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RESEARCH ARTICLE

Heat shock-induced enhanced susceptibility of barley to *Bipolaris sorokiniana* is associated with elevated ROS production and plant defence-related gene expression

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barley; *Bipolaris sorokiniana*; combined stress resistance; heat shock; reactive oxygen species.

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ABSTRACT

- Heat stress alters plant defence responses to pathogens. Short-term heat shock promotes infections by biotrophic pathogens. However, little is known about how heat shock affects infection by hemibiotrophic pathogens like *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*). We assessed the effect of heat shock in *B. sorokiniana*-susceptible barley (*Hordeum vulgare* cv. Ingrid) by monitoring leaf spot symptoms, *B. sorokiniana* biomass, ROS and plant defence-related gene expression following pre-exposure to heat shock.
- For heat shock, barley plants were kept at 49 °C for 20 s. *B. sorokiniana* biomass was assessed by qPCR, ROS levels determined by histochemical staining, while gene expression was assayed by RT-qPCR.
- Heat shock suppressed defence responses of barley to *B. sorokiniana*, resulting in more severe necrotic symptoms and increased fungal biomass, as compared to untreated plants. Heat shock-induced increased susceptibility was accompanied by significant increases in ROS (superoxide, H_2O_2). Transient expression of plant defence-related antioxidant genes and a barley programmed cell death inhibitor (*HvBI-1*) were induced in response to heat shock. However, heat shock followed by *B. sorokiniana* infection caused further transient increases in expression of *HvSOD* and *HvBI-1* correlated with enhanced susceptibility. Expression of the *HvPR-1b* gene encoding pathogenesis-related protein-1b increased several fold 24 h after *B. sorokiniana* infection, however, heat shock further increased transcript levels along with enhanced susceptibility.
- Heat shock induces enhanced susceptibility of barley to *B. sorokiniana*, associated with elevated ROS levels and expression of plant defence-related genes encoding antioxidants, a cell death inhibitor, and PR-1b. Our results may contribute to elucidating the influence of heat shock on barley defence responses to hemibiotrophic pathogens.

INTRODUCTION

Field crops simultaneously face abiotic and biotic stresses that have a profound impact on plant physiology and growth. Abiotic stressors dynamically influence plant defence responses, significantly affecting the outcome of infections (Desaint *et al.* 2021; Zarattini *et al.* 2021). Heat stress is one of the most severe abiotic environmental factors limiting crop growth, productivity, and influencing plant defence against pathogens (Desaint *et al.* 2021). Because of global climate change, unfavourable effects of heat stress on plant resistance are exprected to be more frequent, together with increases in crop diseases and emergence of new pathogens (Kumar *et al.* 2022).

Barley (*Hordeum vulgare* L.) is the fourth most important cereal worldwide, and heat stress fundamentally affects production (Dawood *et al.* 2020). Barley is attacked by pathogens with different lifestyles, such as the biotrophic pathogen barley powdery mildew (*Blumeria hordei* syn: *B. graminis* f. *sp. hordei*), which colonizes susceptible plants and derives nutrients and energy from living host cells (Barna *et al.* 2012). Conversely, necrotrophic pathogens, such as *Pyrenophora teres* f. *teres*, which causes net blotch in barley, derive energy from dead or dying cells (Backes *et al.* 2021). We focused on a hemibiotrophic pathogen *Bipolaris sorokiniana*, which causes common root rot, leaf spot, seedling blight, head blight, and black point of several cereals, including barley (Kumar *et al.* 2002). This disease mainly occurs in warm and humid regions, however, because of global climate change, *B. sorokiniana* now also causes serious infections in temperate areas (Kumar *et al.* 2020). As a hemibiotrophic pathogen, *B. sorokiniana* has an initial biotrophic and a subsequent necrotrophic growth phase. During the biotrophic phase, the fungus breaches the

outer barrier of epidermal cells – cuticle and cell wall – and produces hyphae within invaded, living epidermal cells. Hyphal invasion into the mesophyll layer, followed by epidermal and mesophyll cell death caused by toxins, are characteristics of the necrotrophic phase of *B. sorokiniana* pathogenesis (Kumar *et al.* 2002).

It is still an open question how heat stress influences plant defence responses to pathogens with different lifestyles. Based on the duration of a temperature rise, short-term heat shocks and long-term heatwaves can be distinguished (Jagadish *et al.* 2021). Barley plants exposed to heat stress (36 °C for 30, 60, or 120 min) prior to barley powdery mildew inoculation acquire enhanced susceptibility in both resistant and susceptible cultivars (Schwarzbach 2001). Prolonged heat stress (24, 48, and 120 h at 35 °C) enhances susceptibility to powdery mildew in the susceptible barley line, MvHV 118–17 (Schwarczinger *et al.* 2021). Furthermore, heat stress (48 and 120 h) enhanced powdery mildew symptoms and biomass in both resistant and susceptible cv. Ingrid near isogenic barley lines (Kolozsváriné *et al.* 2022). Continuous high temperature (30 °C) inhibited expression of disease resistance genes (*RPW8.1* and *RPW8.2*) in *Arabidopsis thaliana* and resulted in a reduction in the hypersensitive response (HR) and enhanced spread of the powdery mildew pathogen, *Erysiphe cichoracearum* (Xiao *et al.* 2003). Prolonged heat stress that inhibits HR can result in increased accumulation of biotrophic pathogens in the host-pathogen interactions of tomato-*Cladosporium fulvum* (de Jong *et al.* 2002) and maize-*Puccinia sorghi* (Negeri *et al.* 2013). In rare cases, prolonged heat stress either does not affect or positively influences plant resistance. For example, a powdery mildew resistant barley line (MvHV07-17) maintained resistance during a 5-day heat stress at 35 °C (Schwarczinger *et al.* 2021). Rice with a *Co39* genetic background became resistant to *Magnaporthe oryzae* after plants were exposed to 35 °C (Onaga *et al.* 2017). However, in general, prolonged heat stress has a negative effect on plant disease resistance (de Jong *et al.* 2002; Xiao *et al.* 2003; Negeri *et al.* 2013; Schwarczinger *et al.* 2021; Kolozsváriné *et al.* 2022).

Interestingly, a short-term heat shock can also have a significant impact on plant–pathogen interactions. Submerging plants in water at 48–49 °C for 20 s increased susceptibility of near-isogenic barley lines to barley powdery mildew. In genetically susceptible barley cv. Ingrid, heat shock further increased susceptibility, while powdery mildew-resistant barley lines became susceptible, with elevated pathogen levels and increased disease spread (Barna *et al.* 2014; Künstler *et al.* 2018). A short-term heat shock (49 °C, 45 s) partially suppresses symptomless nonhost resistance of barley to wheat powdery mildew (Künstler *et al.* 2018). In some cases, however, short-term high temperature treatment may increase resistance to plant pathogens. In barley cv. Golden Promise a 50 °C heat shock for 60 s induced resistance to the powdery mildew pathogen *B. hordei* (Vallélian-Bindschedler *et al.* 1998). A short-term heat shock (50 °C, 20 s) in strawberry infected with the fungal pathogen *Podosphaera aphanis* induced defence responses and disease resistance (Sato *et al.* 2018).

Temperature perception and signalling at the cellular level substantially overlaps with pathogen recognition and is generally associated with the production of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). In heat-shocked barley plants infected with the biotrophic powdery mildew, enhanced susceptibility is coupled to a decrease in ROS levels (Barna *et al.* 2014; Künstler *et al.* 2018). Presumably, decreases in ROS in heat-shocked and powdery mildew-inoculated barley facilitate infection. On the other hand, tissue necrosis caused by increased ROS levels during pathogen infections may not favour biotrophic pathogens, but will favour necrotrophic pathogens (Barna *et al.* 2012).

The objective of the present study was to investigate how a short-term heat shock (49 °C, 20 s) influences infection of the hemibiotrophic pathogen *B. sorokiniana* in susceptible barley cv. Ingrid. Furthermore, we assessed: (i) whether heat shock pretreatment and *B. sorokiniana* inoculation is accompanied by changes in ROS levels; and (ii) how expression of certain defence genes changes in response to heat shock followed by *B. sorokiniana* inoculation.

MATERIAL AND METHODS

Plant and pathogen

Barley (*Hordeum vulgare* cv. Ingrid) plants were grown in controlled environmental chambers (20 °C day and 18 °C night, 60% relative humidity, 16-h light/8-h dark photoperiod, with 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light). *B. sorokiniana* strain H-299 (NCBI GenBank accession no. MH697869), originating from barley in Hungary, was used as source pathogen. The fungus was maintained until sporulation in the dark at 18–20 °C in Petri dishes containing Difco potato dextrose agar (PDA) (Becton and Dickinson, Franklin Lakes, NJ, USA) prepared by dissolving 39 g PDA in 1 L distilled water.

Heat shock treatment of barley leaves

The first leaves of 1-week-old intact barley seedlings were immersed in a 49 °C water bath for 20 s, 2 h before *B. sorokiniana* inoculation (Barna *et al.* 2014). Prior to inoculation, untreated plants were maintained in controlled environment chambers under the conditions described above.

Plant inoculation

Heat-shocked or untreated first leaves of 1-week-old Ingrid barley seedlings were inoculated with a *B. sorokiniana* conidial suspension 2 h after heat shock. For the inoculum, sterile tap water was added to 21-day-old sporulating *B. sorokiniana* cultures (6 ml tap water per 9-cm Petri dish) on PDA plates and conidia removed by agitating the mycelium mat with a sterile Drigalski spreader. The conidial suspension was filtered through two layers of sterile cheese cloth and the concentration adjusted to 6×10^4 ml^{-1}. Plants were inoculated with the conidial suspension using a sterile paintbrush and brushing leaves equally. For mock inoculation, leaves were brushed with sterile tap water. After *B. sorokiniana* or mock inoculation, plants were kept in a dark wet chamber for 24 h at 20 °C and 100% humidity, which is a suitable environment for *B. sorokiniana* infection. Following this, plants were returned to the controlled environment chambers under the conditions mentioned above. Formation of leaf spot symptoms on inoculated leaves was documented 7 days after inoculation.

Detection of ROS (superoxide and H_2O_2) by tissue staining methods

Superoxide ($O_2^{\bullet -}$) in barley leaves was detected by histochemical staining with 1 mg·ml^{-1} nitro blue tetrazolium chloride (NBT) (Merck, Darmstadt, Germany) dissolved in 0.01 M potassium phosphate buffer (pH 7.8) by using a vacuum infiltration method according to the procedure of Ádám *et al.* (1989). NBT infiltration was performed 3, 6, 24 and 48 h after inoculation. Infiltrated leaves were incubated for 20 min in daylight and subsequently cleared in 0.15% (w/v) trichloroacetic acid in ethanol/chloroform (4/1, v/v). Cleared leaf samples were stored in glycerol/water (1/1, v/v) until scanning. Cleared leaves were scanned and the resulting image files analysed with ImageJ software (https://imagej.nih.gov/ij/) by quantifying NBT stain intensity as a percentage of maximum pixel intensity.

Changes in H_2O_2 levels were detected by histochemical staining with 1 mg·ml^{-1} 3,3 –diaminobenzidine tetrahydrochloride dihydrate (DAB) (Merck) dissolved in sterile distilled water, and pH adjusted to 3.0 with HCl (Thordal-Christensen *et al.* 1997). DAB infiltration was performed 3, 6, 24 and 48 h after inoculation using vacuum infiltration (Ádám *et al.* 1989). Infiltrated leaves were incubated for 2 h in daylight and cleared as described above. Cleared leaf samples were stored in glycerol/water (1/1, v/v) until scanning. Leaf images were analysed with ImageJ by quantifying DAB staining intensity as a percentage of maximum pixel intensity.

Quantitative analyses of *B. sorokiniana* biomass

To analyse *B. sorokiniana* biomass, *B. sorokiniana* inoculated barley leaves (heat shock or untreated) were sampled (20 primary leaves from individual plants, randomly selected and pooled per treatment) from plants 7 days after inoculation in liquid nitrogen. Collected leaves were ground in liquid nitrogen and total genomic (plant and fungal) DNA isolated from 200 mg leaf tissue using the Plant Genomic DNA Extraction Miniprep System Kit (Viogene BioTek, New Taipei City, Taiwan) according to the manufacturer's instructions. For the quantification of *B. sorokiniana* biomass, a quantitative real-time polymerase chain reaction (qPCR) protocol was applied using the 2X SYBR FAST Readymix reagent (KAPA Biosystems, Wilmington, MA, USA) to detect the presence of the *B. sorokiniana Glyceraldehyde 3-phosphate dehydrogenase* (*BsGAPDH*) gene, together with the barley reference gene *Ubiquitin* (*HvUbi*). For description of primers used in qPCR, see Table S1. The *BsGAPDH* primers used in this study have been designed by us with the aid of the Primer Premier 5 program (Premier Biosoft International, San Francisco, CA, USA), while primers for the barley reference gene *HvUbi* were previously described by Trujillo *et al.* (2006). The PCR reaction mix contained 7.5 µl KAPA SYBR FAST qPCR Master Mix (2×), 0.75 µl 5 µM forward and reverse primers each, 3.5 µl PCR grade water, and 2.5 µl 20-fold diluted DNA in a total reaction volume of 15 µl. DNA amplifications were performed in a Bio-Rad CFX-96 real-time thermocycler (Bio-Rad, Hercules, CA, USA), running a standard program (95 °C for 5 min, 40 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s) followed by a melting curve analysis to determine amplicon specificity using a temperature range of 65–95 °C with increments of 0.5 °C. Three independent biological experiments were performed with three technical replicates per biological sample. In each run, water-only controls were used as negative controls. Normalization of *B. sorokiniana* DNA levels to plant DNA was carried out with the aid of the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008) using the reference gene *HvUbi* as internal control.

Evaluation of barley defence gene expression in response to heat shock and *B. sorokiniana*

To monitor the relative expression of different barley defence genes (*Superoxide dismutase*, *HvSOD*; *Cytosolic Dehydroascorbate reductase*, *HvDHAR Cyt*; *BAX inhibitor-1*, *HvBI-1* and *Pathogenesis related-1b*, *HvPR-1b*) *B. sorokiniana* or mock-inoculated barley leaves (exposed to heat shock or untreated) were sampled (20 primary leaves from individual plants were randomly selected and pooled per treatment) from plants at 0, 3, 6, 9 and 24 h after inoculation in liquid nitrogen. Collected leaves were ground in liquid nitrogen and total RNA was isolated from 100 mg leaf tissue using the Plant Genomic RNA Extraction Miniprep System Kit (Viogene BioTek) according to the manufacturer's instructions. RNA isolation was followed by DNAse I treatment with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) and RNA degradation was checked using formaldehyde agarose gel electrophoresis of total RNA. RNA quantity and quality were assessed with a MaestroNano Spectrophotometer (Maestrogen, Hsinchu City, Taiwan). A total of 1000 ng DNAse I treated total RNA was used for reverse transcription (RT) in each sample. RT was executed with a RevertAid™ H$^-$ cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to instructions of the manufacturer. A pool of randomly selected RNA samples was applied to which no reverse transcriptase was added as a negative control. The relative expression of selected barley defence genes (*HvSOD*, *HvDHAR Cyt*, *HvBI-1* and *HvPR-1b*) was detected with the 2× SYBR FAST Readymix reagent (KAPA Biosystems). The PCR reaction mix contained 7.5 µl KAPA SYBR FAST qPCR Master Mix (2×), 0.75 µl 5 µM forward and reverse primers each, 3.5 µl PCR-grade water, and 2.5 µl 20-fold diluted cDNA in a total reaction volume of 15 µl. Amplification and melting curve analysis were performed as described previously. All reactions were performed using three independent biological experiments with three technical replicates per biological sample. In each run, water-only controls and non-reverse-transcribed RNA were used as negative controls. The primer efficiencies for the genes tested were between 101–105%. Changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008) using a barley ubiquitin gene (*HvUbi*) as reference. Previous research has demonstrated that *HvUbi* is a reliable reference gene for detecting gene expression changes in barley exposed to either heat stress or *B. sorokiniana* infection (Sarkar *et al.* 2019). Testing the suitability of *HvUbi* as a reference gene in our experimental system was performed by analysing the cycle threshold (CT) variation of *HvUbi* expression in response to heat treatments and *B. sorokiniana* infection. Significant changes in CT values were not observed for *HvUbi* transcription in response to treatments. Primers used in qPCR are shown in Table S1. All primers were designed by us with the aid of the Primer Premier 5 program (Premier Biosoft International), except the primers amplifying the reference gene *HvUbi* (Trujillo *et al.* 2006).

Statistical analyses

Statistical analyses were performed using the Statistica 13 software (TIBCO Software, Palo Alto, CA, USA). Pixel intensity values (for NBT and DAB staining) *B. sorokiniana* DNA levels and barley relative gene expression values were log-transformed to achieve normality and homogeneity of variances. ANOVA and Tukey's *post-hoc* test were employed, and differences at $P \leq 0.05$ were considered statistically significant.

RESULTS

Evaluation of *B. sorokiniana* symptoms and biomass in response to heat shock in barley cv. Ingrid

To determine how heat shock influences the defence responses of barley cv. Ingrid to *B. sorokiniana* infection we pretreated the plants at 49 °C for 20 s, 2 h before inoculation. Our results showed that heat shock significantly increase the intensity of necrotic symptoms in barley leaves as compared to control plants kept at 20 °C (Fig. 1a, b). The necrotic symptoms caused by *B. sorokiniana* appeared 4 or 5 days after inoculation in both untreated and heat shocked plants; however, the differences between necrotic symptoms became conspicuous 7 days after inoculation (Fig. 1a, b). In non-inoculated plants, heat shock did not cause any visible effects on growth and development (data not shown). To verify if the increase in the intensity of necrotic symptoms is accompanied by an increase in the biomass of *B. sorokiniana*- (fungal growth), we assessed the relative quantity of the *B. sorokiniana glyceraldehyde 3-phosphate dehydrogenase* (*BsGAPDH*) gene using qPCR. Our results showed that heat shock causes a significant increase in the amount of *B. sorokiniana* biomass as compared to untreated plants held at 20 °C (Fig. 1c). Overall, it seems that heat shock has a significant impact on the defence responses of barley to a hemibiotrophic fungal pathogen, *B. sorokiniana* by increasing both the severity of necrotic symptoms and the amount of fungal biomass.

Changes in levels of ROS following heat shock in barley cv. Ingrid infected with *B. sorokiniana*

Heat stress often leads to the production of ROS in plant tissues (Hameed *et al.* 2012). Furthermore, ROS participate in the hypersensitive resistance response (HR), and also in the development of tissue necrotization during a successful infection (Barna *et al.* 2012). Monitoring ROS during simultaneous heat shock and *B. sorokiniana* infection may provide valuable information on barley defence responses. Accumulation of one of the most important ROS, superoxide ($O_2^{\bullet -}$), was detected by

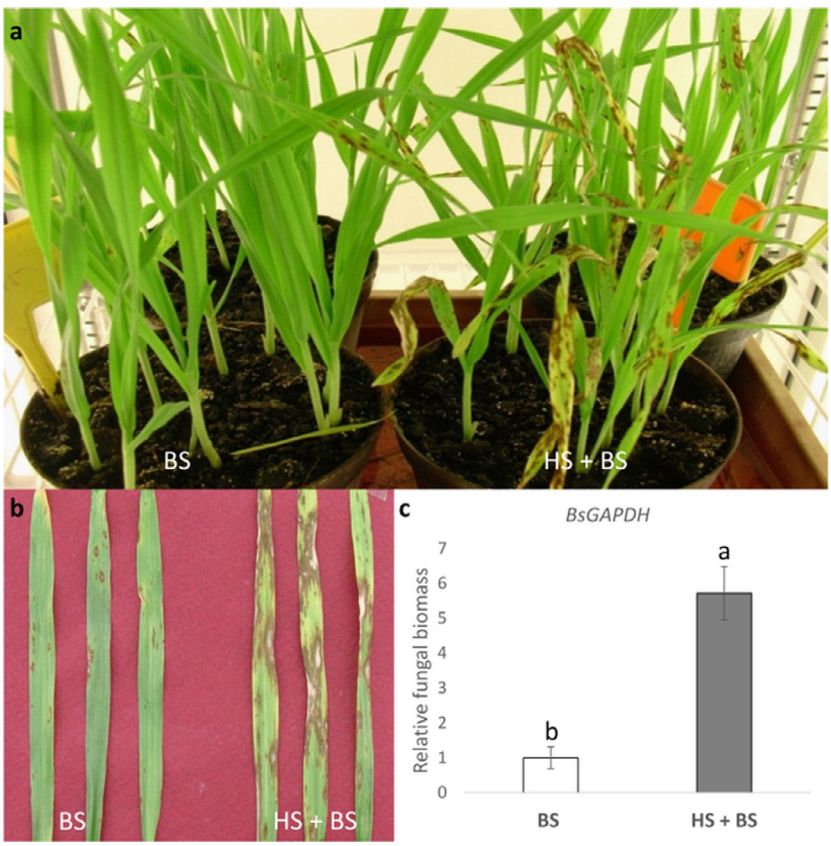

Fig. 1. Symptoms of *Bipolaris sorokiniana* infection (a, b) and biomass (c) in barley (*Hordeum vulgare* cv. Ingrid) in response to heat shock 7 days after *B. sorokiniana* inoculation. Barley plants were exposed to heat shock at 49 °C for 20 s, 2 h before *B. sorokiniana* inoculation. Control plants were kept at 20 °C. Vertical bars indicate relative fungal biomass by assaying the presence of the *B. sorokiniana glyceraldehyde 3-phosphate dehydrogenase* (*BsGAPDH*) gene (c). Photographs are from one representative experiment, which was repeated three times with similar results. The bars show the average of three experiments. Error bars represent standard deviation. Different letters above bar graphs indicate statistically significant differences at $P \leq 0.05$.

NBT histochemical staining in barley leaves 3, 6, 24 and 48 h after *B. sorokiniana* inoculation (HAI). Mock inoculation did not change the levels of superoxide as compared to uninoculated plants. Our results showed that at early time points (3 and 6 HAI) only the combined effect of heat shock and *B. sorokiniana* inoculation results in a detectable increase in superoxide production as compared to heat shock or *B. sorokiniana* treatments alone (Fig. 2, Table 1). On the other hand, at later time points (24 and 48 HAI), heat shock slightly increased the amount of superoxide in uninoculated or mock-inoculated leaves. In addition, *B. sorokiniana* inoculation without heat shock substantially increases the levels of superoxide 24 and 48 HAI as compared to the uninoculated and mock-inoculated plants, and the amount of $O_2^{\bullet -}$ doubled between the first and second day as the *B. sorokiniana* infection progressed. Remarkably, heat shock treatment combined with *B. sorokiniana* inoculation drastically increased superoxide levels as compared to untreated but *B. sorokiniana* inoculated plants, especially 1 day after inoculation (Fig. 2, Table 1). At early time points, a similar trend was observed for another important ROS, H_2O_2, except at 6 HAI, where *B. sorokiniana* inoculation itself could already confer a detectable increase in H_2O_2 (Fig. 3, Table 1). At later time points (24 and 48 HAI) *B. sorokiniana* inoculation without heat shock further increased the levels of H_2O_2 as compared to the uninoculated and mock-inoculated plants. Although heat shock marginally increased the levels of H_2O_2 in uninoculated and mock-inoculated plants, heat shock combined with *B. sorokiniana* inoculation further elevated H_2O_2 levels, especially 2 days after inoculation (Fig. 3, Table 1). In summary, *B. sorokiniana* inoculation increased levels of two important ROS in infected barley plants and heat shock pretreatment further increased ROS generation in *B. sorokiniana*-inoculated plants.

Changes in expression of defence-related genes upon heat shock in barley cv. Ingrid infected with *B. sorokiniana*

To assess the mechanisms of how heat shock enhances *B. sorokiniana* infection in barley we assayed expression of different defence-related barley genes in mock-inoculated and *B. sorokiniana* infected plants previously exposed to high temperature. Since heat shock significantly increased the amount of ROS in *B. sorokiniana* infected samples, we investigated expression of antioxidant genes responsible for regulation of ROS. Superoxide dismutase (SOD, EC 1.15.1.1) is responsible for catalysis of the dismutation of superoxide to H_2O_2, thereby regulating the redox status of plant cells. The expression of barley *HvSOD*, which encodes a cytosolic Cu/ZnSOD, was detected at early time points after inoculation, and our results showed that heat shock significantly induced expression of *HvSOD* in both

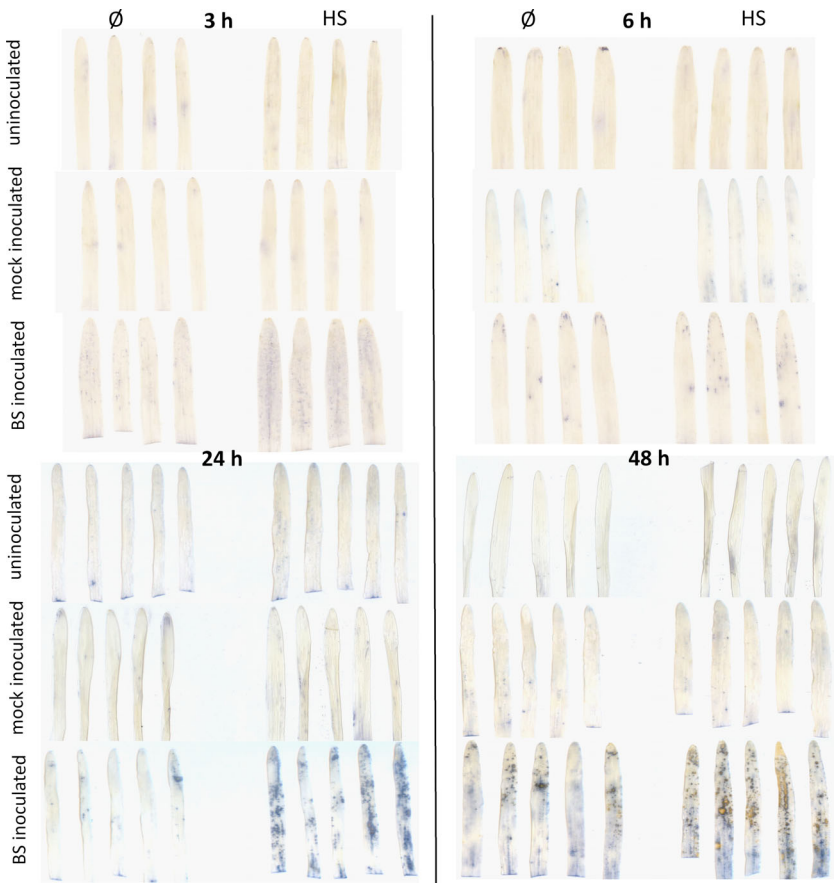

Fig. 2. Accumulation of superoxide ($O_2^{\bullet -}$) in barley (*Hordeum vulgare* cv. Ingrid) leaves 3, 6, 24 and 48 h after inoculation. Uninoculated, mock-inoculated and *Bipolaris sorokiniana*-inoculated barley plants were exposed to heat shock at 49 °C for 20 s 2 h before inoculation. Untreated control plants were held at 20 °C (Ø). Detection of superoxide was visualized by NBT tissue staining. Photographs are from one representative experiment; the experiment was repeated three times with similar results.

Table 1. Superoxide ($O_2^{\bullet-}$) and H_2O_2 accumulation in uninoculated, mock-inoculated and *Bipolaris sorokiniana*-inoculated (BS) barley leaves in response to heat shock, 3, 6, 24 and 48 h after inoculation.

	NBT (superoxide)[a]		DAB (H_2O_2)[a]	
	3 h	6 h	3 h	6 h
Uninoculated				
Ø	3.51 ± 0.41% g	3.32 ± 0.31% g	3.34 ± 0.46% f	3.87 ± 0.48% ef
HS	3.79 ± 0.57% g	3.71 ± 0.48% g	3.40 ± 0.52% f	4.14 ± 0.56% ef
Mock-inoculated				
Ø	3.40 ± 0.32% g	3.42 ± 0.43% g	3.56 ± 0.45% f	3.81 ± 0.33% ef
HS	3.44 ± 0.39% g	3.48 ± 0.37% g	3.61 ± 0.62% ef	4.00 ± 0.22% ef
BS inoculated				
Ø	3.33 ± 0.62% g	4.10 ± 0.40% fg	3.50 ± 0.34% f	5.49 ± 0.40% d
HS	6.28 ± 0.71% e	4.92 ± 0.46% ef	5.28 ± 0.99% de	6.12 ± 0.55% d
	24 h	**48 h**	**24 h**	**48 h**
Uninoculated				
Ø	3.83 ± 0.49% g	3.67 ± 0.53% g	3.74 ± 0.69% ef	3.85 ± 0.41% ef
HS	4.86 ± 0.40% ef	5.19 ± 0.64% ef	5.27 ± 0.38% d	4.94 ± 0.41% de
Mock-inoculated				
Ø	3.71 ± 0.46% g	4.07 ± 0.26% fg	4.01 ± 0.26% ef	4.30 ± 0.45% ef
HS	5.94 ± 0.59% e	5.11 ± 0.34% e	5.30 ± 0.41% d	5.54 ± 0.51% d
BS inoculated				
Ø	10.37 ± 1.11% d	19.01 ± 1.81% c	9.37 ± 1.22% c	12.38 ± 1.25% b
HS	32.11 ± 3.32% a	24.81 ± 2.61% b	14.30 ± 0.95% b	30.84 ± 3.21% a

Numbers represent mean ± SD from three independent biological experiments. Different letters next to mean values indicate statistically significant differences among treatments at $P \leq 0.05$ within the respective staining. HS, plants exposed to heat shock at 49 °C for 20 s, 2 h before inoculation; Ø, untreated plants kept at 20 °C before inoculation.

[a]Levels of superoxide and H_2O_2 in leaves were visualized with NBT and DAB staining. NBT and DAB staining intensities as percentage of maximum pixel intensity values were quantified with ImageJ.

mock-inoculated and *B. sorokiniana*-inoculated plants at 3, 6 and 9 h after inoculation (Fig. 4). The expression of *HvSOD* was further induced in plants treated with heat shock and infected with *B. sorokiniana* at 3 and 24 h after inoculation as compared to mock-inoculated plants (Fig. 4) which correlated with increases in ROS levels at the same time points (Figs 2 and 3, Table 1). *B. sorokiniana* inoculation alone (without heat shock) had no significant effect on the expression of *HvSOD* at all investigated time points (Fig. 4).

The main function of dehydroascorbate reductase (EC 1.8.5.1) is to regenerate ascorbic acid, which is a critical non-enzymatic antioxidant in plants generally recognized as an electron donor of H_2O_2. Our results showed that heat shock induced expression of barley *Cytosolic Dehydroascorbate reductase* (*HvDHAR Cyt*) in both mock- and *B. sorokiniana*-inoculated plants; however, *B. sorokiniana* infection had no specific effect on transcript levels because heat shock treatment did not cause a larger increase in gene expression in infected and heat-shocked samples than in mock-inoculated leaves (Fig. 4).

Expression of the cell death regulator *BAX inhibitor-1* (*HvBI-1*) increased significantly in response to heat shock both in mock-inoculated and *B. sorokiniana*-inoculated plants. However, 24 h after inoculation, a combination of heat shock and *B. sorokiniana* inoculation further increased the expression of *HvBI-1* as compared to the mock -inoculated control (Fig. 4).

In case of all three genes mentioned above (*HvSOD*, *HvDHAR Cyt* and *HvBI-1*) only a transient increase in gene expression was observed following heat shock treatment, reaching a peak at 3 h, while transcript levels decreased at later time points. Heat shock was the primary factor in inducing expression of these genes, as there were only a few time points where additional transcript level increases were observed as a result of *B. sorokiniana* infection and heat shock.

Heat shock slightly, but significantly, induced expression of the *Pathogenesis related-1b* (*HvPR-1b*) gene both in mock- and *B. sorokiniana*-inoculated plants at early time points; however, *B. sorokiniana* infection led to a remarkable increase in *HvPR-1b* transcript levels 24 h after inoculation, and the infection combined with heat shock treatment resulted in a further increase in the expression of *HvPR-1b* (Fig. 4).

DISCUSSION

Heat stress and infection by *B. sorokiniana* are among the most important stresses in non-traditional tropical wheat- and barley-growing areas, and heat stress profoundly affects plant defence to *B. sorokiniana* (Pandey *et al.* 2021). However, due to the effects of global climate change (*i.e.*, increased incidence of heat stress), *B. sorokiniana* is also causing problems nowadays in conventional wheat- and barley-growing areas (Kumar *et al.* 2020).

We intended to investigate the influence of a short-term heat shock (HS, 49 °C for 20 s) in barley on infection by a hemibiotrophic fungal pathogen, *B. sorokiniana*. Our results show that HS treatment applied 2 h before inoculation significantly enhanced susceptibility to BS in barley cv. Ingrid, manifested as increased severity of necrotic symptoms and elevated BS biomass, as compared to untreated controls. In line with our

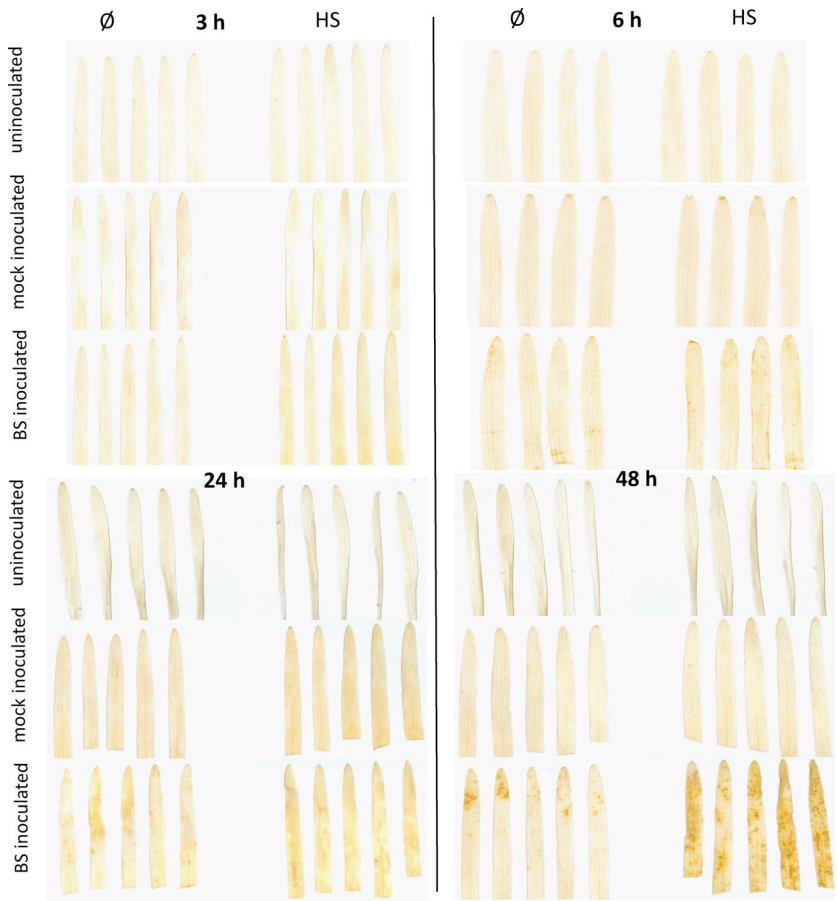

Fig. 3. Accumulation of H_2O_2 in barley (*Hordeum vulgare* cv. Ingrid) leaves 3, 6, 24 and 48 h after inoculation. Uninoculated, mock-inoculated and *Bipolaris sorokiniana*-inoculated barley plants were exposed to heat shock at 49 °C for 20 s 2 h before inoculation. Untreated control plants were held at 20 °C (Ø). Detection of H_2O_2 was visualized by DAB tissue staining. Photographs are from one representative experiment; the experiment was repeated three times with similar results.

results, a similar heat shock enhanced susceptibility of barley cv. Ingrid to the biotrophic barley powdery mildew (*B. hordei*) (Barna *et al.* 2014; Künstler *et al.* 2018). In addition, a heat shock pre-treatment at 40.5 °C for 2 h accelerates development of the powdery mildew pathogen *Pseudoidium neolycopersici* in tomato (Nožková *et al.* 2019). In contrast, heat shock (50 °C for 60 s) induced resistance in barley cv. Golden Promise to barley powdery mildew (Vallélian-Bindschedler *et al.* 1998). Furthermore, heat shock reduced downy mildew disease severity and pathogen development in cucumber (Ding *et al.* 2016). The above-mentioned studies demonstrate the different effects of heat shock in distinct plant–pathogen interactions. However, these data on the effects of short-term heat stresses are difficult to interpret because each of these observations was made in a different type of plant–pathogen interaction, under different environmental conditions (non-identical temperatures, durations of heat shock and time elapsed between heat shock and inoculation), and various modes of heat shock application (pre-treatment before inoculation or elevated temperatures during infection). In fact, heat shock application during infection could be effective in controlling fungal pathogens because spores in leaves of infected plants may lose their infectivity while infected host plants may adapt to such a short-term heat shock (Ding *et al.* 2016; Sato *et al.* 2018). However, in the present study, a heat shock pre-treatment (*i.e.*, heat shock applied before inoculation) enhanced susceptibility of barley to *B. sorokiniana*, similar as previously shown for barley powdery mildew (Barna *et al.* 2014; Künstler *et al.* 2018). It seems that heat shock pretreatments in barley cv. Ingrid have a similar effect on infection by the hemibiotrophic *B. sorokiniana* and the biotrophic barley powdery mildew, probably because both pathogens behave as biotrophs in initial stages of pathogenesis. However, in contrast to powdery mildew, *B. sorokiniana* enters the necrotrophic phase approximately 1 day after inoculation and intense toxin production is observed during this period, leading to vigorous ROS accumulation and appearance of necrotic symptoms (Kumar *et al.* 2002; Barna *et al.* 2012; Ye *et al.* 2019). Previous research has shown that barley lines susceptible to *B. sorokiniana* are also more sensitive to necrotic symptoms induced by the toxin-containing culture filtrate of the fungus (Kumar *et al.* 2001; Király *et al.* 2002). Importantly, in the case of the biotrophic barley powdery mildew, the early accumulation of ROS, such as superoxide ($O_2^{\bullet-}$) and H_2O_2, indicates successful resistance (Hückelhoven *et al.* 1999; Künstler *et al.* 2018). For example, the transient overexpression of *HvGLP4*, encoding a germin like protein that produces H_2O_2, enhanced resistance to barley powdery mildew (Christensen *et al.* 2004). On the other hand, in barley infected with

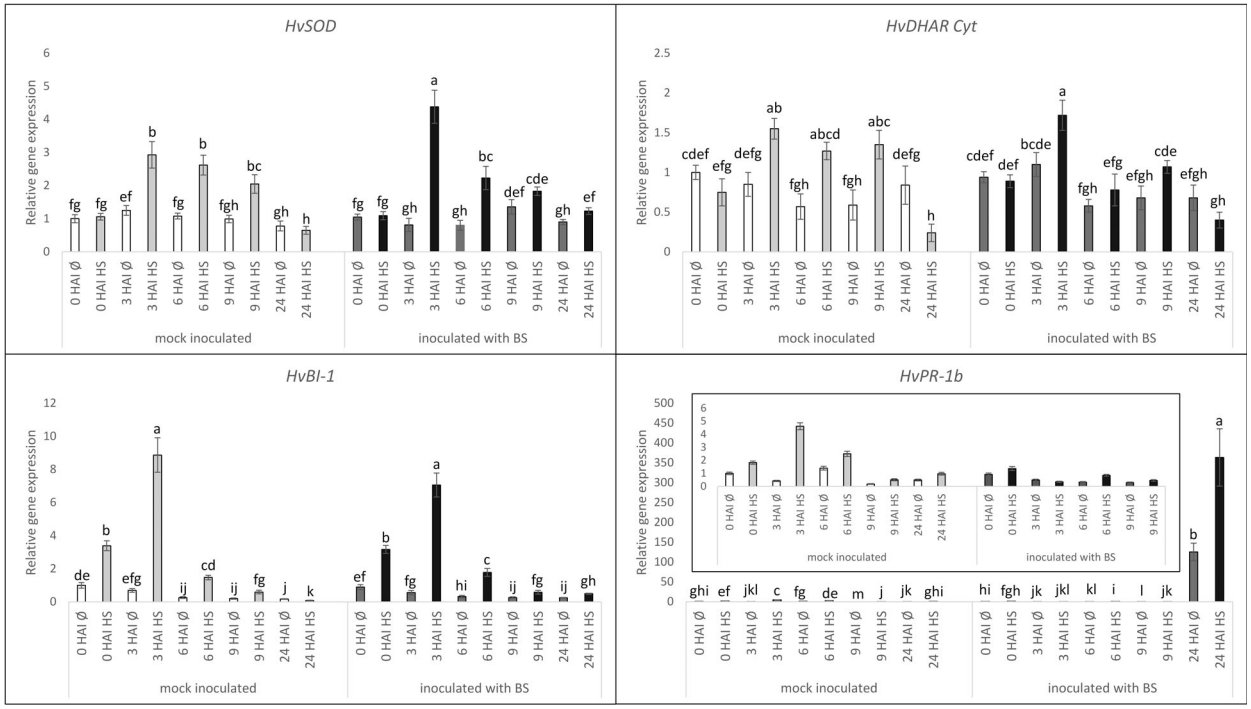

Fig. 4. Changes in expression of different defence-related genes (*Superoxide dismutase*, *HvSOD*; *Cytosolic Dehydroascorbate reductase*, *HvDHAR Cyt*; *BAX inhibitor-1*, *HvBI-1* and *Pathogenesis related-1b*, *HvPR-1b*) in barley (*H. vulgare* cv. Ingrid) at early time points (0, 3, 6 and 24 HAI) in response to heat shock (49 °C at 20 s) applied 2 h before mock or *Bipolaris sorokiniana* inoculation. Untreated control plants were held at 20 °C (Ø). Graphs show the average of three experiments. Error bars represent standard deviation. Different letters above bar graphs indicate statistically significant differences at $P \leq 0.05$. In the case of *HvPR-1b*, the graph is also presented without *B. sorokiniana* inoculated data for 24 HAI (see inset) for better visibility.

the hemibiotrophic *B. sorokiniana* the massive accumulation of H_2O_2 in inoculated leaves is clearly a marker of increased susceptibility (Kumar *et al.* 2001, 2002; Schäfer *et al.* 2004).

In line with these observations, our results showed that *B. sorokiniana* inoculation substantially increased levels of both $O_2^{\bullet-}$ and H_2O_2 1 and 2 days after inoculation, and heat shock combined with *B. sorokiniana* infection further increased the amount of ROS in parallel with enhancement of necrotic symptoms and elevated *B. sorokiniana* biomass. In contrast, barley exposed to heat shock and infected with the biotrophic barley powdery mildew displayed reduced levels of $O_2^{\bullet-}$ and H_2O_2 while becoming more susceptible to the pathogen (Barna *et al.* 2014; Künstler *et al.* 2018). Decreased ROS levels induced by heat shock in powdery mildew-infected barley could be a possible cause of increased susceptibility (Künstler *et al.* 2018). On the other hand, increased ROS accumulation in heat shock-treated and *B. sorokiniana*-inoculated barley may favour a hemibiotrophic pathogen like *B. sorokiniana*. It is known that – in contrast to biotrophy – pathogen-induced ROS accumulation, cell death and tissue necrosis promote pathogen growth and disease development when the pathogen is a necrotroph or has a necrotrophic stage (Barna *et al.* 2012; Ye *et al.* 2019).

High temperature can induce oxidative stress (*i.e.*, excess ROS generation) typically resulting in cell death (Katano *et al.* 2018). Plants have evolved different mechanisms to scavenge ROS production and control excess cell death during *e.g.* heat stress, such as the production of antioxidants and programmed cell death inhibition (Hameed *et al.* 2012). Our results indicate that the expression of antioxidant and programmed cell death inhibitor genes (*HvSOD*, *HvDHAR Cyt*, *HvBI-1*) might be involved in controlling cell and tissue death (necrotic symptoms) that could increase substantially upon heat treatment both in mock- and in *B. sorokiniana*-inoculated barley. Interestingly, however, expression of these genes did not change significantly following *B. sorokiniana* infection alone, at least at the early time points investigated by us, a possible indication that these antioxidant and cell death inhibitor genes do not play a direct role in resistance to *B. sorokiniana*. This is supported by previous research showing that in *B. sorokiniana*-infected barley early induction of the cell death regulator gene *HvBI-1* and an antioxidant (*HvcAPX*, encoding a cytosolic ascorbate peroxidase) is associated with enhanced susceptibility (Király *et al.* 2002; Schäfer *et al.* 2004). In our study, transient but significant increases in transcript levels of *HvSOD* and *HvBI-1* (3 and 24 h after inoculation) correlated with enhanced susceptibility to *B. sorokiniana*, as a result of the combined effect of heat shock and *B. sorokiniana* infection. It seems that the host plant attempts to acquire protection against ROS-mediated necrotization caused by the brutal combined effects of heat shock and *B. sorokiniana* infection through early induction of antioxidants (*e.g. HvSOD*) and programmed cell death inhibitors (*e.g. HvBI-1*). In fact, a slight induction of *SOD* expression was detected in a susceptible wheat cultivar infected with *B. sorokiniana* even in later periods of infection (72 and 96 h after inoculation), presumably associated with the development of tissue necrosis induced by ROS (Alkan *et al.* 2022). Interestingly, when comparing different barley cultivars infected with the fungal pathogen *Pyrephona teres* causing necrotic symptoms, SOD enzyme activity was lowest at early time points after infection

in the most resistant cultivar (Kunos *et al.* 2022), implying that increased SOD activity is associated with susceptibility to necrotrophic and hemibiotrophic pathogens.

The potential roles of the *pathogenesis related-1b* (*HvPR-1b*) gene as a marker of plant defence responses in barley to *B. sorokiniana* were also investigated. Our results showed that early *HvPR1-b* induction (within 24 h) is already evident in response to heat stress; however, *B. sorokiniana* infection leads to a remarkable increase in *HvPR1-b* transcript levels at 24 HAI, and the infection combined with heat stress results in a further increase in the expression of *HvPR1-b*. The enhanced expression of *HvPR1-b* induced by combined stresses correlated with higher *B. sorokiniana* biomass; therefore, *HvPR1-b* presumably does not contribute to plant defence against *B. sorokiniana*. Although an early (within 24 h) activation of *HvPR-1b* expression in barley infected with the biotrophic powdery mildew can be linked to different forms of resistance (Peterhänsel *et al.* 1997; Schultheiss *et al.* 2003), the similar early induction of *HvPR-1b* is known to be correlated with H_2O_2 accumulation and increased susceptibility of barley to necrotic symptoms caused by *B. sorokiniana* (Király *et al.* 2002; Schultheiss *et al.* 2003; Schäfer *et al.* 2004). Furthermore, the upregulation of the *PR1* gene in response to *B. sorokiniana* is also a characteristic of susceptible wheat cultivars (Alkan *et al.* 2022). In agreement with these results, we confirmed that enhanced *HvPR-1b* expression is correlated with susceptibility to *B. sorokiniana* in barley cv. Ingrid and demonstrated that heat shock further enhanced the expression of *HvPR-1b* in *B. sorokiniana*-infected barley in parallel with higher levels of *B. sorokiniana* biomass.

In conclusion, our results suggest that a short-term heat shock induces enhanced susceptibility to *B. sorokiniana* in barley cv. Ingrid, manifested as increased symptom severity and *B. sorokiniana* biomass. Enhanced susceptibility is associated with elevated levels of ROS (superoxide and H_2O_2). The expression of plant defence-related antioxidant genes (*HvSOD*, *HvDHAR Cyt*) and a barley gene encoding a programmed cell death inhibitor (*HvBI-1*) is induced mainly by heat shock. However, heat shock combined with *B. sorokiniana* further increased expression of these genes and transient but significant increases in transcript levels of *HvSOD* and *HvBI-1* correlated with enhanced susceptibility. The expression of the pathogenesis-related *HvPR-1b* gene increased several fold 24 h after *B. sorokiniana* infection; however, heat shock further increased *HvPR-1b* expression, which also correlated with enhanced susceptibility. The present study may help in elucidating how an abiotic environmental factor (heat shock) affects barley defence responses to a hemibiotrophic pathogen like *B. sorokiniana*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Oligonucleotide primers used in qPCR.

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