheat genotypes after infection with *S. nodorum* SnB isolate. Development of disease symptoms on leaves of cultivar Omskaya 35 (Om35) (A), Kazakhstanskaya 10 (Kaz10) (B), and Zhnitsa (C) treated with either *trans*-zeatin (SnB+tZ), salicylic acid (SnB+SA), or ethephon (SnB+ET), and infected with *S. nodorum* SnB (SnB) isolate. Photographs represent the results of a typical variant from a series of experiments. Experiments were carried out on the separated first leaves.

2.3 Redox Status of Plants

To measure hydrogen peroxide (H_2O_2) production and activity of redox enzymes (peroxidase (POD) and catalase (CAT)), plant material (1:5 weight/volume) was homogenized in 0.05 M solution of Na–phosphate buffer (PB), pH 6.2 and incubated at 4 °C for 30 min. Supernatants were separated by centrifugation at 15,000 ×g for 15 min (5415 K Eppendorf, Hamburg, Germany). The H_2O_2 concentration in the supernatant was determined by xylenol orange, using the standard method [25]. POD activity was determined by the oxidation of (o-)phenylenediamine, as described previously [10,21]. Enzymatic activity was expressed as optical density/mg protein per minute. CAT activity was determined using the standard method based on the ability of H_2O_2 to form a stable-colored complex with molybdate salts, as described previously [10,21]. CAT activity was calculated using a calibration curve and expressed in µM H_2O_2/(mg protein per min). Protein content was determined by the Bradford method.

2.4 Isolation of Fungal RNA and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

To study the expressions of genes encoding pathogen NEs and TFs, total RNA (wheat and fungal) was extracted from control and *S. nodorum*-infected wheat seedlings using TRIzol™ reagent (Merck KGaA, Sigma-Aldrich, Darmstadt, Germany), according to the manufacturer's instructions. cDNA synthesis was performed as described previously [10]. Primers for qRT-PCR were designed using a web-based primer designing tool from IDT (https://eu.idtdna.com/PrimerQuest/Home/Index, accessed on 20 September, 2023) (USA). The sequences of all the primers are presented in **Supplementary Table 1**. Quantitative PCR was performed using a set of predefined reagents EvaGreenI (Synthol, Moscow, Russia) and CFX Connect Real-Time PCR Detection System device (Bio-Rad Laboratories, Hercules, CA, USA). To standardize the data, the tubulin gene of the fungus *S. nodorum* (S56922) (**Supplementary Table 1**) was used as an internal reference [26]. The delta–delta Ct method was used to quantify the relative gene expression [27,28]. Three independent biological and three technical replications were performed for each experiment.

2.5 Isolation of Plants miRNA and Performing qPCR

MicroRNA was extracted from control and *S. nodorum*-infected wheat seedlings using a miRNA isolation kit (Biolabmix, Moscow, Russia). RNA concentrations were determined by an ND-1000 spectrophotometer (NanoDrop Technologies LLC, Wilmington, DE, USA). Isolated miRNA from leaf tissues were polyadenylated at 37 °C for 1 hour in a reaction volume containing 0.3 µg RNA and 5 U *Escherichia coli* poly(A) polymerase (New England Biolabs, Ipswich, MA, USA) [29]. First-strand miRNA cDNA was synthesized by mixing 500 ng of poly(A) tailed RNA and 0.5 µg of RTQ primer using M-MLV reverse transcriptase (Synthol, Moscow, Russia). Reverse transcription reagents were incubated at 37 °C for 1 hour.

Table 2. The effect of phytohormones on the expression of the *S. nodorum* SnB isolate *SnToxA* gene during the infection of three wheat genotypes.

Wheat genotypes	Time, hpi (hours post inoculation)	Variant of treatment			
		SnB	SnB+tZ	SnB+SA	SnB+ET
Omskaya 35 (*tsn1/snn3*)	6	0.1 ± 0.05^{c}	0.2 ± 0.06^{c}	0.1 ± 0.05^{c}	0.2 ± 0.04^{c}
	24	0.4 ± 0.06^{b}	0.2 ± 0.06^{c}	0.3 ± 0.07^{b}	0.4 ± 0.02^{bc}
	72	1.0 ± 0.07^{a}	0.6 ± 0.03^{b}	0.7 ± 0.06^{a}	2.5 ± 0.2^{b}
Kazakhstanskaya 10 (*tsn1/Snn3*)	6	0.2 ± 0.06^{b}	0.2 ± 0.06^{b}	0.4 ± 0.04^{ab}	0.8 ± 0.04^{a}
	24	1.0 ± 0.1^{a}	0.2 ± 0.06^{b}	0.9 ± 0.07^{a}	1.8 ± 0.05^{c}
	72	0.4 ± 0.06^{ab}	0.1 ± 0.01^{b}	0.7 ± 0.06^{a}	1.7 ± 0.2^{c}
Zhnitsa (*Tsn1/Snn3*)	6	1.0 ± 0.1^{a}	0.4 ± 0.06^{d}	2.9 ± 0.2^{b}	2.4 ± 0.2^{b}
	24	2.7 ± 0.2^{b}	1.4 ± 0.05^{e}	7.9 ± 0.3^{f}	1.9 ± 0.04^{b}
	72	4.1 ± 0.3^{c}	0.8 ± 0.04^{a}	12.7 ± 0.5^{g}	15.0 ± 1.2^{h}

Designations of samples as in Fig. 1. Different letters indicate statistical differences in values from the control in three lines relating to the same wheat cultivar according to the Duncan's test (n = 6, $p \leq 0.05$).

Table 3. The effect of phytohormones on the expression of the *SnTox3* gene of the *S. nodorum* SnB isolate during infection of three wheat genotypes.

Wheat genotypes	Time, hpi	Variant of treatment			
		SnB	SnB+tZ	SnB+SA	SnB+ET
Omskaya 35 (*tsn1/snn3*)	6	1.0 ± 0.05^{a}	2.5 ± 0.2^{b}	1.2 ± 0.09^{a}	2.4 ± 0.2^{b}
	24	3.0 ± 0.2^{b}	2.6 ± 0.2^{b}	1.5 ± 0.2^{a}	2.3 ± 0.3^{b}
	72	4.0 ± 0.3^{c}	4.5 ± 0.4^{c}	2.5 ± 0.2^{b}	11.5 ± 0.9^{d}
Kazakhstanskaya 10 (*tsn1/Snn3*)	6	1.0 ± 0.05^{a}	1.1 ± 0.09^{a}	1.5 ± 0.2^{d}	2.5 ± 0.2^{b}
	24	2.3 ± 0.2^{b}	2.2 ± 0.2^{b}	1.6 ± 0.2^{d}	1.8 ± 0.2^{b}
	72	6.2 ± 0.5^{c}	3.4 ± 0.2^{e}	4.6 ± 0.3^{e}	14.7 ± 1.1^{ef}
Zhnitsa (*Tsn1/Snn3*)	6	1.0 ± 0.06^{a}	0.5 ± 0.04^{d}	0.1 ± 0.01^{f}	2.6 ± 0.2^{e}
	24	5.0 ± 0.4^{b}	3.6 ± 0.3^{e}	4.9 ± 0.5^{b}	1.0 ± 0.09^{a}
	72	6.0 ± 0.5^{c}	1.6 ± 0.1^{a}	6.6 ± 0.6^{c}	24.0 ± 1.7^{g}

Designations of samples are the same as in Fig. 1. Different letters indicate statistical differences in values from the control in three lines relating to the same wheat cultivar according to the Duncan's test (n = 6, $p \leq 0.05$).

Quantitative PCR for miRNAs was performed by polymerase chain reaction in real-time using microRNA-specific forward primers and the universal reverse primer along with RTQ primer using a CFX Connect real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with EvaGreen I intercalating dye (Synthol, Moscow, Russia). To normalize the results of the miRNA expressions, we used primers for the constitutively expressed 5S rRNA gene. Primers for qRT-PCR were designed using a web database https://www.pmiren.com (accessed on 7 July 2023). The primer sequences are all presented in **Supplementary Table 2**. Primer sequences were validated by the presence of only a single peak on the thermal dissociation (Tm) curve, which was generated by the thermal denaturing protocol. The relative gene expression was calculated using the delta–delta Ct method [30]. Three independent biological and three technical replicates were performed for each experiment.

2.6 Statistical Analysis

All experiments were repeated three times with a different number of biological repetitions from 3 to 10. Experimental data were expressed as mean ± SE, which were calculated using MS Excel (version 16.0.14430.20306, Redmond, WA, USA) in all treatments. The significance of the differences was assessed by ANOVA followed by Duncan's test ($p \leq 0.05$) using STATISTICA 10.0 software (version STA999K347150-W, Tulsa, OK, USA). The treatment variants and the number of repetitions are indicated in the tables and figures.

3. Results

3.1 Phytohormones and Resistance/Susceptibility to NEs SnToxA and SnTox3

To test the effects of the three phytohormones of SA, CK, and ethylene on the induction of the defense responses in different wheat genotypes infected with SnB, the leaf damage areas were measured on the ninth day of the infection in three cultivars treated with phytohormones (Ta-

Table 4. The effect of phytohormones on the expression of the *SnPf2* gene of the *S. nodorum* SnB isolate during infection of three wheat genotypes.

Wheat genotypes	Time, hpi	Variant of treatment			
		SnB	SnB+tZ	SnB+SA	SnB+ET
Omskaya 35 (*tsn1/snn3*)	6	1.0 ± 0.08^a	1.4 ± 0.1^a	0.9 ± 0.08^a	0.8 ± 0.05^a
	24	1.4 ± 0.09^a	1.2 ± 0.1^a	0.7 ± 0.08^c	1.3 ± 0.08^a
	72	1.3 ± 0.09^a	1.6 ± 0.1^b	0.7 ± 0.08^c	0.8 ± 0.02^a
Kazakhstanskaya 10 (*tsn1/Snn3*)	6	1.0 ± 0.08^a	1.5 ± 0.1^b	0.7 ± 0.04^c	1.1 ± 0.1^a
	24	1.0 ± 0.08^a	1.1 ± 0.2^a	0.9 ± 0.05^a	1.0 ± 0.1^a
	72	0.8 ± 0.04^a	1.2 ± 0.2^a	0.5 ± 0.03^c	0.6 ± 0.05^a
Zhnitsa (*Tsn1/Snn3*)	6	1.0 ± 0.08^a	1.5 ± 0.1^b	0.5 ± 0.03^c	0.9 ± 0.06^a
	24	1.6 ± 0.09^b	1.3 ± 0.1^a	1.5 ± 0.09^b	1.7 ± 0.2^b
	72	1.5 ± 0.1^b	1.6 ± 0.2^b	1.5 ± 0.2^b	1.2 ± 0.01^a

Designations of samples as in Fig. 1. Different letters indicate statistical differences in values from the control in three lines relating to the same wheat cultivar according to the Duncan's test (n = 6, $p \leq 0.05$).

ble 1, Fig. 1). Minimal damage areas of 14.2% of the total leaf area were observed in the incompatible interaction in the Om35/SnB variant (Table 1, Fig. 1A). The SnTox3–*Snn3* interaction in the Kaz10/SnB (cultivar/isolate) combination led to the formation of lesions that occupied more than 75% of the total leaf area (Table 1, Fig. 1B). Two interactions SnToxA–*Tsn1* and SnTox3–*Snn3* in the Zhn/SnB (cultivar/isolate) combination resulted in the formation of lesions that occupied more than 85% of the total leaf area (Table 1, Fig. 1C). Such a reaction indicates the important roles of SnToxA and SnTox3 in the development of the disease with compatible interactions [30].

The treatment of three wheat genotypes with *trans*-zeatin significantly reduced the damaged areas to between 3% and 9% of the total leaf area (Table 1). Moreover, *trans*-zeatin had the strongest effect on the cv. Zhnitsa plants (Table 1, Fig. 1C).

Plant treatment with SA also resulted in a reduction in disease symptoms in all three wheat genotypes (Table 1, Fig. 1). In cultivars Om35 and Kaz10, the damaged areas decreased to 12% and 15% of the total leaf area, respectively (Table 1). However, the effect of SA on the cv. Zhnitsa plants were less significant, whereby the damaged areas were only reduced to 25.8% (Table 1). The treatment of plants with ET led to an increase in disease symptoms in all three cultivars (Table 1, Fig. 1). The treatment with ET resulted in the development of lesions on 95% of the leaf area in cultivars Kaz10 and Zhnitsa, which have compatible and specific gene-for-gene interactions with the SnB isolate (Table 1, Fig. 1). However, the lesions only increased up to 75% in the ET-treated cv. Om35, which does not have a compatible gene-for-gene interaction with the SnB isolate (Table 1).

3.2 Phytohormones and Expression of NE Genes and Fungal TF Genes in Plants

The *SnToxA* gene expression increased both during the infection of the sensitive wheat genotype for the cv. Zhnitsa, carrying the *Tsn1* susceptibility gene, and in the insensitive wheat genotypes of the cv. Kaz10 and cv. Om35 (Table 2). However, the abundance of the *SnToxA* mRNA was significantly higher during the infection of the cv. Zhnitsa than during the infection of Kaz10 and Om35, which follows since these do not have the *Tsn1* susceptibility gene in their genome (Table 2). *Trans*-zeatin treatment reduced *SnToxA* gene expression in all three cultivars, regardless of the host genotype (Table 2). ET treatment increased the transcript levels of the *SnToxA* gene in all three cultivars by 2.5–4 times, compared to the untreated plants (Table 2).

The influence of SA on the expression of the *SnToxA* gene depended on the host genotype. In the absence of the compatible SnToxA–*Tsn1* interaction, SA did not affect the expression of this gene; in the presence of the compatible SnToxA–*Tsn1* interaction, during infection of the cv. Zhnitsa, SA increased the expression of the *SnToxA* gene 3-fold compared to untreated plants (Table 2).

During infection, the expression of the *SnTox3* gene increased in both the susceptible and resistant wheat genotypes (Table 3).

Treatment with ET increased the expression of the *SnTox3* gene in all three cultivars by 2.5–4 times compared to untreated plants, regardless of the host genotype (Table 3). In the absence of a compatible interaction, the treatment with *trans*-zeatin to cv. Om35 did not affect the expression of the *SnTox3* gene upon infection; however, this phytohormone reduced the transcript levels of the *SnTox3* gene in the presence of the SnTox3–*Snn3* interaction during the infection of cultivars Kaz10 and Zhnitsa (Table 3). SA treatment reduced the expression of the *SnTox3* gene in all three cultivars (Table 3). This reduction in the gene transcript levels was observed for 72 hours during the infection

Table 5. Influence of phytohormones on *SnStuA* gene expression of *S. nodorum* SnB isolate during infection of three wheat genotypes.

Wheat genotypes	Time, hpi	Variant of treatment			
		SnB	SnB+tZ	SnB+SA	SnB+ET
Omskaya 35 (*tsn1/snn3*)	6	1.0 ± 0.05^{a}	1.5 ± 0.09^{a}	0.7 ± 0.04^{d}	1.0 ± 0.08^{a}
	24	2.4 ± 0.2^{b}	2.1 ± 0.2^{b}	2.5 ± 0.2^{b}	2.8 ± 0.2^{b}
	72	4.4 ± 0.3^{c}	1.2 ± 0.09^{a}	3.1 ± 0.2^{b}	11.8 ± 1.1^{e}
Kazakhstanskaya 10 (*tsn1/Snn3*)	6	1.0 ± 0.05^{a}	0.7 ± 0.04^{c}	0.9 ± 0.06^{a}	1.1 ± 0.06^{a}
	24	2.2 ± 0.3^{b}	1.2 ± 0.09^{a}	2.0 ± 0.1^{b}	1.8 ± 0.2^{b}
	72	1.8 ± 0.1^{b}	1.6 ± 0.08^{b}	1.3 ± 0.09^{a}	4.2 ± 0.5^{d}
Zhnitsa (*Tsn1/Snn3*)	6	1.0 ± 0.04^{a}	0.6 ± 0.05^{d}	4.7 ± 0.4^{c}	2.4 ± 0.3^{e}
	24	6.1 ± 0.5^{b}	2.6 ± 0.2^{e}	4.0 ± 0.3^{c}	2.0 ± 0.2^{e}
	72	3.9 ± 0.2^{c}	3.6 ± 0.3^{c}	10.4 ± 0.6^{f}	8.3 ± 0.7^{g}

Designations of samples as in Fig. 1. Different letters indicate statistical differences in values from the control in three lines relating to the same wheat cultivar according to the Duncan's test (n = 6, $p \leq 0.05$).

Table 6. The effect of phytohormones on the expression of the *SnCon7* gene of the *S. nodorum* SnB isolate during infection of three wheat genotypes.

Wheat genotypes	Time, hpi	Variant of treatment			
		SnB	SnB+tZ	SnB+SA	SnB+ET
Omskaya 35 (*tsn1/snn3*)	6	1.0 ± 0.08^{a}	1.2 ± 0.08^{a}	1.1 ± 0.02^{a}	0.8 ± 0.07^{a}
	24	1.2 ± 0.07^{a}	0.5 ± 0.02^{b}	1.2 ± 0.01^{a}	1.5 ± 0.03^{c}
	72	1.2 ± 0.07^{a}	0.8 ± 0.04^{a}	1.1 ± 0.02^{a}	1.8 ± 0.03^{c}
Kazakhstanskaya 10 (*tsn1/Snn3*)	6	1.0 ± 0.06^{a}	0.9 ± 0.07^{a}	0.9 ± 0.07^{a}	0.7 ± 0.06^{a}
	24	0.9 ± 0.06^{a}	0.8 ± 0.06^{a}	1.3 ± 0.03^{a}	1.3 ± 0.02^{a}
	72	0.7 ± 0.03^{a}	0.9 ± 0.07^{a}	1.2 ± 0.03^{a}	0.9 ± 0.07^{a}
Zhnitsa (*Tsn1/Snn3*)	6	1.0 ± 0.08^{a}	1.1 ± 0.02^{a}	0.8 ± 0.07^{a}	0.8 ± 0.06^{a}
	24	0.9 ± 0.07^{a}	0.8 ± 0.05^{a}	1.1 ± 0.02^{a}	1.0 ± 0.08^{a}
	72	1.4 ± 0.08^{b}	1.1 ± 0.02^{a}	1.4 ± 0.03^{b}	1.2 ± 0.02^{a}

Designation of samples as in Fig. 1. Different letters indicate statistical differences in values from the control in three lines relating to the same wheat cultivar according to the Duncan's test (n = 6, $p \leq 0.05$).

of the Om35 and Kaz10 cultivars (Table 3). However, a decreased expression of the *SnTox3* gene was found only after 6 hours of infection in the cv. Zhnitsa plants treated with SA (Table 3) and could also affect the size of the lesion areas in the cv. Zhnitsa treated with SA (Table 1, Fig. 1).

In our work, we studied the expression of three *S. nodorum* TF genes, *SnPf2*, *SnStuA*, and *SnCon7*, during the infection of diverse wheat genotypes with the treatment of phytohormones.

The host genotype did not affect the expression of the *SnPf2* gene, although the expression changed slightly during infection, increasing by 30–40% in the resistant cv. Om35 cultivar and by 50–60% in the susceptible cv. Zhnitsa (Table 4).

Treatment with ET or *trans*-zeatin did not affect the expression of the *SnPf2* gene during the infection of all three cultivars (Table 4). Treatment with SA somewhat reduced the expression of the TF *SnPf2* gene during infection of all three cultivars (Table 4). This reduction in the gene transcript levels was observed for 72 hours during the infection of Om35 and Kaz10 (Table 4). However, a decreased expression of the *SnPf2* gene was found only after 6 hours of infection in the cv. Zhnitsa plants treated with SA (Table 4).

The expression of the *SnStuA* gene increased to a greater extent during the infection of the susceptible genotype (cv. Zhnitsa), which has two compatible interactions: SnToxA–*Tsn1* and SnTox3–*Snn3*, than in the more resistant genotypes (Table 5).

Treatment with ET increased the *SnStuA* gene expression in all three cultivars, whereas *trans*-zeatin treatment reduced the *SnStuA* gene expression in all three cultivars, regardless of the host genotype (Table 5). The influence of the SA treatment depended on the host genotype. In the presence of a compatible SnToxA–*Tsn1* interaction, SA increased the expression of the *SnStuA* gene; in the absence of a compatible SnToxA–*Tsn1* interaction, SA did not affect the expression of *SnStuA* (Table 5). An increase in the *SnStuA* gene expression during the infection of the cv. Zhnitsa cultivar after treatment with SA could affect the expression of both *SnToxA* and *SnTox3* (Tables 2,3).

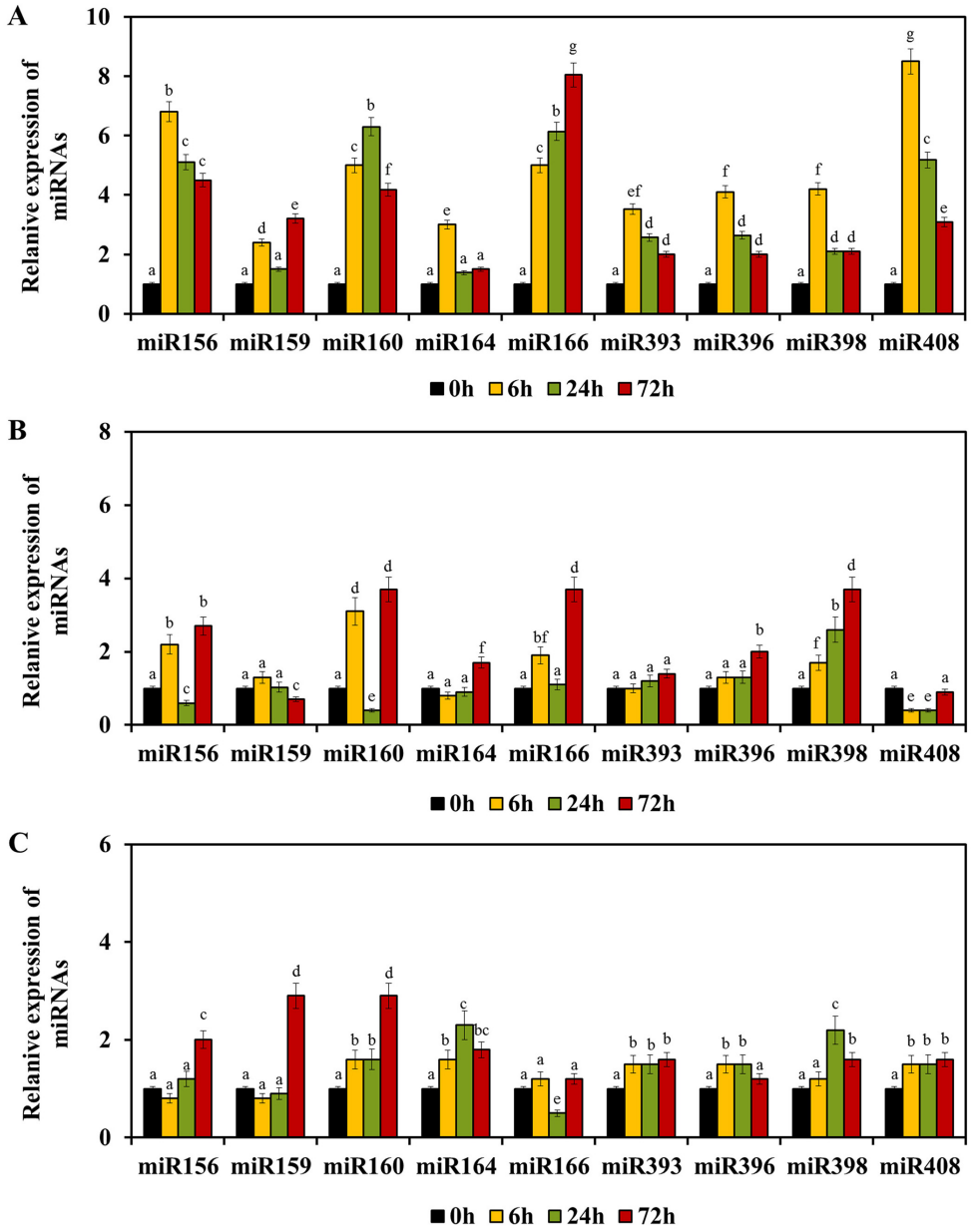

Fig. 2. MicroRNA expression in the diverse wheat genotypes during *S. nodorum* infection. MicroRNA expression in the leaves of the cvs. Omskaya35 (A), Kazakhstanskaya 10 (B), and Zhnitsa (C) were uninfected with *S. nodorum* (0 h) and 6 (6 h), 24 (24 h), and 72 (72 h) hours after infection. Figures present mean ± SE (n = 6). Different letters indicate statistical differences in values from the control on each histogram according to the Duncan's test ($p \leq 0.05$).

The host genotype did not affect the expression of the *SnCon7* gene: it remained practically unchanged during infection (Table 6).

However, phytohormones had a non-specific effect on the expression of this gene (Table 6). Moreover, in the presence of any compatible interaction (cultivars Zhnitsa, Kaz10), the phytohormones did not affect the expression of the *SnCon7* gene (Table 6). In the presence of an incompatible interaction, treatment with ET increased, whereas treatment with SA had no effect, and treatment with *trans*-zeatin reduced the expression of the *SnCon7* gene (Table 6).

3.3 MicroRNA Expression in the Diverse Wheat Genotypes during S. nodorum Infection

In this work, the expression of nine conserved microRNAs (miR156, miR159, miR160, miR164, miR166, miR393, miR396, miR398, and miR408) were studied in three wheat genotypes, cv. Om35, cv. Kaz10, and cv. Zhnitsa, at the early stages of infection with a *S. nodorum* SnB isolate (Fig. 2).

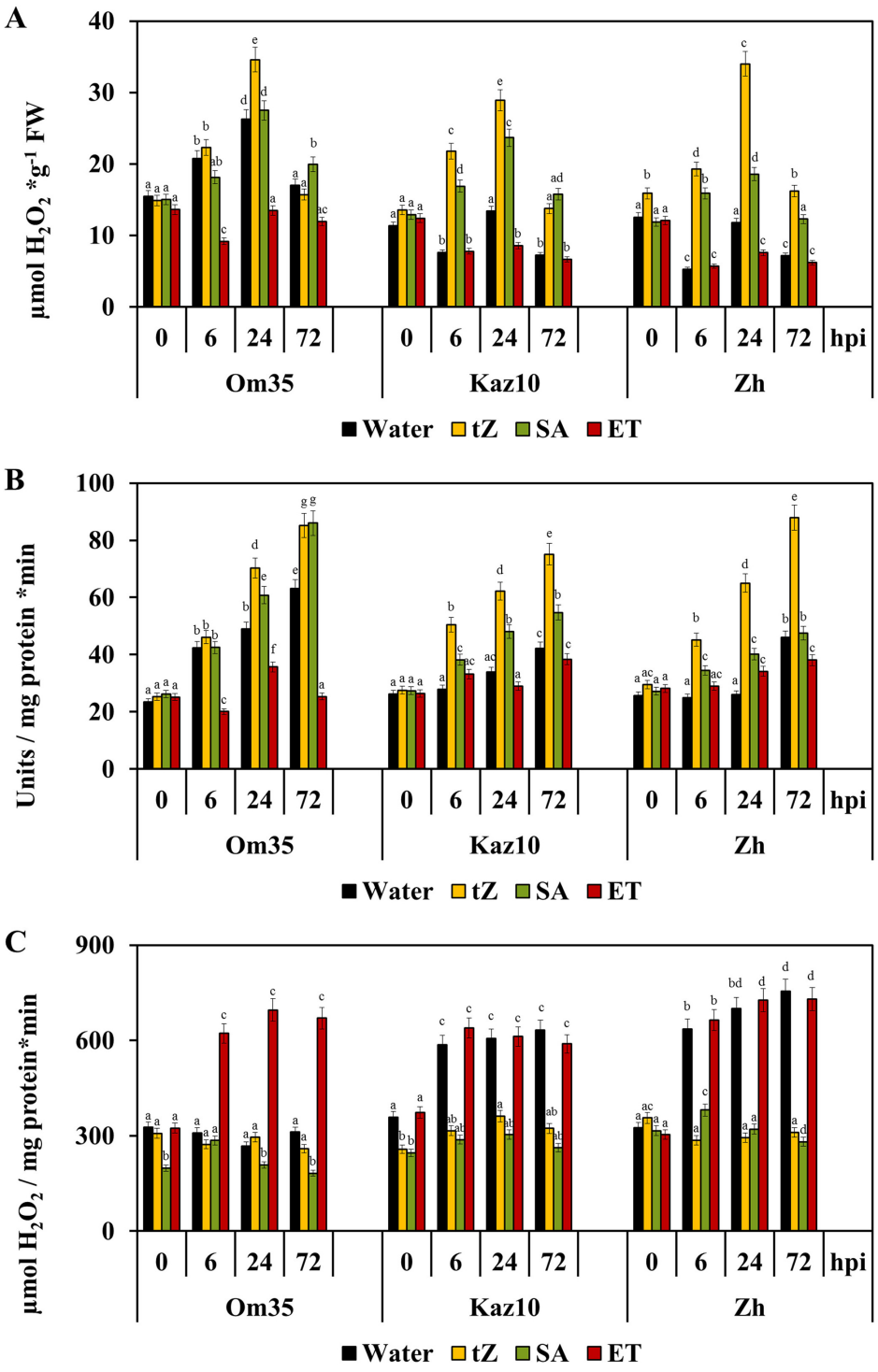

Fig. 3. Effect of phytohormones on the redox status of three wheat cultivars, Omskaya 35 (Om35), Kazakhstanskaya 10 (Kaz10), and Zhnitsa (Zh) during *S. nodorum* infection. Hydrogen peroxide content (H_2O_2) (A); peroxidase (B) and catalase (C) activity in leaves of three wheat cultivars uninfected (0 h), and 6 (6 h), 24 (24 h), and 72 (72 h) hours after *S. nodorum* infection. The samples are indicated as follows: Water—plants untreated with phytohormones; tZ—plants treated with *trans*-zeatin; SA—plants treated with salicylic acid; ET—plants treated with ethephon. Figures present mean ± SE (n = 6). Different letters indicate statistical differences in values from the control for one wheat cultivar on each histogram according to the Duncan's test ($p \leq 0.05$).

Our results showed that the expression of all nine miRNAs was upregulated in the cv. Om35 in the absence of compatible interactions (Fig. 2A). However, the pattern of expression of all miRNAs varied: it differed in the degree of induction and the time of maximum activation (Fig. 2A). The transcript levels of four miRNAs, miR156, miR160,

miR166, and miR408 were increased by 4.5–8.5 times compared to the control (Fig. 2A). The level of expression of three miRNAs, miR393, miR396, and miR398, increased by 2–4 times compared to the control, and the expression of miR159 and miR164 was induced only 2–3 times, compared to the control (Fig. 2A). The expression of six miRNAs (miR156, miR164, miR393, miR396, miR398, and miR408) was maximal after 6 hours of infection before decreasing, although it remained higher than the control level (Fig. 2A). This suggests that they are involved in the induction of the defense responses against *S. nodorum* and the development of PTI [31]. The expressions of miR159 and miR160 had already increased by 2.4 and 5 times, respectively, after 6 hours of infection, yet reached their maximums after 24 hours of infection, while the transcripts level of miR166 reached its maximum after 72 hours of infection (Fig. 2A). This suggests that these miRNAs are involved in the later stages of the defense responses associated with the development of ETI [31]. To test these assumptions, we compared the expression patterns of nine conserved miRNAs in the absence of compatible interactions and in the presence of one or two interactions, i.e., SnToxA–Tsn1 and SnTox3–*Snn3* (Fig. 2).

In the presence of the SnTox3–*Snn3* compatible interaction in the cv. Kaz10, only six miRNAs (miR156, miR160, miR164, miR166, miR396, and miR398) were upregulated (Fig. 2B). However, the degree and timing of induction were different from that in cv. Om35, and the expression of miR159, miR393, and miR408 was downregulated (Fig. 2B), suggesting an effect of NE SnTox3 on these miRNAs. A specific feature of miRNA expression in the cv. Kaz10 was the inhibition of almost all miRNAs, with the exception of miR398, after 24 hours of infection, which corresponds to the time of the active reproduction of the fungus mycelium in wheat tissues [10]. Thus, NE SnTox3 did not affect the expression of miR156 and miR398, yet either completely (miR393 and miR408) or partially (miR164 and miR396) suppressed the expression of miRNAs involved in the PTI. Moreover, NE SnTox3 also completely (miR159) or partially (miR160 and miR166) inhibited the expression of miRNAs involved in the ETI (Fig. 2B).

In the presence of two compatible interactions (SnToxA–*Tsn1* and SnTox3–*Snn3*), the expression of five miRNAs significantly increased in the cv. Zhnitsa. These miRNAs differed from the composition of other miRNAs induced by *S. nodorum* in cv. Kaz 10 in the presence of only the SnTox3–*Snn3* interaction (miR156, miR159, miR160, miR164, and miR398) (Fig. 2C). The degree of induction of the expression of these miRNAs in the cv. Zhnitsa was much lower than in the cv. Om35, and lower than in the cv. Kaz10 (Fig. 2). It should be noted that in the presence of the second SnToxA–*Tsn1* interaction, the expression of miR166 was very strongly inhibited and the miR159 transcripts were again accumulated, although only after 72 hours of infection (Fig. 2C). Thus, based on a comparative analysis of miRNA expressions in two wheat genotypes, cv. Kaz10 and cv. Zhnitsa, it can be assumed that NE SnToxA suppresses the expression of miR166 and induces the expression of miR159 (Fig. 2C).

3.4 Effect of Phytohormones on the Redox Status of Diverse Wheat Genotypes during S. nodorum Infection

Changes in H_2O_2 content and the activities of peroxidase and catalase were studied in three wheat genotypes treated with phytohormones and infected with *S. nodorum* (Fig. 3).

Our results showed that the H_2O_2 content increased by 1.7 times due to an increase in the activity of peroxidases, by more than 2 times, and the absence of an increase in catalase activity in cv. Om35 (Fig. 3). The H_2O_2 content was strongly reduced due to an increase in catalase activity, by about 2 times, and the absence of a significant increase in peroxidase activity 6 and 24 hours after the infection of cv. Kaz10 and cv. Zhnitsa (Fig. 3). Treatment of plants with *trans*-zeatin and SA induced an oxidative burst following an increase in the H_2O_2 content due to an enhancement in peroxidase activity and a decrease in catalase activity in all three wheat genotypes (Fig. 3). However, in the presence of compatible interactions of SnToxA–*Tsn1* and SnTox3–*Snn3*, treatment with SA resulted in a smaller increase in the content of H_2O_2 and had less effect on the activity of peroxidases and catalases than in the absence of such interactions (Fig. 3). Treatment with ET led to a decrease in H_2O_2 content due to a decline in peroxidase activity and an increase in catalase activity in all three cultivars, regardless of the genotype and the presence of compatible interactions (Fig. 3). However, in the presence of compatible interactions SnToxA–*Tsn1* and SnTox3–*Snn3*, treatment with ET reduced the H_2O_2 content in plants to a greater extent than in the absence of compatible interactions (Fig. 3).

3.5 Effect of Phytohormones on the MicroRNA Expression of Diverse Wheat Genotypes during S. nodorum Infection

We studied the treatment effect of CK, SA, and ET on the expression of four miRNAs miR159, miR166, miR393, and miR408 in two wheat genotypes infected with *S. nodorum*.

A comparative analysis of miR159 expression in three diverse wheat genotypes, suggests that the SnTox3–*Snn3* interaction completely repressed the transcription of miR159 (Fig. 2C), while in the presence of the two interactions, SnToxA–*Tsn1* and SnTox3–*Snn3*, the expression of miR159 was induced after 72 hours of infection (Fig. 2C). Such an effect on NEs is possible because some studies have previously described the antagonistic interaction of NEs SnToxA and SnTox3 [7]. Interestingly, the increase in miR159 expression found in cv. Om35 and cv. Zhnitsa after 72 hours of infection did not depend on the phytohormone treatment (Fig. 4A). During further analysis, the differences in miR159 expression were found between wheat

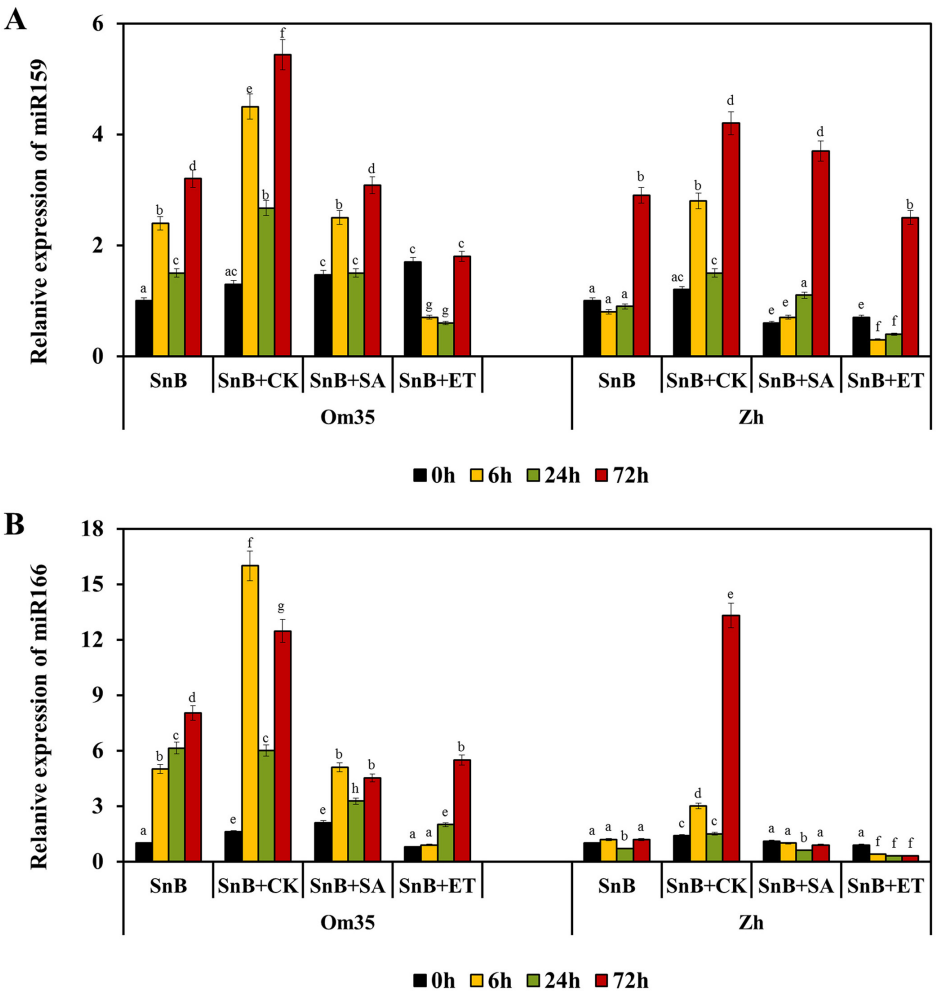

Fig. 4. Effect of phytohormones on miRNA expressions in two wheat cultivars infected with *S. nodorum*. Expression of miR159 (A) and miR166 (B) in cv. Omskaya 35 (Om35) and cv. Zhnitsa (Zh), uninfected (0 h), and 6 (6 h), 24 (24 h), and 72 (72 h) hours after infection with *S. nodorum*. Designations in the figure: SnB—plants untreated with phytohormones and infected with *S. nodorum* (SnB); SnB+tZ—plants treated with *trans*-zeatin and infected with *S. nodorum* (SnB); SnB+SA—plants treated with salicylic acid and infected with *S. nodorum* (SnB); SnB+ET—plants treated with ethephon and infected with *S. nodorum* (SnB). Figures present mean ± SE (n = 6). Different letters indicate statistical differences in values from the control for one wheat cultivar on each histogram according to the Duncan's test ($p \leq 0.05$).

genotypes in the presence or absence of treatment with phytohormones at an early stage of infection, after 6 and 24 hours (Fig. 4A). The expression of miR159 was suppressed in the untreated and SA-treated cv. Zhnitsa plants and in the ET-treated plants of both cultivars after 6 and 24 hours of infection, compared to the control (Fig. 4A). Notably, large or medium lesions developed in all these variants (Table 1). The expression of miR159 was upregulated in cv. Om35 plants and in plants of both cultivars treated with *trans*-zeatin at 6 and 24 hours of infection (Fig. 4A). However, only small lesions developed in the plants with these variants (Table 1).

The results of this work showed that NE SnToxA strongly suppressed the expression of miR166 (Fig. 2C). Treatment of plants with *trans*-zeatin non-specifically increased miR166 expression in both genotypes of wheat cvs. Om35 and Zhnitsy (Fig. 4B). SA and ethylene specifically affected the expression of miR166 depending on the presence of compatible interactions. Thus, in the absence of any interactions in cv. Om35, treatment with SA did not affect miR166 expression after 6 hours of infection, yet reduced transcription after further courses of infection, compared to infected Om35 plants, which had not been treated with phytohormones (Fig. 4B). In the presence of the two interactions, SnToxA–*Tsn1* and SnTox3–*Snn3*, treatment with SA did not affect miR166 expression (Fig. 4B). In the absence of the interactions in cv. Om35, treatment with ET more strongly reduced miR166 expression during the initial period of infection (after 6 and 24 hours), whereas in the presence of the two interactions, ET inhibited miR166 expression throughout the experiment (Fig. 4B).

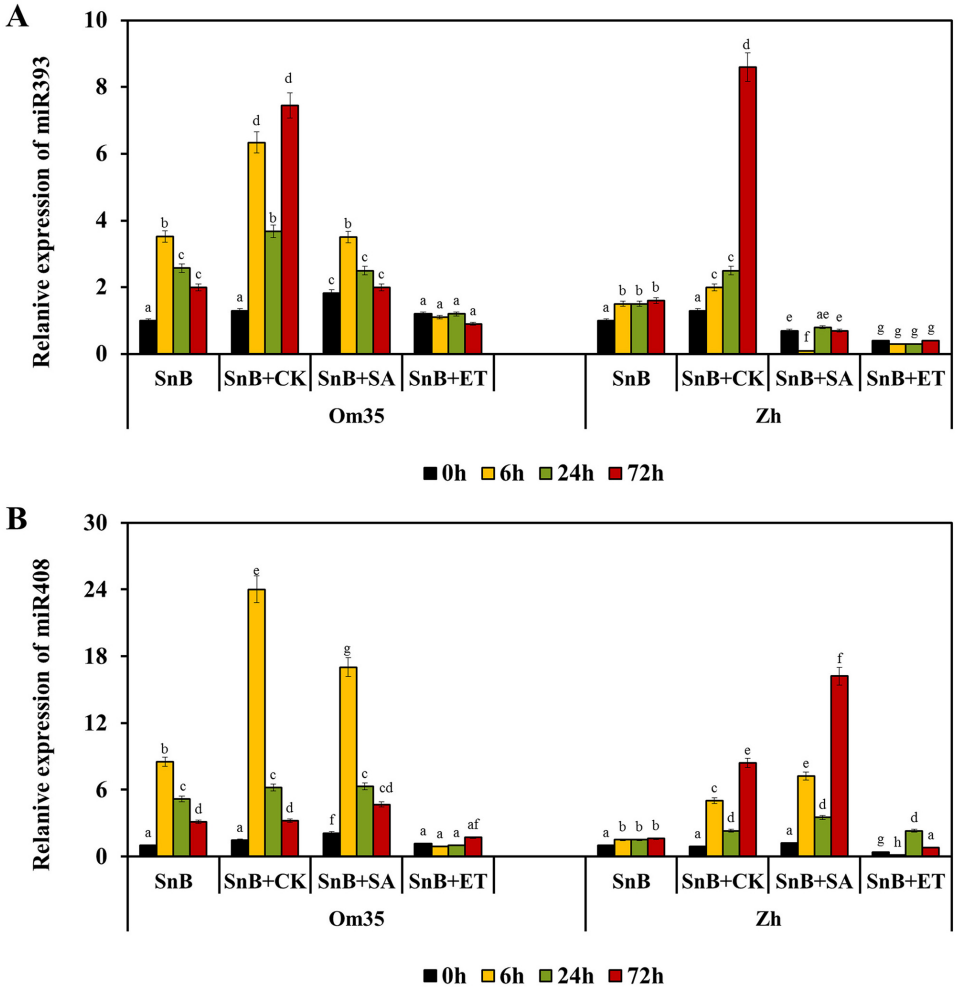

Fig. 5. Effect of phytohormones on miRNA expression in two wheat cultivars infected with *S. nodorum*. Expression of miR393 (A) and miR408 (B) in cvs. Omskaya 35 (Om35) and Zhnitsa (Zh), uninfected (0 h), and 6 (6 h), 24 (24 h), and 72 (72 h) hours after infection with *S. nodorum*. Designations in the figure: SnB—plants untreated with phytohormones and infected with *S. nodorum* (SnB); SnB+tZ—plants treated with *trans*-zeatin and infected with *S. nodorum* (SnB); SnB+SA—plants treated with salicylic acid and infected with *S. nodorum* (SnB); SnB+ET—plants treated with ethephon and infected with *S. nodorum* (SnB). Figures present mean ± SE (n = 6). Different letters indicate statistical differences in values from the control for one wheat cultivar on each histogram according to the Duncan's test ($p \leq 0.05$).

The expression of miR393 was suppressed in the presence of compatible interactions in both cvs. Kaz10 and Zhnitsa (Fig. 2). Treatment of the cvs. Om35 and Zhnitsa plants with *trans*-zeatin increased miR393 expression, whereas ET treatment reduced miR393 expression (Fig. 5A), which is possibly associated with the development of resistance in the first case and with the development of susceptibility in the second case (Table 1). SA treatment affected miR393 expression in plants in a specific way, depending on the presence of the compatible interactions. Thus, in the absence of compatible interactions in the cv. Om35 plants, SA did not affect miR393 expression; the expression of this miRNA increased (Fig. 5A). In the presence of the two interactions SnToxA–*Tsn1* and SnTox3–*Snn3* in the cv. Zhnitsa plants, SA treatment reduced miR393 expression (Fig. 5A). Such an effect by SA in the presence of NE may suggest the interference of effectors in the hormonal signaling pathways of plants and, possibly, an association with a decrease in plant resistance to *S. nodorum* (Table 1).

Our results showed that the increase in miR408 expression is associated with the development of plant resistance against *S. nodorum*, whereas the SnToxA–*Tsn1* and SnTox3–*Snn3* interactions suppressed the expression of this miRNA (Fig. 5B, Table 1).

Treatment of plants with *trans*-zeatin or SA increased the expression of miR408 in both genotypes of wheat (cvs. Om35 and Zhnitsy) (Fig. 5B). However, the maximum expression of miR408 was found in the untreated cv. Om35 plants in the absence of a compatible interaction or those treated with *trans*-zeatin or SA, at the early stage of the disease, i.e., after 6 hours of infection (Fig. 5B). Further-

more, the maximum expression of miR408 was found in the cv. Zhnitsa plants in the presence of two compatible interactions and treated with *trans*-zeatin or SA and after 72 hours of infection with *S. nodorum* (Fig. 5B). Plant treatments with ET non-specifically reduced miR408 expression in both wheat cultivars (Fig. 5B), which may be associated with the development of susceptibility to pathogens, in the case of these treatments (Table 1).

4. Discussion

4.1 Analysis of Phytohormonal Effects on the Susceptibility to NEs SnToxA and SnTox3

In this study, we used one resistant wheat genotype (cv. Om35, which is SnToxA/SnTox3-insensitive) and two susceptible genotypes (cv. Kaz10, which is SnTox3-sensitive and the SnToxA/SnTox3-sensitive strain of cv. Zhnitsa) to study the effects of NE SnToxA and SnTox3 on hormonal signaling pathways by SA, CK, and ethylene. It is well known that pathogenic NEs manipulate hormonal signaling pathways [4,11,12], however, this process remains poorly understood in the wheat–*S. nodorum* pathosystem [7]. Therefore, to deepen the understanding of this interaction, we treated plants with phytohormones SA, CK, and ethephon (the chemical precursor of ethylene). We have previously shown that SA and CK are involved in the process of defense reactions by wheat plants against *S. nodorum*, while ethylene increases the virulence of the pathogen associated with the SnTox3 effector [10]. The treatment of plants with CK or SA resulted in a reduction in disease symptoms, whereas the treatment of plants with ET led to an increase in all three cultivars (Table 1, Fig. 1).

The results of this work showed that CK can regulate resistance in both the presence of NE SnTox3 and NE SnToxA, although nothing specific is known about the effect of CK on the effectors. Unfortunately, the present literature also lacks information on the effect of CK on pathogen effectors; it is only known that effectors can induce CK signaling in plants to promote disease development [4,11]. Despite this, a lot of data has been accumulated on the role of CK in the deployment of plant resistance to various pathogens [32–35], both through the activation of the SA signaling pathway, with the increased expression of SA-related defense genes, and independently of the SA pathway [36]. The effect of SA on the disease symptoms was diminished in the Zhnitsa/SnB variant in the presence of the compatible SnToxA–*Tsn1* interaction (Table 1, Fig. 1). We assume that SnToxA can manipulate the SA signaling pathway. It is known that SA is the main hormone involved in plant immunity, which regulates systemic acquired resistance (SAR) against many pathogens. However, a large number of effectors have already been established that manipulate both SA biosynthesis and the signaling pathway of this hormone [4,11,31]. In addition, it has been shown that SnToxA directly interacts with the PR1 protein, which is the marker protein of the SA pathway [37]. In our work, the positive effect of ET on the progression of infection symptoms (Table 1, Fig. 1) is consistent with the results of other authors, thereby showing that ethylene increases the susceptibility of plants to pathogens [38,39]. Indeed, since the role of SnTox3 in the inhibition of the PTI reaction has previously been shown by manipulating ethylene signaling in plants infected with *S. nodorum* [10], the stronger action of ET on the progress of infection in cultivars Kaz10 and Zhnitsa was possibly associated with the presence of a compatible SnTox3–*Snn3* interaction. However, this fact does not exclude the interaction between ethylene and NE SnToxA. In addition, effectors of various pathogens are known to manipulate the ethylene signaling pathway in plants [40–42].

Thus, our results showed that CK induced a resistance response, whereas ethylene-induced a susceptibility response to Nes: SnToxA and SnTox3. Further, SA regulated the deployment of the resistance to NE SnTox3, yet could not confer the full resistance against NE SnToxA, suggesting that the SnToxA effector could manipulate the SA signaling pathway.

4.2 Analysis of Phytohormonal Effects on the Expression of NE Genes and Fungal TF Genes in Plants

Then, since the mechanism involved in the regulation of the expression of effector genes has not been fully elucidated [12], the influence of three phytohormones on the gene expression of NEs and TFs was studied. Our results showed that *SnToxA* gene expression depended on the wheat genotype (Table 2). Previously, for the Sn5 isolate, the influence of the wheat genotype on the expression of the *SnToxA* gene was shown in the presence of the SnToxA-*Tsn1* compatible interaction, whereby the expression of the *SnToxA* gene was two-fold higher than in the absence of the interaction [43]. The action of CK and ET on the expression of the *SnToxA* gene did not depend on the wheat genotype, in contrast to the effect of SA (Table 2), which confirms our assumption about the dominance of SnToxA NE over the SA signaling pathway. An increase in *SnToxA* gene expression was observed in both the Zhnitsa/SnB variant and in the three ET-treated wheat genotypes infected with SnB (Table 2). In these variants, we found an expansion in the damage zones (Table 1, Fig. 1), which indicates an increase in the virulence of the SnB isolate in these interactions, compared to others. This conclusion is consistent with some observations by previous authors of a positive correlation between the expression level of the *SnToxA* gene and the contribution to the disease by the SnToxA–*Tsn1* interaction [43,44].

The study of the *SnTox3* gene expression showed that, although the expression of the *SnTox3* gene increased in the presence of the SnTox3–*Snn3* interaction more than in its absence, the host genotype did not affect the expression pattern of this gene (Table 3). This is consistent with results by other authors [8]. As in the case of *SnToxA* gene expression, the effect of ET on the *SnTox*3 gene transcript levels

did not depend on the wheat genotype (Table 3). Such increases in the expression of the *SnTox3* gene could lead to an increase in the virulence of the SnB isolate, which could result in an extension of the lesions in the leaves (Table 1, Fig. 1). Previously, we found that in wheat with the susceptible genotype, NE SnTox3 induces ethylene biosynthesis and the expression of TF genes in the ethylene signaling pathway, whereas ET treatment of the wheat with the resistant genotypes made it susceptible [10]. On the contrary, a decrease in the expression of the *SnTox3* gene during treatment with *trans*-zeatin and SA could lead to a weakened virulence in the SnB isolate and reduced lesions in the leaves of the susceptible cultivars (Table 1, Fig. 1). Additionally, we have previously shown that CK and SA play an important role in wheat via the induction of the defense reactions against *S. nodorum*, while NE SnTox3 quickly decreased cytokinin content through oxidative degradation and the inhibition of its biosynthesis in ethylene-dependent and ethylene-independent manners [10]. Different levels of pathogenicity among *S. nodorum* strains and isolates are associated with different levels of NE gene expression [7–9,43,45]. However, the mechanisms underlying this differential expression remain largely unexplored.

Recently, the importance of some TFs as regulatory elements that target key pathogen virulence pathways has been established [13]. It is becoming increasingly clear that TFs play a significant role in the regulation of not only metabolic processes, enzyme biosynthesis, hyphal growth, and sporulation but also the expression of NE genes [13].

It is known that the SnPf2 TF positively regulates the expression of *S. nodorum* NE genes *SnToxA* and *SnTox3* [45]. Deletion of the *SnPf2* gene in *S. nodorum* led to the suppression of the *SnToxA* and *SnTox3* NE gene expressions, which led to the loss of virulence in the wheat pathogen [45]. Our results showed that the host genotype did not affect the expression of the *SnPf2* gene (Table 4). In addition, based on the obtained results, it can be concluded that ethylene and CK both affected the expression of the *SnToxA* and *SnTox3* NE genes, although not through the regulation of the *SnPf2* gene transcription. On the contrary, the pattern of expression of the *SnTox3* and *SnPf2* genes completely coincided in the plants treated with SA, which may indicate a regulatory effect by SA on the expression of the *SnTox3* gene through the regulation of the *SnPf2* TF gene transcription.

The StuA TF is responsible for not only the production of effectors and virulence but also for sporulation, sclerotia formation, melanization, and sexual reproduction [13]. The role of SnStuA in development and virulence has been established in *S. nodorum* [46]. However, only one study has shown that SnStuA is a positive regulator of *SnTox3* and is not required for *SnToxA* expression [46]. Our results indicate that *SnStuA* gene expression is dependent on the plant genotype, which coincides with the results of a previous study [46]. However, specific effects of ethylene and CK on the expression of the *SnStuA* gene were not found (Table 5). Our results showed that the pattern of expression of the *SnToxA*, *SnTox3*, and *SnStuA* genes in plants treated with ET or *trans*-zeatin almost completely matched, which may indicate a regulatory effect by ethylene and CK on the expression of the *SnToxA* and *SnTox3* genes through the regulation of the *SnStuA* gene transcription, independently of plant genotype. On the contrary, the effect of SA on *SnStuA* gene expression depended on the wheat genotype and the presence of NE SnToxA.

SnCon7 is an important factor in controlling hyphal growth and fungal invasion into host tissues [13]. Silencing of SnCon7 in *S. nodorum* reduced the virulence of the isolate and suppressed the expression of the *SnTox3* gene, thereby demonstrating that TFs play a positive regulatory role in the expression of this gene [47]. In addition, it has been proven that SnCon7 binds directly to the promoter region in the *SnTox3* gene and is its direct regulator [47]. The SnCon7 TF also regulated the expression of two other NE genes, *SnTox1* and *SnToxA*, although, in this case, its regulation was indirect [47]. Our results showed that phytohormones regulated the expression of the *SnToxA* and *SnTox3* NE genes, although not by regulating the transcription of the *SnCon7* TF gene.

Thus, ethylene and CK non-specifically affected the expression of the *SnToxA* and *SnTox3* NE genes through the regulation of the *SnStuA* TF gene expression. Treatment with *trans*-zeatin decreased the expressions of the NE and TF genes, whereas they were increased by ET treatment. This effect can be explained by the fact that CK stimulates wheat resistance by suppressing the transcription of the NE and TF genes in the pathogen, while ethylene suppresses plant resistance by stimulating the transcription of the NE and TF genes in the pathogen. In the absence of a compatible SnToxA–*Tsn1* interaction, SA either did not affect or reduced the expression of the *SnToxA* and *SnTox3* NE genes and the *SnPf2* and *SnStuA* TF genes. These alterations can be associated with a positive effect of SA on the development of resistance reactions. In the presence of a compatible SnToxA–*Tsn1* interaction, SA specifically regulated the expression of the *SnTox3* gene through its influence on the expression of the *SnPf2* and *SnStuA* TF genes. Additionally, it, possibly, specifically regulated the expression of the *SnToxA* gene through its influence on the expression of the *SnStuA* gene. These results suggest that SnToxA can hijack and manipulate the SA signaling pathway during fungal growth and development. Our conclusions are also based on the results obtained by other authors, which showed an increase in the PR1 content (a marker protein of the SA pathway) in sensitive wheat genotypes infiltrated with SnToxA [48]. At the same time, the fine mechanisms involved in the regulation of virulence factors remain unexplored. We assume that RNA interference and small RNAs may be involved in this process.

4.3 Analysis of MicroRNA Expression in Plants Infected with S. nodorum

It is known that all the conserved miRNAs studied in this work (miR156, miR159, miR160, miR164, miR166, miR393, miR396, miR398, and miR408) regulate growth and development processes in plants and are also involved in the response to abiotic and biotic stresses [16,31,49]. Our results showed that all nine miRNAs studied in the present research were activated in resistant cv. Om35, which had been infected by *S. nodorum* (Fig. 2A). In the literature, the induction of miR156, miR164, miR393, and miR396 expression is associated with an increase in the resistance of wheat to powdery mildew *Blumeria graminis* [50]; an increase in miR156 expression in rice is associated with the development of resistance to *Rhizoctonia solani* [51]; induction of miR164 expression in wheat was observed in the development of resistance to *Puccinia striiformis* [52]; rice miR398 triggered resistance against *Magnaporthe oryzae* [15]. Indeed, miR166 regulates the resistance to fungal pathogens, such as *Fusarium oxysporum*, *Erysiphe graminis*, along with bacteria and viruses [53]. However, our results on the expression of nine conserved miRNAs in three wheat genotypes infected with *S. nodorum* clearly show that NEs SnToxA and SnTox3 influence the expression of four miRNAs (miR159, miR166, miR393, and miR408), thereby implying their involvement in the induction of resistance in wheat plants against *S. nodorum*. On the contrary, the miRNA expression results do not provide a clear answer to the question of how the NEs affect the other five miRNAs (miR156, miR160, miR164, miR396, and miR398) and their involvement in the induction of resistance or susceptibility to specific pathogens. To answer this question, further studies are required that employ a larger number of genotypes for both plants and pathogens.

The results of this work showed that the expression of miR160, miR166, and miR393 was upregulated in the resistant cultivars and downregulated by NEs in the susceptible cultivars (Fig. 2). Most likely, the inhibition of expression is associated with the suppression of the auxin signaling pathway and regulation of the SA signaling pathway by these miRNAs [31,53,54]. Thus, miR393 affects the transcripts of the F-box auxin receptor genes and subsequently confers enhanced resistance against *P. syringae* bacteria in Arabidopsis [31]. Furthermore, miR160 suppressed the auxin signal by targeting genes encoding auxin response factors 10 (StARF10) and StARF16 in potato plants infected with *Phytophthora infestans*, which thereby regulated the signaling pathway of salicylic acid (SA) and activated the expression of the PR1 marker gene [54]. In addition, rice lines overexpressing miR160a showed resistance to *M. oryzae* due to increased H_2O_2 accumulation [16]. Some studies have shown that miR166 affects auxin homeostasis and increases plant drought tolerance [53,55]. It has been established that auxin weakens the immune response in plants, and SA is one of the main phytohormones involved in plant immunity [56]. The literature describes a model of the influence of SA on auxin responses, where SA suppresses the expression of the TIR1/ABF F-box receptor complex, which results in the stabilization of the AUX/IAA auxin repressor protein and the repression of auxin signaling and responses [57]. In addition, we have previously shown that infecting the resistant cv. Om35 with *S. nodorum* activated the SA signaling pathway in plants, increased the content of H_2O_2, and led to the accumulation of *PR1* gene transcripts [10]. Thus, in the pathosystem, wheat-*S. nodorum* miR160 and miR393 are most likely regulated by the interaction between the auxin and SA signaling pathways.

The results of this work showed that miR159 and miR408 expression increased in the resistant cultivar and was suppressed by NEs in susceptible cultivars, while miR398 expression was induced by *S. nodorum*, regardless of the wheat genotype (Fig. 2). The functions of miR398 and miR408 during a pathogen attack in plants are associated with the regulation of redox metabolism [15]. Thus, miR398b increased the activity of superoxide dismutase (SOD) in *M. oryzae*-infected rice plants, thereby raising the concentration of H_2O_2, which induced the expression of *PR1* and *PR10* defense genes and plant resistance [31,58]. Recently, it has been shown that miR408 targets the catalase genes in wheat, which are enzymes involved in the degradation of H_2O_2 [59]. Moreover, SOD and peroxidase are the targets for miR159 [60]. Previously, we have shown a decrease in catalase activity in the resistant cv. Om35 infected with *S. nodorum*, which led to the accumulation of H_2O_2 and the development of protective reactions [61]. Thus, in the pathosystem wheat–*S. nodorum*, miR408, and miR398 were found to regulate ROS accumulation.

In addition, there is evidence that miR166 and miR159 in cotton are exported to pathogenic hyphae to inhibit the expression of virulence genes by the pathogen *Verticillium dahlia* [62]. Our results showed that miR159 was downregulated by SnTox3 NE, while miR166 was downregulated by SnToxA (Fig. 2).

Thus, we suggest that the role of conserved miRNAs in the induction of resistance by wheat plants to the pathogen *S. nodorum* consists of the regulation of redox metabolism and hormonal signaling pathways in the infected plants. Therefore, the next step in the work was to study the effect of phytohormones CK, SA, and ethylene on redox metabolism and miRNA expression in infected plants.

4.4 Analysis of Phytohormonal Effects on the Redox Status of Plants Infected with S. nodorum

In this regard, we studied the effect of phytohormones SA, CK, and ethylene on the redox status of diverse wheat genotypes infected with *S. nodorum*. Earlier, we found that the resistance of the *T. aestivum* to the pathogen *S. nodorum* was determined by the intensive generation of ROS, mainly H_2O_2, due to an increase in POD activity and a de-

crease or absence of an increase in CAT activity in the initial stage of infection [61]. It is known that peroxidases, together with NADPH oxidases, are involved in the generation of H_2O_2 in the apoplast in the plant-microbial interaction, while catalases degrade H_2O_2 [63].

The results showed that CK had a non-specific effect on the induction of an oxidative burst, while the effect of SA and ethylene depended on the presence of a compatible interaction. The SA treatment caused an oxidative burst in plants, although the SA effect decreased in the presence of SnToxA (Fig. 3). We observed a simultaneous increase in *SnToxA* expression and a decrease in H_2O_2 generation in cv. Zhnitsa treated with SA and infected with *S. nodorum* (Table 2, Fig. 3). Based on these results, we assume that SnToxA inhibited the ability of SA to cause the oxidative burst by mainly stimulating the catalase activity and decreasing the peroxidase activity. Conversely, our assumptions are based on the ability of SnToxA to increase the activity of catalases and suppress the activity of peroxidases, thereby reducing the oxidative burst [21,64]. Alternatively, it is known that the salicylate-dependent response is associated with an oxidative burst but, initially, with the activation of the NADPH-oxidase and apoplastic peroxidases and the inhibition of catalase activity [63]. In addition, catalase is a virulence factor in aggressive isolates of *S. nodorum* and it was found that the expression of the SNOG_03173.1 gene encoding fungal catalase was upregulated in the presence of a compatible SnToxA–*Tsn1* interaction [21,65]. Thus, based on the obtained results and data in the literature, it is possible to describe a model whereby *S. nodorum*, in the presence of the wheat genotype with the dominant *Tsn1* allele, responds to an enhanced SA content by increasing the NE SnToxA activity. In turn, SnToxA opposes the development of defense reactions mediated by SA: it increases catalase activity, reduces the oxidative burst, and inhibits the PR1 protein [21,66].

Furthermore, based on the results, whereby the *SnTox3* gene expression increased (Table 3), with simultaneous suppression of the oxidative explosion (Fig. 3) in different wheat genotypes treated with ET, we assume that SnTox3 enhanced the ethylene-induced inhibition of ROS generation through the effect on the redox enzymes and genes of the salicylate signaling pathway. Our assumptions are based on previously obtained results, showing that the SnTox3–*Snn3* interaction inhibited H_2O_2 production in wheat at the early stage of infection by affecting NADPH oxidases, peroxidases, superoxide dismutase, and catalase [21,64]. Moreover, using various *S. nodorum* isolates, it was shown that NE SnTox3 reduced the oxidative burst, activated the biosynthesis and signaling pathway of ethylene, decreased the transcript level of the *PR1* and *PR2* genes, and inhibited transcription of the *TaWRKY13* gene markers of the salicylate signaling pathway [10]. We have proven that the salicylate signaling pathway plays a major role in the induction of defense reactions in wheat plants against *S. nodorum* in the presence of the SnTox3–*Snn3* interaction and at an early stage of infection [10].

We assume that the effect of NEs on redox enzymes may be related to the ability of NEs to manipulate hormonal signaling pathways and to regulate the expression of plant miRNAs, which are responsible for fine-tuning transcription in the whole plant.

4.5 Analysis of Phytohormonal Effects on the MicroRNA Expression in Plants Infected with S. nodorum

To test this assumption, we studied the effect of CK, SA, and ET treatments on the expression of four miRNAs, miR159, miR166, miR393, and miR408, in two wheat genotypes cv. Om35 (incompatible interaction) and cv. Zhnitsa (two compatible interactions: SnToxA–*Tsn1* and SnTox3–*Snn3*), during infection with SnB isolate. Although the regulatory role of the miRNAs in phytohormone crosstalk during growth and development is well studied, there is much less information about the role of miRNAs in the regulation of phytohormonal crosstalk under biotic stress [15,49].

Our results suggest that the induction of miR159 expression was associated with the development of resistance by the plant to *S. nodorum* and could trigger the CK signaling pathway, although miR159 expression was suppressed by NE SnTox3, possibly via the ethylene signaling pathway [10]. It should be noted that we have previously shown the role of NE SnTox3 in reducing the content of CK in infected plants due to oxidative degradation by the enzyme cytokinin oxidase (CKX) [10], while CKX is a target for miR159 [60]. Presumably, NE SnTox3 can regulate the content of CK by influencing miR159.

Little is known about the role of miR166 in plant immunity; the role of this miRNA is manifested mainly in growth and development as well as under abiotic stress [53]. The miR166 family members (miR166a–miR166g) have several major target genes that encode HD-ZIP III transcription factors, such as PHABULOSA (PHB), PHAVOLUTA (PHV), REVOLUTA (REV), ATHB-8, and ATHB-15 [55]. Targets of miR166 ATHB-8 and ATHB-15 are associated with the regulation of secondary cell wall differentiation and lignification [53], which may be important during pathogen attacks, to strengthen the cell wall and form the protective barrier. Moreover, miR166 plays important roles in auxin and abscisic acid (ABA) interactions in monocots: the downregulation of miR166 enhances plant abiotic stress resistance via the regulation of ABA homeostasis [55]. However, an increase in miR166 expression, associated with the development of resistance, has been observed in poplar, wheat, soybean, and other plants affected by bacterial, fungal, or viral diseases caused by *Botryosphaeria dothidea*, *Erysiphe graminis*, *Fusarium oxysporum*, or *Mungbean Yellow Mosaic India Virus* (MYMIV) [53]. In our work, an increase in miR166 expression was also associated with the development of wheat

resistance against *S. nodorum*. The effect of CK, SA, and ethylene on miR166 expression may be associated with crosstalk between ABA and auxins, or CK, SA, and ethylene. This issue requires further study.

In addition, an increase in the expressions of miR159 and miR166 72 hours after the start of the infection may be associated with the export of these miRNAs to fungal hyphae for the specific suppression of fungal target genes. Recently, it has been shown that miR159 and miR166 in cotton, penetrate the hyphae of the fungus *V. dahliae* and suppress the expression of genes encoding the enzymes isotrichodermin C-15 hydroxylase (HiC-15) and Ca^{2+}-dependent cysteine protease (Clp-1), respectively, which led to a decrease in pathogen virulence and increased plant resistance [62].

As is known, miR393 suppresses the auxin signaling pathway, which blocks immune responses in the plant [31]. The SnToxA NE and hormone ethylene downregulate miR393, most likely to activate the auxin signaling pathway. Moreover, miR393 is a mediator of an ABA signal in the regulation of auxin signals during stress responses [16,49], which can lead to antagonism of ABA and SA signals during the development of pathogenic resistance [3]. However, this issue requires further study.

The miR408 is a highly conserved miRNA, which is involved in the regulation of plant growth, development, and stress responses. Indeed, miR408 regulates the growth and development of different plants by downregulating its targets, encoding blue copper (Cu) proteins, and transporting Cu to plastocyanin (PC), which affects photosynthesis, and ultimately, promotes grain yield. In addition, miR408 improves tolerance to stress by downregulating target genes and enhancing cellular antioxidants, thereby increasing the antioxidant capacity of plants [67]. In addition, in wheat, it has recently been shown that miR408 is involved in plant development and the response to various stresses by regulating the level of catalase gene transcripts [59].

The results of this work, firstly, suggest that the miR408 expression is regulated by the phytohormones, CK and SA, during the development of protective reactions of wheat against *S. nodorum*, associated with the generation of H_2O_2 and inhibition of catalase activity. Our conclusions are based on the analysis of miRNA targeting sites in *TaCAT* genes, which was performed previously [59]. The analysis showed that *TaCAT2-A* exons were targeted by four miRNAs, including *Tae-miR408*. In addition, the expression of this *TaCAT2-A* gene was induced by *S. nodorum* [59]. Secondly, the host target of SnToxA was previously found to be plastocyanin, which is part of the electron transport chain involved in photosynthesis [68]. Further, plastocyanin is indirectly activated by miR408 to increase photosynthesis [59]. Finally, our results showed that the expression of miR408 is reduced specifically through the SnToxA–*Tsn1* interaction, and it may be associated with the regulation of plastocyanin activity.

5. Conclusions

The nine conserved miRNAs studied in this work played a role in the development of wheat resistance against *S. nodorum*, through the regulation of hormonal signaling pathways, plant redox metabolism, and interaction with pathogen effectors. Moreover, the *S. nodorum* NEs of SnToxA and SnTox3 can manipulate hormonal signaling pathways by influencing plant microRNAs to regulate susceptibility by the plant. Thus, SnTox3 mainly suppressed the expression of three miRNAs miR159, miR393, and miR408. Additionally, the SnToxA NE suppressed miR166 expression. On the contrary, treatment with CK and SA increased the expression of miR159 and miR408, while treatment with CK increased the expression of miR393 and miR166. ET treatment inhibited the expression of miR159, miR408, miR393, and miR166. Suppression of miP159 expression by SnTox3 was most likely associated with the activation of the ethylene signaling pathway. In addition, SnToxA hijacked the SA signaling pathway and manipulated it for fungal growth and development. Furthermore, SnToxA and SnTox3 suppressed the expression of miR408, which was regulated by CK and SA, during the development of the protective reactions by wheat against *S. nodorum*, in response to the generation of H_2O_2 and inhibition of catalase activity. Fungal TFs SnPf2 and SnStuA could be indirectly involved in the regulation of these processes through the regulation of the NE gene expressions since they changed their expression pattern under the influence of phytohormones. The results of this work show for the first time the role of microRNAs in the development of wheat resistance against *S. nodorum* and the effect of *S. nodorum* NEs—SnToxA and SnTox3—on the activity of plant microRNAs. These results will further our understanding of the complex interactions between *S. nodorum* and wheat plants associated with NEs and wheat susceptibility genes. Future research is needed to improve our knowledge of the mechanisms underlying the development of wheat resistance against *S. nodorum*.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

Conceptualization, SV and IM; methodology, TN and GB; software, TN and GB; validation, TN; formal analysis, IM and SV; investigation, TN and GB; writing—original draft preparation, SV and TN; writing—review and editing, IM and SV; funding acquisition, TN. All authors have read and agreed to the published version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbe1504022.

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