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Effect of the host-specific toxin SnTOX3 from *Stagonospora nodorum* on ethylene signaling pathway regulation and redox-state in common wheat

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The fungus *Stagonospora nodorum* Berk. is the causative agent of Septoria nodorum blotch (SNB) of wheat. The most important factors of *Stagonospora nodorum* virulence include numerous fungal necrotrophic effectors (NEs) encoded by *SnTox* genes. They interact with the matching products of host susceptibility genes (*Snn*). SnTox-*Snn* interactions are mirror images of classical gene-for-gene interactions and lead to the development of disease. We have studied the SnTox3-*Snn3* interaction, resulting in the development of infection on leaves and formation of extensive lesions. The mechanism of SnTox3 action is likely to be linked to the regulation of redox metabolism and the influence on ethylene synthesis in the wheat plants, although the molecular mechanisms are not fully unveiled. To characterize the SnTox3-*Snn3* interaction, we used *S. nodorum* isolates differing in the expression of the NEs genes *SnTox3* (SnB (Tox3$^+$), Sn4VD (Tox3$^-$)) and two soft spring wheat (*Triticum aestivum* L.) cultivars, contrasting in resistance to the SNB agent and differing in the allelic composition of the susceptibility locus *Snn3-B1*: Kazakhstanskaya 10 (susceptible) and Omskaya 35 (resistant). We carried out a comparative assessment of the transcriptional activity patterns of genes responsible for ethylene biosynthesis (*TaACS1*, *TaACO*) and signaling pathway (*TaEIL1*, *TaERF1*) by real-time PCR and estimated the redox state of wheat plants infected with different isolates of *S. nodorum* by spectrometry. The induction of ethylene biosynthesis and signaling has been shown to result from gene-for-gene interaction between *Snn3-B1* and SnTox3. The results of plant redox status estimation showed that ethylene inhibited accumulation of hydrogen peroxide in SnTox3-sensitive genotypes by regulating the operation of various pro-/antioxidant enzymes at the transcriptional and posttranslational levels. Our results suggest that NE SnTox3 influences ethylene biosynthesis and signaling, thereby regulating redox metabolism in infected wheat plants as necessary for successful host colonization at the initial phases of infection, which ultimately leads to extensive lesions due to fast pathogen reproduction.

Key words: *Stagonospora nodorum*; *Triticum aestivum*; polymerase chain reaction; real-time polymerase chain reaction; necrotrophic effectors; ethylene; redox-metabolism; gene-for-gene interaction; nonspecific resistance.

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Влияние хозяин-специфичного токсина SnTOX3 патогена *Stagonospora nodorum* на сигнальный путь этилена и редокс-статус растений мягкой яровой пшеницы

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Важнейший фактор вирулентности возбудителя септориоза пшеницы *Stagonospora nodorum* Berk. – многочисленные некротрофные эффекторы (НЭ) гриба (SnTox), взаимодействующие с продуктами генов восприимчивости хозяина (*Snn*). Взаимодействия SnTox-*Snn* осуществляются по типу ген-на-ген и ведут к развитию болезни. В настоящей работе изучено взаимодействие SnTox3-*Snn3*, результатом которого является развитие инфекции на листьях с образованием обширных зон поражения. Предположительно, механизм действия SnTox3 связан с регуляцией редокс-метаболизма и влиянием на синтез этилена у растений пшеницы, однако молекулярные механизмы до конца не раскрыты. Для характеристики взаимодействия SnTox3-*Snn3* в работе были использованы изоляты *S. nodorum*, различающиеся по экспрессии гена *SnTox*: SnБ (Tox3$^+$) и Sn4ВД (Tox3$^-$), и два сорта мягкой яровой пшеницы (*Triticum aestivum* L.), контрастные по устойчивости к возбудителю септориоза и различающиеся по аллельному составу локуса восприимчивости *Snn3-B1*: Казахстанская 10 (восприимчи-

вая) и Омская 35 (устойчивая). Проведена сравнительная оценка характера транскрипционной активности генов биосинтеза (*TaACS1*, *TaACO*) и сигнального пути этилена (*TaEIL1*, *TaERF1*) методом полимеразной цепной реакции (ПЦР) в реальном времени и оценен редокс-статус растений пшеницы, инфицированных различными изолятами *S. nodorum* с помощью спектрофотометрических методов. Показано, что индукция биосинтеза и сигнального пути этилена происходила в результате взаимодействия по типу ген-на-ген *Snn3-B1*-SnTox3. Результаты оценки редокс-статуса растений показали, что этилен подавлял накопление пероксида водорода в чувствительных к SnTox3 генотипах за счет регуляции работы различных ферментов про-/антиоксидантной системы на транскрипционном и посттрансляционном уровнях. Таким образом, полученные результаты предполагают, что НЭ SnTox3 влиял на биосинтез и сигнальный путь этилена с целью регуляции редокс-метаболизма инфицированных растений пшеницы для успешной колонизации хозяина на начальных этапах инфицирования, что впоследствии приводило к обширным зонам поражения за счет быстрого размножения патогена.
Ключевые слова: *Stagonospora nodorum*; *Triticum aestivum*; полимеразная цепная реакция; полимеразная цепная реакция в реальном времени; некротрофный эффектор; этилен; редокс-метаболизм; взаимодействие ген-на-ген; неспецифическая устойчивость.

Introduction

Wheat, a staple crop, has been attacked by various kinds of leaf spot diseases in recent decades, and Septoria nodorum blotch (SNB) ranks among the most injurious ones. It is caused by the fungal pathogen *Stagonospora nodorum* Berk. Yield losses inflicted by this pest reach up to 30 % in susceptible wheat cultivars under permissive environmental conditions (Bertucci et al., 2014). Studies of SNB have been intensively conducted over the past three decades, but there is still no clear understanding of the mechanisms that underlie wheat resistance/susceptibility to infection, on the one hand, and pathogen virulence, on the other hand (Fraaije et al., 2002; Bertucci et al., 2014; Winterberg et al., 2014; Phan et al., 2016; Shi et al., 2016).

It has been shown that among the most important factors of virulence of *S. nodorum* are the numerous necrotrophic effectors (NEs), formerly referred to as host-specific (selective) toxins (Phan et al., 2016; McDonald, Solomon, 2018). The interaction in the wheat–*S. nodorum* pathosystem is of the gene-for-gene type (McDonald, Solomon, 2018). These relationships are confirmed by the fact that products of the pathogen virulence genes (=host-specific toxins) (SnTox) cause compatibility, i. e. disease expansion, when interacting with products of the host plant susceptibility genes (*Snn*) (Phan et al., 2016). The effect of each SnTox-*Snn* interaction is incomplete and is complemented by other interactions. To date, eight SnTox-*Snn* interactions are known, while only three genes encoding NEs (*SnToxA*, *SnTox1*, and *SnTox3*) have been cloned from the pathogen, and only two susceptibility genes (*Tsn1* and *Snn1*) have been cloned from wheat (Phan et al., 2016; Shi et al., 2016).

The genetics of the relationship between wheat and *S. nodorum* is very complex, race-specific resistance explaining only about 40 % of phenotypic manifestations (Shi et al., 2016). In addition, it was shown in the last four years that some of the characterized SnTox-*Snn* interactions causing susceptibility are aimed at manipulating nonspecific plant defense pathways associated with redox metabolism, secondary metabolism and pathogenicity-related proteins (Winterberg et al., 2014; Phan et al., 2016; Shi et al., 2016).

The *Snn3-B1*-SnTox3 interaction plays a significant role in SNB development (Shi et al., 2016; McDonald, Solomon, 2018). It is assumed that the result of this interaction is the development of infection on leaves with the formation of extensive lesions, which is associated with the influence of SnTox3 on the generation of reactive oxygen species (ROS); unfortunately, the mechanism underlying this effect remains obscure (Winterberg et al., 2014). Nevertheless, a recent study shows that SnTox3 induces methionine accumulation and ethylene synthesis in wheat plants within 24 h after infection (Winterberg et al., 2014).

One of the earliest plant responses to the penetration of a pathogen is known to be local ROS generation, which plays an important role in the development of systemic resistance (Barna et al., 2012). Currently, the mechanisms regulating apoplastic ROS synthesis during immune response are intensively studied but still insufficiently understood. Recent studies have shown that the pro-/antioxidant state of plants is under the strict control of plant hormones involved in the formation of defense reactions during stress (Barna et al., 2012). They include ethylene, whose role in biotic stress is complex and depends on the type of pathogen and plant species (Vleesschauver et al., 2010; Barna et al., 2012). Earlier, we showed the negative role of ethylene in the development of wheat plant resistance to *S. nodorum* (Veselova et al., 2016). Unfortunately, the mechanisms of action of plant hormones, including ethylene, on ROS generation under biotic stress are poorly known (Barna et al., 2012).

In this regard, the aim of this work was a comparative assessment of the transcriptional pattern of genes involved in ethylene biosynthesis and signaling pathway genes and of the redox state of wheat plants infected with *Stagonospora nodorum* isolates differing in NE *SnTox3* expression.

Materials and methods

The objects of the study were two cultivars of soft spring wheat (*Triticum aestivum* L.) contrasting in resistance to *S. nodorum* Berk.: susceptible cv. Kazakhstanskaya 10 (Kaz10) and resistant cv. Omskaya 35 (Om35). The pathogen objects were two isolates of the fungus *S. nodorum*: Sn4VD (Republic of Belarus) and SnB (Republic of Bashkortostan). Fungi were grown on potato-glucose agar (PGA). Plants were hydroponically grown on 10 % solution of Hoagland–Arnon nutrient medium in a KS-200 SPU growth chamber (Russia) at

20/24 °C (night/day) at the irradiance 146 W/m^2 FAR (Osram lamps L 36W/77) and the 16-h photoperiod for seven days. The assessment of seedling resistance of cultivars was carried out by the lawns method, as described in (Veselova et al., 2016). The resistance/susceptibility of cultivars was assessed from the lesion area seven days after inoculation with *S. nodorum* isolates. The development of SNB symptoms on wheat leaves was photographed with an SP-800UZ Image Stabilization camera (Olympus, Indonesia). The lesion area was measured with ImageJ program (rsbweb. nih.gov/ij/download.html) and expressed as percent leaf area infected. The degree of damage was also assessed according to the International scale based on the percentage of the affected area of plant organs: RR (0–5 %) – caltivars with very high and high resistance; R (up to 10–15 %) – resistant caltivars; M (up to 25 %) – slightly susceptible caltivars; S (up to 40–65 %) – susceptible caltivars; SS (over 65 %) – caltivars with very high and high susceptibility.

DNA was isolated from wheat seedlings and 7-day fungus culture by the phenol-detergent method (Maniatis et al., 1984). *SnTox3* gene (FJ823644) identification in *S. nodorum* isolates was performed by PCR with gene-specific primers (5′→3′): F–CGAGCTGATATCCCGTTTGA; R–GGGACAGTGACAATAGGTAAGG (Winterberg et al., 2014); primers for the housekeeping gene *tubulin* (S56922) (Fraaije et al., 2002) being used as an internal control for the presence of fungal DNA. Analysis of *SnTox3* gene expression in different isolates of *S. nodorum* during inoculation of wheat plants was performed with the same primers using semi-quantitative PCR. Total RNA was isolated with Trizol reagent (Sigma, Germany) according to manufacturer's recommendations from leaves of susceptible wheat cv. Kaz10 and resistant cv. Om35 fixed in liquid nitrogen after their inoculation with a pathogen. To obtain cDNA based on RNA from the studied samples, reverse transcription reaction was performed using reverse transcriptase in accordance with manufacturer's protocol (Synthol, Russia). PCR with the cDNA template was performed in a TP4-PCR-01-Tertsik type PCR machine (DNK-Tekhnologia, Russia).

The allelic state of the *Snn3-B1* locus was determined by PCR with primers for the *Xcfd20* and *Xgwm234* microsatellite markers (Bertucci et al., 2014). The sequences of primers *Xcfd20* (5′→3′): F–TGATGGGAAGGTAATGGGAG; R–ATCCAGTTCTCGTCCAAAGC; of primers *Xgwm234* (5′→3′): F–GAGTCCTGATGTGAAGCTGTTG; R–CTCATTGGGGTGTGTACGTG (Bertucci et al., 2014). In all cases, PCR products were resolved in 7 % PAAG stained with ethidium bromide using the Gene Ruler DNA Ladder (Fermentas). Gels were photographed using a GelDoc XR documenting system (Bio-Rad, USA).

To reveal the effect of SnTox3 on the biosynthesis and signaling pathway of ethylene, part of the wheat seedlings were treated with 1.5 mM solution of ethephone (ET) (2-chloroethylphosphonic acid), an ethylene-releasing compound (Sigma, Germany) (Veselova et al., 2016), 24 h before inoculation with various *S. nodorum* isolates, while the remainder of the wheat seedlings were treated with 50 μM aminoethoxyvinylglycine (AVG), ethylene biosynthesis inhibitor (Sigma, Germany). After the treatment, the vessels were closed and kept in the dark. The content of hydrogen peroxide (H_2O_2) and the activities of peroxidase enzymes (PO), oxalate oxidase (OXO), and catalase were measured 24 and 72 h after inoculation with *S. nodorum* isolates as previously described (Veselova et al., 2018).

Total RNA was isolated from control and experimental wheat leaves with Trizol reagent according to manufacturer's (Sigma, Germany) recommendations. Prior to the isolation, the leaves were fixed in liquid nitrogen 24 h after their inoculation with *S. nodorum*. Analysis of the expression of genes for oxidoreductases and genes involved in the biosynthesis and signaling pathways of ethylene was performed by quantitative real-time PCR with an iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, USA) and SYBR Green I intercalating dye (Sintol, Russia). To normalize the expression results of the studied genes, primers for the *RLI* gene for constitutively expressed RNA inhibitor protein (RNase L inhibitor-like) (AY059462) were used (Gimenez et al., 2011). Changes in the expression of the gene of interest were estimated by the level of normalized gene expression calculated with the iCycler iQ5 Real-Time Detection System Software (Bio-Rad, USA). Primers for the genes encoding NADPH oxidase (*TaRboh*, AY561153) (Giovanini et al., 2006), superoxide dismutase (SOD) (*TaSod*, JX398977.1) (Giovanini et al., 2006), anionic peroxidase (*TaPrx*, TC151917) (Maksimov et al., 2014), aminocyclopropane synthase (ACC synthase – *TaACS1*, U35779) (Subramaniam et al., 1996), aminocyclopropane oxidase (ACC oxidase – *TaACO*, KF900072) (primer sequences (5′→3′): F–TGTCCATCGCCTCCTTCTA; R–CGAACACGAACCTTGGGTAT; transcription factor of the ethylene signaling pathway EIN3-LIKE1 (ETHYLENE INSENSITIVE3- LIKE1 (EIL1) – *TaEIL1*, KU030837, *Arabidopsis* orthologue gene *AtEIN3*) (Liu et al., 2016) and the transcription factor of the primary response to ethylene ERF1 (ETHYLENE RESPONSE FACTOR1 – *TaERF1*, EF583940) (Dong et al., 2010) were used in this study.

All experiments were carried out three times with three biological and three analytical replications ($n = 9$ in total), except for the measurements of infected area, which were performed in not less than 30 biological replications ($n = 90$ altogether). The Figures 1–3 and Tables 1–3 report mean values and their confidence intervals calculated from their standard errors. Significance of differences between experimental variants was estimated by Student's *t*-test at the confidence level $p \leq 0.05$.

Results

***SnTox3* gene and *Snn3-B1* susceptibility locus.** Two *S. nodorum* isolates, SnB and Sn4VD, were tested for the presence/absence of the *SnTox3* gene by PCR. The gene was found in both (Fig. 1, *a*).

However, analysis of the transcriptional activity of this NE gene showed no expression in the avirulent Sn4VD isolate and accumulation of *SnTox3* transcripts after inoculation of the susceptible cv. Kaz10 and the resistant cv. Om35 with the virulent isolate SnB (see Fig. 1, *b*). PCR diagnostics of the allelic state of the *Snn3-B1* locus was performed in two soft spring wheat cultivars, Kaz10 and Om35. For this purpose, specific primers for two *Xcfd20* and *Xgwm234* microsatellite markers flanking the *Snn3-B1* locus were used (Bertucci et al., 2014; Shi et al., 2016). The null allele was not found in these varieties. However, the cultivars differed in the allelic

composition of the *Snn3-B1* locus (see Fig. 1, *c*). In particular, the *Xcfd20* marker was represented by two alleles in both cultivars, and *Xgwm234* was represented by one allele in Om35 and by two in Kaz10 (see Fig. 1, *c*).

Cultivar/isolate combinations. It was shown previously that cv. Om35 was the most resistant among several cultivars of soft spring wheat, whereas Kaz10 was the least resistant to the hemibiotrophic fungus *S. nodorum* (Veselova et al., 2016). Those observations were made using isolate SnB. In this work, the following cultivar/isolate combinations were studied: Kaz10/SnB (S/Tox3$^+$), Kaz10/Sn4VD (S/Tox3$^-$), Om35/SnB (I/Tox3$^+$), Om35/Sn4VD (I/Tox3$^-$), where S is the Tox3-sensitive cultivar, I is the Tox3-insensitive cultivar, Tox3$^+$ is the isolate expressing the toxin gene, Tox3$^-$ is the isolate not expressing the toxin gene.

A complete compatibility reaction was detected in the S/Tox3$^+$ cultivar/isolate combination (Kaz10/SnB), where inoculation with the pathogen led to the formation of large lesions covering up to 80 % of the total leaf area (Table 1). Resistance reactions were observed in the remaining cultivar/isolate combinations (Om35/SnB, Kaz10/Sn4VD, Om35/Sn4VD) (see Table 1). Pretreatment of both Tox3-sensitive (Kaz10) and Tox3-insensitive (Om35) plants with ET increased their susceptibility only to the SnB isolate (Tox3$^+$) but did not affect the susceptibility to the Sn4VD isolate (Tox3$^-$) (see Table 1). Pretreatment of Tox3-insensitive plants (Om35) with ethylene biosynthesis inhibitor AVG did not affect their defense response, regardless of the isolate that was used for plant inoculation: SnB (Tox3$^+$) or Sn4VD (Tox3$^-$) (see Table 1). Treatment of Tox3-sensitive plants (Kaz10) with AVG increased their resistance to the SnB isolate but did not affect the resistance to the Sn4VD isolate (see Table 1).

The biosynthesis and signaling pathway of ethylene in infected plants. Analysis of the transcriptional activity of ethylene biosynthesis genes (*TaACS* for ACC synthase and *TaACO* for ACC oxidase) and signaling pathway genes (*TaEIL1* and *TaERF1*) showed an increase in the mRNA contents of these genes during the compatibility reaction developed in susceptible plants (Kaz10/SnB) and in plants treated with ET (Kaz10/SnB + ET, Om35/SnB + ET) (Table 2). In Tox3-sensitive plants (Kaz10), the transcripts contents

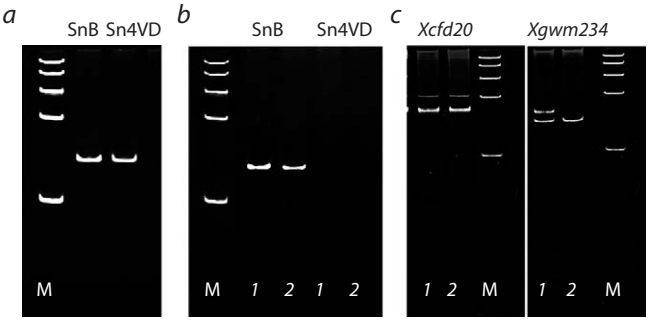

Fig. 1. Identification of the *SnTox3* gene in two *S. nodorum* isolates: SnB and Sn4VD (*a*); analysis of transcriptional activity of the *SnTox3* gene in infection of two soft spring wheat cultivars (*b*); identification of alleles of the *Snn3-B1* locus in these cultivars using primers for SSR markers (*Xcfd20* and *Xgwm234*) by PCR (*c*).

Lanes: *1* – Kazakhstanskaya 10; *2* – Omskaya 35; M – DNA molecular weight ladder 100–1000 bp.

of the ethylene biosynthesis genes and the gene coding for *TaERF1* (transcription factor involved in the primary response to ethylene) increased about three to four fold, and the mRNA content of the *TaEIL1* gene (coding for the main regulatory factor of the ethylene signaling pathway) increased 14–18 times on the 24th hours after inoculation (see Table 2). However, in Tox3-insensitive plants (Om35/SnB + ET cultivar/isolate combination), the accumulation of mRNAs of the genes responsible for biosynthesis and signaling pathway of ethylene was lower than in other cultivar/isolate combinations, which led to a compatibility reaction (see Table 2).

When the incompatibility reaction developed in Tox3-insensitive plants in the Om35/SnB, Om35/Sn4VD cultivar/isolate combinations, suppression or absence of the accumulation of transcripts of genes involved in ethylene biosynthesis and signaling pathway was found regardless of ET or AVG treatments (see Table 2). When the incompatibility reaction developed in Tox3-sensitive plants, either inoculated with Sn4VD isolate (Tox3$^-$) (Kaz10/Sn4VD, Kaz10/Sn4VD + AVG, Kaz10/Sn4VD + ET) or with SnB isolate (Tox3$^+$) (Kaz10/SnB + AVG), there was no accumulation of mRNAs of the *TaACS*, *TaACO*, or *TaERF1* genes and the accumula-

Table 1. Reaction of two wheat cultivars with different allelic states of the *Snn3-B1* locus to inoculation with *S. nodorum* SnB (Tox3$^+$) and Sn4VD (Tox3$^-$) isolates

Kazakhstanskaya 10			Omskaya 35		
Cultivar/isolate	Damage area, %	Group*	Cultivar/isolate	Damage area, %	Group*
Kaz10/SnB	80.0 ± 3.0	SS	Om35/SnB	10.9 ± 2.5	R
Kaz10/SnB+ET**	94.9 ± 2.3	SS	Om35/SnB+ET	57.2 ± 2.6	S
Kaz10/SnB+AVG**	18.6 ± 2.2	M	Om35/SnB+AVG	9.1 ± 1.9	R
Kaz10/Sn4VD	8.4 ± 2.2	R	Om35/Sn4VD	1.6 ± 0.5	RR
Kaz10/Sn4VD+ET	8.1 ± 1.8	R	Om35/Sn4VD+ET	1.9 ± 1.1	RR
Kaz10/Sn4VD+AVG	6.5 ± 2.6	R	Om35/Sn4VD+AVG	1.6 ± 0.5	RR

* RR (0–5 %) – cultivars with very high and high resistance; R (5–15 %) – resistant cultivars; M (15–25 %) – slightly susceptible cultivars; S (25–65 %) – susceptible cultivars; SS (65–100 %) – cultivars with very high and high susceptibility.

** Plants were treated with either ethephon (ET) or ethylene biosynthesis inhibitor (AVG) 24 h before inoculation with *S. nodorum*.

Table 2. Transcriptional analysis of ethylene biosynthesis and signaling pathway genes measured in Kazakhstanskaya 10 and Omskaya 35 wheat cultivars contrasting in SNB resistance 24 h after inoculation with *S. nodorum* isolates

Variant of treatment	Gene			
	TaACS	*TaACO*	*TaEIL1*	*TaERF1*
		Kazakhstanskaya 10		
Kaz10	100	100	100	100
Kaz10/SnB	311 ± 60	237 ± 30	1400 ± 200	356 ± 70
Kaz10/SnB+AVG	143 ± 23	77 ± 3	389 ± 0	100 ± 20
Kaz10/SnB+ET	350 ± 34	223 ± 10	1810 ± 270	450 ± 34
Kaz10/Sn4VD	128 ± 28	110 ± 10	322 ± 10	102 ± 10
Kaz10/Sn4VD+AVG	117 ± 9	63 ± 15	390 ± 60	146 ± 10
Kaz10/Sn4VD+ET	121 ± 10	65 ± 6	375 ± 20	107 ± 7
		Omskaya 35		
Om35	100	100	100	100
Om35/SnB	86 ± 10	47 ± 10	77 ± 6	92 ± 10
Om35/SnB+ET	169 ± 8	190 ± 15	325 ± 54	262 ± 35
Om35/SnB+AVG	102 ± 20	54 ± 10	115 ± 20	178 ± 40
Om35/Sn4VD	97 ± 10	143 ± 10	133 ± 20	54 ± 2
Om35/Sn4VD+ET	125 ± 6	95 ± 7	156 ± 10	199 ± 2
Om35/Sn4VD+AVG	108 ± 20	206 ± 20	129 ± 20	134 ± 5

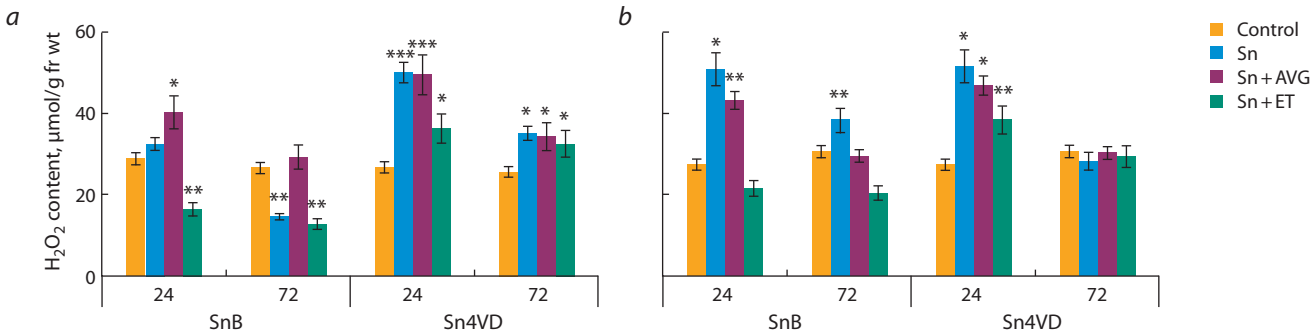

Fig. 2. Hydrogen peroxide contents in leaves of cultivars Kazakhstanskaya 10 (*a*) and Omskaya 35 (*b*) 24 and 72 h after inoculation with *S. nodorum* SnB (Tox3$^+$) and Sn4VD (Tox3$^-$) isolates.

Control – uninfected plants; Sn – infection with *S. nodorum* isolates; Sn + AVG – infection + AVG treatment; Sn + ET – infection + ET treatment. Asterisks show statistically significant differences from the control group, and different numbers of asterisks indicate differences between the variants significant at $p \leq 0.05$.

tion of *TaEIL1* mRNA was low as compared to control plants (see Table 2).

Redox state of infected plants. We studied the components of the pro-/antioxidant system in order to elucidate the role of SnTox3 in the regulation of redox metabolism in the host plant via the biosynthesis and signaling pathway of ethylene in suppressing defense reactions in infected plants. The compatibility reactions in susceptible plants (Kaz10/SnB) and in plants treated with ET (Kaz10/SnB+ET, Om35/SnB+ET) were characterized by a decrease in H_2O_2 content (Fig. 2, *a*, *b*) due to elevated activity of catalase (CAT) (Fig. 3, *e*, *f*), reduced peroxidase (PO) (see Fig. 3, *a*, *b*) and oxalate oxidase (OXO) (see Fig. 3, c, *d*) activities, and lack of transcript accumulation of the genes encoding oxidoreductases NADPH oxidase (*TaRbohF*), SOD (*TaSod*), and PO (*TaPrx*) (Table 3). However, in Tox3-insensitive plants (Om35/SnB + ET cultivar/isolate combination), the suppression reaction of oxidative burst was less pronounced than in other combinations leading to a compatibility reaction (see Fig. 2, 3, Table 3).

The incompatibility reactions in the Om35/SnB, Om35/Sn4VD, Kaz10/Sn4VD cultivar/isolate combinations and in AVG-treated plants from all combinations were characterized by an increase in H_2O_2 generation (see Fig. 2, *a*, *b*) due to a decrease or absence of increase in CAT activity, a drastic increase in the activity of PO and OXO (see Fig. 3), and the accumulation of *TaRbohF*, *TaSod*, and *TaPrx* transcripts at the early stage of infection (24 h) (see Table 3), which led to the development of a hypersensitive-type response and arrest

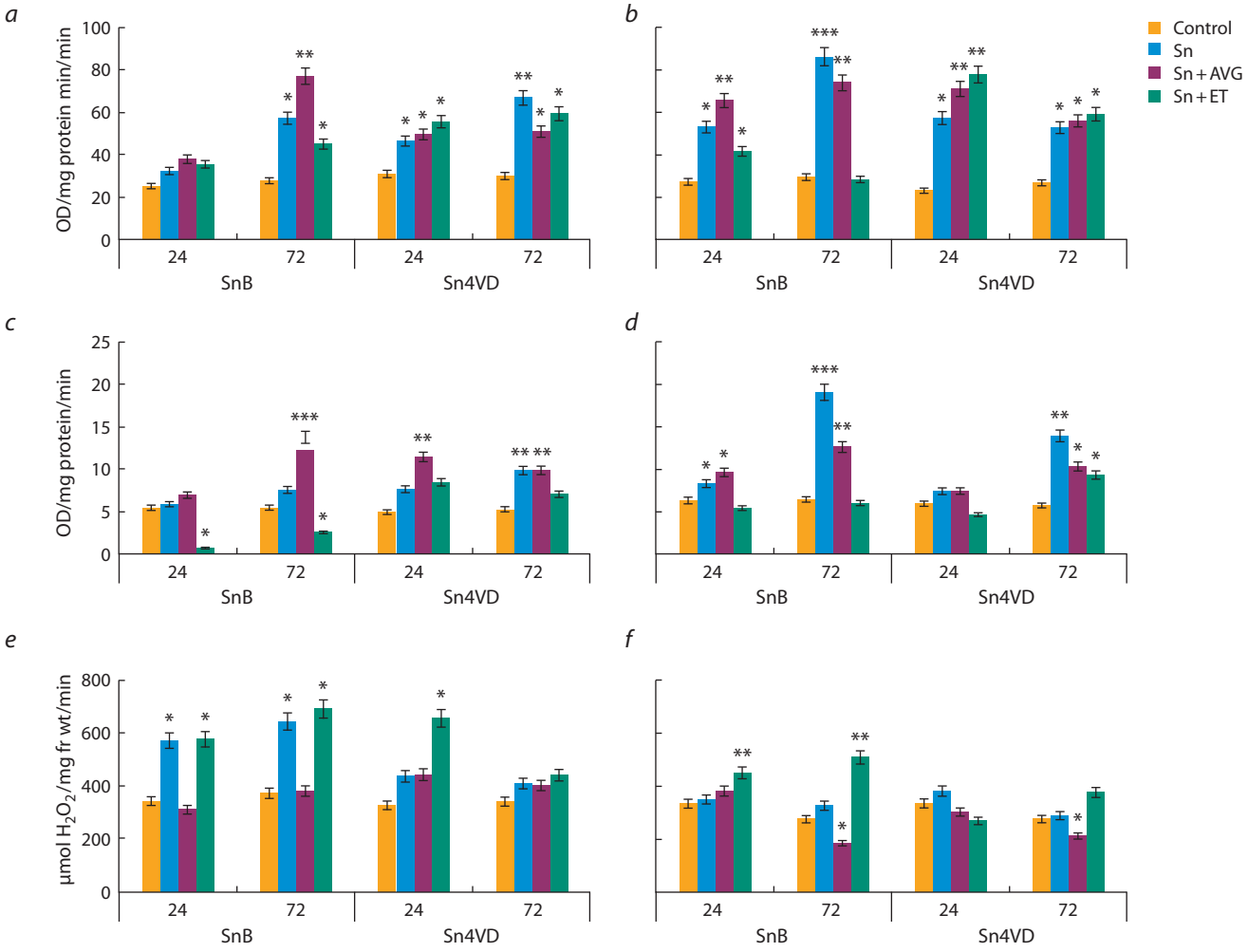

Fig. 3. Enzyme activities in leaves 24 and 72 h after inoculation with *S. nodorum* SnB (Tox3$^+$) and Sn4VD (Tox3$^-$) isolates: (*a*, *b*) peroxidase; (*c*, *d*) oxalate oxidase; (*e*, *f*) catalase; (*a*, *c*, *e*) cv. Kazakhstanskaya 10; (*b*, *d*, *f*) Omskaya 35.

Designations follow Fig. 2.

Table 3. The results of transcriptional analysis of oxidoreductase genes registered 24 h after inoculation with *S. nodorum* isolates in wheat cultivars Kazakhstanskaya 10 and Omskya 35 characterized by different levels of resistance to the disease

Variant of treatment*	Pathogen isolate					
	SnB (Tox3$^+$)			Sn4VD (Tox3$^-$)		
	Gene					
	TaRboh	*TaSod*	*TaPrx*	*TaRboh*	*TaSod*	*TaPrx*
	Kazakhstanskaya 10					
Control	100	100	100	100	100	100
Sn	78 ± 3	88 ± 10	71 ± 3	160 ± 21	144 ± 8	223 ± 20
Sn + AVG	111 ± 20	169 ± 20	163 ± 30	145 ± 40	110 ± 3	210 ± 50
Sn + ET	15 ± 1	81 ± 7	104 ± 20	152 ± 37	114 ± 2	251 ± 50
	Omskya 35					
Control	100	100	100	100	100	100
Sn	126 ± 10	264 ± 20	365 ± 26	160 ± 20	196 ± 9	264 ± 20
Sn + AVG	136 ± 20	242 ± 40	311 ± 31	129 ± 10	178 ± 18	170 ± 20
Sn + ET	83 ± 10	118 ± 9	136 ± 15	132 ± 30	159 ± 10	181 ± 34

* Treatment options are designated as in Fig. 2.

of pathogen growth (see Table 1). Treatment with ET did not affect the nature of the response to inoculation with the Sn4VD isolate in either Tox3-sensitive or insensitive plants (see Fig. 2, 3, Table 3).

Discussion

At present, it is known that *S. nodorum* produces eight NEs associated with the virulence of pathogen isolates (Phan et al., 2016; Shi et al., 2016). The main NEs are SnToxA, SnTox1, SnTox3 toxins, which are considered to be the key factors of the virulence of pathogen strains and isolates, as shown in experiments with mutant *S. nodorum* strains and different wheat lines sensitive and insensitive to these effectors (Phan et al., 2016; Shi et al., 2016).

In our study, high transcriptional activity of the *SnTox3* gene was found in the virulent isolate SnB (Fig. 1, *b*). The avirulent isolate Sn4VD did not express this gene (see Fig. 1, *b*), which may be indicative of SnTox3 inactivation in this isolate (Tan, Oliver, 2017). Here we studied two cultivars of soft spring wheat contrasting in resistance to *S. nodorum* and differing in the allelic composition of the *Snn3-B1* locus (see Fig. 1, *c*). This difference is presumed to be associated with their sensitivity and insensitivity to NE SnTox3 (Shi et al., 2016). In the referred study, two BG220 and Sumai3 wheat lines carrying different alleles of the *Snn3-B1* locus showed different degrees of sensitivity to NE SnTox3. Despite the fact that the *Snn3-B1* null allele was not detected in cv. Om35, this cultivar was insensitive or weakly sensitive to SnTox3. This may indicate a large deletion in the locus between the *Xcfd20* and *Xgwm234* markers, which is in agreement with the literature data (Shi et al., 2016). Thus, out of 17 Sumai3 mutants insensitive to SnTox3, the *Snn3-B1* null allele was detected only in 5 of them: two lines harbored null alleles for three microsatellite markers, *Xgwm234*, *Xmag705* and *Xcfb306*, and three lines had a null allele for one microsatellite marker *Xcfb306* (Shi et al., 2016). Thus, four different cultivar/isolate combinations were selected with two isolates of *S. nodorum* SnB ($Tox3^+$) and Sn4VD ($Tox3^-$) and two cultivars of spring common wheat with different genotypes to study the role of SnTox3 in the development of infection.

On the one hand, the main function of NE SnTox3 is the formation of lesion zones on the wheat leaves of sensitive genotypes by hijacking host's nonspecific signaling defense pathways and manipulating them for pathogen growth and propagation (Winterberg et al., 2014). Furthermore, SnTox3 has been shown to increase ethylene synthesis in infected plants (Winterberg et al., 2014).

To elucidate the role of SnTox3 in ethylene biosynthesis and signaling pathways, we treated part of wheat plants of two cultivars, Kaz10 and Om35, with ethylene chemical precursor ET and ethylene biosynthesis inhibitor AVG. The results showed that both the elevated susceptibility of the ET-treated plants to *S. nodorum* and elevated resistance of the AVG-treated plants depended on the pathogen genotype: $Tox3^+$ in SnB or $Tox3^-$ in Sn4VD (see Table 1). This observation suggests that NE SnTox3 acts as a virulence factor and affects the plant defensive system by regulating ethylene biosynthesis and signaling pathways. It has been shown that ethylene production by some pathogens is closely associated with their virulence (Ma K.-W., Ma W., 2016). For instance, the XopD effector of the pathogenic bacterium *Xanthomonas euvesicatoria* manipulates the ethylene signaling pathway, affecting transcription factor ERF4 (Ma K.-W., Ma W., 2016), and the necrotrophic fungus *Cochliobolus miyabeanus* induces the ethylene signaling pathway in rice to produce and secrete ethylene as an effector to accelerate infection (Shen et al., 2018).

In our work, the analysis of the transcriptional activity of the genes controlling the biosynthesis and signaling pathway of ethylene also showed that the activation of genes involved in this pathway in infected plants depended on the pathogen isolate genotype and the sensitivity of the wheat genotype to NE SnTox3 (see Table 2). These results suggest that the biosynthesis and signaling pathway of ethylene are induced in the gene-for-gene interaction between *Snn3-B1* and SnTox3.

Interestingly, in SnTox3 insensitive plants (Om35/SnB + ET cultivar/isolate combination), ET treatment increased plant sensitivity to this NE (see Table 3), suggesting that such a reaction could result from a mutation in genes regulated by the *Snn3-B1*-SnTox3 interaction (Shi et al., 2016). However, the activation of genes for ethylene biosynthesis and the signaling pathway in the Om35/SnB + ET combination was weaker than in case of the compatible interaction in plants sensitive to SnTox3 (Kaz10/SnB, Kaz10/SnB + ET) (see Table 2). This suggests that resistant plants possess a mechanism for efficient suppression of ethylene biosynthesis and signaling pathway to induce defense responses that are inhibited by ethylene. For example, ethylene inhibited salicylic acid (SA) biosynthesis and suppressed the expression of the SA-mediated signaling pathway marker genes *PR-1* and *PR-2* in *Arabidopsis* plants infected with *Pseudomonas syringae* (Chen et al., 2009), as well as in wheat plants infected with *S. nodorum* (Veselova et al., 2016). Silicon blocked ethylene production by the pathogen *C. miyabeanus*, which improved the resistance of rice plants (Shen et al., 2018). Thus, the obtained results prove the influence of SnTox3 on the biosynthesis and signaling pathway of ethylene in the course of *Snn3-B1*-SnTox3 interaction according to the gene-for-gene type with ultimate suppression of the defense reactions of wheat plants to facilitate colonization.

Our previous studies showed that ethylene provided comfortable conditions for the penetration and development of *S. nodorum* in wheat plant tissues at the initial stage of infection due to the regulation of redox metabolism and reduction of H_2O_2 generation (Veselova et al., 2016, 2018). On the contrary, the accumulation of ROS in wheat plants at the initial stage of infection with the pathogen *S. nodorum* determined the resistance of the cultivar, inducing the expression of the genes encoding pathogenicity-related proteins (Veselova et al., 2016, 2018). The change in the redox state of infected wheat plants in our experiments completely depended on the activation or inhibition of the biosynthesis and signaling pathway of ethylene, and this effect was due to the *Snn3-B1*-SnTox3 interaction of the gene-for-gene type (see Fig. 2, 3). Our results demonstrate that ethylene suppresses H_2O_2 accumulation in plants sensitive to SnTox3 via increasing CAT activity, reducing PO and OXO activities, and lowering the transcript contents of genes encoding NADPH oxidase and SOD, in consistency with literature data (Golemiec et al., 2014; Ma et al., 2017) and our earlier results (Veselova et al., 2018).

Formerly, it was shown that NE SnTox3 regulated genes involved in redox metabolism and the formation of necrosis (Winterberg et al., 2014), but the mechanisms effecting the influence of SnTox3 on ROS generation remain obscure. It is known from the literature that specific effectors of pathogens can induce hypersensitive response in plant cells and suppress the oxidative burst in plants during the infection process in various ways (Jwa, Hwang, 2017). For example, two cytoplasmic effectors of *Phytophthora sojae* interact with catalases to regulate H_2O_2 concentration. The Pep1 effector of *U. maydis* interacts with POX12 maize peroxidase *in vivo* and suppresses early immune responses in maize (Hemetsberger et al., 2012).

Conclusion

The results of our work suggest that the pathogen effector SnTox3 influences biosynthesis and signaling pathway of ethylene in order to regulate the redox metabolism of infected wheat plants in the way promoting successful colonization of the host at initial stages of infection, which subsequently gives rise to extensive damage lesions due to fast pathogen reproduction.

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