Changes in benzoxazinoid contents and the expression of the associated genes in rye (Secale cereale L.) due to brown rust and the inoculation procedure

Magdalena Święcicka¹, Marta Dmochowska-Boguta², Waclaw Orczyk², Agnieszka Grądzielewska³, Anna Stochmal⁴, Mariusz Kowalczyk⁴, Leszek Bolibok⁵, Monika Rakoczy-Trojanowska⁶,*

¹ Department of Plant Genetics, Breeding and Biotechnology, Institute of Biology, Warsaw University of Life Sciences (SGGW), Warsaw, Poland, ² Department of Genetic Engineering, Plant Breeding and Acclimatization Institute–National Research Institute, Radzików, Błonie, Poland, ³ Department of Horticultural Plant Genetics and Breeding, Institute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences in Lublin, Lublin, Poland, ⁴ Department of Biochemistry and Crop Quality, Institute of Soil Science and Plant Cultivation—State Research Institute, Pulawy, Poland, ⁵ Department of Forest Silviculture, Institute of Forest Sciences, Warsaw University of Life Sciences (SGGW), Warsaw, Poland

* monika_rakoczy_trojanowska@sggw.edu.pl

Abstract

Benzoxazinoids (BXs) are secondary metabolites with diverse functions, but are primarily involved in protecting plants, mainly from the family Poaceae, against insects and fungal pathogens. Rye is a cereal crop that is highly resistant to biotic stresses. However, its susceptibility to brown rust caused by Puccinia recondita f. sp. secalis (Prs) is still a major problem affecting its commercial production. Additionally, the genetic and metabolic factors related to this disease remain poorly characterized. In this study, we investigated whether and to what extent the brown rust infection and the inoculation procedure affect the contents of specific BXs (HBOA, GDIBOA, DIBOA, GDIMBOA, DIMBOA, and MBOA) and the expression of genes related to BX (ScBx1–5, ScIgl, and Scglu). We revealed that treatments with water and a urediniospore suspension usually downregulate gene expression levels. Moreover, HBOA and DIBOA contents decreased, whereas the contents of the remaining metabolites increased. Specifically, the MBOA content increased more after the mock treatment than after the Prs treatment, whereas the increase in GDIBOA and GDIMBOA levels was usually due to the Prs infection, especially at two of the most critical time-points, 17 and 24 h post-treatment. Therefore, GDIBOA and GDIMBOA are glucosides that are important components of rye defence responses to brown rust. Furthermore, along with MBOA, they protect rye against the stress associated with the inoculation procedure used in this study.
Introduction

Benzoxazinoids (BXs) are secondary metabolites synthesized mainly by species belonging to the family Poaceae, including rye (Secale cereale L.). Several studies have confirmed that BX biosynthesis comprises several steps [1–6]. First, indole-3-glycerol phosphate is converted to indole, which is then transformed to indolin-1-one. Next, three monooxidations result in the synthesis of 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). Other reactions include the glycosylation of DIBOA to produce 2-O-β-glucoside (GDIBOA) and O-methylation to generate 4,7-dimethoxy-2-(3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl)oxy)-3,4-dihydro-2H-1,4-benzoxazin-3-one glucoside (GHDMBOA). Hydroxylations convert GDIBOA and GDIMBOA to DIBOA and DIMBOA, respectively.

Several genes controlling BX biosynthesis have been isolated and sequenced. The species with the most characterized Bx genes is maize [2, 4–6]. In rye, nine genes, ScBx1–ScBx7, ScGT, and Scglu reportedly control reactions corresponding to most of the BX biosynthesis reactions determined in maize [7–12]. Recently, Rakoczy-Trojanowska et al. [13] and Wlazło et al. [14] proved that another gene, ScIgl, has the same function as ScBx1 in late developmental stages.

Benzoxazinoids are primarily important components of plant defence strategies against biotic and abiotic stresses. They may also help regulate flowering time, auxin metabolism, iron uptake, and aluminium tolerance [15] as well as control root–microbe interactions via a global regulatory function related to root secondary metabolism [16]. Although the role of BXs in defence against insects has been broadly studied and well documented, their association with disease resistance is not obvious [1–3]. Additionally, the correlation between BX content and composition and disease resistance may depend on the infection site and pathogen characteristics. For example, in the case of northern corn leaf blight caused by the hemi-biotrophic fungal pathogen Helminthosporium turcicum, the BX concentration of the midstalk leaves is positively correlated with resistance and negatively with symptom development [17]. Similarly, in wheat seedlings, reactions to stem rust and head blight are negatively correlated with BX contents [18]. In contrast, maize responses to anthracnose caused by Colletotrichum graminicola are not correlated with the BX concentration [19]. As in the case of the passive defence mechanism, the induced BX-based defence is also ambiguous and determined by specific factors (e.g., plant–pathogen interactions and pathogen virulence). Ahmad et al. [20] reported that an infection by Exserohilum turcicum stimulates the accumulation of apoplastic BX during the early infection stages. Moreover, Song et al. [21] proved that the inoculation of maize with the arbuscular mycorrhizal fungus Glomus mosseae, which minimizes the symptoms of sheath blight triggered by the necrotrophic fungus Rhizoctonia solani, significantly increases the DIMBOA content in the roots, whereas an inoculation with R. solani alone does not have a similar effect. Furthermore, an infection by the necrotroph Septoria tritici results in the hydrolysis of DIMBOA glucoside, whereas an infection by Drechslera teres, which is a necrotroph incompatible with wheat, only slightly decreases the DIMBOA glucoside concentration, and an infection by the obligate parasite Puccinia recondita does not alter the DIMBOA glucoside concentration [22]. Yang et al. [23] reported that the resistance to northern corn leaf blight is associated with a decrease in the abundance of BX secondary metabolites.

Brown rust (BR) of rye, which is caused by the obligate biotrophic basidiomycete P. recondita f. sp. secalis (Prs) (Roberge ex Desmaz), is one of the most important diseases of rye in Central and Eastern Europe [24, 25]. Yield losses due to BR can be up to 40% under natural conditions [26], but can be as high as 80% following an early infection [27]. Several rye R
genes (i.e., Pr genes) associated with defence responses to Prs have been described, including Pr1–5, Pr-d–f, Pr-i–l, Pr-n, and Pr-p–t, all of which are dominantly inherited [28].

There is no available information regarding whether BXs influence BR resistance. Moreover, how a BR infection affects BX synthesis, including the expression of the associated genes, remains relatively unclear. Nevertheless, Rakoczy-Trojanowska et al. [29] suggested a possible relationship between BX and BR. Specifically, they determined that a single nucleotide polymorphism (ScBx4_1583) in ScBx4 (encoding a cytochrome P450 monooxygenase) is stably associated with BR resistance.

The objective of this study was to determine whether a Prs infection and the subsequent disease development as well as the inoculation procedure itself influence the expression levels and profiles of the following seven genes: ScBx1–ScBx5, ScIgl, and Scglu, of which, the first six control the production of six BXs [2-hydroxy-4H-1,4-benzoxazin-3-one (HBOA), GDIBOA, DIBOA, GDIMBOA, DIMBOA, and 6-methoxybenzoxazolinone (MBOA)] and the last one mediates the hydroxylation of glucosides to aglucones. We were also interested in clarifying if the BX content is associated with the plant–pathogen interaction at a given time-point.

Results

Analysis of pathogenesis in a preliminary experiment

Four plant–pathogen interaction profiles were observed during the disease development following the inoculation of rye cv. Słowiańskie (Fig 1). Profile i (infection site with developed appressoria indicating an effective pathogen infection) was assigned to 30.9% of the infection sites on leaves collected at 4 h post-inoculation (hpi) and 53.8% of the infection sites at 8 hpi. Profile ii [developed haustoria mother cells (HMCs) indicating the advancement of infection stages] was detected in 1.8%, 8.4%, and 41.7% of the samples at 12, 16, and 20 hpi, respectively. Profile iii (micronecrosis symptoms due to the plant resistance response) (Fig 2B) was detected for 0.5% and 8.1% of the infection sites at 48 and 72 hpi, respectively. None of the infection sites were assigned as profile iv (micronecrosis symptoms without HMCs).

Analysis of pathogenesis in the main experiment

All infection sites were designated as profile i at 8 hpi (Fig 2A). Profiles i and ii were detected at 17 and 24 hpi. Despite the very similar responses in the detached leaf test, at the seedling
Fig 2. (A) Plant–pathogen interaction profiles for the seedlings of rye inbred lines: L318, D33, and D39. The data represent mean and standard derivation. *** $p < 0.001$ according to the ANOVA and LSD post-hoc tests. (B) Example of profile iii in line D33 at 48 hours post inoculation (hpi) as determined with a fluorescence microscope. Sp: spore; GT: germ tube; A: appressorium; HMC: haustorial mother cell; M: micronecrosis. Bar = 100 μm.

https://doi.org/10.1371/journal.pone.0233807.g002
stage, the lines differed from each other in terms of plant–pathogen interaction profiles. Specifically, the percentage of infection sites designated as profile i for L318, D33, and D39 was 36.8% (standard deviation, sd = 10.2), 42.70% (sd = 13.3), and 46.4% (sd = 4.5), respectively, at 17 hpi and 26.2% (sd = 16.1), 29.7% (sd = 12.2), and 25.2% (sd = 20.7), respectively, at 24 hpi. The percentage of infection sites scored as profile ii for L318, D33, and D39 was 63.2% (sd = 10.2), 57.3% (sd = 13.3), and 53.6% (sd = 4.5), respectively, at 17 hpi and 73.8% (sd = 16.1), 70.3% (sd = 12.2), and 74.8% (sd = 20.7), respectively, at 24 hpi. All four profiles were observed at 48 hpi. At this time-point, similar profile i rates were observed for all lines (16%, sd = 4.4; 22.4%, sd = 15.5 and 23.1%, sd = 10.6 for L318, D33, and D39, respectively). Additionally, the profile ii rates for lines D33 and D39 (34.8%, sd = 12.2 and 34.4%, sd = 11.6, respectively) were approximately half of that of line L318 (71.9%, sd = 8.5). The profile iii rate was similarly high in lines D33 (38.1%) and D39 (38.6%), but was much lower in line L318 (12.2%, sd = 10.2). Profile iv was observed for only D33 and D39 (4.8%, sd = 4.2 and 3.8%, sd = 4.6, respectively).

The infection type of inoculated lines L318, D33, and D39 was scored as 3 (medium-sized uredinia with chlorosis), 2 (medium-sized uredinia with necrosis), and 1 (small uredinia with necrosis), respectively. Accordingly, of the analysed lines, L318 was the most susceptible to the Prs infection (Fig 3) which is consistent with the results obtained previously for plants grown in the field (S9 Table).

Dissecting the effect of the treatment procedure

 Influence of the treatment with water and an aqueous suspension of Prs urediniospores on gene expression levels. In most cases, the early [8 h post-treatment (hpt)] response of ScBx1–5 to the mock and Prs treatments was a decrease in expression levels (relative to the corresponding expression levels of the untreated plants) (Fig 4 and S1–S3 Tables). Regarding ScIgl and Scglu, the water and Prs urediniospore treatments either decreased or increased their expression levels at 8 hpt, but a significant increase with the untreated control level was detected for only the mock-treated D33 seedlings. Usually, the decrease in gene expression after the first 8 hpt was greater for the seedlings infected with Prs than for the mock-treated seedlings.

At subsequent time-points, the gene expression levels remained generally unchanged (especially ScBx1 and ScBx2) (Fig 4). However, the following three patterns of temporal gene expression changes were observed (with a total frequency of 45.2%): (1) similar level at two subsequent time-points and an increase at 48 hpt (the most frequent pattern); (2) further decrease up to 17 hpt and/or 24 hpt, followed by an increase at the last time-point; and (3) alternating increase and decrease. The greatest increase in expression level (more than 6-fold compared with the initial level) was detected for Scglu in the Prs-treated seedlings of the most resistant line, D39, at 48 hpt (Fig 4F). The greatest decrease in expression level was observed for Scglu in line D33 after the mock and Prs treatments at all time-points (Fig 4B and 4E). With a few exceptions, the greatest increase in gene expression was observed at the last time-point.

 Influence of the treatment with water and an aqueous suspension of Prs urediniospores on the BX content. In contrast to the gene expression changes, the BX contents usually increased at 8 hpt for the mock- and Prs-treated seedlings (relative to the BX contents of the untreated plants) (Fig 5 and S4–S6 Tables). A clear decrease in content was observed for HBOA in all three lines, for DIBOA and DIMBOA in line D33 subjected to mock and Prs treatments, and for DIBOA in lines L318 and D39 treated with Prs. At the first time-point, the greatest increase in BX contents was observed for MBOA in mock-treated L318 seedlings (Fig
The largest decrease in the DIBOA content was detected in the Prs-treated D33 seedlings (Fig 5E).

During the next 40 h, the BX level remained relatively unchanged in a few cases. However, the following five BX content profiles were more frequently detected (61.1%): (1) continued decrease; (2) an increase at 17 hpt and/or 24 hpt, followed by a decrease at the last time-point; (3) stable level up to 24 hpt, followed by a decrease at 48 hpt; (4) stable level up to 24 hpt,
Dissecting the effect of brown rust

**Influence of the Prs infection on gene expression.** A comparison of the gene expression in mock- and Prs-treated plants at 8, 17, 24, and 48 hpt revealed that in most cases (58.3%), the infection decreased expression levels (Fig 6 and S7 Table). On average, 41.6% of the differences were significant. Specifically, 21.4%, 46.4%, and 57.1% of the differences were significant for lines L318, D33, and D39, respectively. Among the positive differences, 31.4% were significant, with 0.0%, 18.2%, and up to 60% of the positive differences confirmed as significant for lines L318, D33, and D39, respectively. The line with the most genes exhibiting upregulated expression was D39, especially at the first time-point (Fig 6C). In line D33, upregulated expression was observed only for ScBx1 at 8 hpt and ScBx2 at 48 hpt (Fig 6B). None of the analysed genes had significantly upregulated expression levels in line L318 following the Prs infection (Fig 6A). Of the three investigated lines, D39 was the most responsive, with Prs upregulating and
downregulating expression levels (depending on the gene and time-point). Line D33 exhibited a moderate response level to Prs, whereas L318 exhibited the weakest response. The intensity of the reactions corresponded with the infection types. The expression levels of ScBx3 in D39, ScBx5 in D33, and Scglu in L318 only decreased, but the upregulated or downregulated expression of the remaining genes in response to Prs depended on the rye genotype and the time after the infection. The greatest increases and decreases in expression levels were determined for Scglu in line D39 at 17 and 48 hpt, respectively (Fig 6C).

Influence of the Prs infection on the BX contents. The infection of rye plants with Prs affected the contents of most BXs, with decreases rather than increases more commonly observed (70.8%) (Fig 7 and S8 Table). Among the negative differences, 70.6% were significant, with 82.4%, 55.6%, and 75.0% of these differences revealed as significant in lines L318, D33, and D39, respectively. One-third of the positive differences were significant, with 28.6%, 0.0%, and 62.5% of these differences confirmed as significant in L318, D33, and D39, respectively.

The most and least responsive lines were D39 and D33, respectively. The time-points with the most changes in BX contents and the most cases of increased contents were 17 and 24 hpt. The GDIBOA and GDIMBOA contents usually increased in response to Prs, whereas the opposite pattern was observed for the other examined BXs. Generally, similar reactions to Prs were observed in all lines. Specifically, at a given time-point, the contents of a BX either increased or decreased in all lines. An exception was GDIBOA in D33. Relative to the corresponding level in the mock-treated control, the GDIBOA content in D33 decreased at the first and second time-points, but increased at 24 and 48 hpt (Fig 7B). However, in lines L318 and

Fig 5. Synthesis patterns of BXs in untreated, mock-treated (abc) and Prs-treated (def) plants of rye ILs L318 (ad), D33 (be), D39 (cf) at four examined time-points, 8, 17, 24, and 48 hpt. The data represent mean value with standard derivation, * indicates statistically significant at p < 0.05 (based on the Mann-Whitney U test).

https://doi.org/10.1371/journal.pone.0233807.g005
Fig 6. Differences in the ScBx1–5, ScIgl, and Scglu expression levels between Prs- and mock-treated rye IL L318 (a), D33 (b), and D39 (c) plants. The data represent mean value with standard derivation, * indicates statistically significant at p < 0.05 (based on the Mann-Whitney U test).

https://doi.org/10.1371/journal.pone.0233807.g006
D39, the GDIBOA contents increased at the first three time-points and then decreased (Fig 7A and 7C). Overall, the greatest increase in BX content was detected for GDIBOA in line L318 at 17 hpt (Fig 7A), whereas the largest decrease in BX content was recorded for DIBOA in line D39 at 17 hpt (Fig 7C).

Fig 7. Differences in the BX synthesis levels between Prs- and mock-treated rye IL L318 (a), D33 (b), and D39 (c) plants. The data represent mean value with standard derivation, * indicates statistically significant at p < 0.05 (based on the Mann-Whitney U test).

https://doi.org/10.1371/journal.pone.0233807.g007
Discussion

Plant secondary metabolites are associated with diverse processes, including resistance to pathogens. In some cereal species, mainly rye, wheat, and maize, BXs are the main class of secondary metabolites involved in responses to fungal pathogens [3,15]. Nevertheless, their role in protecting plants against diseases remains relatively uncharacterized [23]. Even less is known about the induced defence, especially regarding gene expression levels. Moreover, there are only a few reports describing the relationship between infections by biotrophic fungi and changes in BX biosynthesis and its regulation [e.g., 20, 23]. Accordingly, we decided to clarify if and how the synthesis of six BXs (HBOA, GDIBOA, DIBOA, GDIMBOA, DIMBOA, and MBOA) and the expression of seven genes (ScBx1–5, ScIgl, and Scglu) are affected by a BR infection, which is one of the most important rye diseases. Additionally, we were also interested in how the infection procedure per se influences both gene expression and metabolite synthesis.

The investigated genes included ScBx1–5 as well as ScIgl and Scglu. The ScIgl gene has the same function as ScBx1 in the late developmental stages [13,14]. The Scglu gene encodes a β-glucosidase that hydrolyses stable glucosides to reactive aglucones following the plant tissue destruction due to herbivores or pathogens [6–8, 30]. We assumed that the structural disorganization of plant cells caused by Prs during the formation of haustoria [31–33] might modify Scglu expression. This is consistent with the results published by Gomez-Anduro et al. [34], who proved that in maize, Zmbglu2 expression is negatively influenced by mechanical damage. Additionally, changes to intracellular tensions caused by the formation of haustoria should lead to the release of glycosides from vacuoles and their interaction with β-glucosidases.

To determine the effects of treatments with water and an aqueous suspension of fungal spores on gene expression and BX contents, we compared the data obtained for mock- and Prs-treated plants with the corresponding data for the untreated plants. The effects of the mock and Prs treatments were compared at four time-points to clarify the influence of the pathogen infection on the gene expression and BX contents. To the best of our knowledge, such an approach that considers all elements of the infection procedure has not been applied in similar types of studies to date. Researchers have typically used two treatments (mock and pathogen treatments) [e.g., 35–37] or they have compared pathogen-infected plants with untreated plants [e.g., 38, 39]. Thus, they were unable to assess the impact of the treatment procedure, which is particularly important for analyses of secondary metabolism. Moreover, some of the conclusions of these earlier investigations may not be legitimate.

Dissecting the effect of the treatment procedure

Influence of the treatment with water and an aqueous suspension of Prs urediniospores on gene expression. Considering the specificity of secondary metabolism, we assumed that the procedures for the mock treatment and infection with Prs include elements that most likely affect BX metabolism and the expression of the associated genes. The underlying mechanism may be associated with the micro-wounds caused by brushing, which may increase jasmonic acid (JA) levels and induce the BX defence pathway. The role of JA in BX biosynthesis has been well documented [40, 41].

The effects of the treatments in this study (regardless of whether the plants were sprayed with water or an aqueous suspension of urediniospores) were very noticeable. Regarding gene expression, the Prs infection and treatment with water most commonly resulted in downregulated expression levels, which were generally comparable for both stresses. With a few exceptions, the differences between the expression levels in the untreated and stressed plants at the first time-point (8 hpt) were slightly greater for the mock-treated plants, implying that in the...
early stage after the treatments, the changes in gene expression levels were primarily due to the treatment per se and not the pathogen. Our findings regarding ScBx1 expression differ from those of a study by Ding et al. [36], in which the expression of maize Bx1, which is an orthologue of ScBx1 [10], was usually upregulated in the shoots of plants treated with different elicitors from the pepper pathogen Phytophthora capsici and pepper root exudates to induce plant defence mechanisms at a similarly early time-point (12 hpt). During the next 40 h, the gene expression levels remained relatively stable. Only in slightly more than a quarter of the cases were other profiles observed. We conclude that the treatment procedures, regardless of whether the plants were mock- or Prs-treated, induce a strong and rapid reaction, but they do not cause further changes. Although we expected to observe additional changes as the BR infection progressed, they occurred only sporadically and were mainly associated with ScIgl expression at 48 hpt in lines L318 and D33. Similarly, Ahmad et al. [20] postulated that in maize, Igl expression is induced by stress. In the current study, downregulated ScIgl expression was most often observed. Additionally, in some cases (L318, D33, and D39 at 8 hpt), the mock treatment increased ScIgl expression, whereas the Prs infection had the opposite effect.

**Influence of the treatment with water and an aqueous suspension of** Prs **urediniospores on BX contents.** Only two BXs, HBOA and DIBOA (with few exceptions), underwent content changes that were similar to the gene expression changes. In contrast, completely different changes were observed for three other BXs, GDIBOA, GDIMBOA, and MBOA. Furthermore, the changes in the DIMBOA content depended on the genotype, with both stresses increasing the content in L318 and D39, but decreasing the content in D33. The Prs spore treatment usually resulted in a greater change to BX contents regardless of the rye genotype. The exception was the MBOA content, which increased considerably more in the mock-treated plants. On the basis of these observations, we hypothesize that three BXs, GDIBOA, GDIMBOA, and MBOA, are crucial components of rye defence reactions, with the first two (especially GDIMBOA) protecting plants against BR, and MBOA providing protection against the treatment procedure per se.

**Dissecting brown rust effects**

**Effect of the** Prs **infection on gene expression.** To elucidate the effects of a fungal infection, the gene expression level differences between the mock- and Prs-treated plants were analysed at four time-points. The Prs infection mainly negatively influenced gene expression. However, the direction of the changes depended on the genes (i.e., ScBx2 and ScBx4 expression levels were more frequently upregulated, whereas ScBx5 and ScIgl expression levels were downregulated), time-points (i.e., upregulated and downregulated expression levels were mostly detected at 8 and 17 hpt, respectively), and genotypes (i.e., most of the instances of upregulated expression occurred in D39). At the first time-point, the ScBx1, ScBx2, and ScBx4 expression levels increased in all lines. Nevertheless Yang et al. [23] concluded there is a negative relationship between the expression levels of the maize Bx1 and Bx2 genes and resistance to northern corn leaf blight. This discrepancy may be explained by the fact the Yang et al. [23] study involved the fungus *E. turcicum*, which is characterized by a different etiology and is more invasive than *Prs*. Moreover, La Hovary [9] subjected rye seedlings to wounding and the reported ScBx1 and ScBx2 expression levels at 6 hpt are similar to our observed expression levels, but a JA treatment downregulated the expression of both genes (relative to the corresponding expression levels in the mock-treated plants). The frequency of upregulated expression levels corresponded with the defence reaction type, with the highest number of instances observed in the least susceptible line, D39. In contrast, the most susceptible line, L318, had no significantly increased expression levels. Additionally, D39 was the only line in which ScBx1,
ScBx2, and Scglu expression levels were significantly upregulated in the infected plants in a coordinated manner at the first and last time-points. The ScBx4 expression level increased in the infected D39 seedlings at all time-points. A published association analysis [29] revealed a significant role for ScBx4 in the resistance of mature rye plants to BR under field conditions. In the current study, we confirmed the importance of this gene for the BR resistance in much younger rye plants, but only for the resistant genotype. Therefore, the previously identified polymorphism, ScBx4_1583, may be associated with non-race-specific adult plant and seedling stage resistance.

Another unique observation in IL D39 was that the expression levels of Scglu increased significantly in the infected plants (compared with the levels in the mock-treated controls) at the first, third, and fourth time-points. The Scglu and ScBx4 expression levels similarly increased only in line D39. This should result in an increase in β-glucosidase production and, consequently, enhanced efficiency of glucoside hydrolysis. Contrary to the expectation, the induction of Scglu expression was not accompanied by increased aglucone contents at the first and third time-points. At the last time-point, the DIBOA and DIMBOA contents increased admittedly, but not significantly. These findings suggest the Scglu expression level is not related to the accumulation of both aglucones, at least at the examined time-points. In lines D33 and L318, the Scglu expression level usually decreased, which is consistent with the results of a study by Gomez-Anduro et al. [34], in which salt stress and mechanical damage negatively influenced the expression of the maize glu2 gene.

**Effect of the Prs infection on BX contents.** Similar to the gene expression changes, the BX contents decreased (even more frequently) after the BR infection relative to the corresponding levels in the mock-treated plants at four time-points. In contrast to the observed gene expression, there were no clear relationships between the type of reaction and the defence reaction type. Nevertheless, two common features were detected. First, the decreased abundance of DIBOA and DIMBOA was a natural consequence of the downregulated expression of Scglu. Interestingly, at the most critical time-point, 24 hpt, the decrease in the DIBOA content was inversely related to the disease resistance of the ILs. Regarding DIMBOA, its content decreased the most in D39, although the decreases in the other two lines were similar. Consistent with our findings, Yang et al. [23] reported that in maize, the resistance to northern corn leaf blight caused by E. turcicum is related to a decrease in the abundance of DIMBOA. In contrast, Ahmad et al. [20] reported that E. turcicum induces the accumulation of apoplastic BXs during the early infection stages, which may inhibit the ability of the fungus to penetrate plant tissues. Similarly, Yu et al. [42] speculated that maize plants resist infections by the biotroph Sporisorium reilianum f. sp. zeae via DIMBOA synthesis and Song et al. [21] demonstrated the positive association between DIMBOA accumulation and the resistance to sheath blight disease caused by the necrotrophic fungus R. solani.

The second common feature was that the Prs infection in our study decreased the contents of two glucosides, GDIBOA and GDIMBOA, at a key time-point (24 hpt), during which pathogen growth intensifies and the growing conditions change. Therefore, our results regarding these two glucosides differ completely from those obtained by Yang et al. [23], who proved that in addition to DIMBOA contents, the abundance of GDIMBOA (and two other aglucones) is negatively correlated with the resistance to northern corn leaf blight. However, our findings are similar to those reported by Oikawa et al. [43], who proved that southern corn leaf blight caused by Bipolaris maydis, which has a pathogenesis similar to that of Prs, increases the content of another glucoside, 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside.

An additional and important question we wanted to answer was whether the stress-induced upregulated and downregulated gene expression levels were accompanied by the same changes
to BX contents. In many cases, these processes were coordinated (e.g., downregulated ScIgl expression followed by a decrease in the DIMBOA content in L318 at the first time-point; downregulated ScBx5 expression followed by a decrease in the DIBOA and DIMBOA contents in D33 at 24 hpt; and the upregulated expression of ScBx1, ScBx2, and ScBx4 followed by an increase in the GDIMBOA content in line D39 at 8 hpt). However, a lack of correlation was also relatively frequently detected. It is unknown why downregulated ScBx5 expression is correlated with a decrease in HBOA accumulation. The ScBx5 gene controls BX biosynthesis downstream of HBOA. One possible explanation for the effects of the changes to ScBx5 expression may involve an unknown type of feedback. Another unexplained phenomenon was the simultaneous decrease in the DIBOA content and an increase in both glucosides (GDIBOA and GDIMBOA). Specifically, a lack of coordination between gene expression levels and BX contents was apparent in line D39. The HBOA, DIBOA, DIMBOA, and MBOA contents in D39 decreased in response to Prs, whereas the expression of the genes controlling their biosynthesis (especially at the first and last time-points) increased. It is also unclear why in line D39 the Scglu expression level increased significantly, but the abundance of GDIBOA and GDIMBOA decreased at 8, 24, and 48 hpt. The few published articles relevant to the current study describe how the fungal-induced changes in Bx gene expression and BX contents are synchronized. For example, a study by Ding et al. [36] examined the activation of BX synthesis and selected defence gene expression after treatments with elicitors from the pepper pathogen P. capsici and pepper root exudates. The authors proved that in many cases, upregulated or downregulated Bxl expression is not accompanied by a similar change to metabolite contents. For example, at 48 h after the treatment with a spore lysis suspension, the Bx1 expression level decreased, whereas DIBOA and MBOA accumulation increased and the DIMBOA content was unchanged. At 24 h after the treatment with the spore culture suspension, Bx1 was expressed at lower levels in the infected plants than in the untreated control plants, but the DIBOA and DIMBOA contents exhibited the opposite pattern. Nevertheless, they revealed a strong correlation between Bx1 expression and the DIMBOA content, which differs considerably from our study. Accordingly, in rye, the changes to gene expression and BX contents appear to occur independently or they are not temporally coordinated. The accumulation of specific transcripts is most probably sufficient for ensuring an increase in metabolite production, specifically GDIBOA and GDIMBOA in plants infected with Prs and MBOA in mock-treated plants.

The data presented herein indicate that some BXs (mainly GDIBOA and GDIMBOA) are substantial components of induced rye defences against BR. The significant absolute and relative increases in their contents occur at key moments for disease development, which is when HMCs start to form, pathogen growth is extensive, and necrotic lesions are developing. Although the abundance of three other BXs, namely DIBOA, DIMBOA, which may be involved in maize innate immunity against Setosphaeria turcica as extracellular signals for the PAMP-induced callose [20], and MBOA, increases as early as 8 hpt, these BXs are probably not associated with immunity because they are induced by both Prs and water. Additionally, the MBOA content steadily increased up to 48 hpt, implying this BX has a substantial role in rye responses to stresses related to the infection procedure. In contrast, HBOA appears to minimally affect the resistance mechanisms mobilized by Prs.

In summary, a Prs infection and the treatment procedure itself affect both gene expression and BX contents in rye. The key components of the defence response of all analysed rye ILs against BR are as follows: ScBx1, ScBx2, ScBx4, and Scglu as well as GDIBOA and GDIMBOA. The changes in the gene expression levels and BX contents are usually positively associated with disease resistance. The intensity of the reaction depends on the genotype, with the most
resistant lines mobilizing their defence mechanisms more effectively, in a more coordinated manner, and earlier than the less resistant lines.

Our research is an important step toward characterizing the molecular mechanisms underlying rye defences against BR and for evaluating inoculation procedures. Furthermore, our data may be useful for future transcriptome- and metabolome-based selection of rye germplasm with enhanced resistance to BR. However, there are still many ambiguities that must be clarified, the most important of which is why the BR infection and disease development and the infection procedure cause such different changes to individual metabolites and genes. Future studies should also examine why the treatments with water and *Prs* urediniospores have the opposite effects on gene expression and/or BX contents at the same time-points.

**Material and methods**

**Plant materials**

The following three rye inbred lines (ILs) were analysed: D33 and D39 (bred by Danko Plant Breeders Ltd., Poland) as well as L318 (bred by the Department of Plant Genetics, Breeding and Biotechnology, Warsaw University of Life Sciences). The ILs were selected based on the BX contents measured after a natural vernalization period. The highest DIBOA content was detected for L318, followed by D33 and then D39. Under field conditions, BR resistance was highest for D33, followed by D39 and then L318 [44; S9 Table].

Plants were cultivated in 24-well trays filled with a mixture of peat and perlite under controlled conditions (22˚C with a 16-h light/8-h dark photoperiod). Twelve plants (one replicate) were grown in a single tray segment (7 cm diameter). Experiments were performed with three biological replicates.

**Pathogen**

Rye plants were infected with *Prs* single spore isolate No. 1.1.6, which was selected based on a preliminary experiment involving detached-leaf inoculations with 30 isolates. The selected isolate was characterized by the least compatible and most uniform host–pathogen reaction in the tested lines (S10 Table). The detached-leaf test was conducted as described by Hsam *et al.* [45]. For details, please refer to the Supporting Information.

**Establishing the essential time-points for plant–pathogen interactions**

The seedlings of susceptible rye cultivar Słowińskie were inoculated with *Prs* single spore isolate No. 1.1.6. Leaf samples were collected at 4, 8, 12, 16, 20, 24, 36, 48, and 72 hpi and stained with calcofluor white as described by Orczyk *et al.* [46]. Briefly, samples were fixed for 24 h with an ethanol:dichloromethane (3:1) solution supplemented with 0.15% trichloroacetic acid, after which they were rinsed twice with 50% ethanol, twice with 0.05 M sodium hydroxide, three times with water, and once with 0.1 M Tris. They were then stained with calcofluor white (3.5 mg/ml).

The stained leaf fragments were examined with the Diaphot fluorescence microscope (Nikon) for the presence of germinating spores, HMCs, and micronecrosis symptoms. Additionally, the number of infection sites was calculated. Observations were made in 80 on average (but not less than 30) infection sites per leaf sample. For selecting time-points for additional analyses of plant–pathogen interactions, the germinating spores and appressoria at infection sites were counted. The following four infection site profiles were used to reflect plant–pathogen interactions: (i) appressoria; (ii) appressoria and HMCs; (iii) appressoria, HMCs, and micronecrosis; and (iv) appressoria and micronecrosis. The analysis of pathogenesis and the
percentage of profiles in the preliminary experiment with cv. Słowiańskie (Fig 1) were calculated according to: Eqs 1, 2, 3 and 4. The analysis of pathogenesis and the percentage of profiles in the main experiment with lines L318, D33 and D39 (Fig 2) were calculated according to: Eqs 5, 6, 7 and 8.

\[
\text{Percentage of profile } i = \frac{n_i}{n_{gs} + n_i + n_{ui} + n_{iii} + n_{iv}} \times 100\% 
\]

\[
\text{Percentage of profile } ii = \frac{n_{ui}}{n_{gs} + n_i + n_{ui} + n_{iii} + n_{iv}} \times 100\% 
\]

\[
\text{Percentage of profile } iii = \frac{n_{iii}}{n_{gs} + n_i + n_{ui} + n_{iii} + n_{iv}} \times 100\% 
\]

\[
\text{Percentage of profile } iv = \frac{n_{iv}}{n_{gs} + n_i + n_{ui} + n_{iii} + n_{iv}} \times 100\% 
\]

Where: \(n_{gs}\) – number of infection sites with germinating spores, \(n_i\) – number of infection sites with appressoria, \(n_{ui}\) – number of infection sites with appressoria and HMC, \(n_{iii}\) – number of infection sites with appressoria, HMC and micronecrosis, \(n_{iv}\) – number of infection sites with appressoria and micronecrosis.

The number of infection sites with germinating spores and the rates of the four profiles were used to select the following time-points for further analyses of plant–pathogen interactions: 8, 17, 24, and 48 hpi. The first time-point (8 hpi) was selected because of the considerable abundance of appressoria at established infection sites. The 17 and 24 hpi time-points were associated with HMC formation and intense pathogen growth, respectively. The final time-point (48 hpi) corresponded to the beginning of the resistance reaction. Leaf samples were collected at the selected time-points, stained, and analysed regarding plant–pathogen interaction profiles.

**Prs and mock treatment**

Spores from a single spore isolate of *Prs* were suspended in water with Tween 20 for the subsequent inoculation of three 12-day-old rye IL seedlings. The plants were sprayed with a spore solution or water containing Tween 20 (mock treatment), after which the applied solutions were spread on the leaf surface with a brush. Untreated plants grown under the same conditions as the inoculated and mock-treated plants served as an additional control. Immediately after the inoculation or mock treatment, multiple pots with plants were incubated for 24 h at 18˚C in boxes covered with black plastic material to maintain dark and humid conditions. The
plants were then transferred to growth chambers (with conditions as described above). The plants were cultivated, inoculated, and histologically analysed as described by [32].

**Sampling of plant materials**

At 8, 17, 24, and 48 hpt, the collected plant material for a biological replicate was divided into two equal parts, with one part used for RNA isolation, and the second part analysed for BX content. Additionally, leaf fragments were collected to characterize the infection type. The number of infection sites for each plant–pathogen interaction profile was recorded. Infection types were determined at 15 days post-inoculation (dpi) based on the following 6-point scale [47]: 0 = immune (no visible reaction); 0; = resistant (chlorotic or necrotic flecking) 1 = resistant (minute uredinia surrounded by chlorosis or necrosis); 2 = moderately resistant (small to medium-size uredinia, surrounded by chlorosis or necrosis); 3 = moderately susceptible (medium to large uredinia surrounded by chlorosis); and 4 = susceptible (medium to large uredinia with little or no chlorosis).

The tissues for gene expression and biochemical analyses were frozen. The tissues designated for biochemical analyses were lyophilized (Alpha model 2–4 LDplus; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

**RNA isolation and reverse transcription for qRT-PCR**

The expression levels of the following genes were analysed: ScBx1–5 (GenBank: KF636825–KF636828 and KF620524), ScIgl (GenBank: MN120476), and Scglu (GenBank: AY586531.2). Total RNA was extracted from 100 mg untreated, mock-treated, and Prs-treated aerial parts of rye plants with the GeneMATRIX Universal RNA Purification Kit (version 1.2) (Eurx, Gdańsk, Poland). The isolated RNA was dissolved in 40 μl RNase-free water, after which the RNA integrity and concentration were measured with the NanoDrop 2000 spectrophotometer. To avoid genomic DNA contamination, the RNA was treated with Turbo DNase (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was then used as the template to synthesize cDNA with the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). The resulting cDNA was diluted with RNase-free water.

**Expression analysis of the selected candidate genes**

A qRT-PCR assay was completed in a 96-well plate, with three biological and two technical replicates. Each assay included one candidate gene (ScBx1–5, ScIgl, or Scglu) and the HvAct reference gene [chosen based on results of the earlier experiments including three reference genes: actin, glyceraldehyde phosphate dehydrogenase (GADPH), and cell division control protein, AAA-superfamily of ATPases]. The qRT-PCR was completed with the LightCycler 96 Real Time System (Roche, Basel, Switzerland), with the following program: 95˚C for 600 s; 32 cycles of 95˚C for 10 s, 57˚C for 10 s, and 72˚C for 15 s; 95˚C for 10 s, 55˚C for 60 s, and 97˚C for 1 s. The total volume of the reaction mixture was 20 μl, which contained 4 μl cDNA, 1 μl each gene-specific primer (5 mM), 4 μl RNase-free water, and 10 μl FastStart Essential DNA Green Master (Roche). Candidate gene expression levels were normalized against the HvAct expression level according to the 2−ΔΔCt method [48]. Details regarding the gene-specific primers are provided in S11 Table.

**Microscopic analysis**

The microscopic analysis of pathogenesis-related processes was performed as described by [32].
Biochemical analysis

The contents of the following six BXs were assayed as previously described [29,44]. For details, please refer to the Supporting Information.

Evaluation of the stress effect

To evaluate the effect of the infection by Prs urediniospores, the gene expression and BX levels were compared between the inoculated and untreated plants at specific time-points. To dissect the effect of the Prs infection, the difference between the gene expression/BX levels of the infected and mock-treated plants was calculated for each time-point. Positive and negative differences were considered to indicate increases and decreases, respectively.

Supporting information

S1 Table. Relative gene expression level of ScBx1—ScBx5, ScIgl, and Scglu in untreated seedlings of rye inbred lines, L318, D33, and D39.

S2 Table. Relative gene expression level of ScBx1—ScBx5, ScIgl, and Scglu in Prs- and mock-treated seedlings of rye inbred lines, L318, D33, and D39 at four time-points, 8, 17, 24, and 48 hpt.

S3 Table. The differences in gene expression level of ScBx1—ScBx5, ScIgl, and Scglu between Prs-treated, mock-treated and untreated rye seedlings (dissecting treatment procedure effect).

S4 Table. BX synthesis level in untreated seedlings of rye inbred lines, L318, D33, and D39.

S5 Table. BX synthesis level in Prs- and mock-treated seedlings of rye inbred lines, L318, D33, and D39 at four time-points, 8, 17, 24, and 48 hpt.

S6 Table. The differences in BX synthesis level between Prs-treated, mock-treated and untreated rye seedlings (dissecting treatment procedure effect).

S7 Table. The differences in gene expression level of ScBx1—ScBx5, ScIgl, and Scglu between Prs- and mock-treated rye seedlings (dissecting brown rust effect).

S8 Table. The differences in BX synthesis level between Prs- and mock-treated rye seedlings (dissecting brown rust effect).

S9 Table. Characteristics of rye inbred lines, L318, D33, and D39, chosen for experiments.

S10 Table. Resistance reaction of three rye inbred lines, L318, D33, and D39, determined based on detached-leaf test.
S11 Table. Primers used in qRT-PCR reaction.

S1 File.

Acknowledgments

We thank Edanz Group (https://en-author-services.edanzgroup.com/) for editing a draft of this manuscript.

Author Contributions

Conceptualization: Magdalena Święcicka, Monika Rakoczy-Trojanowska.

Formal analysis: Anna Stochmal.

Investigation: Magdalena Święcicka.

Methodology: Marta Dmochowska-Boguta, Wacław Orczyk, Agnieszka Grądzielewska, Mariusz Kowalczyk.

Software: Leszek Bolibok.

Supervision: Monika Rakoczy-Trojanowska.

Validation: Wacław Orczyk, Monika Rakoczy-Trojanowska.

Visualization: Magdalena Święcicka, Marta Dmochowska-Boguta.

Writing – original draft: Magdalena Święcicka, Monika Rakoczy-Trojanowska.

References

1. Makowska B, Bakera B, Rakoczy-Trojanowska M. The genetic background of benzoxazinoid biosynthesis in cereals. Acta Physiol. Plant. 2015; 37:176. https://doi.org/10.1007/s11738-015-1927-3

2. Frey M, Schullehner K, Dick R, Fiesselmann A, Gierl A. Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. Phytochemistry. 2009; 70:1645–1651. https://doi.org/10.1016/j.phytochem.2009.05.012

3. Niemeyer HM. Hydroxamic acids derived from 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one: key defense chemicals of cereals. J. Agric. Food Chem. 2009; 57(5):1677–1696. https://doi.org/10.1021/jf8034034

4. Meihls LN, Handrick V, Glauser G, Barbier H, Kaur H, Haribal MM, et al. Natural variation in maize aphid resistance is associated with 2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one glucoside methyltransferase activity. Plant Cell. 2013; 25:2341–2355. https://doi.org/10.1105/tpc.113.112409

5. Handrick V, Robert CAM, Ahern KR, Zhou S, Machado RAR, Maag D, et al. Biosynthesis of 8-O-methylated benzoxazinoid defence compounds in maize. The Plant Cell Online. 2016; 28:1682–1700. https://doi.org.10.1105/tpc.16.00065

6. Wouters FC, Blanchett B, Gershenzo J, Vassão DG. Plant defense and herbivore counter-defense: benzoxazinoids and insect herbivores. Phytochem. Rev. 2016; 15:1127–1181. https://doi.org/10.1007/s11101-016-9481-1

7. Nikus J, Daniel G, Jonsson LM. Subcellular localization of beta-glucosidase in rye, maize and wheat seedlings. Physiol. Plant. 2001; 111(4):466–472. https://doi.org/10.1034/j.1399-3054.2001.1110406.x

8. Sue M, Nakamura C, Nomura T. Dispersed benzoxazinone gene cluster: molecular characterization and chromosomal localization of glucosyltransferase and glucosidase genes in wheat and rye. Plant Physiol. 2011; 157:985–997. https://doi.org/10.1104/pp.111.182378

9. La Hovary C. Allelochemicals in Secale cereale: Biosynthesis and molecular biology of benzoxazinones. 2011; https://repository.lib.ncsu.edu/bitstream/handle/1840.16/6844/etal.pdf?sequence=2

10. Bakera B, Makowska B, Groszyk J, Niziołek M, Orczyk W, Bolibok-Brągoszewska H, et al. Structural characteristics of ScBx genes controlling the biosynthesis of hydroxamic acids in rye (Secale cereale L.). J. Appl. Genet. 2015; 56:287–298. https://doi.org/10.1007/s13353-015-0271-z
11. Groszyk J, Kowalczyk M, Yanushevskaya Y, Stochmal A, Rakoczy-Trojanowska M, Orczyk W. Identification and VIGS-based characterization of Bx1 ortholog in rye (Secale cereale L.). PLoS ONE. 2017; 12: e0171506. https://doi.org/10.1371/journal.pone.0171506

12. Tanvir F, Dionisio G, Adhikari KB, Fomsgaard IS, Gregersen PL. Biosynthesis and chemical transformation of benzoxazinoids in rye during seed germination and the identification of a rye Bx6-like gene. Phytochemistry. 2017; 140:95–107. https://doi.org/10.1016/j.phytochem.2017.04.020

13. Rakoczy-Trojanowska M, Świącicka M, Rymuszka J, Stochmal A, Kowalczyk M. (2018a) New aspects of the genetic background of benzoxazinoid biosynthesis in rye (Secale cereale L.). EUCARPIA cereal section / IWIIW2 meetings, March 19–22, 2018. Polydöme, Clermont-Ferrand, France. PI-23

14. Wiazło A, Świącicka M, Koter M.D, Kręciński T, Bolibok L, Stochmal A, et al. Genes ScCBS1 and ScCGL—a Competitors or Cooperators? Genes 2020; 11(2):223. https://doi.org/10.3390/genes11020223

15. Zhou S, Richter A, Jander G. Beyond Defense: Multiple Functions of benzoxazinoids in maize metabolism, plant and cell. Physiology. 2018; 59(8):1528–1537. https://doi.org/10.1093/ppc/pcy064

16. Cotton TEA, Petriaq P, Cameron DD, Al Meselmani M, Schwarzenbach R, Rolfe SA, et al. Metabolic regulation of the maize rhizobiome by benzoxazinoids. The ISME Journal. 2019; 13:1647–1658. https://doi.org/10.1038/s41396-019-0375-2

17. Toldi ET, Relationship between DIMBOA content and Helminthosporium turcicum resistance in maize. Növénytermelés. 1984; 33:213–218.

18. Zheng YQ, Zhao Y, Dong FS, Yao JR, Hurle K. Relationship of DIMBOA content in wheat seedlings and its resistance to plant pathogens. Allelopathy J. 2005; 15:137–143.

19. Lyons PC, Nicholson RL. Evidence that cyclic hydroxamate concentrations are not related to resistance of corn leaves to anthracnose. Can. J. Plant Pathol. 1989; 11:215–220. https://doi.org/10.1080/07060668909501102

20. Ahmad S, Veyrat N, Gordon-Weeks R. Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. Plant Physiol. 2011; 157:317–327. https://doi.org/10.1104/pp.111.180224

21. Song YY, Cao M, Xie LJ, Liang XT, Su YJ, et al. Induction of DIMBOA accumulation and systemic defense responses as a mechanism of enhanced resistance of mycorrhizal corn (Zea mays L.) to sheath blight. Mycorrhiza. 2011; 21:721–731. https://doi.org/10.1007/s00572-011-0380-4

22. Weibull J, Niemeyer HM. Changes of DIMBOA-Glc content in wheat plants upon infection by three plant pathogenic fungi. Physiol. Mol. Plant Pathol. 1995; 47:201–212. https://doi.org/10.1006/pmpp.1995.1052

23. Yang P, Praz C, Li B, Singla J, Ro¸bert CAM, Kessel B, et al. Fungal resistance mediated by maize wall-associated kinase ZmWAK-RLK1 correlates with reduced benzoxazinoid content. New Phytologist. 2019; 221(2):976–987. https://doi.org/10.1111/nph.15419

24. Roux SR, Wehling P. Nature of mixed infection type 2(5) observed in rye (Secale cereale L.) plants carrying the Pr1 leaf-rust resistance gene. J Kulturpflanzen; 2010; 62:29–34.

25. Miedane T, Klocke B, Flath K, Geiger HH, Weber WE. Diversity, spatial variation, and temporal dynamics of virulences in the German brown rust (Puccinia recondita f.sp. secalis) population in winter rye. Eur. J. Plant Pathol. 2012; 132:23–35. https://doi.org/10.1007/s10658-011-9845-8

26. Wehling P, Lizn A, Hackauf B, Roux SR, Ruge B, Klocke B. Leaf-rust resistance in rye (Secale cereale L.). 1. Genetic analysis and mapping of resistance genes Pr1 and Pr2. Theor. Appl. Genet. 2003; 107:432–438. http://doi.org/10.1007/s00122-003-1263-7

27. Solodukhina OV. Genetical Characterization of Rye Accessions with Regard to Leaf Rust Resistance. Russ. J. Genet. 2002; 38:399–407. https://doi.org/10.1023/A:1015202303392

28. Roux SR, Hackauf B, Ruge-Wehling B, Linz A, Wehling PG. Exploitation and comprehensive characterization of leaf-rust resistance in rye. Vortr Pflanzenzüchtung 2007; 71:144–150.

29. Rakoczy-Trojanowska M, Krajewski M, Bocianowski J, Schollenberger M, Wakułniski W, Milczarek P. Identification of single nucleotide polymorphisms associated with brown rust resistance, a-amylase activity and pre-harvest sprouting in rye (Secale cereale L.). Plant Mol. Biol. Rep. 2017a; 35:366–378. https://doi.org/10.1007/s11105-017-1030-6

30. Sue M, Yamazaki K, Yajima S, Nomura T, Matsukawa T, Iwamura H. Molecular and structural characterization of hexameric β-D-glucosidases in wheat and rye. Plant Physiol. 2006; 141:1237–1247. https://doi.org/10.1104/pp.106.077693

31. Bolton MD, Kolmer JA, Garvin DF. Wheat leaf rust caused by Puccinia triticina. Mol. Plant Pathol. 2008; 9:563–575. https://doi.org/10.1007/j.1364-3703.2008.00487.x

32. Dmochowska-Boguta M, Alaba S, Yanushevskaya Y, Plechota U, Lasota E, Nadolska-Orczyk A, et al. Pathogen-regulated genes in wheat isogenic lines differing in resistance to brown rust Puccinia triticina. BMC Genomics. 2015; 16:742. https://doi.org/10.1186/s12864-015-1932-3
33. Liu G, Tian D, Shi C, Wang DM. Autophagy is induced in haustorial mother cells of Puccinia triticina and is necessary for plant infection. Eur. J. Plant Pathol. 2017; 147:833–843. https://doi.org/10.1007/s10658-016-1047-y

34. Gómez-Andújar G, Ceniceros-Ojeda EA, Casados-Vázquez LE, Bencivenni C, Sierra-Beltrán A, Murillo-Amador B, et al. Genome-wide analysis of the beta-glucosidase gene family in maize (Zea mays L. var B73). Plant Mol. Biol. 2011; 77:159–83. https://doi.org/10.1007/s11103-011-9800-2

35. Zhang H, Yang Y, Wang C, Liu M, Li H, Fu Y, et al. Large-scale transcriptome comparison reveals distinct gene activations in wheat responding to stripe rust and powdery mildew. BMC Genomics. 2014; 15:898. https://doi.org/10.1016/j.pbiol.2015.08.030

36. Ding X, Yang M, Huang H, Chuan Y, He X, Li C, et al. Priming maize resistance by its neighbors: activating 1,4-benzoxazine-3-ones synthesis and defense gene expression to alleviate leaf disease. Front. Plant Sci. 2015; 6:830. https://doi.org/10.3389/fpls.2015.00830

37. Neugebauer KA, Bruce M, Todd T, Trick HN, Fellers JP. Wheat differential gene expression induced by different races of Puccinia triticina. PLoS One. 2018; 13:e0198350. https://doi.org/10.1371/journal.pone.0198350

38. Ding X, Yang M, Huang H, Chuan Y, He X, Li C, et al. Priming maize resistance by its neighbors: activating 1,4-benzoxazine-3-ones synthesis and defense gene expression to alleviate leaf disease. Front. Plant Sci. 2015; 6:830. https://doi.org/10.3389/fpls.2015.00830

39. Xingquan Z, Changyou W, Ali ME, Hong Z, Xinlun L, Weian L, et al. PROFILING gene expression patterns of stripe rust (Puccinia striiformis f.sp. tritici) resistance gene in new wheat germplasm. Pak. J. Bot. 2010; 42:4253–4266.

40. Oikawa A, Ishihara A, Iwamura H. Induction of HDMBOA-Glc accumulation and DIMBOA-Glc4-O-methyltransferase by jasmonic acid in poaceous plants. Phytochemistry. 2002; 61:331–337. https://doi.org/10.1016/S0031-9422(02)00225-X

41. Tzin V, Hojo Y, Strickler SR, Bartsch LJ, Archer CM, Ahern KR, et al. Rapid defense responses in maize leaves induced by Spodoptera exigua caterpillar feeding. J. Exp. Bot. 2019; 68(16):4709–4723. https://doi.org/10.1093/jxb/erx274

42. Yu T, Wang Z, Jin X, Liu X, Kan S. Analysis of gene expression profiles in response to Sporisorium reilianum f. sp. zeae in maize (Zea mays L.). Electron. J. Biotechnol. 2014; 17(5):230–237. https://doi.org/10.1016/j.ejbt.2014.07.006

43. Oikawa A, Ishihara A, Tanaka C, Mori N, Tsuda M, Iwamura H. Accumulation of HDMBOA-glc is induced by biotic stresses prior to the release of MBOA in maize leaves. Phytochemistry. 2004; 65:2995–3001. https://doi.org/10.1016/j.phytochem.2004.09.006

44. Rakoczcy-Trojanowska M, Orczyk W, Krajewski P, Bocianowski J, Stochmal A, Kowalczyk M. ScBx gene based association analysis of hydroxamate content in rye (Secale cereale L.). J. Appl. Genet. 2017b; 58:1–9. https://doi.org/10.1007/s13353-016-0356-3

45. Hsam SLK, Peters N, Paderina EV, Felsenstein F, Oppitz K, Zeller FJ. Genetic studies of powdery mildew resistance in common oat (Avena sativa L.) II. Cultivars and breeding lines grown in Western Europe and North America. Euphytica. 1997; 96:421–427. https://doi.org/10.1023/a:1016015223.1998.00227.x

46. Orczyk W, Dmochowska-Boguta M, Czembor HJ, Nadolska-Orczyk A. Spatiotemporal patterns of oxidative burst and micronecrosis in resistance of wheat to brown rust infection. Plant Pathol. 2010; 59:567–575. https://doi.org/10.1111/j.1365-3059.2010.02297.x

47. Murphy HC. Physiologic specialization in Puccinia coronata avenae. United States Department of Agriculture, Technical Bulletin No. 433, Washington, D.C 1935.

48. Livak KL, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Method, Methods (San Diego, Calif.) 2001; 25(4):402–408. https://doi.org/10.1016/meth.2001.1262