



Systemic response to *Fusarium graminearum* and *culmorum* inoculations: changes in detoxification of flag leaves in wheat

Ágnes Gallé¹ · Alina Pelsőczy¹ · Dániel Benyó^{1,2} · Anna Podmaniczki² · Ágnes Szabó-Hevér³ · Péter Poór¹ · Beáta Tóth³ · Edit Horváth^{1,2} · László Erdei¹ · Jolán Csiszár¹

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Abstract

Fusarium graminearum and *F. culmorum* cause the most widespread wheat disease Fusarium head blight (FHB). The present study describes that the *Fusarium* inoculation of the wheat spikes caused systemic changes in the key elements of the antioxidant/detoxification defence system in the flag leaf during the grain filling period in wheat lines differing in biotic stress susceptibility to explore changes in some components of the response. According to our data, the inoculation with both *F. graminearum* and *F. culmorum* at the anthesis changed significantly the activities of superoxide dismutase (SOD) and guaiacol peroxidase (POD) enzymes, as well as the glutathione transferase (GST) activity in the flag leaves of the selected wheat lines approx. two weeks later after the infection. In silico approach supported the expressional up-regulation of various *GST* genes upon *Fusarium* infection. Based on our results, *GST* sequences TaGSTF26 and TaGSTU120 were among the series of important stress response genes, which were transcriptionally up-regulated, thus possibly playing a role in the systemic response to *Fusarium* infection, where TaGSTF26 might have an important role in the successful defence. These *GST*s can serve as effective markers of the detoxification process for breeders and plant protection in the future.

Keywords Antioxidant enzymes · *Fusarium graminearum* · *Fusarium culmorum* · Flag leaf · Glutathione transferase · Wheat

Introduction

Among the several factors which lead to a decrease in the expected yield, Fusarium head blight (FHB) is of high importance, as it is the most widespread ear wheat disease (Goswami and Kistler, 2004). FHB can be caused by a variety of fungi, including different *Fusarium* species (Champeil et al. 2004). This disease causes total or partial ear premature senescence followed by a reduction in both crop

yields and grain quality (Blandino et al. 2011). *F. graminearum* and *F. culmorum*, the most important FHB agents, are also the main causes of the accumulation of the type-B trichothecenes deoxynivalenol (DON, Blandino et al. 2011; Bottalico and Perrone, 2002). Besides DON (also known as vomitoxin), *F. graminearum* and *F. culmorum* both produce other trichothecene mycotoxins, like zearalenone, nivalenol, dideoxynivalenol, and 3-acetyldeoxynivalenol. These toxins disrupt normal cell function by inhibiting protein synthesis and cause many diseases in the fed animals (e.g. diarrhoea, emesis, alimentary haemorrhaging and contact dermatitis) or in the infected plants (hypersensitive reaction) (Bennett and Klich, 2003; Yu et al. 2008). Thus, the better understanding of factors that influence *Fusarium* disease development and resistance in wheat have great importance.

The common way to control and reduce the FHB caused by *Fusarium* species is the fungicide application, which is mostly effective but often expensive. A better and more sustainable solution would be the development of FHB-resistant cultivars, which is a high priority breeding objective for wheat breeding programs (Zhou et al. 2005). To fulfil these

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✉ Ágnes Gallé
gallea@bio.u-szeged.hu

- ¹ Department of Plant Biology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, Szeged 6726, Hungary
- ² Institute of Plant Biology, Biological Research Centre, Temesvári krt. 62, Szeged 6726, Hungary
- ³ Cereal Research Non-Profit Ltd, Alsóikötő sor 9, Szeged 6726, Hungary

aims, it is necessary to understand the mechanisms behind the susceptibility and tolerance of different wheat lines (Bernardo et al. 2007). Moreover, this knowledge could serve the ability to select effective stress markers under different *Fusarium* infections for breeding and plant protection.

Wheat plants respond to *Fusarium* infection by inducing defences, including local and systemic reactions. Elements of these processes are parts of plant ‘immunity’, which may lead to resistance against the pathogen. Several classes of systemic response genes have been reported to induce tolerance to FHB in wheat (Sabbagh et al. 2017). One group of genes referred to defence response genes encode proteins with a detoxifying activity whose expression often increases as part of the plant host defence response to pathogen attack. As a result of *F. graminearum* infections, large number of the glutathione transferases (GSTs) were parts of the groups of those differentially expressed genes (Pan et al. 2018), which code proteins with detoxification activity.

Plant glutathione transferases constitute a very ancient protein superfamily which play a role in the detoxification of toxic substances by their conjugation with glutathione, the attenuation of oxidative stress (Gullner et al. 2018). GSTs can be divided into fourteen groups, the two largest plant-specific groups: tau and phi classes participate mainly in conjugating reactions and possess high affinity towards a broad spectrum of harmful compounds such as stress metabolites and toxins (Csiszár et al. 2016). GSTs are widely involved in cellular processes by recognizing and transporting a variety of electrophilic compounds of exogenous or endogenous origins and fulfils other cellular roles, e.g. the zeta class functions in tyrosine catabolism (Csiszár et al. 2016). Wheat GST genes were firstly collected and groups were identified by Gallé et al. (2009). At that time, the full genome sequences of wheat were hardly available, and expressed sequence tags (and the assembly of them: tentative consensus sequences, TIGR Gene Indices, Quackenbush et al. 2000) were used for making a family tree. Ninety-six GST sequences were identified using homology searching. A decade later, the GST sequences were collected by Wang et al. (2019) using Ensembl wheat genome and found 330 wheat GSTs. GSTs were renamed according to their position in the wheat genome. In this study, new names (according to their position in the genome) of the genes (and proteins) were used to analyse their systemic role in the detoxification process upon *Fusarium* infection.

Numerous expression analyses proved that distinct groups of GSTs are markedly induced in the early phase of microbial infections (Gardiner et al. 2010), but the long-term changes and the role of GST in systemic responses received less scientific attention, especially in flag leaves.

In the present study, we aimed to identify candidates of systemic response, and particularly gene products, which are connected to late stress response and show systemic

activation/expression after different *Fusarium* infections of wheat spikes. Key elements of the antioxidant/detoxification defence system such as GSTs were investigated in the flag leaf during the grain filling period in various resistant and susceptible wheat lines. Furthermore, we aimed to connect some information about the possible regulation factors beneath the results, to give insights to the signalling network of wheat systemic response.

Materials and methods

Plants and infection conditions

To figure out whether the *Fusarium graminearum* or *F. culmorum* inoculation caused systemic changes, one resistant and three variously susceptible wheat lines (*Triticum aestivum* L.) were used (Table 1). The FHB-resistant spring wheat landrace ‘Sumai 3’ is highly resistant against FHB and was developed in China (Niwa et al. 2014; Bai and Shaner, 1994). The other wheat lines originated from a crossing inbred population of GK Mini Manó/Nobeokabozu. GK Mini Manó is a resistant line against stem rust (carrying the resistance gene *Sr36*) bred in the Cereal Research Non-Profit Ltd, Szeged, Hungary. Nobeokabozu is a recombinant, FHB-resistant line from Japan, with ‘WSY’, Wangshubai/Suma i3/Yangangfanzhou ancestors.

For our experiments, four field-grown wheat lines were selected. Three of them originated from the crossing GK Mini Manó/Nobeokabozu, and one other line, which was the inbred line of one ancestor of Nobeokabozu: Sumai 3.

The wheat lines were inoculated with *F. graminearum* and *F. culmorum* at or immediately after the anthesis, in the premilk stage described earlier by Szabó-Hevér et al. (2014). The sampling was performed in the dough stage from the five wheat lines on the fields of GK (Fig. 1).

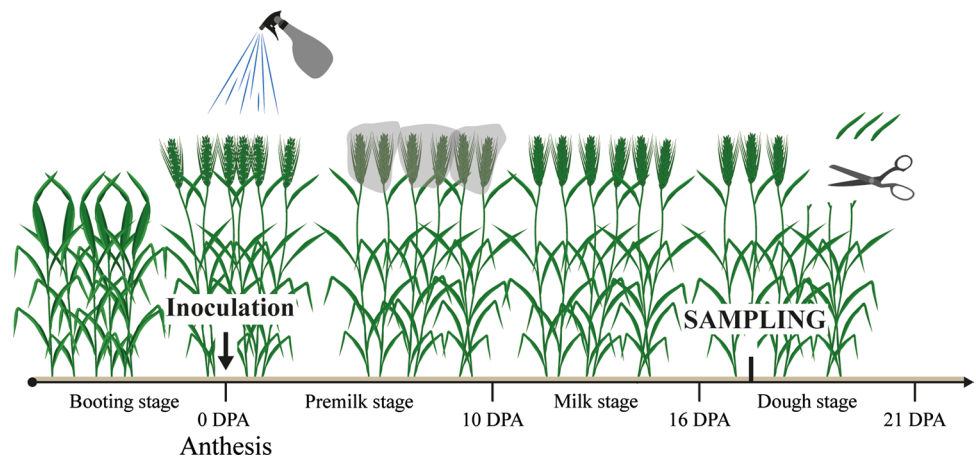
Measurements of the activity of key antioxidant and detoxification enzymes

For the enzyme measurements, tissue homogenization and extraction steps were carried out at 4 °C. Crude protein extracts were prepared by homogenizing 0.2 to 0.5 g of flag

Table 1 Used wheat lines and the predicted FHB tolerance according to previous results

Wheat line on plot number	Predicted FHB tolerance
2147	Sensitive
2148	Sensitive
2149	Sensitive
Sumai 3	Resistant/tolerant

Fig. 1 Time of the inoculation with *F. graminearum* or *F. culmorum* and the time of sampling day. DPA refer to days post-anthesis. Sampling was performed at the dough stage, 18 days after the inoculation



leaf tissues in 2 mL of extraction buffer (0.1 M phosphate buffer pH 7.0, containing 1 mmol L⁻¹ phenylmethylsulfonyl fluoride and 1% polyvinylpyrrolidone). The homogenate was then centrifuged at 10,000 g for 15 min, and the supernatant was used for enzyme assays.

Activities of antioxidant enzymes were performed as was described earlier in Gallé et al. (2009). SOD activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin in light. One unit (U) of SOD was calculated as the amount causing a 50% inhibition of NBT reduction in light. The enzyme activity was measured spectrophotometrically (KONTRON, Milano, Italy) at 560 nm, and results were expressed in terms of the specific activity (U mg⁻¹ protein). CAT activity was determined by the decomposition of H₂O₂ and was measured spectrophotometrically by following the decrease in absorbance at 240 nm. One U means the amount of H₂O₂ (in μmol) decomposed in 1 min. POD activity was determined by monitoring the increase in absorbance at 470 nm during the oxidation of guaiacol. The amount of enzyme producing 1 μmol min⁻¹ of oxidized guaiacol was defined as 1 U. GR activity was determined by measuring the absorbance increment at 412 nm when 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) was reduced by glutathione (GSH), generated from glutathione disulphide (GSSG). The specific activity was calculated as the amount of reduced DTNB, in μmol min⁻¹ protein mg⁻¹ ($\epsilon_{420} = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Activity GST, the key detoxification enzyme, was determined spectrophotometrically (KONTRON, Milano, Italy) by using an artificial substrate, 1-chloro-2,4-dinitrobenzene (CDNB). Reactions were initiated by the addition of CDNB, and the increase in absorbance at 330 nm was detected. One U is the amount of enzyme producing 1 μmol conjugated product in 1 min ($\epsilon_{340} = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

The protein contents of the leaf extracts were determined by the method of Bradford (1976). All chemicals were originated from Sigma-Aldrich (St. Louis, MO, USA).

RNA purification, cDNA synthesis and expression analyses with quantitative real-time PCR

RNA was extracted from flag leaves harvested at the dough stage (17–18 DPA) according to Chomczynski and Sacchi (1987), as was earlier described in Gallé et al. (2009). DNase digestions were applied (Thermo Scientific, Waltham, MA, USA). First-strand cDNA was synthesized using MMLV reverse transcriptase (Thermo Scientific, Waltham, MA, USA). Primers were designed using Primer Express (<https://www.thermofisher.com>) and Primer 3 (bioinfo.ut.ee) software. Primer pairs were shown by Gallé et al. (2009) with one exception (*TaGSTU145*: F: 5'-CCGTGCTCGCTTGGAT-3', R: 5'-GGCCTGAGTCTGTGTGTTTGT-3'). *TaGST* genes were selected according to previous results, literature and expression analysis (Gallé et al. 2009; Wang et al. 2019). *TaGSTU120*, *TaGSTU184*, *TaGSTF26* and *TaGSTZ7* (formal *TaGSTU1B*, *TaGSTU1C*, *TaGST19E50* and *TaGSTZ*, respectively) showed up-regulation in flag leaves due to osmotic and/or drought stress in various stress-tolerant wheat lines (Gallé et al. 2009). *TaGSTF41* (formal *TaGSTA2*) showed highly significant similarity to a pathogen-induced GST (Dudler et al. 1991). Transcript sequencing data originated from Genevestigator (<https://genevestigator.com/gv/>) confirmed the importance of *TaGSTU145* (formal *TaGSTU2*) in *F. graminearum* infection-induced response reactions.

The expression rate of GST genes was monitored by quantitative real-time PCR (BioRad, Hercules, CA, USA) using SYBR green probes (Thermo Scientific, Waltham, MA, USA). Each reaction was repeated at least three times. QRT-PCR was initiated with denaturation at 95 °C for 10 min followed by 41 cycles of denaturation at 95 °C for 15 s and annealing, extension at 60 °C for 1 min. The specificity of the reaction was determined by a melting curve analysis of the product performed immediately after the final PCR cycle by increasing the temperature from 55 to 90 °C (0.2 °C 0.2 s⁻¹). The wheat elongation factor α subunit

(TaEF-1 α) and a gene with unknown function (TaNP-1) were used as reference genes as high and low controls, respectively (Jukanti et al., 2006).

Data were calculated using the $2^{(-\Delta\Delta C_t)}$ formula (Livak and Schmittgen 2001): the additive effect of concentration, gene and replicate, was minimized by subtracting the C_t number of the target gene from that of the reference genes, which yielded ΔC_t . This value was subtracted from all other ΔC_t values, which yield the $\Delta\Delta C_t$ (Yuan and Stewart, 2005). The 2147 untreated sample's transcript amounts were taken as an arbitrary unit.

Expression analysis and heat map construction

Expression data of TaGST transcripts in response to different *Fusarium* infections was retrieved from Genevestigator (<https://genevestigator.com/gv/>; Hruz et al. 2008). Perturbations were followed; fold change in expression as compared to respective untreated/control sample was retrieved and used to generate the heat map (Gallé et al. 2019).

Statistical analysis

Samples were taken in three biological repetitions in two different years. Data presented as means \pm SD resulted from at least three independent measurements. Statistical analysis was carried out with Sigma Plot 11.0 software (Systat Software Inc., Erkrath, Germany). Statistical analyses were performed using Duncan's multiple range comparison test after ANOVA, and differences were considered significant if $P \leq 0.05$.

Results

Spikes of the four selected wheat lines were exposed to *F. graminearum* and *F. culmorum* inoculations after the beginning of the anthesis. Inoculation resulted in visible symptoms of FHB on the wheat spikes of 2147, 2148 and 2149 lines, while did not cause any visible symptoms on Sumai 3 landrace 18 days after the inoculation (data not shown).

The different *Fusarium* infections altered the activity of antioxidant enzymes at the dough stage in the flag leaves of wheat lines. Among the control/untreated wheat lines, the Sumai 3 exhibited the lowest SOD, CAT and POD activities and the highest SOD activity measured in wheat line 2149 (Fig. 2). Differences were detectable between the SOD activity of the untreated and *F. culmorum* inoculated wheat spikes in 2147 and 2148 wheat lines (Fig. 2a). Inoculation with *F. culmorum* resulted in higher SOD activity in these lines, while *F. graminearum* induced SOD in Sumai 3 (Fig. 2a). CAT activity differed between control and both *Fusarium* inoculated samples (the treatment decreased the

CAT activity) in 2148 and 2149 wheat lines, and no changes were measured in Sumai 3 after both fungi inoculations (Fig. 2b). POD activity in the flag leaves of the four lines was elevated by both *Fusarium* infections in almost every sample (Fig. 2c). *F. graminearum* inoculation resulted in differences in GR activity in 2147, 2148 and Sumai 3 lines; *F. graminearum* increased it in the two susceptible lines, and enhanced GR activity was measured in flag leaves of Sumai 3 after *F. culmorum* inoculation (Fig. 2d).

The GST activity towards CDNB artificial substrate differed between the four lines; the conjugating activity of GSTs was higher in Sumai 3's flag leaf than in the other three wheat lines both in treated and untreated samples (Fig. 3). Inoculation with *F. culmorum* increased the GST activity in wheat line 2147, while *F. graminearum* in 2147 and 2149 wheat lines (Fig. 3).

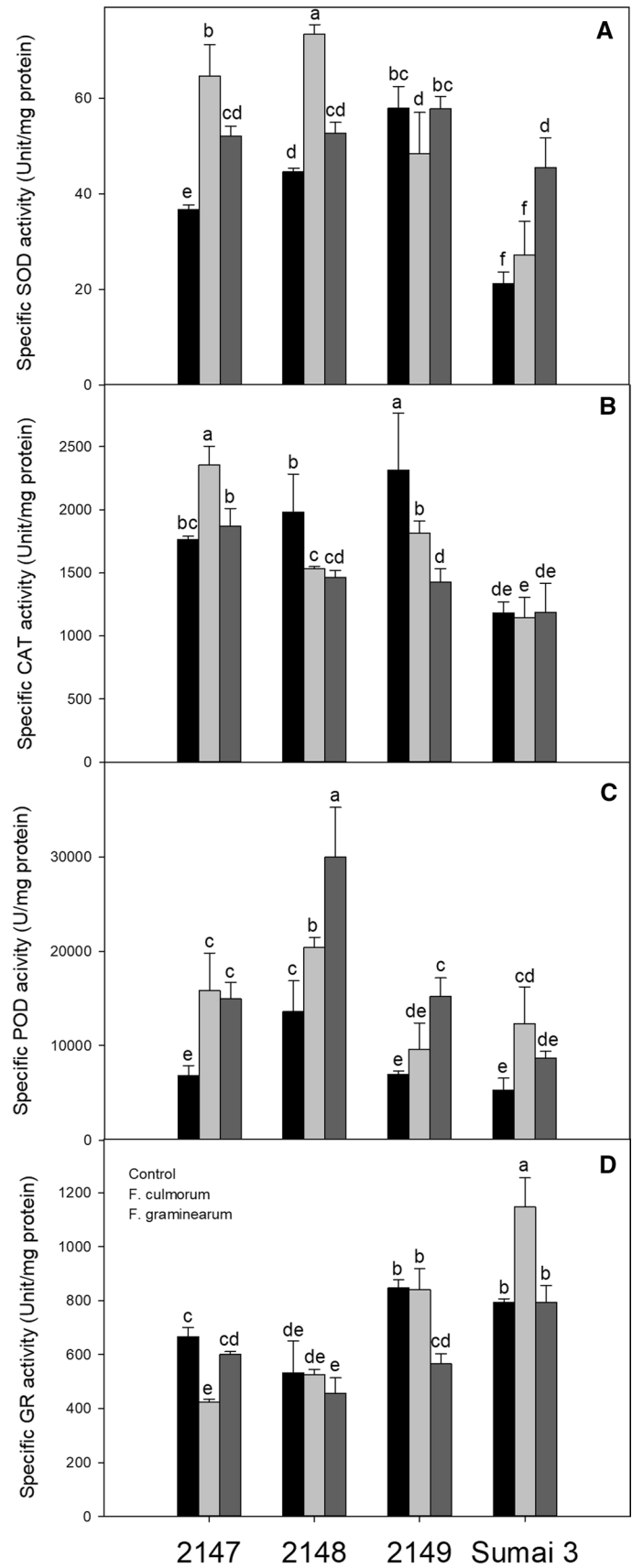
Different *Fusarium* infections can cause also changes in the GST transcript amounts which were confirmed by several in silico available microarray and transcriptome sequencing data (Supplement Fig. 1). Both phi and tau group GST genes (e.g. TaGSTF3 and TaGSTU3, new names: TaGSTF59 and TaGSTU188) were transcriptionally up-regulated by *F. graminearum* infection. According to transcriptome sequencing data, other GSTs (e.g. TaGSTU145) were also up-regulated in wheat exposed to *F. graminearum* (data not shown).

Based on our results, *Fusarium* infection caused several changes in the transcript amount of the selected GST genes (Fig. 4). We could detect differences between TaGSTF26's transcript amounts of the control and the *Fusarium* sp. inoculated samples in all four wheat lines (Fig. 4). The expression of TaGSTU120 was elevated in 2148 and 2149 wheat lines wheat line, especially upon *F. graminearum* infection. Besides the changes detected in TaGSTF26, TaGSTF58 and TaGSTU145 showed *Fusarium*-caused increased expression in 2147. Based on the gene expression analysis, TaGSTU41, TaGST184 and TaGSTZ7 showed the least changes after fungi inoculation in the selected wheat lines.

Discussion

Several studies reported about candidates stimulated in plant systemic biotic stress response; however, more information is available about the elements of local, innate responses (Kachroo and Robin, 2013; Gao et al. 2021). As a network of proteins is related to the innate resistance to *Fusarium* infection, systemic response is considered to be quantitatively governed by numerous resistance genes located on different chromosomes. At least 18 of the 21 wheat chromosomes have been reported to be associated with resistance (Jahoor et al. 2004; Golkari et al. 2007). To understand the signal perception, transduction and reaction of the host plant, the

Fig. 2 Changes in superoxide dismutase SOD; **a**, catalase CAT; **b**, peroxidase POD; **c** and glutathione reductase GR; **d** activities in the flag leaves of wheat lines 2147, 2148, 2149 and Sumai 3. Wheat spikes were inoculated with *F. graminearum* or *F. culmorum* mean \pm SD, n=4). Data were analysed by one-way ANOVA and Duncan’s test. Distinct letters were used to sign mean values considered to be significantly different upon $P < 0.05$



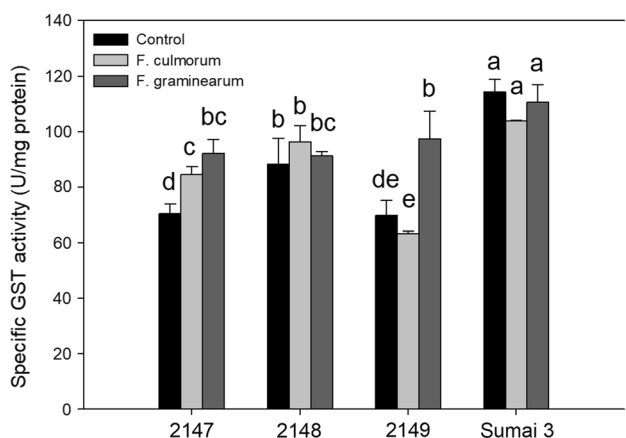


Fig. 3 Changes in the activity of glutathione S-transferase GST in the flag leaves of wheat lines 2147, 2148, 2149 and Sumai 3. Wheat spikes were inoculated with *F. graminearum* or *F. culmorum* mean \pm SD, $n=4$). Data were analysed by one-way ANOVA and Duncan's test. Distinct letters were used to sign mean values considered to be significantly different upon $P < 0.05$

components of the defence reactions have been extensively investigated with both protein and transcript profiling.

Searching for proteins involved in the plant's response to FHB (anti-fungal candidates) dates back to 2000 (Doohan et al. 2000; Pritsch et al. 2000; Zhou et al. 2005). Among the several response genes, proteins with an antioxidant function such as SOD, dehydroascorbate reductase and GSTs were induced after inoculation with *F. graminearum*, indicating an oxidative burst of H_2O_2 inside the tissues infected by FHB (Zhou et al. 2005). But there was less information about the up-regulation of these genes or gene products in the distal part of the wheat plants (e.g. flag leaf). According to our results, it can be concluded that flag leaves of susceptible wheat lines showed higher SOD and POD and decreased CAT activity compared to the resistant wheat line, which tendencies in the antioxidant enzyme activities were dependent on *Fusarium* species.

Besides the elimination of oxidative stress metabolites, the detoxification of fungal toxins (mycotoxins) is a task of host GSTs also in fungus-infected plants. The hemibiotrophic *F. graminearum* and *F. culmorum* are producing trichothecenes toxins. Treatment of barley spikes with DON led to the marked up-regulation of gene transcripts encoding, e.g. GSTs. The GSH conjugating activity towards deoxynivalenol was proven as the formation of DON-GSH conjugates was also observed. These results showed that detoxification by GSH-conjugation may reduce the impact of trichothecenes in crops (Gardiner et al. 2010; Gullner et al. 2018). Numerous transcriptome investigations reported that distinct groups of GSTs are markedly induced in the early phase of bacterial, fungal and viral infections (Gullner et al. 2018). Up-regulation of GSTs has been observed

in FHB-resistant as well as FHB-susceptible wheat material following *Fusarium* infection, but increased levels of specific GST family members have been observed in wheat and barley in association with FHB resistance (Foroud et al. 2012; Kugler et al. 2013; Huang et al. 2016; Dhokane et al. 2016; Pan et al. 2018). First of all, Erayman et al. (2015) found one FHB-induced GST in the susceptible line, while six more in the moderately susceptible ones. In contrast, Pan et al. (2018) identified differentially expressed genes up-regulated by *F. graminearum* and found a group of 261 genes. Among the transcripts up-regulated after *Fusarium* inoculation in three resistant genotypes, but showed no significant changes in the susceptible ones, there were three GSTs. GSTs can modulate the redox state of GSH, thus participate in the regulation of early signalling events in biotic stresses such as fungal infections (Pan et al. 2004, 2018; Mou et al. 2003). The primary activity of GSTs is the detoxification of toxic substances by their conjugation with GSH, thus can eliminate the harmful by-products of biotic- and oxidative stress. Further, GSTs may participate in hormone transport, and some GSTs display glutathione peroxidase activity, and these GSTs can detoxify toxic lipid hydroperoxides that accumulate during infections (Csiszár et al. 2016; Gullner et al. 2018; Gallé et al. 2019). These findings underline the importance of GSTs not only in the successful defence reactions, but also in the early signalling in infected tissues. However, less information is available about the role of GSTs in systemic reactions.

According to our results, systemic responses were observed in the activity and expression of some stress-related GSTs in the flag leaves of the selected wheat plants. *Fusarium* resistance was in correlation with higher basal GST activity in the flag leaves of different wheat lines after the inoculation of wheat spikes. At the same time, inoculation of wheat spikes with *Fusarium*, especially with *F. graminearum* resulted in higher GST activity in the flag leaves of susceptible wheat lines.

GST isoenzymes differ in their catalytic activity against CDNB artificial substrate. Some of the GST homodimers especially in the tau and phi groups (e.g. TaGSTU2-2, the new name of the gene: TaGSTU145) exhibit high GSH conjugating activity against CDNB (Cummins et al., 2003; Thom et al. 2002). The catalytic activity against stress metabolites or analogues can give a detailed definition of the roles of each member of GST isoenzyme family under stress conditions. TaGSTU1-1 (new names of the genes: TaGSTU120 and TaGSTU184) and TaGSTF6 (the new name of the gene: TaGSTU58) show relatively high conjugating activity against crotonaldehyde (stress metabolite analogue) and peroxidase activity against cumene hydroperoxide (lipid peroxide model substrate), which underlines the importance of these isoenzymes under oxidative stress conditions. The induction of GST isoenzymes or coding genes

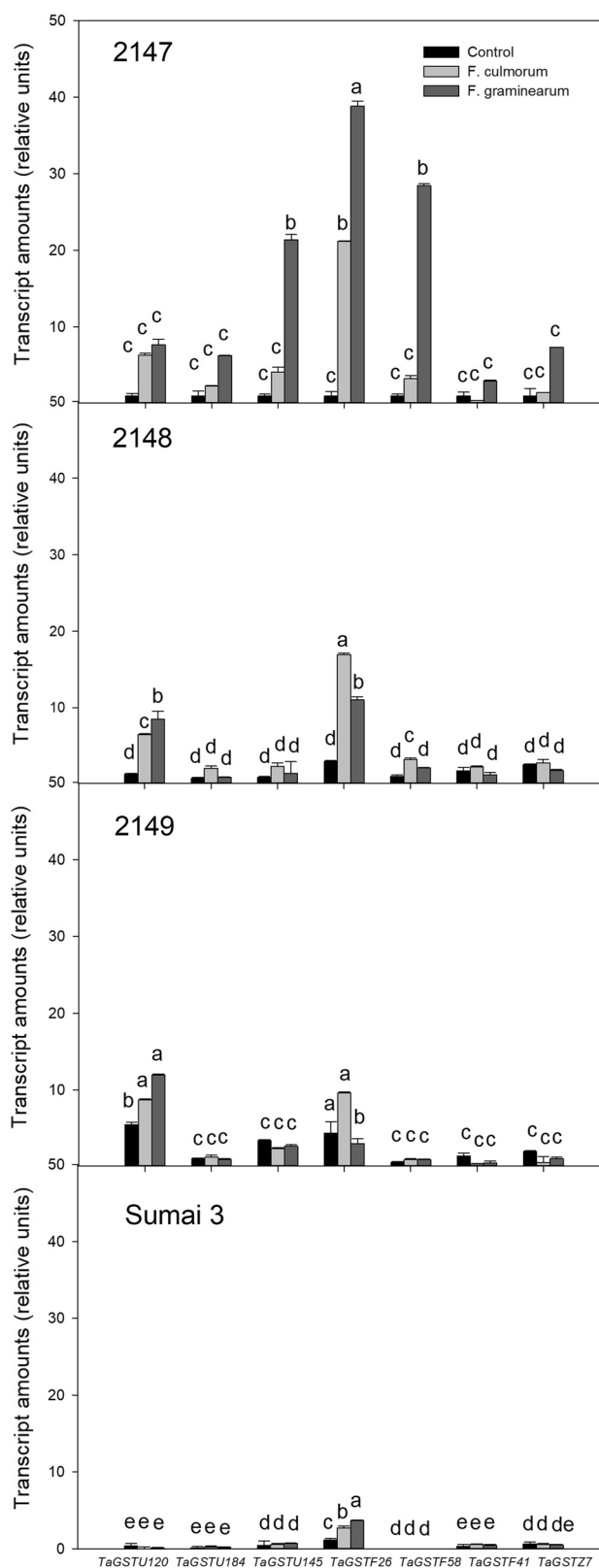
Fig. 4 Changes in the transcript levels of selected glutathione S-transferase GST genes (tau group: TaGSTU120, TaGSTU184 and TaGSTU145; phi group: TaGSTF26, TaGSTF58, TaGSTFA2 and TaGSTZ71) in the flag leaves of wheat lines 2147 **a**, 2148 **b**, 2149 **c** and Sumai 3 **d**. Wheat spikes were inoculated with *F. graminearum* or *F. culmorum* mean ± SD, n=4). Data were analysed by one-way ANOVA and Duncan’s test. Distinct letters were used to sign mean values considered to be significantly different upon $P < 0.05$

can provide further information about their possible roles under stress and can serve as a marker of stress responses of plants. Earlier it was found that TaGSTU120, TaGSTU184, TaGSTF26 and TaGSTZ7 were up-regulated in flag leaves due to osmotic and/or drought stress in various stress-tolerant wheat lines (Gallé et al. 2009). In addition, TaGSTU41 was shown also as a significant pathogen-induced GST in wheat (Dudler et al. 1991). Transcript sequencing data originated from Genevestigator confirmed this importance of TaGSTU145 in *F. graminearum* infection-induced response reactions of wheat. Based on our result, TaGSTU120, TaGSTU184, TaGSTU145, TaGSTF26, TaGSTU41 and TaGSTZ71 were likely to be involved in FHB systemic resistance in flag leaves of wheat lines. TaGSTF26 was transcriptionally up-regulated in both resistant and sensitive lines after both *Fusarium* inoculations, while TaGSTU120 was only induced in the sensitive wheat lines. The observed transcriptional differences between the resistant and sensitive lines indicate less severe stress reaction in the resistant, Sumai 3 wheat line, which can be a result of lower extended infection. At the same time, there cannot be found any relevant differences between the effects of two *Fusarium* species on the expression of GSTs in the selected wheat lines.

Wang et al. (2019) collected the cis regulatory elements of the GST genes in wheat. According to their findings the cis regulating elements in the 5’ flanking region of the selected GST genes were gathered (Supplement Table 1). Methyl jasmonate and light-responsive elements were abundantly found in the promoter region of those GST genes, which showed different expressions due to *Fusarium* infection. Earlier the role of jasmonates was demonstrated in the induction of rapid systemic responses of Arabidopsis plants (Truman et al. 2007), and it was found that GSTs are regulated by jasmonic acid in wheat (Li et al. 2013).

Based on our results, TaGSTU120 and TaGSTF26 were among the series of important response genes, which were transcriptionally up-regulated, thus can play a role in the systemic response to *Fusarium* inoculation, where TaGSTF26 might have an outstanding role in the successful defence in the case of *Fusarium* infection. These GSTs can serve as effective markers of the detoxification process for breeders and plant protection in the future.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s42976-022-00272-3>.



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Declarations

Conflict of interest Ágnes Gallé is member of the Cereal Research Communications Editorial Board.

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