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EXPRESSION OF THE *ToxA* AND *PtrPf2* GENES OF THE PHYTOPATHOGENIC FUNGUS *PYRENOPHORA TRITICI-REPENTIS* AT THE BEGINNING OF THE INFECTION PROCESS

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❀ **Background.** *Pyrenophora tritici-repentis* causing a tan spot of wheat produces host-specific toxins. **Materials and methods.** Two *P. tritici-repentis* isolates with different ability to cause necrosis on the leaves of wheat cultivar Glenlea (nec$^+$ and nec$^-$) and with different expression level of *ToxA* and *PtrPf2* (factor transcription gene) *in vitro* were used for analysis. *ToxA* gene expression in *P. tritici-repentis* isolates *in planta* was characterized using quantitative PCR. **Results.** The expression of the *ToxA* gene in *P. tritici-repentis* ToxA$^+$ isolates significantly increased when infected the wheat leaves compared to ToxA expression results obtained *in vitro*. The levels of *ToxA* expression in both isolates differed significantly after 24, 48 and 96 h after inoculation, however, the dynamics of the trait change over time were similar. However, the highest *ToxA* expression in the virulent (nec$^+$) isolate in contrast with the avirulent (nec$^-$) isolate was observed at a point of 48 h. Whereas the expression of regulating transcription factor *PtrPf2 in planta* differed imperceptibly from expression *in vitro* throughout the observation period. **Conclusion.** Obviously, the role of the fungal transcription factor in regulating the effector gene expression weakens *in planta*, and other mechanisms regulating the expression of pathogen genes at the biotrophic stage of the disease develop.

❀ **Keywords:** *Pyrenophora tritici-repentis*; tan spot of wheat; gene of sensitivity to Ptr ToxA *Tsn1*, effector gene *ToxA*; gene of transcription factor *PtrPf2*.

ЭКСПРЕССИЯ ГЕНОВ *ToxA* И *PtrPf2* ФИТОПАТОГЕННОГО ГРИБА *PYRENOPHORA TRITICI-REPENTIS* В НАЧАЛЕ ИНФЕКЦИОННОГО ПРОЦЕССА

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❀ Для анализа уровня экспрессии гена *ToxA*, кодирующего синтез белкового некроз-индуцирующего токсина Ptr ToxA, и гена фактора транскрипции *PtrPf2* фитопатогенного гриба *Pyrenophora tritici-repentis in planta* были выбраны два изолята, различающихся способностью вызывать некроз на листьях восприимчивого сорта пшеницы Glenlea (nec$^+$ и nec$^-$) и уровнем экспрессии этих генов *in vitro*. Показано, что ген некротрофного эффектора *ToxA* дифференциально экспрессируется у изолятов *P. tritici-repentis* в разные временные периоды после инокуляции сорта Glenlea, имеющего доминантную аллель гена *Tsn1*, которая контролирует чувствительность к некроз-индуцирующему токсину Ptr ToxA. Уровень экспрессии *ToxA* резко увеличивается в процессе заражения пшеницы изолятами *P. tritici-repentis* ToxA$^+$ по сравнению с результатами, ранее полученными *in vitro*. Причем, у вирулентного (nec$^+$) изолята наблюдали более сильную экспрессию гена через 48 ч после инокуляции по сравнению с авирулентным (nec$^-$) изолятом. Уровни экспрессии *ToxA* в образцах существенно различались через 24, 48 и 96 ч после инокуляции, однако динамика изменения признака у обоих изолятов во времени была одинаковой. Другой характер изменчивости экспрессии гена наблюдали для фактора транскрипции *PtrPf2*, регулирующего экспрессию *ToxA*: экспрессия этого гена в растении мало отличалась от экспрессии в культуре, два изолята лишь незначительно различались в точке максимальной экспрессии *ToxA*, то есть через 48 ч. Очевидно, роль грибного фактора транскрипции в регуляции экспрессии гена эффектора в растении незначительна, и в силу вступают другие механизмы регуляции экспрессии генов патогена на биотрофной стадии развития болезни.

❀ **Ключевые слова:** *Pyrenophora tritici-repentis*; желтая пятнистость пшеницы; ген чувствительности к токсину Ptr ToxA *Tsn1*, ген эффектор *ToxA*; ген фактора транскрипции *PtrPf2*.

INTRODUCTION

Tan spot of wheat is a disease that appeared in the 1940s and since then has covered almost the entire global territory of wheat cultivation. The harmfulness of disease caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechsler is associated with its ability to produce host-specific toxins that induce leaf necrosis and chlorosis on susceptible wheat cultivars. The fungus *P. tritici-repentis* is known to produce the host-specific phytotoxins Ptr ToxA and Ptr ToxB, which are proteins that induce necrosis and chlorosis on susceptible wheat varieties and are considered the main pathogenic factors. Another host-specific phytotoxin is a low-molecular-weight Ptr ToxC, non-proteinaceous compound [1, 2]. The Ptr ToxA and Ptr ToxB toxins are encoded by the *ToxA* and *ToxB* genes, respectivel. The gene-specific primers have been constructed for detection of their presence in the fungal genome. Until now, the race structure of pathogen populations was determined by infecting differentiator varieties which distinguished 8 races by the combination of three phytotoxins in fungal isolates [3, 4]. However, the number of races may be greater due to the discovery of new necrosis-inducing toxins [5–9]; therefore, the phenotypic assessment of isolates assigned to a particular race may not coincide with their genetic characteristics. For example, it was revealed that in Russian populations there are isolates with *ToxA* gene ($ToxA^+$) but do not induce necrosis in susceptible varieties (nec^-). In this regard, it was hypothesized that this phenomenon is due to the absence or low level of the *ToxA* gene expression [10].

P. tritici-repentis is known to produce the Ptr ToxA toxin, which induces necrosis only on the leaves of wheat varieties with a dominant allele of the *Tsn1* gene in the genome, which controls sensitivity to the Ptr ToxA toxin [11]. The *Tsn1* gene is structurally similar to plant R-genes of resistance to disease; it includes the S/TPK (serine/threonine specific protein kinase) and NBS-LRR (nucleotide binding site and leucine-rich repeat) domains [12].

The relationship between the expression of *ToxA* in *P. tritici-repentis* isolates in culture and their ability to induce necrosis in susceptible cultivars, as well as the mechanisms of regulation of the expression of this effector gene are fragmentary. The *ToxA* gene in the genome of *P. tritici-repentis* has the nature of a foreign element transferred from another fungal pathogen *Parastagonospora nodorum* (Berk.) Quaedvl., Verkley & Crous, which causes a common disease – *Septoria nodorum* blotch [13]. In 2018, the first report of the detection of a transcription factor gene *PtrPf2* in *P. tritici-repentis* isolates, which encodes a product regulates the expression of the *ToxA* gene, appeared. This gene turned out to be an ortholog of the *PnPf2* gene, a transcription factor for the *SnToxA* and *SnTox3* effectors of *Parastagonospora nodorum* [14].

Previously, we analyzed two groups of *P. tritici-repentis* isolates from different populations of the pathogen based on constitutive expression of the *ToxA* effector gene and the *PtrPf2* transcription factor gene. For the first time, the intra- and interpopulation variability of the pathogen was demonstrated in terms of the expression of *ToxA* and *PtrPf2 in vitro* [15].

The aim of study was to determine the expression of the *ToxA* and *PtrPf2* genes in two isolates of the *P. tritici-repentis* pathogen in the tissues of a susceptible wheat cultivar with a dominant *Tsn1* gene allele at the early stages of fungal infection.

MATERIALS AND METHODS

To analyze the expression of fungal genes during wheat infection, we selected two monoconidial isolates from the South Kazakhstan population (Almaty, 2018), Ptr1 and Ptr10 with the *ToxA* effector gene and differing in level of expression *in vitro*, which was estimated previously [15].

Cultivation of *P. tritici-repentis* strains, induction of conidia formation, and inoculation of wheat plants were performed according to the described methods [16]. The virulence of the isolates was assessed by their ability to induce necrosis on the leaves of seedlings of the susceptible wheat cultivar Glenlea with a dominant *Tsn1* allele, using a five-point scale [17]. The phytopathological test was performed at least twice.

A modified technique was used to study the expression of *P. tritici-repentis* genes in the tissues of a wheat plant during disease development [18]. For this purpose, the leaf fragments of seven-day wheat seedlings of the cultivar Glenlea were placed in a Petri dish on the surface of a 2% agar medium containing 70 mg/L benzimidazole and were

fixed by agar blocks. A drop of 10 μl of a conidia suspension with a concentration of 3500 conidia/ml was applied to each leaf. Three Petri dishes were prepared with 10 leaf fragments and were simultaneously infected with a conidia suspension of each isolate, and then were incubated in chamber at 22 °C with 12 h light photoperiod (1500 lm). The samples for subsequent analysis of gene transcriptional activity were collected 24, 48, and 96 hours after inoculation. Ten to fifteen 3 × 6 mm segments of plant tissue were cut out from the point of pathogen inoculation, placed in a tube, and immediately frozen at −20 °C for subsequent RNA isolation.

RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Germany). cDNA was synthesized by RT-PCR on a total RNA template (1−2 μg) using an MMLV RT kit (Evrogen, Russia).

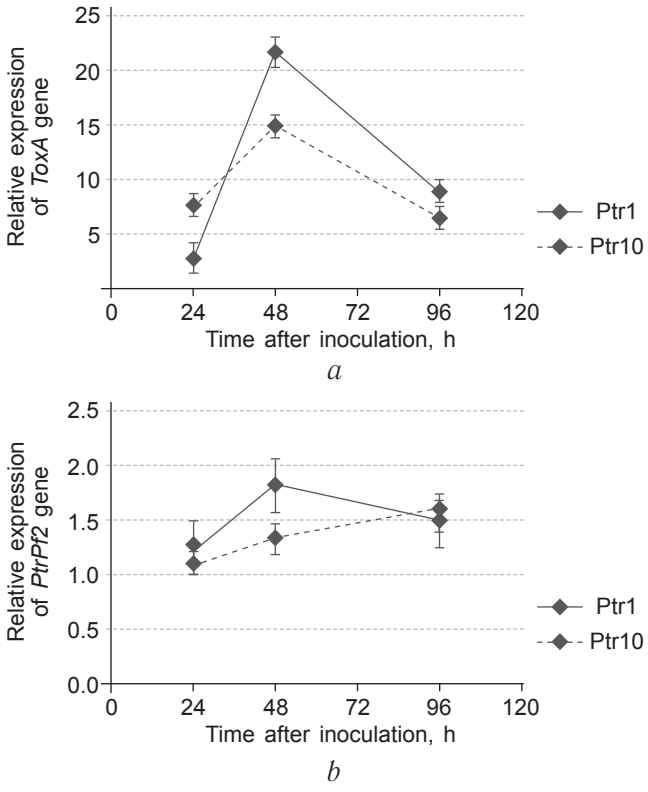
The expression of the *ToxA* and *PtrPf2* genes in *P. tritici-repentis* isolates in plant tissues at different time intervals after infection was assessed using quantitative PCR (qPCR) with gene-specific primers [14]. The *Act1* gene was used as a reference control. qPCR reactions were performed in 20 μl containing 4 μl of 5 × qPCRmix-HS SYBR master mix (Evrogen, Russia), 500 nM of each primer, and 2 μl of cDNA solution using the following amplification protocol: 50 °C for 2 min; 95 °C for 15 min; [95 °C for 15 s; 62 °C for 60 s] × 40 on a CFX96 RealTime System thermal cycler (Bio-Rad, USA) threefold. Primary data were processed using Bio-Rad CFX Manager 1.6 software. The relative gene expression was calculated using the formula $R = 2^{-\Delta\Delta Ct}$ [19].

RESULTS

As a result of the inoculation of the susceptible wheat cultivar Glenlea with two ToxA$^+$ *P. tritici-repentis* isolates, it was found that the Ptr1 isolate caused a necrotic reaction with a score of 3−4 points and is thus considered virulent (nec$^+$), while the Ptr10 isolate affected wheat with a 1−2-point necrotic reaction and is thus considered low virulent/avirulent (nec$^-$). According to our data, the relative expression of *ToxA* and *PtrPf2* genes *in vitro* was 0.67 ± 0.01 and 0.90 ± 0.03 respectively for the Ptr1 isolate, and 0.92 ± 0.1 and 1.00 ± 0.05 for Ptr10 [15].

As a result of the experiment performed according to the methods described previously on whole plants, in the total cDNA extracted from non-inoculated plants the target genes *ToxA* and *PtrPf2* were not amplified, while these genes were detected in the infected plants. The Figure depicts graphically the expression of *ToxA* and *PtrPf2* genes *in planta* over 4 days.

As a result of penetration of the fungus into the plant tissue, the level of *ToxA* gene expression increased rapidly in comparison with constitutive expression, more than 4-fold in the Ptr1 isolate and 7-fold in the Ptr10 isolate (24 hours after inoculation). 48 hours after inoculation, the maximum relative expression level of *ToxA in planta* was noted, and after 96 hours, there was a decrease in relative expression of *ToxA* gene (see Figure, *a*). At the same time, the relative expression of the *PtrPf2* transcription factor gene did not change 24 hours after inoculation and only increased slightly after 48 hours compared with expression *in vitro* and remained practically unchanged during 4 days of monitoring *in planta* (see Figure, *b*).



Relative expression of *ToxA* (*a*) and *PtrPf2* (*b*) genes in *Pyrenophora tritici-repentis* Ptr1 and Ptr10 isolates with *Act1* as the reference gene in the infected leaves of wheat cultivar Glenlea at different periods after inoculation

DISCUSSION

The relative expression of the *ToxA* and *PtrPf2* genes in individual isolates of phytopathogenic fungi are an important characteristic of the pathogenic properties and can be used to analyze the interaction of genes in pathosystems. The presence or absence of the expression of *ToxA* gene, which is responsible for the synthesis of the necrosis-inducing protein toxin Ptr ToxA, can be detected from the phenotypic manifestation of the reaction of common wheat plants with the dominant *Tsn1* allele of the susceptibility to infection with *P. tritici-repentis* ToxA$^+$ isolates. However, many researchers have registered cases of lack of necrosis induction by ToxA$^+$ isolates [5, 6, 13, 20–23]. Attempts were made to explain this observation in the context of gene mutation, but the *ToxA* nucleotide sequence in many ToxA$^+$nec$^-$isolates of *P. tritici-repentis* turned out to be extremely conservative, which is typical for a foreign genetic element that has recently entered the genome of the fungus [13]. The structure of the *ToxA* gene, recently found in the genomes of other pathogens of wheat and barley *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur and *P. teres* Drechsler, is also characterized by low variability [22, 24, 25].

The expression of the effector genes in phytopathogenic fungi is known to be determined by a network of signaling genes, including transcription factors, which have evolved under specific environmental conditions. There is still insufficient information about the regulation of genes encoding necrotrophic effectors. The 37 superfamilies of DNA-binding domains known for all organisms and 12 superfamilies have been found in fungi [26, 27]. Among them, three types of proteins were found to be specific for the fungal kingdom, of which the zinc finger transcription factor encoded by the *PnPf2* gene was found in *Parastagonospora nodorum*, and its ortholog, the *PtrPf2* gene, was revealed in *P. tritici-repentis*. These are the transcription factors *PnPf2* and *PtrPf2* of the effector genes *SnToxA* and *PtrToxA* of two fungal pathogens *Parastagonospora nodorum* and *P. tritici-repentis*, respectively [14].

It has been demonstrated that *ToxA* expression in *P. tritici-repentis* isolates increases significantly during plant infection at the initial stages and is under the control of the *PtrPf2* transcription factor gene [14]. The authors noted maximum expression of *ToxA* on day 3 after infection of the susceptible cultivar, while *PtrPf2* was expressed uniformly throughout the monitoring period (from day 3 to day 10) [14]. Our results showed a similar picture of maximum expression of the *ToxA* gene 48 hours after plant infection and uniform expression of *PtrPf2* within 4 days of observation. Moreover, the *ToxA* expression in the virulent isolate was higher than in the low virulent one *in planta* at all measurement time points, while the *PtrPf2* expression in both isolates in the plant did not show significant differences. Thus two *P. tritici-repentis* isolates differed significantly from each other in the relative expression of the *ToxA* gene in the tissues of the susceptible wheat cultivar at different time points, although the dynamic of the variability of this trait between them were similar. Inter-strain differences in the expression of the effector gene associated with the manifestation of the disease were also found in other phytopathogenic fungi. For example, in two isolates of *Stagonospora nodorum* (Berk.) E. Castell. & Germano, differences in the expression level of the *SnToxA* gene were revealed 26 hours after inoculation of a susceptible wheat cultivar by more than two times, and higher expression levels were associated with an increase in the disease in the wheat–*S. nodorum* pathosystem [28]. The influence of the expression of necrotrophic effectors on the disease manifestation was also revealed in other works [28, 29]. Three necrotrophic effectors, *SnToxA, SnTox1*, and *SnTox3,* have been studied well in the wheat–*Parastagonospora nodorum* pathosystem, which can influence each other through expression-suppressing epistasis. For example, the expression of the *SnTox3* gene can be suppressed by the *SnTox1* gene [29]. The effect of the *Tsn1–ToxA* interaction on disease manifestation can vary greatly depending on the genotype of the wheat cultivar with *Tsn1* gene. In particular, a significant role of the Ptr ToxA toxin was not revealed on durum wheat cultivars, and, conversely, a strong effect of the necrotrophic effector *Parastagonospora nodorum SnToxA* was noted upon inoculation of Tsn1$^+$ cultivars [30].

The role of gene expression as a major cause of variability in virulence, in addition to differences in the gene nucleotide sequence, has been revealed for isolates *Zymoseptoria tritici* (Roberge ex Desm.) Quaedvl. & Crous [31].

Our results and the above examples from works on the analysis of gene expression of fungal effectors in plants confirm the idea proposed by many authors that the main mechanism influencing the dynamics of racial composition in populations of phytopathogenic fungi probably do not involve a change in the frequencies of alleles of genes related to virulence but rather to variability in the regulation of gene expression of effectors, depending both on the genotype of the host plant and on various environmental conditions.

CONCLUSION

Expression of the *ToxA* gene increases dramatically during infection of the susceptible wheat cultivar Glenlea with isolates of *P. tritici-repentis* ToxA$^+$ compared with expression *in vitro*. *P. tritici-repentis* isolates are characterized by differential expression of *ToxA* in the plant, as the levels of *ToxA* expression in both isolates differed significantly 24, 48, and 96 hours after inoculation; however, the dynamics of the trait change over time was the same. The virulent isolate showed stronger *ToxA* expression 48 hours after inoculation compared with the avirulent isolate.

Another pattern of gene expression variability was noted for the transcription factor *PtrPf2* which regulates *ToxA* expression, as the expression of this gene in the plant did not differ much from that in the culture; the two isolates differed only slightly at the point of maximum *ToxA* expression, that is, 48 hours after inoculation. Thus, the hypothesis about the existence of a relationship between the level of *PtrPf2* expression *in vitro* and the ability of isolates to induce necrosis on leaves of a susceptible cultivar [15] was not justified. It is obvious that the role of fungal transcription factors in the regulation of the expression of effector genes *in planta* is insignificant, and other mechanisms of regulation of the expression of pathogen genes at the biotrophic stage of the disease come into force.

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