Title of reported project:

Nonhost resistance to plant viruses – role of temperature, reactive oxygen species and salicylic acid

Research background and main aims of the project

Plants generally display two fundamental types of disease resistance. Host resistance is usually plant cultivar / pathogen race-specific (Mysore and Ryu, 2004), conferred by resistance (*R*) genes and is less durable, since pathogens eventually lose/mutate their corresponding effectors to evade detection by host *R* gene-encoded proteins (Dangl et al., 2013). On the other hand, nonhost resistance (i.e. resistance to pathogens adapted to other host plants) can act against all races of a given pathogen and occurs in all cultivars of a plant species. It is a rapid, durable, usually symptomless form of plant defense considered as a key means of effective pathogen control (Gill et al., 2015; Lee et al., 2017). Understanding its mechanisms is crucial for breeding cultivars displaying effective disease resistance (Fonseca and Mysore, 2019; Pruitt et al. 2021).

Reactive oxygen species (ROS) have a dual function during plant disease resistance to e.g. viruses. In low concentrations, ROS transmit resistance signals, while in high amounts they may damage both the plant and pathogen (Levine et al., 1994; Torres et al., 2005; Pogány et al., 2009; Hafez et al., 2012; Hernández et al., 2016). Accumulation of the plant hormone salicylic acid (SA) is regulated by ROS (Neuenschwander et al., 1995; Leon et al., 1995), and SA has a pivotal role in coordinating plant defense to biotrophic pathogens, including viruses (Gaffney et al., 1993; Delaney et al., 1994; Cole et al., 2004; Dziurka et al., 2016). Furthermore, temperature has a profound influence on the outcome of plant-pathogen interactions, with higher than normal temperatures very often inhibiting disease resistance to e.g. viruses (Samuel, 1931; Király et al., 2008; Wang et al., 2009).

Our previous research demonstrated that a heat shock-driven reduction in plant ROS (superoxide and hydrogen peroxide) deteriorates the host and nonhost resistance of barley (*Hordeum vulgare*) to powdery mildews (Barna et al., 2014; Künstler et al., 2018). We have also shown that both the host resistance of tobacco (*Nicotiana tabacum*) to *Tobacco mosaic virus* (TMV) and *in planta* superoxide levels are suppressed in plants exposed to higher (30 C°) temperatures (Király et al., 2008).

Importantly, the molecular and pathophysiological mechanism(s) of nonhost resistance of plants to viruses is still a largely unexplored research area. Furthermore, studying the influence of heat stress on plant (virus) disease resistance responses could become a pivotal issue, considering recent trends in global climate change.

The main aim of this project was to investigate whether ROS accumulation during optimal temperatures and salicylic acid, a plant hormone affected by ROS, have a role in maintaining nonhost resistance of plants to virus infections?

1/ Exploring the currently unknown role of optimal (as opposed to high) temperatures and reactive oxygen species (ROS) in maintaining symtomless (Type I) nonhost resistance of barley to *Tobacco mosaic virus* (TMV).

2/ Clarifying whether ROS accumulation during optimal temperatures and salicylic acid, a plant hormone affected by ROS, contribute to the nonhost resistance of tobacco (*Nicotiana* spp.) to viruses adapted to other hosts (e.g. *Barley stripe mosaic virus*, BSMV; *Tobacco necrosis virus*, TNV).

Results

Roles of heat exposure and the presence of BSMV in symtomless (Type I) nonhost resistance of barley cv. Ingrid to TMV

Previously it was shown that *Tobacco mosaic virus* (TMV) can replicate in leaves of barley (*Hordeum vulgare*), a nonhost plant, and even cause systemic infection without any visible symptoms. TMV replication and movement was dependent primarily on high temperatures (30 °C) and enhanced by co-infection with viruses adapted to barley, e.g. *Barley stripe mosaic virus* (BSMV) (Hamilton and Dodds, 1970; Dodds and Hamilton, 1972).

In our earlier work we have successfully applied heat shock treatments (49 °C, 20-45 sec, 30-120 min before pathogen inoculation) to suppress the symptomless (Type I) nonhost resistance of barley cv. Ingrid to wheat powdery mildew (Király et al., 2013; Künstler et al., 2018).

Therefore, we wanted to clarify if a similar heat shock (49 °C, 20 sec, 120 min / 2 h/ before pathogen inoculation) could also impair symptomless (Type I) nonhost resistance of barley cv. Ingrid to a virus, TMV? Since BSMV enhances TMV replication in barley (see above), we also used cv. Ingrid plants grown from BSMV-infected seeds (BSMV is seed-transmissible). We tested two different BSMV isolates (BSMV-Braunschweig and BSMV-Hu /Hungarian isolate/) and found that BSMV-Hu replicates far better in barley cv. Ingrid. Therefore, we decided to use BSMV-Hu in future experiments.

We found that from 4-7 days after inoculation (DAI) onwards, TMV (U1 strain) levels in heat shock-exposed barley (cv. Ingrid containing BSMV) became close to 2 times higher as in controls **(Fig. 1)**. Interestingly, visible symptoms (leaf tip necrosis at 15 DAI) were detectable in most plants (kept at 20 °C, with/without a heat shock) but only if plants were older (15 days old /15 DPP/ at the time of heat shock/virus infection but not at 7 DPP). Therefore, from this point we used barley plants for heat treatments and TMV inoculation at 7 DPP. Importantly, a pre-exposure to heat shock could increase TMV levels regardless of the development of leaf tip necrosis. These results suggest that a heat shock can indeed significantly impair symptomless nonhost resistance of barley to a virus like TMV.

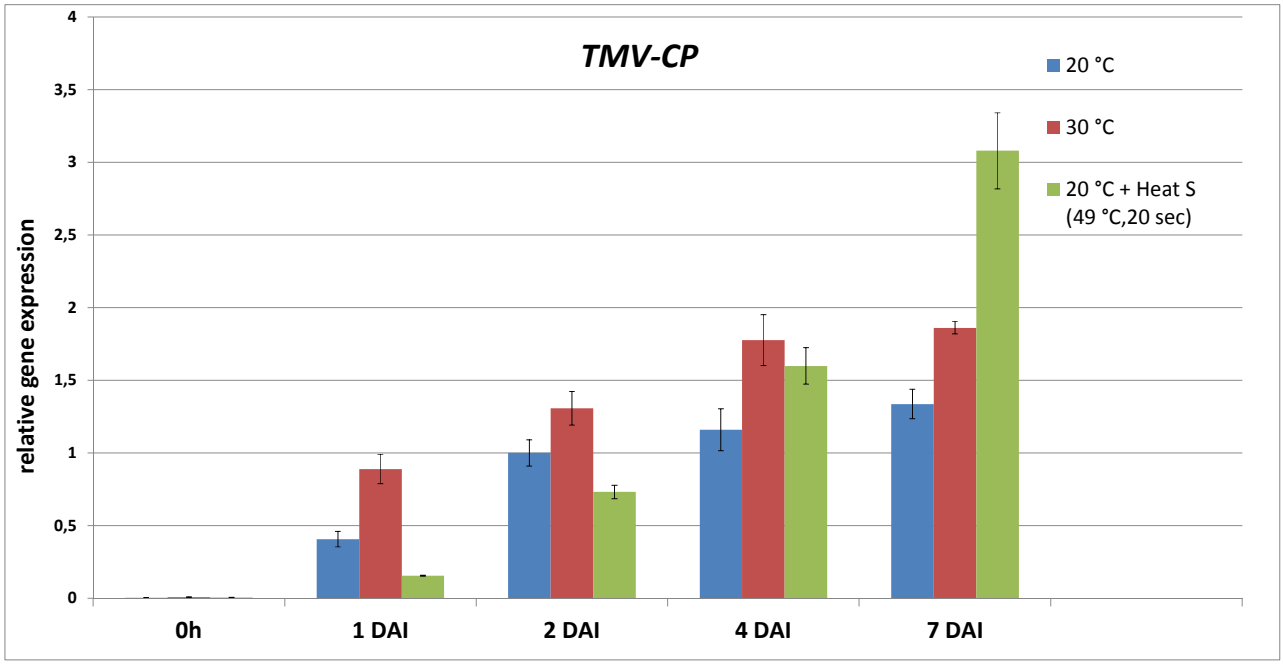


Figure 1 Accumulation of *Tobacco mosaic virus* (TMV) in barley cv. Ingrid containing *Barley stripe mosaic virus* (BSMV), following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 1, 2, 4 and 7 days after TMV inoculation (DAI). Expression of the TMV coat protein gene (*TMV-CP*) was assayed by real time RT-qPCR and normalized to that of the reference gene *HvUbi*.

To reproduce the experiments of Dodds et al. (described above), the effect of the presence of BSMV on suppressing nonhost resistance of barley (cv. Ingrid) to TMV at constant high temperatures (30 °C, vs. 20 °C as a control) was also tested. Interestingly, when plants were exposed to 30 °C for 3 days before virus inoculation, TMV levels sharply declined **(Fig. 2)**.

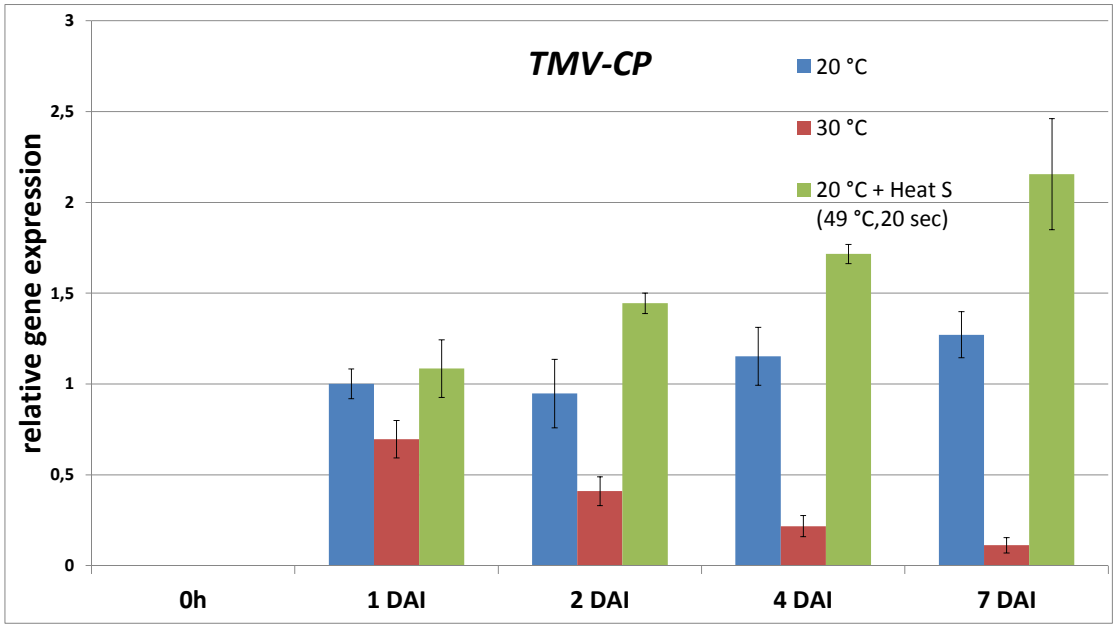


Figure 2 Accumulation of *Tobacco mosaic virus* (TMV) in barley cv. Ingrid containing *Barley stripe mosaic virus* (BSMV), following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 days or constantly kept at 20 °C, at 1, 2, 4 and 7 days after TMV inoculation (DAI). Expression of the TMV coat protein gene (*TMV-CP*) was assayed by real time RT-qPCR and normalized to that of the reference gene *HvUbi*.

On the other hand, when plants were exposed to 30 °C only 3 hours before virus inoculation, nonhost resistance to TMV seemed to be significantly reduced, as TMV levels were ca. 50-100 % higher than during 20 °C at all investigated time points **(Fig. 1)**. This could indicate that if plants have a longer time (e.g. 3 days) to adapt to higher temperatures, nonhost resistance to e.g. virus infections is likely also maintained, while in case of a sudden heat stress (e.g. 30 °C only 3 hours before virus inoculation or a heat shock) the plant defense system may partially collapse, manifested as a partial breakdown of nonhost resistance to e.g. viruses like TMV.

In order to see if nonhost resistance to TMV is also impaired in heat-stressed barley without the presence of BSMV, we repeated our previous TMV infection assays in heat-stressed cv. Ingrid barley plants free of BSMV. Our results revealed that heat stress exposure can significantly reduce barley nonhost resistance to TMV even in the absence of BSMV, as TMV levels were at least 50-100 % higher in heat-stressed plants than during 20 °C at most investigated time points (1 to 7 DAI). Interestingly, however, in this case the reduction of nonhost resistance to TMV was especially pronounced in plants exposed to 30 °C for 3 hours, rather than to a heat shock (49 °C, 20 sec) **(Fig. 3)**.

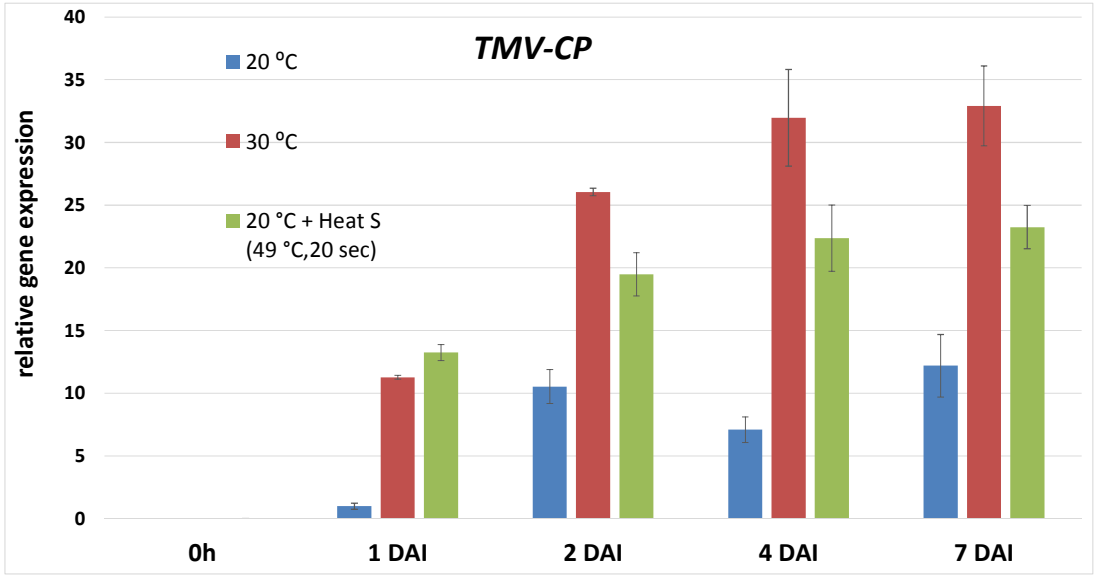


Figure 3 Accumulation of *Tobacco mosaic virus* (TMV) in barley cv. Ingrid, following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 1, 2, 4 and 7 days after TMV inoculation (DAI). Expression of the TMV coat protein gene (*TMV-CP*) was assayed by real time RT-qPCR and normalized to that of the reference gene *HvUbi*.

To confirm the presence of infective TMV (as opposed to only viral RNA) in the originally inoculated barley leaves by a biotest, we have back-inoculated tobacco (*Nicotiana tabacum* cv. Xanthi NahG) first with TMV-infected barley cv. Ingrid containing BSMV (exposed/not exposed to heat stress). Back inoculation resulted in development of hypersensitive local necrotic lesions (HR) in tobacco and lesion numbers correlated with TMV levels assayed in both back-inoculated tobacco **(Fig. ..4..)** and the original (inoculum source) barley leaves, when assayed by RT-qPCR.

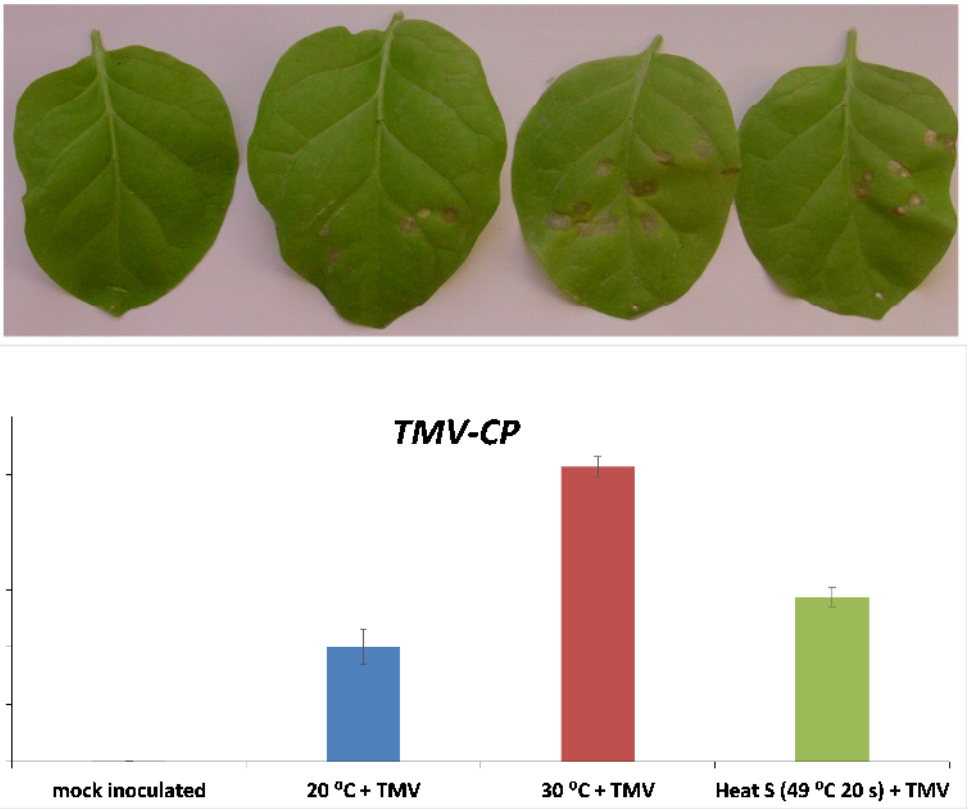


Figure 4 Accumulation of *Tobacco mosaic virus* (TMV) in leaves of *Nicotiana tabacum* cv. Xanthi *NahG* tobacco back inoculated with barley cv. Ingrid containing *Barley stripe mosaic virus* (BSMV). Back inoculation was conducted 4 days after inoculation of BSMV-containing cv. Ingrid barley with TMV (see Fig. 1). Tobacco leaves photographed at 10 days after back inoculation. Hypersensitive necrotic lesions (HR) indicate the presence of TMV. mock inoculated = no TMV; 20 °C = barley plants constantly kept at 20 °C; 30 °C = pre-exposure to 30 °C for 3 hours before inoculation; Heat S (49 °C 20 s) = pre-exposure to a heat shock (49 °C for 20 sec) before inoculation. Expression of the TMV coat protein gene (*TMV-CP*) was assayed by real time RT-qPCR and normalized to that of the tobacco reference gene *NtAct*.

We have also repeated the above-mentioned back inoculation experiment in case of TMV-infected but BSMV-free cv. Ingrid barley (exposed/not exposed to heat stress). This test similarly confirmed the presence of TMV in the originally inoculated BSMV-free cv. Ingrid barley leaves. In essence, the experiments described above demonstrated that cv. Ingrid barley plants indeed contain infective, replicating TMV, regardless of the presence or absence of BSMV.

Roles of defense gene expression in symtomless (Type I) nonhost resistance of barley cv. Ingrid to TMV

A heat stress-elicited breakdown of nonhost resistance to e.g. viruses like TMV in barley could result from a partial collapse of the plant defense system. To test this hypothesis, we monitored transcription of several defense-related barley genes by real time RT-qPCR: a pathogenesis-related gene (*HvPR-1b*), a gene encoding NADPH oxidase responsible for production of the reactive oxygen species (ROS) superoxide and host disease resistance (*HvRBOHF2*), and *HvSOD1* (superoxide dismutase) and *HvBI-1* (BAX-inhibitor), genes encoding an antioxidant and a cell death regulator, respectively.

Interestingly, in barley cv. Ingrid containing BSMV, infected with TMV and either exposed/not exposed to heat stress (see above) expression of all the investigated defense-related genes (*HvPR-1b, HvRBOHF2, HvSOD1, HvBI-1*) correlated with TMV levels (assayed at 1 to 7 DAI), rather than nonhost resistance **(Fig. 5)**. These results indicate a partial defense collapse in response to heat stress (at least at these relatively later time points), where these barley genes might function as stress/susceptibility markers, rather than defense components.

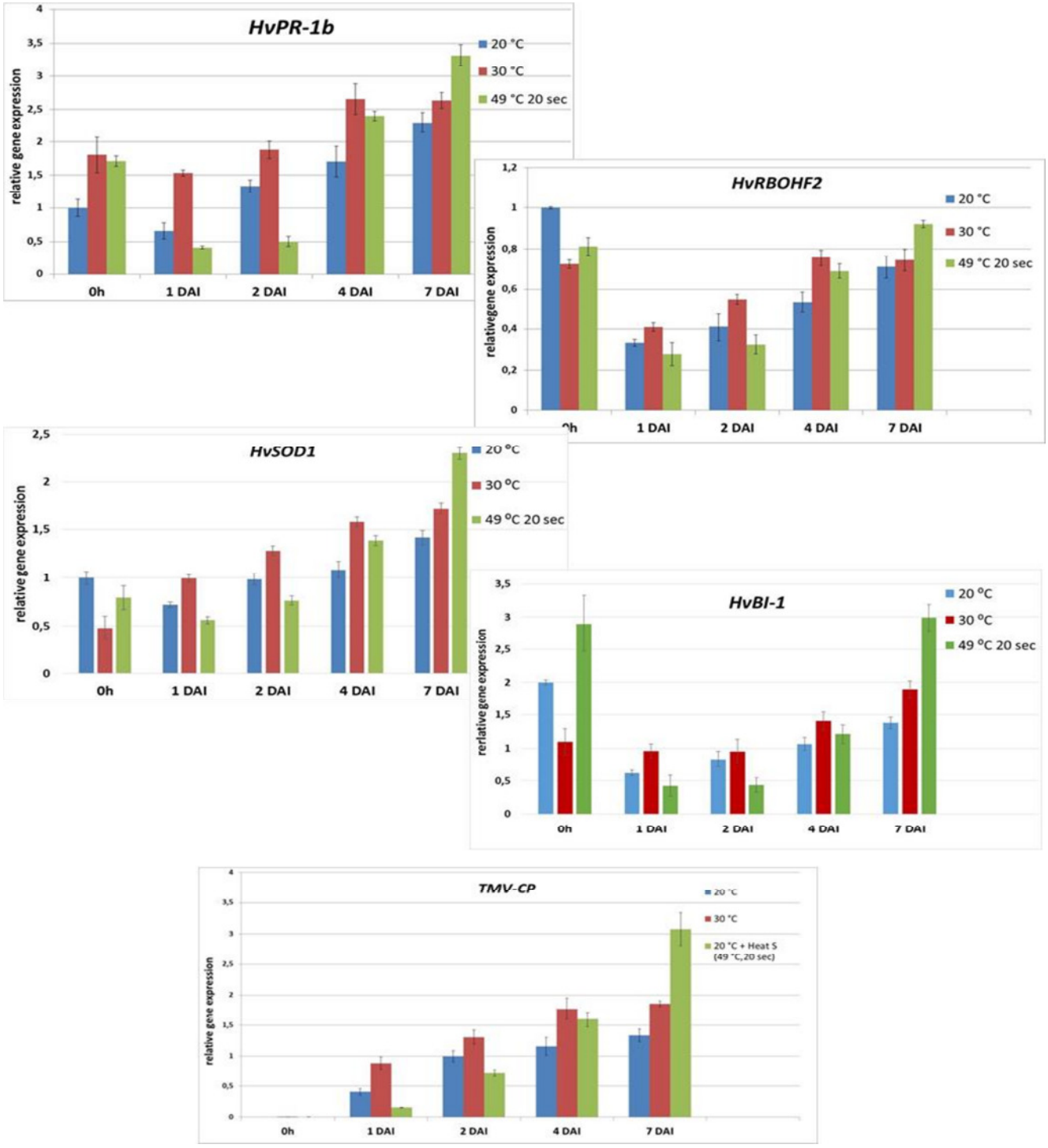
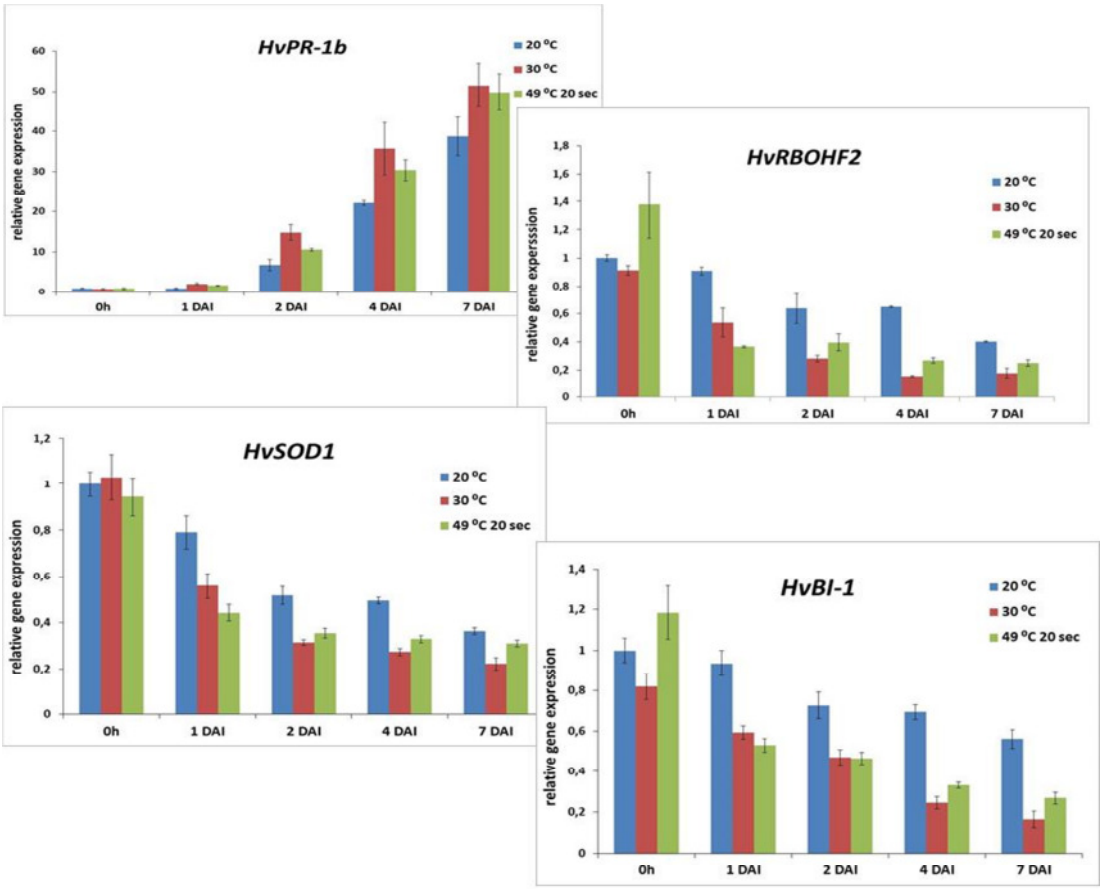


Figure 5 Expression of a pathogenesis-related (*HvPR-1b*), a NADPH oxidase (*HvRBOHF2*), a superoxide dismutase (*HvSOD1*), and a BAX-inhibitor gene (*HvBI-1*) in barley cv. Ingrid containing *Barley stripe mosaic virus* (BSMV), following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 1, 2, 4 and 7 days after *Tobacco mosaic virus* (TMV) inoculation (DAI). Gene expression was assayed by real time RT-qPCR, normalized to that of the reference gene *HvUbi* and to mock-inoculated samples. For comparison, expression of the TMV coat protein gene (*TMV-CP*) in the same samples (see Fig. 1) is also included.

To clarify if heat stress-elicited breakdown of nonhost resistance to TMV in BSMV-free barley could also result from a partial collapse of the plant defense system, we monitored transcription of the above-mentioned defense-related barley genes (*HvPR-1b, HvRBOHF2, HvSOD1, HvBI-1*) by real time RT-qPCR. As in our earlier experiments with BSMV-containing cv. Ingrid barley, the expression of *HvPR-1b* correlated with TMV levels, rather than nonhost resistance, indicating a function of this barley gene as a stress/susceptibility marker. Interestingly, however, expression of the remaining defense genes (*HvRBOHF2, HvSOD1, HvBI-1*) displayed an inverse correlation with TMV levels, i.e. an association with nonhost resistance **(Fig. 6)**. This implies that, at least to a certain extent, defense responses could have been retained in BSMV-free, TMV-infected barley, even at a relatively late stage of pathogenesis (1 to 7 DAI). In fact, these results might also indicate a less severe stress in these plants than in BSMV-containing barley.



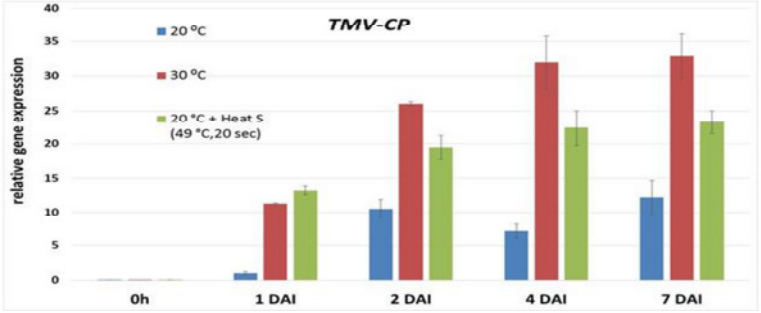
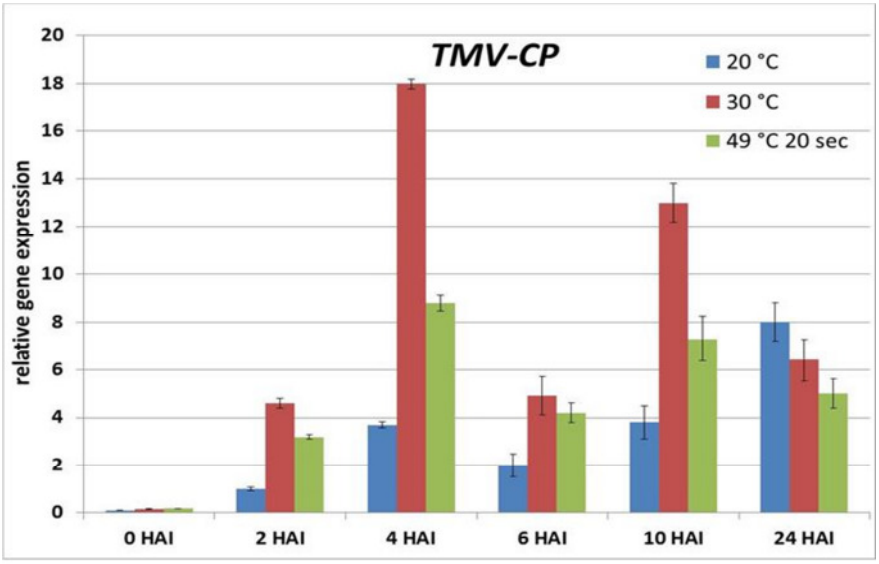


Figure 6 Expression of a pathogenesis-related (*HvPR-1b*), a NADPH oxidase (*HvRBOHF2*), a superoxide dismutase (*HvSOD1*), and a BAX-inhibitor gene (*HvBI-1*) in barley cv. Ingrid, following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 1, 2, 4 and 7 days after *Tobacco mosaic virus* (TMV) inoculation (DAI). Gene expression was assayed by real time RT-qPCR, normalized to that of the reference gene *HvUbi* and to mock-inoculated samples. For comparison, expression of the TMV coat protein gene (*TMV-CP*) in the same samples (see Fig. 3) is also included.

In order to see if the above-mentioned defense-related genes may contribute to the establishment of nonhost resistance to TMV in an early stage of pathogenesis, we assayed TMV levels and the expression of *HvPR-1b, HvRBOHF2, HvSOD1* and *HvBI-1* in BSMV-free and BSMV-containing barley cv. Ingrid (TMV-infected and exposed/not exposed to heat stress) within the first few hours of pathogenesis (i.e. 1, 2, 3, 6, 24 HAI). We found that the symptomless nonhost resistance of cv. Ingrid barley to TMV (at normal temperatures vs. heat-stress) was indeed evident as early as 2 to 6 HAI, regardless of the presence or absence of BSMV (assayed by RT-qPCR) **(Fig. 7)**. This is in line with our published results showing a similar early timing of symptomless host resistance to *Potato virus X* (PVX) in tobacco (Király et al., 2021). Importantly, expression of all investigated defense-related genes (*HvPR-1b, HvRBOHF2, HvSOD1, HvBI-1*) generally correlated with nonhost resistance to TMV at these early timepoints (within 24 HAI), suggesting that these defense genes indeed take part in the establishment of nonhost resistance to TMV already within this early stage of pathogenesis, regardless of the presence or absence of BSMV **(Fig. 8** and **Fig. 9)**.



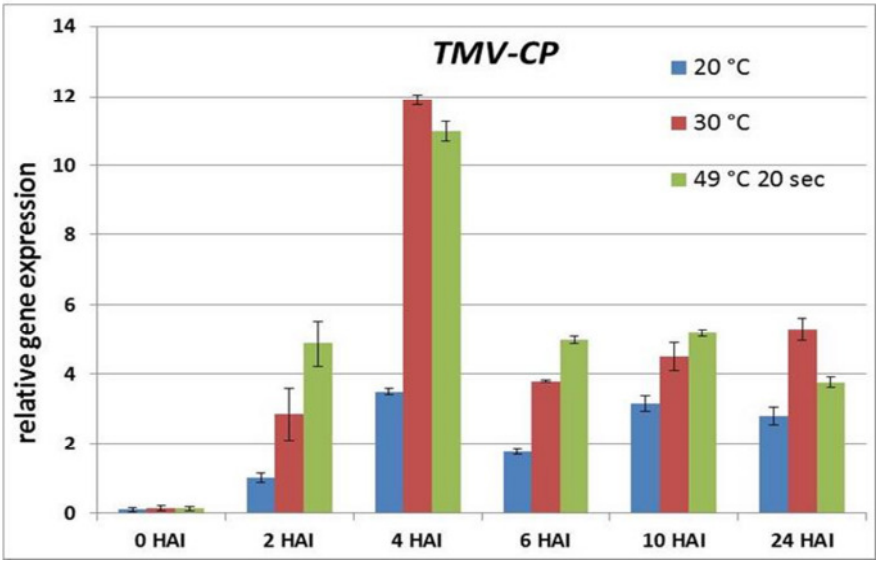


Figure 7 Accumulation of *Tobacco mosaic virus* (TMV) in barley cv. Ingrid containing *Barley stripe mosaic virus* (BSMV) (upper panel) or free of BSMV (lower panel), following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 2, 4, 6, 10 and 24 hours after TMV inoculation. Expression of the TMV coat protein gene (*TMV-CP*) was assayed by real time RT-qPCR and normalized to that of the reference gene *HvUbi*.

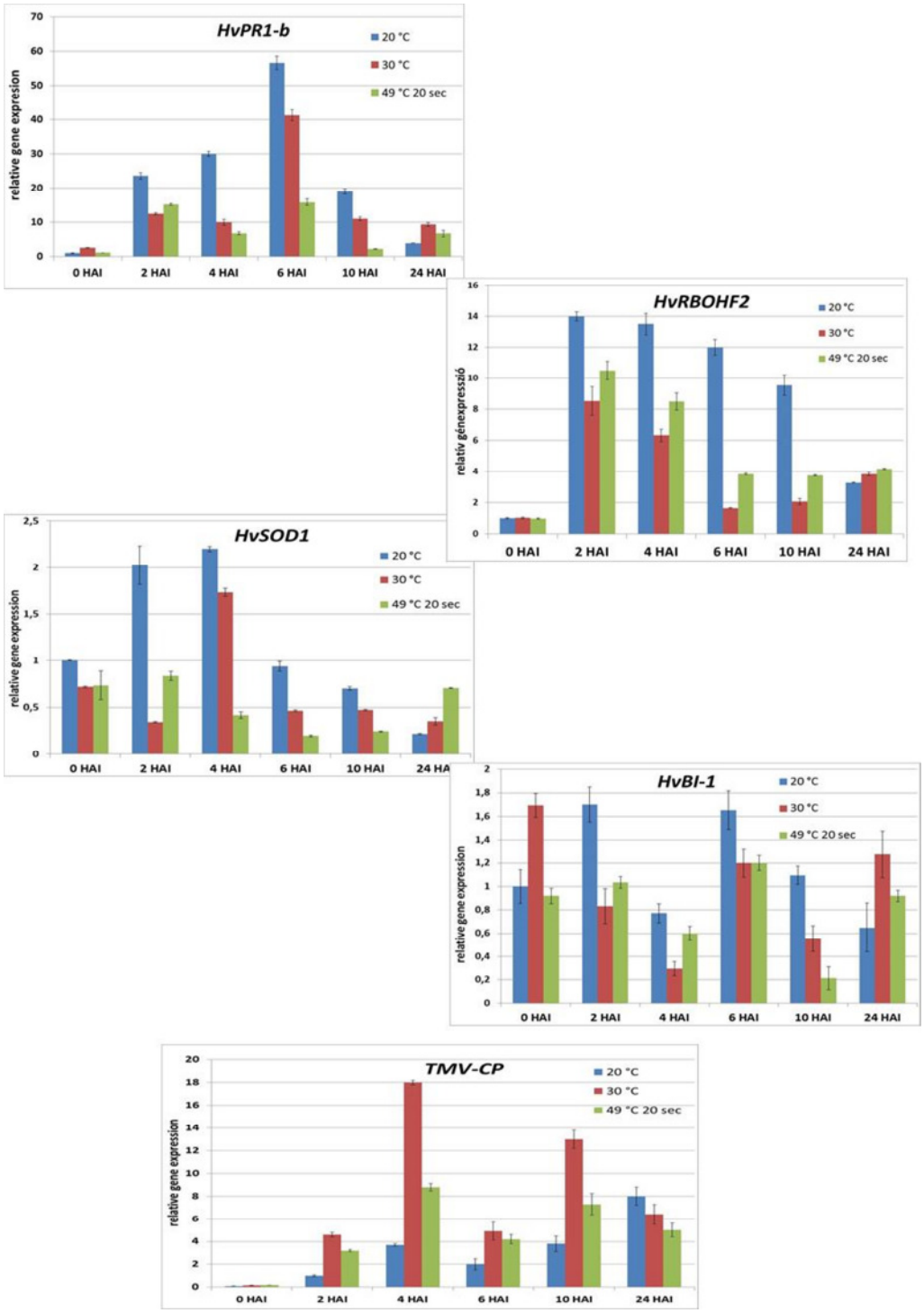


Figure 8 Expression of a pathogenesis-related (*HvPR-1b*), a NADPH oxidase (*HvRBOHF2*), a superoxide dismutase (*HvSOD1*), and a BAX-inhibitor gene (*HvBI-1*) in barley cv. Ingrid containing *Barley stripe mosaic virus* (BSMV), following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 2, 4, 6, 10 and 24 hours after *Tobacco mosaic virus* (TMV) inoculation (DAI). Gene expression was assayed by real time RT-qPCR, normalized to that of the reference gene *HvUbi* and to mock-inoculated samples. For comparison, expression of the TMV coat protein gene (*TMV-CP*) in the same samples (see Fig. 7 upper panel) is also included.

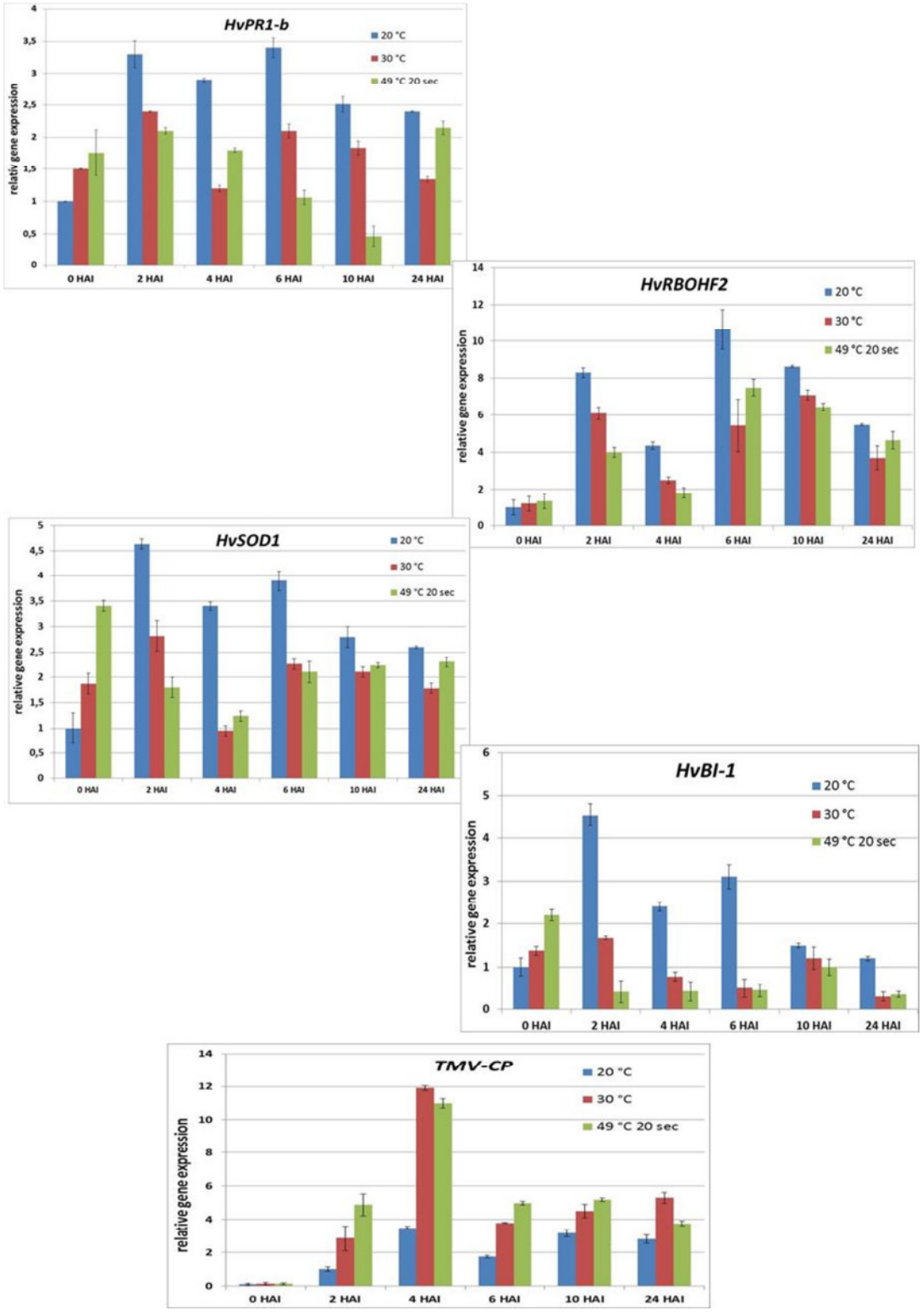


Figure 9 Expression of a pathogenesis-related (*HvPR-1b*), a NADPH oxidase (*HvRBOHF2*), a superoxide dismutase (*HvSOD1*), and a BAX-inhibitor gene (*HvBI-1*) in barley cv. Ingrid, following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 2, 4, 6, 10 and 24 hours after *Tobacco mosaic virus* (TMV) inoculation (DAI). Gene expression was assayed by real time RT-qPCR, normalized to that of the reference gene *HvUbi* and to mock-inoculated samples. For comparison, expression of the TMV coat protein gene (*TMV-CP*) in the same samples (see Fig. 7 lower panel) is also included.

Roles of reactive oxygen species (ROS) in symtomless (Type I) nonhost resistance of barley cv. Ingrid to TMV

To further monitor nonhost resistance-related defense processes in TMV-infected, heat-stressed barley, we started experiments where the early accumulation of ROS (superoxide /$O_2^{\cdot-}$/ and hydrogen peroxide /H_2O_2/) is detected by tissue staining methods (Nitro Blue Tetrazolium /NBT/ and Diaminobenzidine /DAB/ staining, respectively). Plant resistance to virus infections is, at least partially, conferred by an early ROS accumulation, especially that of superoxide (Doke and Ohashi, 1988; Rossetti and Bonatti, 2001; Bacsó et al., 2011; Király et al., 2008, 2021).

We found that at 1, 2, 3 and 6 HAI superoxide accumulation was slightly but significantly lower in heat-stressed barley cv. Ingrid leaves containing BSMV and infected with TMV, as compared to controls (kept at 20 °C), suggesting a role of superoxide in nonhost resistance of barley to TMV **(Fig. 10)**. However, H_2O_2 levels did not change significantly in TMV infected barley plants, regardless whether or not they were exposed to heat stress, suggesting that 1/ DAB-detection is not sensitive enough to detect ssubtle changes in H_2O_2 In order to confirm the finding that superoxide levels indeed correlate with nonhost resistance of barley to TMV we validated our NBT tissue staining results (superoxide accumulation in TMV-infected barley cv. Ingrid leaves containing BSMV and exposed/not exposed to heat stress) by quantitative image analysis using the Image J program (https://imagej.nih.gov/ij/). This revealed that in heat-stressed barley cv. Ingrid leaves containing BSMV and infected with TMV superoxide levels were only ca. 30-50 % of those in control plants (kept at 20 °C) **(Fig. 10)**.

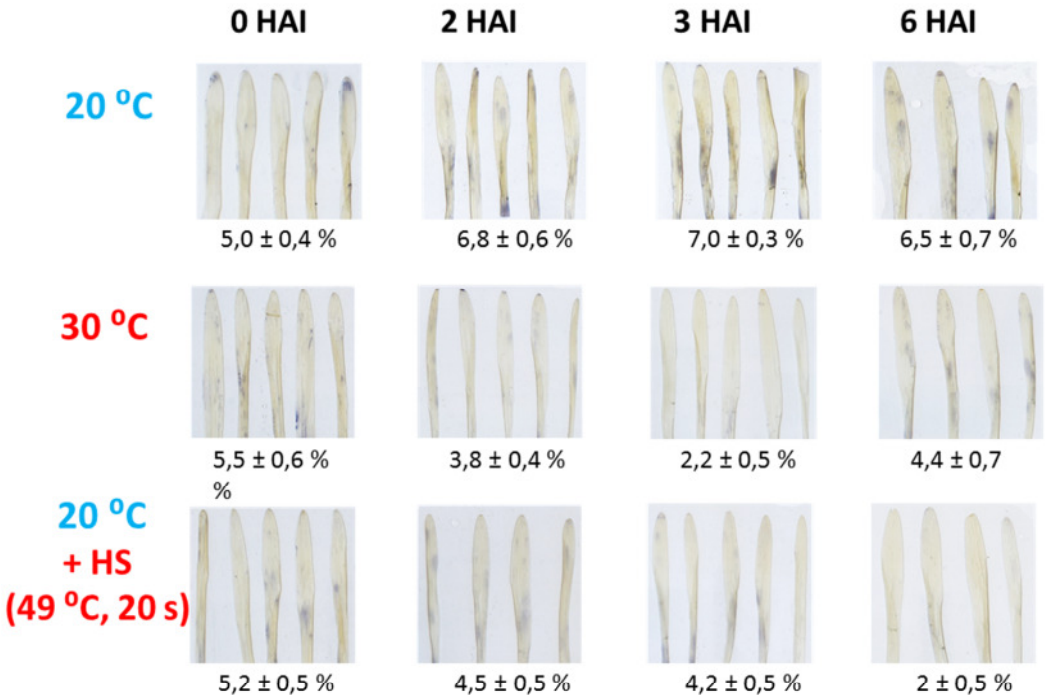


Figure 10 Absence of an early burst of superoxide ($O_2^{\cdot-}$) in *Tobacco mosaic virus* (TMV) infected cv. Ingrid barley containing *Barley stripe mosaic virus* (BSMV) and exposed to heat shock pre-treatments (30 °C for 3 hours before TMV inoculation; 49 °C for 20 seconds, at 2 hours before TMV inoculation) or constantly kept at 20 °C. Superoxide production detected by nitro blue tetrazolium chloride (NBT) staining. Qantification of NBT-staining was conducted by ImageJ (% leaf area stained with NBT). HAI = hours after inoculation.

We have also repeated the above-mentioned experiment (NBT tissue staining to detect superoxide accumulation) in TMV-infected but BSMV-free cv. Ingrid barley (exposed/not exposed to heat stress). In BSMV-free, heat-stressed barley cv. Ingrid leaves infected with TMV superoxide levels were also ca. 30-50 % of those in control plants (kept at 20 °C) **(Fig. 11)**. This test confirmed the finding that superoxide levels indeed correlate with nonhost resistance of barley to TMV at an early stage of virus infection (1-6

HAI), regardless of the presence or absence of BSMV. Importantly, expression of defense genes related to superoxide production/metabolism (*HvRBOHF2, HvSOD1*) are markers of both early ROS (superoxide) accumulation and nonhost resistance to TMV (compare Figures 8 and 9 to Figs 10 and 11).

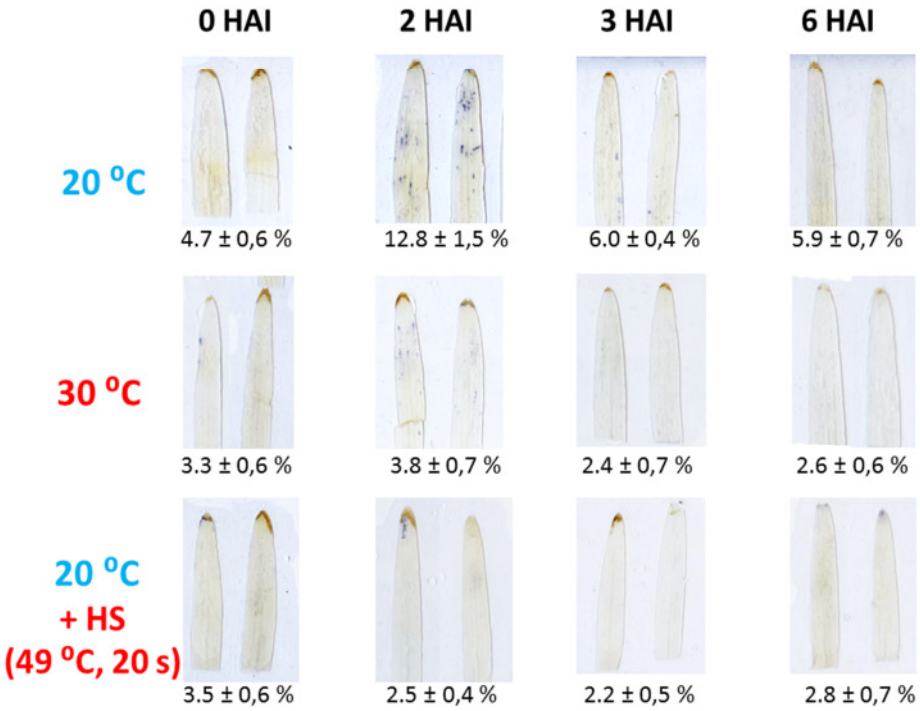


Figure 11 Absence of an early burst of superoxide ($O_2^{\cdot-}$) in *Tobacco mosaic virus* (TMV) infected cv. Ingrid barley exposed to heat shock pre-treatments (30 °C for 3 hours before TMV inoculation; 49 °C for 20 seconds, at 2 hours before TMV inoculation) or constantly kept at 20 °C. Superoxide production detected by nitro blue tetrazolium chloride (NBT) staining. Qantification of NBT-staining was conducted by ImageJ (% leaf area stained with NBT). HAI = hours after inoculation.

If ROS (superoxide) indeed contributes to the nonhost resistance of barley to TMV then the inhibition of superoxide accumulation by e.g. antioxidant treatments should at least partially inhibit nonhost resistance (i.e. increase TMV levels). The infiltration of the antioxidant enzymes superoxide dismutase (SOD, 2500 U/ml) and catalase (CAT, 5000 U/ml) into barley (cv. Ingrid, BSMV-free) leaves immediately after inoculation has significantly increased TMV levels as compared to control (buffer-infiltrated) plants. This increase in TMV titers was comparable to that caused by heat shock (49 °C, 20 sec) pre-treatments: at 2 and 5 DAI both antioxidant and heat shock treatments alone resulted in at least 50-100 % higher TMV levels **(Fig. 12)**. Remarkably, simultaneous application of heat shock and SOD-CAT infiltration caused an even higher (almost an order of magnitude) increase in TMV titers **(Fig. 12)** and often the appearance of visible, HR-type local necrotic symptoms **(Fig. 13)**.

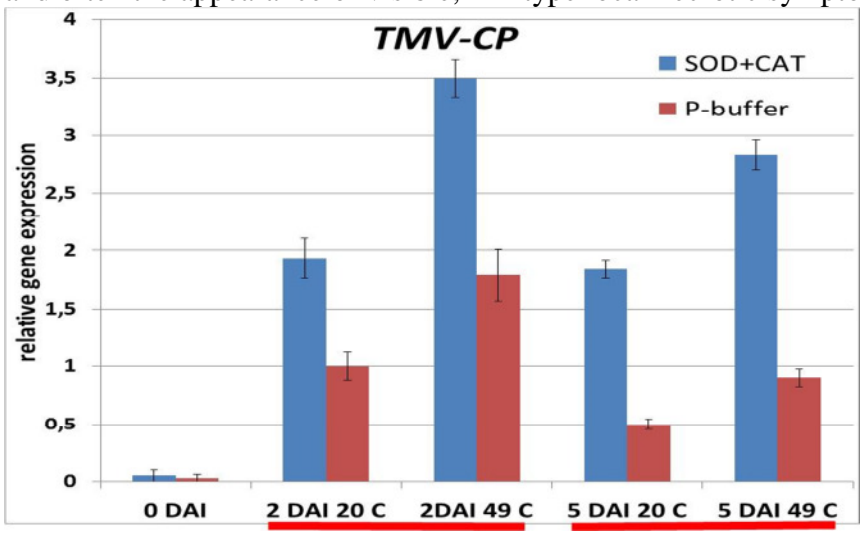


Figure 12 *Tobacco mosaic virus* (TMV) accumulation in cv. Ingrid barley, 2 and 5 days after virus inoculation (DAI), in response to a heat shock pre-treatment (49 °C for 20 seconds, at 2 hours before TMV inoculation, 20 °C = control treatment) and leaf infiltration of antioxidants (SOD, 2500 U/ml + CAT, 5000 U/ml; control infiltration: 10 mM K-phosphate buffer, pH 7,5). Expression of the TMV coat protein gene (*TMV-CP*) was assayed by real time RT-qPCR and normalized to that of the reference gene *HvUbi*.

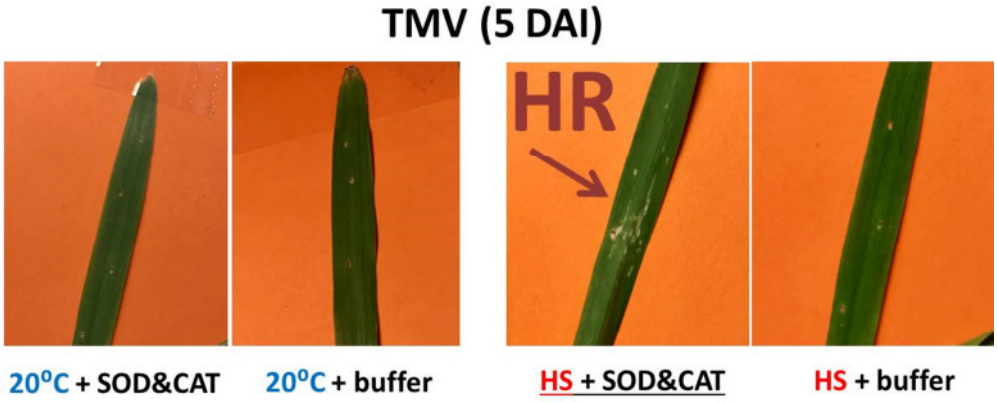


Figure 13 Combined application of heat shock pre-treatment (49 °C for 20 seconds, at 2 hours before TMV inoculation, 20 °C = control treatment) and leaf infiltration of antioxidants (SOD, 2500 U/ml + CAT, 5000 U/ml; control infiltration: 10 mM K-phosphate buffer, pH 7,5) in cv. Ingrid barley inoculated with *Tobacco mosaic virus* (TMV) may cause the appearance of visible necrotic lesions resembling a hypersensitive response (HR) and indicating programmed cell death (PCD). DAI = days after TMV inoculation.

Interestingly we found that even in those cases where a simultaneous application of heat shock (49 °C, 20 sec) and SOD-CAT infiltration did not result in visible, HR-type local necrotic symptoms, death of individual mesophyll cells and their chloroplasts could be detected in TMV-inoculated barley leaves (**Fig. 14**).

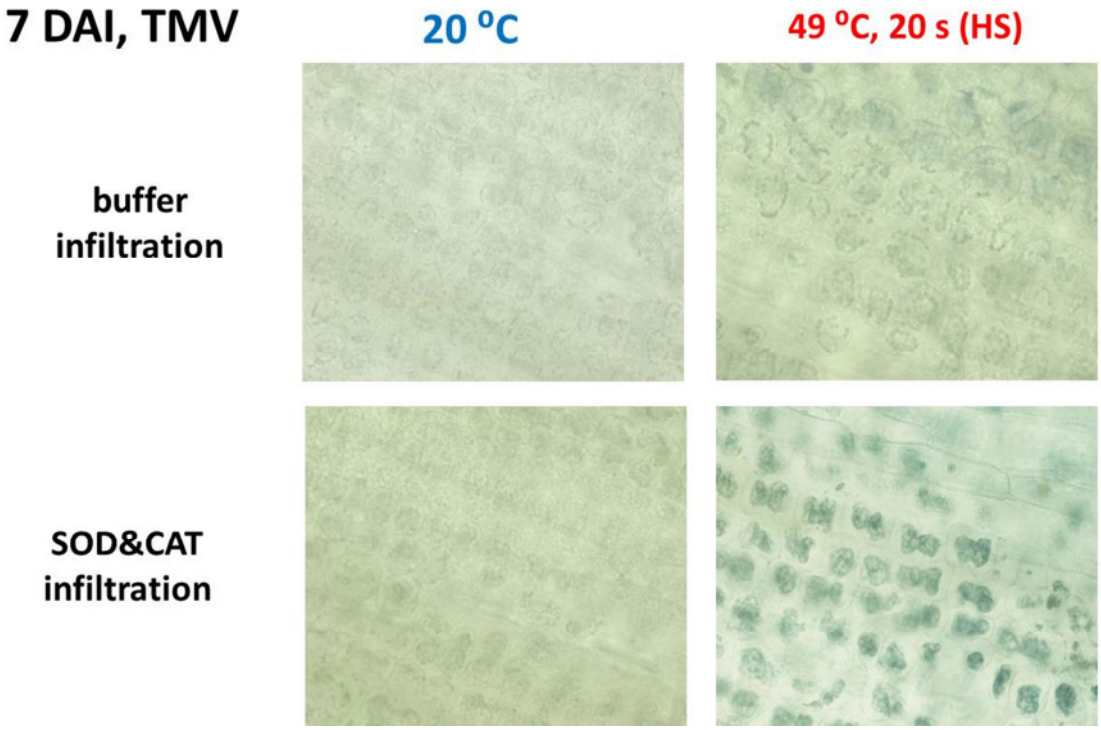


Figure 14 Heat shock pre-treatment (49 °C for 20 seconds, 2 hours before virus inoculation, 20 °C = control treatment), especially when combined with leaf infiltration of antioxidants (SOD, 2500 U/ml + CAT, 5000 U/ml; control infiltration: 10 mM K-phosphate buffer, pH 7,5) in cv. Ingrid barley inoculated with *Tobacco mosaic virus* (TMV) may cause death of mesophyll cells/chloroplasts even in the absence of a visible hypersensitive response (HR). Barley cell death visualized by Evans Blue staining. DAI = days after TMV inoculation.

Our results also revealed that at 2 DAI symptomless non-host resistance to TMV was correlated with enhanced expression of barley defense genes responsible for superoxide production/resistance (*HvRBOHF2*), and programmed cell death (PCD) regulation (*HvBI-1*) **(Fig. 15)**. However, at a later time point (5 DAI) *HvRBOHF2* and *HvBI-1* induction was rather associated with a compromised non-host resistance to TMV (HR), i.e. at later stages of pathogenesis these genes seem to function as stress markers **(Fig. 15)**.

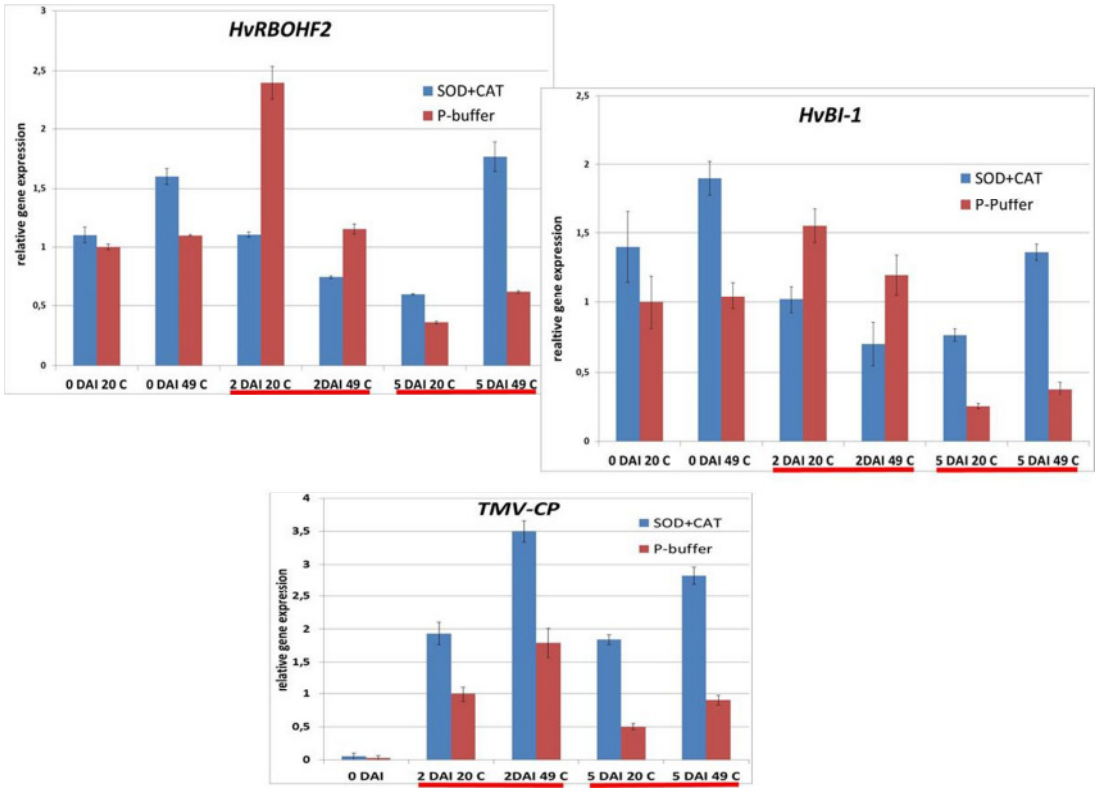


Figure 15 Expression of defense-related genes responsible for ROS (superoxide) production/resistance (*HvRBOHF2*) and PCD regulation (*HvBI-1*) in cv. Ingrid barley, 2 and 5 days after *Tobacco mosaic virus* (TMV) inoculation (DAI), in response to heat shock pre-treatments (49 °C for 20 seconds, at 2 hours before virus inoculation, 20 °C = control treatment) and leaf infiltration of antioxidants (SOD, 2500 U/ml + CAT, 5000 U/ml; control infiltration: 10 mM K-phosphate buffer, pH 7,5). Gene expression was assayed by real time RT-qPCR and normalized to that of the reference gene *HvUbi* and to mock-inoculated samples. For comparison, expression of the TMV coat protein gene (*TMV-CP*) in the same samples (see Fig. 7 lower panel) is also included.

The above results demonstrated that the synergistic effect of heat shock and antioxidants in suppressing nonhost resistance of barley to TMV indeed correlates with the appearance of HR-type local cell and tissue death (i.e. programmed cell death, PCD) and point to a functional role of ROS (superoxide) in maintaining this symptomless nonhost resistance response. Previously we have shown a similar effect in barley, where suppression of symptomless, nonhost resistance to wheat powdery mildew by the same heat shock and antioxidant treatments correlated with the appearance of HR symptoms (Künstler et al., 2018).

Heat exposure may compromise symtomless (Type I) nonhost resistance of barley to TMV in several cultivars

The first early experiments indicating that heat exposure may suppress symptomless, nonhost resistance to TMV used the traditional barley cv. Black Hulless (Hamilton and Dodds, 1970; Dodds and Hamilton, 1972). Therefore, we wanted to clarify if exposure to a sudden heat stress (either 30 °C for 3

hours or a 49 °C, 20 sec heat shock) can indeed suppress the symptomless, nonhost resistance to TMV not only in cv. Ingrid barley but also in the cv. Black Hulless?

We found that heat shock pre-treatments in the barley cv. Black Hulless also resulted in significantly (at least 50-100 %) higher TMV accumulation (assayed by RT-qPCR), as compared to non-pretreated controls kept at constant 20 °C **(Fig. 16)**. Furthermore, our experiments indicated that in TMV-infected cv. Black Hulless barley – similar to cv. Ingrid – the enhanced expression of 3 out of 4 investigated defense-related genes (*HvRBOHF2, HvSOD1, HvBI-1* but not *HvPR-1b*) correlate with nonhost resistance to TMV at 1 to 7 DAI (data not shown). These results suggest that, at least to a certain extent, defense responses could be retained in TMV-infected cv. Black Hulless barley even at a relatively late stage of pathogenesis.

We have also assayed expression of a barley defense gene encoding for a heat shock protein (*HSP90-1*), since our recent results indicate that *HvHSP90-1* expression is significantly (at least 50 %) down-regulated in a heat-pretreated cv. Ingrid barley line (mlo5) in parallel with a compromised symptomless host resistance to barley powdery mildew (Kolozsváriné Nagy et al., 2022). Indeed, our assays revealed that *HvHSP90-1* expression is also significantly declined in TMV-infected cv. Black Hulless barley (at 1-7 DAI), along with a heat shock-induced suppression of symptomless nonhost resistance to TMV **(Fig. 16)**.

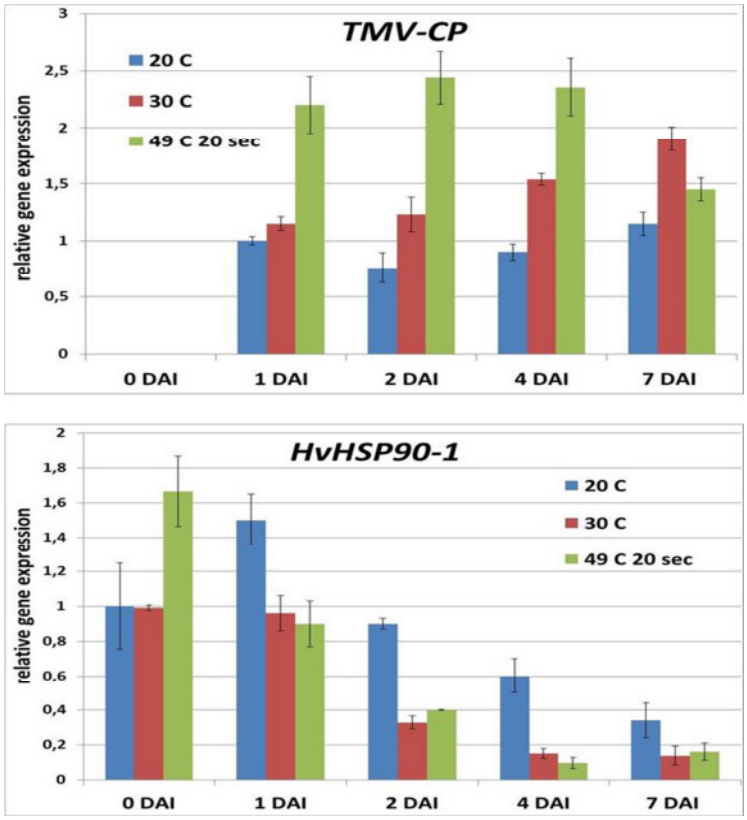


Figure 16 Accumulation of *Tobacco mosaic virus* (TMV) (upper panel) and expression of a defense gene encoding for a heat shock protein (*HvHSP90-1*) (lower panel) in barley cv. Black Hulless, following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 1, 2, 4 and 7 days after TMV inoculation (DAI). Expression of the TMV coat protein gene (*TMV-CP*) and *HvHSP90-1* was assayed by real time RT-qPCR and normalized to that of the reference gene *HvUbi* and to mock-inoculated samples.

We have repeated the above-mentioned experiments (monitoring defense gene expression in response to heat shock pretreatments and TMV infection) in a barley line (MvHV07-17) that is known to display symptomless host resistance to barley powdery mildew and tolerance to drought (Schwarczinger et al., 2021; Mészáros et al., 2020). In fact, we have obtained similar results as with cv. Black Hulless, i.e. heat shock pre-treatments resulted in significantly (at least 50-100 %) higher TMV accumulation and

reduced defense gene expression, while at normal temperatures (20 °C) virus resistance and defense gene expression (including that of *HvHSP90-1*) was retained in TMV-infected MvHV07-17 barley **(Fig. 17)**.

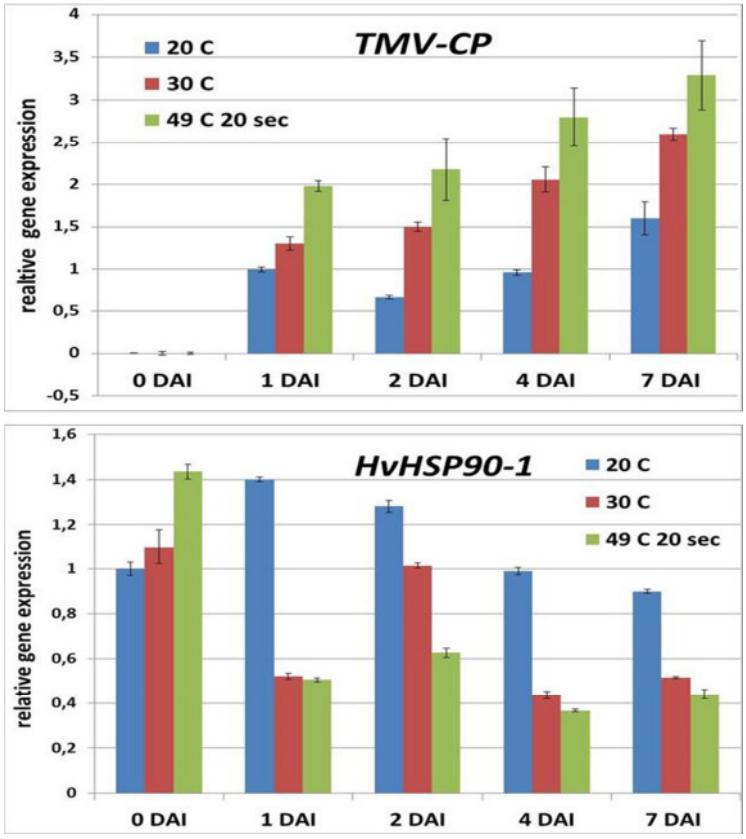


Figure 17 Accumulation of *Tobacco mosaic virus* (TMV) (upper panel) and expression of a defense gene encoding for a heat shock protein (*HvHSP90-1*) (lower panel) in the barley line MvHV07-17, following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 1, 2, 4 and 7 days after TMV inoculation (DAI). Expression of the TMV coat protein gene (*TMV-CP*) and *HvHSP90-1* was assayed by real time RT-qPCR and normalized to that of the reference gene *HvUbi* and to mock-inoculated samples.

In summary, heat shock pre-exposure may compromise symtomless (Type I) nonhost resistance of barley to TMV at least in three different cultivars (Ingrid, Black Hulless, MvHV07-17), pointing to a general role of optimal (as opposed to high) temperatures and reactive oxygen species (ROS) in maintaining symtomless (Type I) nonhost resistance of barley to viruses like TMV.

Glutathione may take part in suppressing symtomless (Type I) nonhost resistance of barley cv. Ingrid to TMV at later stages of infection

Our results demonstrated that the infiltration of antioxidant enzymes (SOD and CAT) can significantly suppress nonhost resistance of barley cv. Ingrid to TMV (see Fig. 12) and confirm the role of antioxidants in maintaining partial susceptibility of barley to nonadapted pathogens (Künstler et al., 2018). We wanted to clarify if a non-enzymatic antioxidant like glutathione may also take part in suppressing the nonhost resistance of barley to TMV (i.e. in maintaining partial susceptibility)? Levels of reduced and oxidized glutathione (GSH and GSSG) were assayed by HPLC separation and electronspray ionization mass spectrometric analyses (HPLC-ESI/MS), as described by us earlier (Künstler et al., 2019). In fact, our experiments revealed that GSH levels indeed significantly increase during heat shock-induced suppression of symtomless (Type I) nonhost resistance of barley cv. Ingrid to TMV at later stages of virus infection (1 to 7 DAI) **(Fig. 18)**.

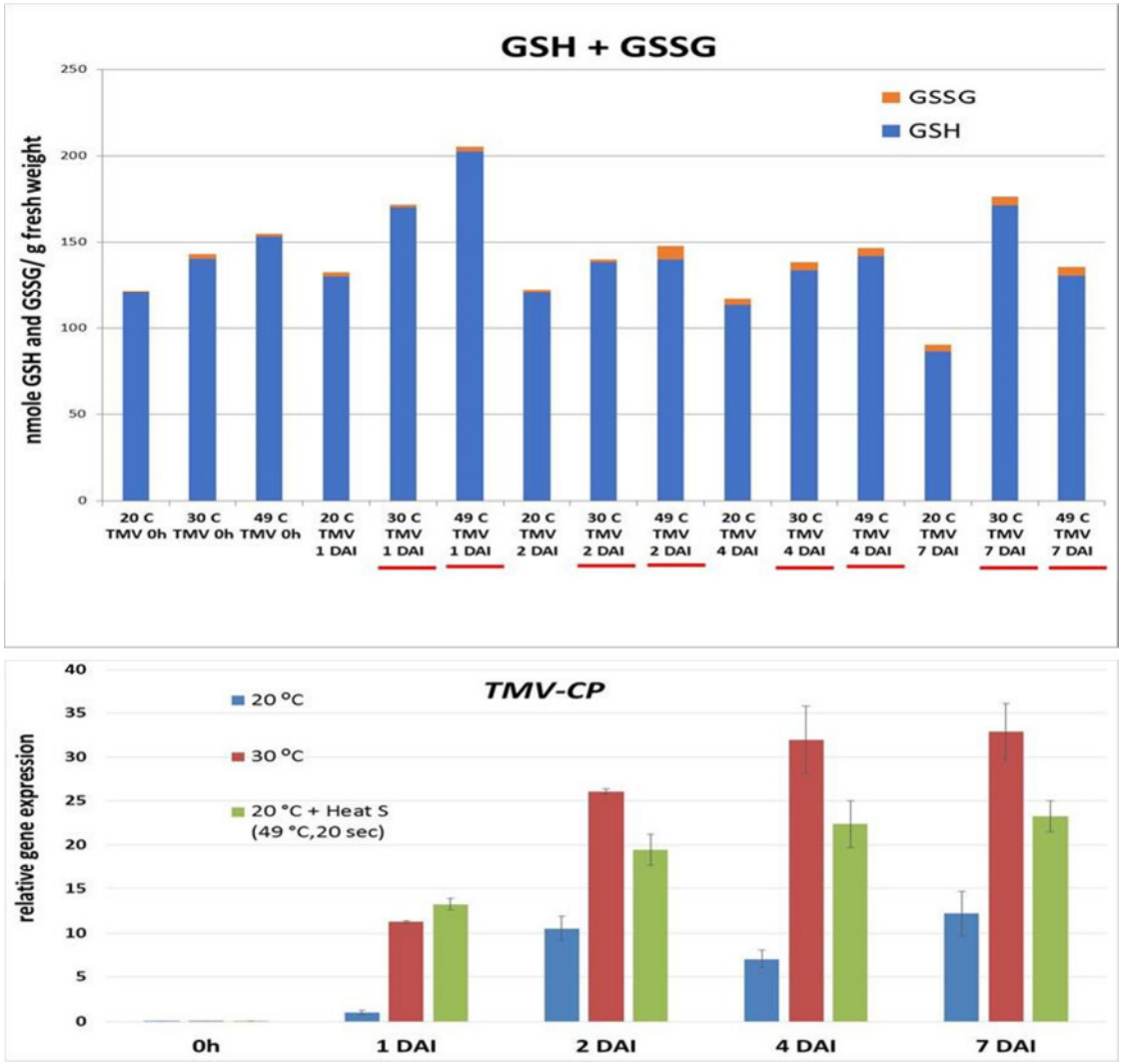


Figure 18 Levels of reduced and oxidized glutathione (GSH and GSSG) in barley cv. Ingrid, following pre-exposure to a heat shock (49 °C for 20 sec), or to 30 °C for 3 hours or constantly kept at 20 °C, at 1, 2, 4 and 7 days after *Tobacco mosaic virus* (TMV) inoculation (DAI). Glutathione was assayed by HPLC separation and electronspray ionization mass spectrometric analyses (HPLC-ESI/MS). For comparison, expression of the TMV coat protein gene (*TMV-CP*) in the same samples is also included.

The above results are in line with earlier findings demonstrating that glutathione contents increase significantly in wild type cv. Ingrid barley susceptible to barley powdery mildew (*Blumeria hordei*) in an advanced stage of pathogenesis (7 DAI) (Harrach et al., 2008). However, glutathione may potentially play a role in modulating/signaling e.g. nonhost resistance to TMV at early time points after inoculation. In addition to its antioxidative role, glutathione is also known as a central regulator of plant resistance to pathogens, including viruses, its presence being indispensable for the proper (early) timing of plant defense responses (Gullner et al., 1999; Vanacker et al. 1999; Ghanta et al., 2011, 2014; Künstler et al. 2019, 2020; Zechmann, 2020). Our future goal is to investigate whether high levels of glutathione at early infection stages are required for nonhost resistance of barley to e.g. TMV?

Roles of temperature, ROS and SA in symptomless (Type I) nonhost resistance of tobacco to BSMV

We attempted to use tobacco (*Nicotiana tabacum*) as a model to elucidate whether viruses adapted to cereals (e.g. *Barley stripe mosaic virus*, BSMV) can, under certain conditions, replicate in plants that are normally nonhosts for these pathogens. The plant hormone salicylic acid (SA) plays a pivotal role in host resistance of e.g. tobacco to TMV (Gaffney et al., 1993). First we wanted to investigate whether the plant hormone salicylic acid (SA) contributes to the possibly symptomless (Type I) nonhost resistance of

tobacco to BSMV by using a tobacco line accumulating low SA (cv. Xanthi NN nahG) (Gaffney et al., 1993). To assess if SA may have a role in nonhost virus resistance of tobacco, we have inoculated wild type and nahG cv. Xanthi tobacco with a Hungarian BSMV isolate (BSMV-Hu).

No symptoms developed up to 14 DAI, suggesting the operation of symptomless (Type I) nonhost resistance to BSMV. At 4 and 7 DAI, expression of the defense marker gene *NtPR-1a* markedly decreased in SA-deficient (NahG) plants, as compared to wild type cv. Xanthi NN tobacco which might indicate a suppression of nonhost resistance to BSMV. However, we could not detect any BSMV in these plants by real time RT-qPCR and later it turned out that our BSMV-Hu isolate has lost its infectivity. Therefore, at present, we are in the process of testing a new BSMV isolate from Germany (PV-0330) but so far we could not obtain a sufficient amount of infective material 8BSMV-inoculated barley leaves) in order to continue the experiments described above.

We have created tobacco F_1 plants with a potentially impaired nonhost resistance to e.g. BSMV by crossing SA-deficient NahG tobacco with transgenic lines (cv. SR1, lines C8 and F9) that overexpress the alfalfa ferritin gene *MsFer* (Deák et al., 1999). All F_1 plants obtained from the above mentioned crosses tested positive for presence/expression of both parental transgenes (*PpNahg* and *MsFer*) by RT-PCR. Ferritin-overproducing plants are deficient in the ROS hydroxyl radical ($OH^{\cdot}$) and, according to our recent results, can partially suppress a symptomless resistance to *Potato virus X* (PVX) (Király et al., 2021). Therefore, F_1 tobacco plants that are both SA-deficient and overexpress ferritin (i.e. ROS-deficient) could possibly display an impaired nonhost resistance to e.g. BSMV, if SA and ROS indeed play a role in this type of symptomless defense to nonadapted pathogens. Based on our project results obtained with barley/TMV, it can be also anticipated that the nonhost resistance of these ROS/SA-deficient F_1 tobaccos to e.g. BSMV could further deteriorate in response to heat shock pre-treatments.

Other, published results related to the project

In collaboration with a research group at the University of Missouri (USA), we have been involved in investigating resistance responses to members of the *Tombusviridae* group. One of these viruses is *Tobacco necrosis virus* (TNV), causing HR-associated (Type II) nonhost resistance (NHR), rather than symptomless NHR, in most plant species including tobacco (Price, 1940). The hypersensitive response (HR) is manifested as small necrotic lesions indicating localization of the invading virus. As opposed to several other *Tombusviridae* viruses, we found that the TNV coat protein elicits nonhost HR but cannot suppress virus gene-silencing, the first line of plant defense to pathogenic infections. Results of this work have been published this past year (**Adhab et al., 2019**).

TMV, the virus causing symptomless (Type I) nonhost resistance e.g. in barley, may elicit HR-associated (Type II) host resistance in several other plant species, including tobacco. According to previous research, this type of plant defense is governed by salicylic acid and requires glutathione (GSH) for its normal function – enhancing GSH biosynthesis leads to improved resistance to bacterial and fungal infections through elevated SA levels (Ghanta et al., 2011, 2014). Our published results (Künstler et al., 2019) have demonstrated that GSH overproduction confers SA accumulation and elevated resistance also to a virus pathogen, TMV. We have further shown that in tobacco GSH may even compensate for SA deficiency to maintain HR-associated host resistance to a virus: elevation of endogenous GSH levels in SA-deficient NahG tobacco, both genetically and by exogenous treatments, restores wild type levels of TMV resistance (**Künstler et al., 2019**). However, the exact mechanism of interplay between GSH and SA in nonhost plant virus resistance is still largely unknown.

We have successfully published a review article on roles of various sulfur-containing compounds in plant disease resistance, including nonhost resistance to e.g. viruses (**Künstler et al., 2020**).

Our group has published a paper in 2021 demonstrating the functional contribution of ROS (superoxide and $OH^{\cdot}$) to the symptomless host resistance (so called extreme resistance) of tobacco to *Potato virus X* (PVX) (**Király et al., 2021**). Treatments with antioxidants (SOD, CAT) or crossing extreme resistant tobacco with ROS ($OH^{\cdot}$) deficient, ferritin-overproducing plants resulted in a partial suppression of this symptomless virus resistance.

In 2022 we have published a paper demonstrating that near-isogenic lines of the barley cv. Ingrid show enhanced susceptibility to barley powdery mildew mildew (*B. hordei*) infection in response to a pre-exposure to high-temperature stress (35 °C from 30 s to 5 days) (**Kolozsváriné Nagy et al., 2022**). This work confirms that heat stress pre-exposure of barley (in particular, cv. Ingrid) may indeed suppress resistance to different type of pathogens (e.g. viruses and fungi).

We have also published a work in 2022 showing that foliar applications of the culture filtrate of a rhizobacterium (*Bacillus amyloliquefaciens*) results in enhanced systemic, symptomless resistance of tomato plants to TMV that correlates with elevated defense (*PR*) gene expression and a tight regulation of *in planta* ROS production (**Abdelkhalek et al., 2022**).

In collaboration with a polish research group (Warsaw University of Life Sciences), we have recently shown that enhanced susceptibility of a ROS (superoxide) deficient *Arabidopsis thaliana* mutant (*rbohD*-) to *Turnip mosaic virus* (TuMV) is associated with reduced contents of total cellular and apoplastic glutathione (**Otulak-Kozieł et al., 2023**).

The role and interplay of GSH and SA in nonhost plant virus resistance is still largely unexplored. However, in framework of an international collaboration, we could demonstrate that the role of salicylic acid in maintaining an HR-type nonhost resistance of *N. edwardsonii* var. Columbia to *Tobacco necrosis virus* (TNV) is indeed associated with high levels of glutathione in various subcellular compartments (**Király et al., 2023**).

Summary

We have shown in three different barley (*Hordeum vulgare*) cultivars that a pre-exposure to heat shock (either 30 °C for 3 hours or 49 °C, 20 sec) results in a significant suppression of symptomless nonhost resistance to *Tobacco mosaic virus* (TMV), regardless whether or not the plants contain an adapted barley virus, BSMV. Importantly, we confirmed the presence of infective TMV in the originally inoculated barley leaves (regardless of the presence/absence of BSMV). In TMV-inoculated barley either pre-exposed/not exposed to heat shock, expression of defense-related genes (*HvRBOHF2, HvSOD1, HvBI-1*) generally correlated with nonhost resistance to TMV at both early (1-24 HAI) and late (1-7 DAI) stages of infection. Reactive oxygen species (superoxide) accumulation was significantly lower in TMV-infected heat-shocked barley cv. Ingrid leaves at early timepoints (2-6 HAI), suggesting a role of superoxide in symptomless nonhost resistance of barley to TMV. We found a synergistic effect of heat shock and antioxidants (SOD+CAT) in suppressing nonhost resistance of barley to TMV, confirming the functional role of ROS (superoxide) in this symptomless antiviral defense response. Furthermore, our experiments suggest a role of the non-enzymatic antioxidant glutathione in maintaining heat shock-induced suppression of symtomless (Type I) nonhost resistance of barley cv. Ingrid to TMV at later stages of virus infection (1 to 7 DAI).

Conclusions and future perspectives of our research project

It is worth mentioning that heat shock pre-exposure suppressed nonhost resistance to TMV also in a barley line (MvHV07-17) that is known to display symptomless host resistance to barley powdery mildew and tolerance to drought (Schwarczinger et al., 2021; Mészáros et al., 2020). In fact, MvHV07-17 barley can maintain its powdery mildew resistance even at a constant temperature of 35 °C for up to five days (Schwarczinger et al., 2021). Therefore, monitoring and characterizing the symptomless, multi-stress resistance responses of crops like MvHV07-17 barley is of primary importance and should be a part of contemporary plant breeding programs.

Although the molecular/pathophysiological mechanisms of (symptomless) nonhost resistance of plants to viruses remains a largely unexplored research area, our project results suggest the importance of temperature, ROS and plant hormones like salicylic acid in these defense processes. Therefore, studying the influence of heat stress on plant (virus) disease resistance responses should and will become a pivotal issue, considering recent trends in global climate change.

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