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PII: S0981-9428(18)30210-9

DOI: 10.1016/j.plaphy.2018.05.010

Reference: PLAPHY 5257

To appear in: *Plant Physiology and Biochemistry*

Received Date: 20 March 2018

Revised Date: 4 May 2018

Accepted Date: 6 May 2018

Please cite this article as: Andrá. Künstler, Rená. Bacsó, Ré. Albert, Balá. Barna, Zoltá. Király, Y.M. Hafez, Jó. Fodor, Ildikó. Schwarczinger, Lóá. Király, Superoxide ($O_2^{\cdot-}$) accumulation contributes to symptomless (type I) nonhost resistance of plants to biotrophic pathogens, *Plant Physiology et Biochemistry* (2018), doi: 10.1016/j.plaphy.2018.05.010.

Superoxide ($O_2^{\cdot-}$) accumulation contributes to symptomless (type I) nonhost resistance of plants to biotrophic pathogens

András Künstler[1], Renáta Bacsó[1], Réka Albert, Balázs Barna, Zoltán Király, Yaser Mohamed Hafez[b], József Fodor, Ildikó Schwarczinger, Lóránt Király*

Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, H-1022 Budapest, Herman Ottó str. 15, Hungary

[b]Present address: EPCRS Excellence Center & Plant Pathology and Biotechnology Lab, Agricultural Botany Department, Faculty of Agriculture, Kafr-El-Sheikh University, 33516, Kafr-El-Sheikh, Egypt

[1]A. Künstler and R. Bacsó contributed equally to this work and are considered as co-first authors

*Corresponding author: kiraly.lorant@agrar.mta.hu

Running head: Superoxide in plant nonhost resistance

Keywords: superoxide; NADPH oxidase; symptomless (type I) nonhost resistance; hypersensitive response; heat shock; antioxidants; biotrophic pathogens

Abbreviations: Bgh, *Blumeria graminis* f. sp. *hordei*; *Bgt*, *Blumeria graminis* f. sp. *tritici*; BI-

1, BAX inhibitor-1; CAT, catalase; DAI, days after inoculation; ETI, effector-triggered immunity; HAI, hours after inoculation; H_2O_2, hydrogen peroxide; HR, hypersensitive response; NBT, nitro blue tetrazolium chloride; $O_2^{\cdot -}$, superoxide; PAMP, pathogen-associated molecular pattern; PCD, programmed cell/tissue death; PTI, PAMP-triggered immunity; ROS, reactive oxygen species; SOD, superoxide dismutase;

Competing interest statement: Authors have no competing interests to declare

Abstract

Nonhost resistance is the most common form of disease resistance exhibited by plants against most pathogenic microorganisms. Type I nonhost resistance is symptomless (i.e. no macroscopically visible cell/tissue death), implying an early halt of pathogen growth. The timing/speed of defences is much more rapid during type I nonhost resistance than during type II nonhost and host ("gene-for-gene") resistance associated with a hypersensitive response (localized necrosis, HR). However, the mechanism(s) underlying symptomless (type I) nonhost resistance is not entirely understood. Here we assessed accumulation dynamics of the reactive oxygen species superoxide ($O_2^{\cdot-}$) during interactions of plants with a range of biotrophic and hemibiotrophic pathogens resulting in susceptibility, symptomless nonhost resistance or host resistance with HR. Our results show that the timing of macroscopically detectable superoxide accumulation (1-4 days after inoculation, DAI) is always associated with the speed of the defense response (symptomless nonhost resistance vs. host resistance with HR) in inoculated leaves. The relatively early (1 DAI) superoxide accumulation during symptomless nonhost resistance of barley to wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) is localized to mesophyll chloroplasts of inoculated leaves and coupled to enhanced NADPH oxidase (EC 1.6.3.1) activity and transient increases in expression of genes regulating superoxide levels and cell death (superoxide dismutase, *HvSOD1* and BAX inhibitor-1, *HvBI-1*). Importantly, the partial suppression of symptomless nonhost resistance of barley to wheat powdery mildew by heat shock (49 °C, 45 sec) and antioxidant (SOD and catalase) treatments points to a functional role of superoxide in symptomless (type I) nonhost resistance.

1. Introduction

Plants are generally resistant to a wide range of potential pathogens present in the environment meaning that nonhost resistance operating in all cultivars of a given plant species is effective against all races of a particular pathogen (Heath, 2000; Gill et al., 2015; Lee et al., 2017). Due to its durability, nonhost resistance has been considered as a potential means of effective pathogen control. Understanding its mechanisms is crucial for breeding disease resistant cultivars (Gill et al., 2015). Nonhost resistance is manifested in several obstacles, including presence/absence of e.g. plant surface topology features required to initiate pathogen growth, preformed barriers like the cell wall, cuticle (surface waxes), actin microfilaments, products of glucosinolate metabolism, and induced defense responses, e.g. lignin accumulation, production of antimicrobials like phytoalexins and induction of pathogenesis-related (PR) proteins (Thordal-Christensen 2003; Mysore and Ryu, 2004; Gill et al., 2015; Lee et al., 2017 and references herein).

The typical form of nonhost resistance (type I, without macroscopic symptoms) could result from the initial plant defense response against microbial invaders involving recognition of pathogen-associated molecular patterns (PAMPs), also called PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Boller and Felix, 2009; Niks and Marcel, 2009). Although adapted pathogens suppress this reaction in their hosts by the activity of effector proteins, the typical form of host resistance (i.e. race-cultivar specific “gene-for-gene” resistance) may develop as a second line of plant defense. This is also known as effector-triggered immunity (ETI), mediated by the activity of pathogen effectors recognized by plant resistance (R) proteins (Jones and Dangl, 2006; Dangl et al., 2013). The consequence of ETI is the elicitation of a resistance reaction, often associated with localized programmed cell/tissue death (PCD) at infection sites (hypersensitive response, HR), ultimately limiting pathogen

spread (Hammond-Kosack and Jones, 1997). In fact, nonhost resistance can be also associated with an HR (type II nonhost resistance) implying a role of ETI in both host and nonhost resistance (Mysore and Ryu, 2004; Gill et al., 2015; Lee et al., 2017 and references herein). However, the symptomless (no macroscopic HR) type I nonhost resistance is probably the most common among nonhost-pathogen interactions (Mysore and Ryu, 2004). During type I nonhost resistance the plant does not show any visible symptoms after inoculation with a nonadapted pathogen, implying that pathogen growth is halted early, as a consequence of preformed and/or inducible defenses, including PTI. In contrast, during the slower type II nonhost resistance, as in many cases of host resistance (ETI), an HR (localized necrosis) is triggered because the pathogen can disarm the first layers of defense and is recognized by the plant only in later stages of pathogenesis (Mysore and Ryu, 2004). HR during both nonhost and host resistance share similar signaling processes, e.g. the accumulation of reactive oxygen species (ROS). Importantly, ROS have a dual role during plant defense to infections: 1) higher ROS concentrations confer inhibition/killing of invading pathogens along with promoting PCD of infected plant cells (HR) and oxidative cross linking of cell wall components (penetration resistance) 2) low ROS concentrations act as signals inducing antioxidants and pathogenesis-related genes in plant tissues adjacent to infection sites (Levine et al., 1994; Thordal-Christensen et al., 1997; Dat et al., 2000; Torres et al., 2005; Hafez et al., 2012).

The first reports on the role of the ROS hydrogen peroxide (H_2O_2) during type II (HR-associated) nonhost resistance found enhanced H_2O_2 accumulation during HR-associated nonhost resistance to plant pathogenic bacteria (*Pseudomonas* spp.) (Bestwick et al., 1998; Yoda et al., 2009). Further research emphasized the pivotal role of H_2O_2 generated in plant cell organelles (peroxisomes, chloroplasts) during HR-associated nonhost resistance to bacteria (Zurbriggen et al., 2009; Rojas et al., 2012). The role of the ROS superoxide ($O_2^{\cdot-}$; its

dismutation by e.g. superoxide dismutases [EC 1.15.1.1] generates H_2O_2, Halliwell and Gutteridge, 2015) in HR-associated nonhost resistance has been also documented. During type II (HR-associated) nonhost resistance in the *Capsicum annuum*/*Xanthomonas campestris* pv. *vesicatoria* interaction, both $O_2^{\cdot-}$ and H_2O_2 accumulate much earlier than during HR-associated host resistance to a different *X. campestris* pathovar (Kwak et al., 2009). Furthermore, inactivation of genes (encoding NADPH oxidase and Rac GTPase) that determine *in planta* superoxide generation leads to suppression of HR-associated nonhost resistance to bacterial and oomycete pathogens (Yoshioka et al., 2003; Moeder et al., 2005; An et al., 2017).

As regards the role of ROS in symptomless (type I) nonhost resistance, it is known that accumulation of H_2O_2 but not $O_2^{\cdot-}$ is induced during nonhost resistance of barley to wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*, *Bgt*) at cellular sites of attempted fungal penetration in the leaf epidermis (Hückelhoven et al., 2001a). A similar pattern of localized H_2O_2 accumulation was also associated with symptomless nonhost resistance of cowpea (*Vigna unguiculata*) to the cucurbit powdery mildew *Erysiphe cichoracearum* (Mellersh et al., 2002). These results suggest a role for H_2O_2 in directly inhibiting pathogen penetration at the epidermis during symptomless (type I) nonhost resistance to powdery mildews. However, $O_2^{\cdot-}$ generated in plant tissues distal to pathogen attack might also influence defense signaling during symptomless nonhost resistance. Trujillo et al. (2004a) found that $O_2^{\cdot-}$ was detectable in epidermal cells distal from attacked cells in barley and wheat exhibiting nonhost resistance to the powdery mildews *Bgt* and *B. graminis* f. sp. *hordei* (*Bgh*), respectively, suggesting a role for $O_2^{\cdot-}$ in the signaling process leading to macroscopically symptomless (type I) nonhost resistance.

We have shown previously that macroscopically symptomless host resistance (without HR) can be induced to biotrophic and hemibiotrophic pathogens (powdery mildews, rusts,

bacteria) by external treatments (riboflavin-methionine, xanthine-xanthine oxidase) that generate $O_2^{\cdot-}$ one to three days after inoculation. However, the same treatments applied later induce host resistance with HR (El-Zahaby et al., 2004). This is in line with the enhanced accumulation of $O_2^{\cdot-}$ and H_2O_2 correlating with HR development during bacteria-induced host resistance, a process accompanied by a drop in antioxidant levels evident e.g. in chloroplasts (Grosskinsky et al., 2012). In fact, symptomless vs. HR-associated host resistance of barley to its powdery mildew correlates with an earlier vs. later $O_2^{\cdot-}$ accumulation in mesophyll chloroplasts beneath infection sites (Hückelhoven and Kogel, 1998). In addition, we have recently demonstrated that the graft-transmissible, symptomless host resistance of cherry pepper (*Capsicum annuum* var. *cerasiforme*) to its powdery mildew (*Leveillula taurica*) is coupled to constitutive, NADPH oxidase-associated $O_2^{\cdot-}$ accumulation (Albert et al., 2017).

To elucidate the possible role of $O_2^{\cdot-}$ in symptomless (type I) nonhost resistance, here we assess the dynamics of superoxide accumulation during several plant-pathogen interactions (infections by [hemi]biotrophic pathogens) that result either in susceptibility, symptomless nonhost resistance or host resistance with an HR. We further focus on the functional role of $O_2^{\cdot-}$ during symptomless nonhost and HR-type host resistance of barley to powdery mildews. Our results show that the timing of macroscopically detectable $O_2^{\cdot-}$ accumulation in inoculated tissues is always associated with the speed of the defense response (symptomless nonhost resistance vs. host resistance with an HR). Importantly, the partial suppression of symptomless nonhost resistance of barley to wheat powdery mildew (*Bgt*) by heat shock and antioxidant treatments points to a functional role of $O_2^{\cdot-}$ in symptomless (type I) nonhost resistance.

2. Materials and methods

176 **2.1 Plants, pathogens and inoculation**

177

178 The barley (*Hordeum vulgare*) cv. Ingrid (wild type, *Mlo*), and near isogenic backcross
179 lines Ingrid *Mla12*, and Ingrid *mlo5* were kindly supplied by Ralph Hückelhoven (Technical
180 University of Munich, Germany). Their generation was described previously (see e.g. Harrach
181 et al. 2008 and references herein). The barley cv. Botond, wheat (*Triticum aestivum*) cvs.
182 Buzogány and MV-Emma and potato (*Solanum tuberosum*) cvs. Hópehely and White Lady
183 are commercially available in Hungary. The grapevine (*Vitis vinifera*) cvs. Nimrang and
184 Kishmish vatkana were a kind gift of Pál Kozma (University of Pécs, Hungary). Plants were
185 grown under greenhouse conditions (18-23 °C, 16 h photoperiod with a supplemental light of
186 160 µmol m^{-2} s^{-1}, 75-80 % relative humidity).

187 The barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) used in this study (race
188 A6) was kindly supplied by Ralph Hückelhoven (Technical University of Munich, Germany).
189 Race 77 of wheat leaf rust (*Puccinia triticina*, syn. *P. recondita* f. sp. *tritici*) (El-Zahaby et al.,
190 2004) and the K-39 isolate of the potato late blight pathogen (*Phytophthora infestans*) (a gift
191 of József Bakonyi, Plant Protection Institute, CAR, HAS, Budapest, Hungary) were used.
192 Isolates of wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*), barley leaf rust (*Puccinia*
193 *hordei*) and grapevine powdery mildew (*Erysiphe necator*) pathogens used in the present
194 study were collected and isolated in greenhouses of the Plant Protection Institute, CAR, HAS,
195 Budapest, Hungary.

196 Barley and wheat powdery mildews (*B. graminis* f. sp. *hordei, Bgh* and *B. graminis* f.
197 sp. *tritici, Bgt*) were maintained on susceptible host plants (barley cv. Ingrid *Mlo* and wheat
198 cv. Buzogány, respectively) in growth chambers (20 °C, 60% relative humidity, 16 h
199 photoperiod of 100 µmol m^{-2} s^{-1}). Barley and wheat leaf rusts (*P. hordei* and *P. triticina*) and
200 *E. necator* were maintained on their susceptible hosts (barley cv. Ingrid *Mlo*, wheat cv.

Buzogány and grapevine cv. Nimrang, respectively) under greenhouse conditions described above. *P. infestans* was maintained on a selective pea-broth agar (PBA) at 20 °C.

Barley and wheat powdery mildews (*Bgh* and *Bgt*) were inoculated onto primary leaves of 7 day-old barley and wheat plants to give an inoculation density of ca. 50 conidia mm^{-2} as described by Harrach et al. (2008). In barley inoculated with *Bgh* and *Bgt* fungal structures were visualized for light microscopy with Pelikan blue staining by incubating leaves in 10% (v/v) blue ink (Pelikan AG) dissolved in 25% (v/v) acetic acid for 1 min (Hückelhoven and Kogel, 1998). For microscopic imaging of fungal structures and $O_2^{\cdot-}$ accumulation in barley leaf tissues an Olympus BX51 light microscope was used. Barley and wheat leaf rusts (*P. hordei* and *P. triticina*) were inoculated onto primary leaves of 5 day-old barley and wheat plants by applying uredospore suspensions in 1% (w/v) starch (ca. 20-25 mg uredospores per 100 ml suspension). Grapevine powdery mildew (*E. necator*) was inoculated to susceptible host plants by touching the adaxial epidermis of fully expanded leaves with sporulating colonies on the surface of source leaves (Hoffmann et al., 2008). *P. infestans* was inoculated onto potato leaves with a filtered sporangial suspension (50 000 sporangia ml^{-1}) essentially as described (Cohen and Reuveni, 1983).

2.2 Detection of superoxide ($O_2^{\cdot-}$) and NADPH oxidase enzyme activity

Superoxide ($O_2^{\cdot-}$) accumulation in barley leaves inoculated with *Bgt* or *Bgh* was detected by histochemical staining with 0.1 % (w/v) nitro blue tetrazolium chloride (NBT) (Sigma Aldrich Co.) by vacuum infiltration according to the procedure of Ádám et al. (1989). Infiltrated leaf samples were incubated under daylight for 20 min and subsequently cleared in a solution containing 0.15 % (w/v) trichloroacetic acid in ethanol: chloroform 4:1 (v/v) and stored in 50 % (v/v) glycerol until photography (Hückelhoven and Kogel, 1998). $O_2^{\cdot}$

accumulation (percentage of NBT-stained area per leaf) was quantified by using the ImageJ program (https://imagej.nih.gov/ij/).

NADPH oxidase (EC 1.6.3.1) enzyme activity in barley leaves either un-inoculated or inoculated with *Bgt* or *Bgh* was determined as described by Ádám et al. (1997) and Xia et al. (2009) with modifications. Samples were homogenized in four volumes of extraction buffer (50 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 1 mM ascorbic acid, 1 mM EDTA, 0.6% [w/v] PVP and 1 mM PMSF [phenylmethane sulfonyl fluoride]). Pellets obtained by ultracentrifugation were resuspended in 0.5 ml extraction buffer before immediate use in photometric assays at 530 nm. 50 µl supernatant was added to 2 ml assay buffer (0.2 mM NADPH, 0.3 mM NBT and 50 mM HEPES, pH 6.8). In order to detect NADPH oxidase specific activity, horseradish superoxide dismutase (SOD, EC 1.15.1.1, 40 units ml^{-1}) (Sigma Aldrich Co.) was added to the reaction mixture and the obtained activity was subtracted from that measured without SOD.

2.3 Heat shock and treatments with antioxidants (superoxide dismutase and catalase)

Heat shock treatment of barley leaves was accomplished essentially as described by Barna et al. (2014). Leaves of 7 day-old intact barley plants were immersed in 49 °C water for 45 sec 30 min before inoculation with *Bgt* or *Bgh*, to allow sufficient time for drying of leaf surfaces.

Simultaneous infiltration of superoxide dismutase and catalase (SOD and CAT [EC 1.11.1.6], 2500 and 5000 units ml^{-1}, equivalent to 0.8 and 1.4 mg protein ml^{-1}, respectively) (Sigma Aldrich Co.) into barley leaves was conducted immediately after inoculation, according to Hafez and Király (2003).

2.4 Gene expression analysis

Expression of genes that regulate superoxide accumulation and cell death (superoxide dismutase, *HvSOD1* and BAX inhibitor-1, *HvBI-1*) was monitored in barley leaves either un-inoculated or inoculated with *Bgt* or *Bgh* by reverse transcription (RT) and quantitative (real time) PCR (qPCR). Total RNA extraction from un-inoculated and inoculated leaves was done from 200 mg fresh leaves/sample homogenized in liquid nitrogen with the aid of a minicolumn kit according to instructions of the manufacturer (Viogene). Subsequent reverse transcription (RT) was conducted by using a RevertAid^{TM} H^{-} cDNA Synthesis Kit (Thermo Fisher Scientific). qPCR reactions were run in a DNA Engine Opticon 2 thermocycler (MJ Research) by employing the 2× SYBR FAST Readymix Reagent (KAPA Biosystems) as previously described (Hafez *et al*., 2012) except that expression of a barley ubiquitin gene (*HvUbi*, GenBank accession M60175) was used as an internal control.

Oligonucleotide primers used in RT-qPCR for amplifying barley (*H. vulgare*) sequences were the following: 5´-ACCCTCGCCGACTACAACAT-3´ (5′ primer) and 5´-CAGTAGTGGCGGTCGAAGTG-3´ (3′ primer) for a 240 bp ubiquitin cDNA fragment (*HvUbi*, GenBank M60175); 5´-TCAAGGGCACCATCTTCTTC-3´ (5′ primer) and 5´-TTTCCGAGGTCACCAGCAT-3´ (3′ primer) for a 214 bp superoxide dismutase cDNA fragment (*HvSOD1* or *HvCSD1*, GeneBank KU179438, TC109315); 5´-ATGTTCTCGGTGCCAGTCT-3´ (5′ primer) and 5´- GGGCGTGCTTGATGTAGTC -3´ (3′ primer) for a 409 bp BAX inhibitor-1 cDNA fragment (*HvBI-1*, GenBank AJ290421). All oligonucleotide primers, except those for *HvUbi* (Proels *et al*., 2010), were designed with the aid of the Primer Premier 5 program (PREMIER Biosoft International).

2.5 Statistical analysis

Three independent biological experiments were conducted in each case with three replicates per treatment. For NADPH oxidase enzymatic activity assays and gene expression analysis by RT-qPCR, each biological sample contained at least six leaves collected from different barley plants. Statistically significant differences from un-inoculated control plants were calculated by Student's *t*-test (at $p \leq 0.05$ and $p \leq 0.01$).

3. Results

3.1 A relatively early accumulation of superoxide ($O_2^{\cdot-}$) is a characteristic of symptomless nonhost resistance of plants to (hemi)biotrophic pathogens

In initial experiments we have compared accumulation patterns of superoxide ($O_2^{\cdot-}$) in several plant-pathogen combinations that result in either susceptibility, symptomless (type I) nonhost resistance or host resistance with a hypersensitive response (HR, local necrotic lesions). All of the investigated plant-pathogen combinations involved biotrophic pathogens (powdery mildews [*Blumeria* and *Erysiphe* spp.], rusts [*Puccinia* spp.]) or the hemibiotrophic potato late blight fungus (*Phytophthora infestans*). $O_2^{\cdot-}$ accumulation was determined by histochemical staining of inoculated leaves 1,2 3 or 4 days after inoculation (DAI). Table 1 demonstrates that accumulation of $O_2^{\cdot-}$ occurred during both symptomless nonhost resistance and host resistance with HR but not in cases of host susceptibility with typical disease symptoms, where superoxide was never detected. Importantly, accumulation of $O_2^{\cdot-}$ always occurred earlier during symptomless nonhost resistance, as compared to HR-accompanied host resistance.

Fig.1a depicts the association of symptomless nonhost resistance of barley (cv. Ingrid *Mla12*) to wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*, *Bgt*) with an early (1 DAI) accumulation of $O_2^{\cdot-}$, as compared to barley displaying host resistance with HR to barley powdery mildew (*Blumeria graminis* f. sp. *hordei*, *Bgh*), where significant amounts of $O_2^{\cdot-}$ were not detectable at 1 DAI. On the other hand, at 2 DAI massive $O_2^{\cdot-}$ accumulation in barley leaves was evident both during symptomless nonhost resistance to *Bgt* and HR-accompanied host resistance to *Bgh*. In barley leaves cv. Ingrid (wild type, *Mlo*) that are susceptible to *Bgh* $O_2^{\cdot-}$ accumulation was not detected up to 2 DAI (Fig. 1a). The association of symptomless nonhost resistance of barley to *Bgt* and $O_2^{\cdot-}$ accumulation was demonstrated in three different near isogenic lines of barley cv. Ingrid (*Mla12, Mlo* and *mlo5*). In fact, in all of these plant-pathogen interactions the simultaneous infiltration of SOD and CAT (enzymes responsible for dismutation of $O_2^{\cdot-}$ to H_2O_2 and degradation of H_2O_2, respectively) immediately after inoculation with *Bgt* significantly reduced NBT staining, demonstrating the specificity of NBT for $O_2^{\cdot-}$ detection in *Bgt*-infected barley leaves (Fig. 1b).

3.2 Superoxide accumulation during symptomless nonhost resistance of barley to wheat powdery mildew is localized to mesophyll cells (chloroplasts) of inoculated leaves

In order to localize the sites of superoxide ($O_2^{\cdot-}$) accumulation during symptomless nonhost resistance of barley to *B. graminis* f. sp. *tritici* (*Bgt*), NBT-staining (infiltration) applied to infected leaves was investigated on the cellular level. Infiltration of the NBT solution into leaf intercellular spaces (Ádám et al., 1989; Hückelhoven and Kogel, 1998), as opposed to immersion of leaves (e.g. Grosskinsky et al., 2012), likely enables a more uniform detection of $O_2^{\cdot-}$ in the entire leaf, including the mesophyll. We focused on mesophyll tissues for two reasons 1) during HR-accompanied host resistance of barley (cv. Pallas *Mla12*) to

Bgh, superoxide accumulation has been shown to occur in chloroplasts of mesophyll cells adjacent to infection sites relatively late, at 2 DAI (concomitant with HR-development) but not at 1 DAI (Hückelhoven and Kogel, 1998), 2) during symptomless host resistance of barley (cv. Pallas *Mlg*) to *Bgh*, superoxide accumulation has been also shown to occur in chloroplasts of mesophyll cells adjacent to infection sites but already at 1 DAI (Hückelhoven and Kogel, 1998). Importantly, the macroscopically symptomless host resistance of *Mlg* barley to *Bgh* is mechanistically similar to symptomless nonhost resistance of barley to *Bgt* (Hückelhoven and Kogel, 1998; Trujillo et al., 2004b). Furthermore, we have demonstrated a similar pattern of relatively early superoxide accumulation on a macroscopic scale not only in barley leaves displaying nonhost resistance to *Bgt* but also in several other plant-pathogen interactions resulting in symptomless (type I) nonhost resistance (see Table 1). Therefore, we thought that the relatively early (1 DAI) accumulation of superoxide during symptomless nonhost resistance of barley to *Bgt* might also be localized to chloroplasts of mesophyll cells. Indeed, at 1 DAI superoxide accumulation was clearly visible in mesophyll chloroplasts during symptomless nonhost resistance of barley (cv. Ingrid *Mla12*) to *Bgt* but not during HR-accompanied host resistance of the same barley line to *Bgh* (Fig. 2).

3.3 Superoxide accumulation during symptomless nonhost resistance of barley to wheat powdery mildew is accompanied by enhanced NADPH oxidase activity and distinct gene expression changes

Previous observations indicate that superoxide-generating NADPH oxidases contribute to plant disease resistance responses, including resistance to powdery mildews in *Arabidopsis thaliana* and barley (Berrocal-Lobo et al., 2010; Proels et al., 2010). In order to test the possible contribution of NADPH oxidases to the relatively early, elevated superoxide ($O_2^{\cdot-}$)

accumulation during symptomless nonhost resistance of barley to *B. graminis* f. sp. *tritici* (*Bgt*), we assayed NADPH oxidase enzymatic activity in un-inoculated and powdery mildew-inoculated leaves of barley (cv. Ingrid *Mla12*) displaying symptomless nonhost resistance to *Bgt* and HR-accompanied host resistance to *B. graminis* f. sp. *hordei* (*Bgh*). We found that the temporal pattern of NADPH oxidase activity mirrors that of $O_2^{\cdot-}$ accumulation. NADPH oxidase activity was several times higher at 1 DAI during symptomless nonhost resistance to *Bgt* than during HR-accompanied host resistance to *Bgh* and in uninoculated control plants. However, at 2 DAI, barley NADPH oxidase activity was similarly high during both forms of resistance, as compared to un-inoculated controls (Fig. 3).

In order to detect gene expression changes in barley specific to NADPH oxidase-related $O_2^{\cdot-}$ accumulation during symptomless nonhost resistance to *Bgt*, we assayed expression of genes that regulate (i.e. suppress) superoxide accumulation and cell death (superoxide dismutase, *HvSOD1* and BAX inhibitor-1, *HvBI-1*, respectively). With both genes a transient increase in expression occurred 24 hours after inoculation (HAI) in nonhost-resistant leaves, the same time point when $O_2^{\cdot-}$ accumulation and elevated NADPH oxidase-activity were also apparent. In case of HR-accompanied host resistance to *Bgh*, elevated *HvSOD1* and *HvBI-1* expression was evident from 12 HAI while at 24 HAI the same high levels of gene expression were detected as during symptomless nonhost resistance to *Bgt*. Interestingly, however, elevated expression of *HvSOD1* and *HvBI-1* was largely retained at later time points (48 and 72 HAI) during HR-accompanied host resistance to *Bgh*, as opposed to symptomless nonhost resistance to *Bgt* (Fig. 4).

3.4 Inhibition of superoxide accumulation can suppress symptomless nonhost resistance of barley to wheat powdery mildew

If superoxide ($O_2^{\cdot-}$) indeed contributes to symptomless nonhost resistance of e.g. barley to wheat powdery mildew (*B. graminis* f. sp. *tritici*, *Bgt*), *in planta* inhibition of $O_2^{\cdot-}$ accumulation should at least partially suppress this form of resistance. We have shown earlier that symptomless host resistance of barley to its own powdery mildew (*B. graminis* f. sp. *hordei*, *Bgh*) induced by treatments with another ROS, H_2O_2, can be suppressed by superoxide dismutase (SOD) and catalase (CAT) (Hafez and Király, 2003). Therefore we thought that application of the same experimental approach (i.e. simultaneous infiltration of SOD and CAT into inoculated barley leaves) might suppress the symptomless nonhost resistance of barley to wheat powdery mildew (*Bgt*) due, at least in part, to inhibition of superoxide accumulation. However, SOD and CAT treatments could not suppress symptomless nonhost resistance of barley to *Bgt*, as judged by the complete absence of macroscopic symptoms of susceptibility (i.e. colony growth of powdery mildew) (data not shown), although the same SOD and CAT treatments significantly reduced superoxide accumulation (Fig. 1b).

In order to demonstrate that inhibition of superoxide accumulation may indeed lead to suppression of symptomless nonhost resistance of barley to *Bgt*, we considered application of a short heat pre-treatment (heat shock) that has been shown to cause a slight decrease in H_2O_2 and suppression of symptomless and HR-accompanied host resistance of barley cv. Ingrid to *Bgh* (Barna et al., 2014). We reasoned that such a heat shock might suppress the resistance of barley to *Bgh*, at least in part, by reducing superoxide accumulation. Accordingly, exposing barley leaves to a heat shock (immersion in 49 C° water for 45 seconds before inoculation with *Bgh*) caused not only a partial suppression of symptomless and HR-accompanied host resistance to *Bgh* of two barley cv. Ingrid lines (*mlo5* and *Mla12*, respectively) but also a significant decline of superoxide accumulation before the appearance of powdery mildew disease symptoms (Fig. 5). Based on these results it seemed plausible that the same heat shock could at least partially suppress symptomless nonhost resistance of barley to *Bgt*. However,

heat shock alone, just as SOD and CAT treatments (see above), was not sufficient to cause a suppression of this nonhost resistance on a macroscopic scale (i.e. powdery mildew symptoms did not appear) (data not shown). On the other hand, a combination of heat shock and antioxidant (SOD and CAT) treatments seemed to lead to a suppression of symptomless nonhost resistance of barley to *Bgt*, as judged by the development of weak powdery mildew symptoms (mycelial growth, fungal colonies) on treated and inoculated leaves (Fig. 6). The appearance of HR-type local necrotic lesions within mycelia-covered leaf parts is likely due to limited pathogen spread in barley cells surrounding certain sites of *Bgt* penetration, indicating that suppression of symptomless nonhost resistance of barley to *Bgt* was only partial (Fig. 6). In order to show that the appearance of weak powdery mildew symptoms in barley was indeed due to the growth of *Bgt*, we back-inoculated the mycelia and conidia isolated from barley leaves to *Bgt*-susceptible wheat plants that developed visible powdery mildew symptoms (data not shown). These results demonstrated that symptomless nonhost resistance of barley to *Bgt* can be suppressed (i.e. partially converted to susceptibility), partly at least, by inhibiting the accumulation of $O_2^{\cdot-}$.

Fig. 7a depicts the combined effect of heat shock and antioxidants (SOD and CAT) on symptomless nonhost resistance to *Bgt* in three near isogenic lines of barley cv. Ingrid (*Mlo*, *Mla12* and *mlo5*). Mycelial growth of *Bgt* was slightly but clearly enhanced in leaves of all three barley lines exposed to heat shock, as compared to untreated controls (full nonhost resistance). Importantly, however, the simultaneous infiltration of leaves with SOD and CAT further enhanced fungal growth in heat shock pre-treated barley, pointing to a possible contribution of $O_2^{\cdot-}$ to nonhost resistance. In fact, results presented in Fig. 7b demonstrate that suppression of symptomless nonhost resistance of barley to *Bgt* by heat shock and antioxidants (SOD and CAT) was always coupled to a reduced accumulation of $O_2^{\cdot-}$.

4. Discussion

We assessed the dynamics of accumulation of the ROS superoxide ($O_2^{\cdot-}$**)** during interactions of plants with a range of biotrophic and hemibiotrophic pathogens that result either in susceptibility, symptomless (type I) nonhost resistance or host resistance with an HR. Accumulation of superoxide in infected leaves always occurred earlier during symptomless nonhost resistance, as compared to host resistance with HR, while it was never detected at early stages of susceptibility. Therefore, our results suggest that an earlier $O_2^{\cdot-}$ accumulation might be a pivotal factor governing the development of symptomless nonhost resistance vs. the slower HR-type host resistance. This is supported by previous data showing that during several cases of nonhost vs. host resistance of a given plant species (see plant-pathogen combinations in Table 1) the timing of pathogen restriction is correlated with $O_2^{\cdot-}$ accumulation assayed in this study (Niks, 1983; Hückelhoven et al., 1999; Vleeshouwers et al., 2000; Neu et al., 2003; Trujillo et al., 2004a; Bolton et al., 2008; Hoffmann et al., 2008). However, in case of symptomless nonhost resistance of wheat to *Puccinia hordei*, the correlation between pathogen restriction and $O_2^{\cdot-}$ accumulation may be less tight, as resistance has been shown to develop already by 2 DAI (Niks, 1983), while we could detect $O_2^{\cdot-}$ accumulation only at 3 DAI. It might be possible that superoxide production in this particular nonhost-pathogen combination is a secondary effect; alternatively, the nonhost resistance of the wheat cultivar used in our experiments (´MV-Emma´) develops at a slower rate but in concert with $O_2^{\cdot-}$ accumulation.

$O_2^{\cdot-}$ was the first ROS implicated in orchestrating HR-type host resistance to oomycete, bacterial and viral pathogens (Doke, 1983; Doke and Ohashi, 1988; Ádám et al., 1989). Furthermore, we have shown previously that symptomless host resistance to (hemi)biotrophic pathogens (powdery mildews, rusts, bacteria) can be induced by externally generated $O_2^{\cdot-}$

relatively early, 1 to 3 DAI, while the same treatments applied later induce host resistance with HR (El-Zahaby et al., 2004). In similar experiments, symptomless host resistance to *Tobacco mosaic virus* could be induced if susceptible tobacco plants were treated with a $O_2^{\cdot-}$-generating riboflavin-methionine solution early, at two hours after inoculation (Bacsó et al., 2011). The functional role of $O_2^{\cdot-}$ in symptomless host resistance is suggested e.g. by the work of Shang et al. (2010) demonstrating that absence of *Cucumber mosaic virus* in ‘‘dark green islands’’ of systemically infected leaf tissues correlates with $O_2^{\cdot-}$ accumulation. Furthermore, we have recently demonstrated that the graft-transmissible, symptomless host resistance of cherry pepper (*Capsicum annuum* var. *cerasiforme*) to powdery mildew (*Leveillula taurica*) is coupled to constitutive $O_2^{\cdot-}$ accumulation even in uninfected plants (Albert *et al*., 2017). Taken together, the above-mentioned data and our present results, linking a relatively early $O_2^{\cdot-}$ accumulation to symptomless nonhost resistance, point to a role of $O_2^{\cdot-}$ in inducing fast, efficient and symptomless plant disease resistance responses probably by inhibiting/killing pathogens and/or participating in defense signaling.

Our results showed that the relatively early (1 DAI) $O_2^{\cdot-}$ accumulation during symptomless nonhost resistance of barley to wheat powdery mildew (*B. graminis* f. sp. *tritici*, *Bgt*) is localized to chloroplasts of mesophyll cells in inoculated leaves, while at the same time point $O_2^{\cdot-}$ was not detected in mesophyll chloroplasts during HR-associated host resistance to barley powdery mildew (*B. graminis* f. sp. *hordei*, *Bgh*). Interestingly, symptomless host resistance of barley to *Bgh* also correlates with a similar early (1 DAI) $O_2^{\cdot-}$ accumulation in mesophyll chloroplasts close to infection sites (Hückelhoven and Kogel, 1998). Although an antimicrobial effect of this $O_2^{\cdot-}$ accumulation is possible, it seems also likely that a relatively early ROS ($O_2^{\cdot-}$) signaling associated with chloroplasts might be a characteristic of symptomless resistance responses of barley to powdery mildew infections. The central role of chloroplast-associated ROS bursts in early (basal) resistance responses to

pathogenic infections is suggested by localized $O_2^{\cdot-}$ and H_2O_2 accumulation detectable already before HR development during bacteria-induced host resistance and accompanied by elevated antioxidant capacity e.g. in chloroplasts (Grosskinsky et al., 2012). In contrast, susceptibility to the necrotroph *Botrytis cinerea* in advanced stages of infection is coupled to massive H_2O_2 accumulation in host cells and a severe degeneration of chloroplasts (Simon et al., 2013). Importantly, Zabala et al. (2015) has shown that during PAMP-triggered immunity to *Pseudomonas syringae* pv. *tomato* DC3000 an early chloroplastic ROS burst occurs within 5-6 HAI. However, in case of susceptibility chloroplast-targeted bacterial effectors inhibit photosynthetic electron transport leading to decreased ROS production at this early stage. The ROS signal ($O_2^{\cdot-}$ and H_2O_2) could spread from chloroplasts to the apoplast through activation of $O_2^{\cdot-}$-generating NADPH oxidases, and from there to adjacent cells, leading to pathogen resistance and/or programmed cell death (see e.g. Zurbriggen et al., 2010). Interestingly, in barley and wheat exhibiting nonhost resistance to powdery mildews (*Bgt* and *Bgh*, respectively) $O_2^{\cdot-}$ can be detected in plasma membranes/cell walls of a few epidermal cells distal from attacked cells, suggesting a role for $O_2^{\cdot-}$ in the signaling process leading to macroscopically symptomless (type I) nonhost resistance (Trujillo et al., 2004a). It is possible that the strong $O_2^{\cdot-}$ accumulation in mesophyll chloroplasts that we detected in barley displaying nonhost resistance to *Bgt* is responsible for amplifying the weaker epidermis-derived signals described by Trujillo et al. (2004a).

We found that the temporal pattern of NADPH oxidase enzymatic activity mirrors that of the relatively early vs. late $O_2^{\cdot-}$ accumulation in barley displaying symptomless nonhost resistance to *Bgt* and HR-accompanied host resistance to *Bgh*, respectively. This implies that a substantial amount of $O_2^{\cdot-}$ formed during these resistance responses is derived from NADPH oxidases, enzymes that are mainly responsible for $O_2^{\cdot-}$ production during successful plant defenses to (hemi)biotrophic pathogens (e.g. Levine et al., 1994; Berrocal-Lobo et al., 2010;

Proels et al., 2010; Xiao et al., 2017). As regards the role of NADPH oxidases in nonhost resistance, silencing of two NADPH oxidase genes (*NbRBOHA* and *NbRBOHB*) in *Nicotiana benthamiana* lead to a reduction of ROS ($O_2^{\cdot-}$ and H_2O_2) and weakening of HR-associated nonhost resistance to *Phytophthora infestans* (Yoshioka et al., 2003). Similar results were obtained in tobacco where NADPH oxidase regulation was impaired by overexpression of a dominant negative form of the rice *OsRac1* gene; HR-associated nonhost resistance to *Pseudomonas syringae* pv. *maculicola* ES4326 was suppressed (Moeder et al., 2005). Furthermore, An et al. (2017) recently demonstrated that histone acetyltransferase (Elongator) genes control the symptomless nonhost resistance of *Arabidopsis thaliana* to bacterial infections in part by conferring expression of a NADPH oxidase gene (*AtRBOHD*) and accumulation of ROS. In barley, the only NADPH oxidase gene so far with a documented role in disease resistance is *HvRBOHF2* which is required for host resistance to powdery mildew (*Bgh*), inhibiting pathogen penetration at the epidermis (Proels et al., 2010). We found that expression of *HvRBOHF2* does not change significantly during symptomless nonhost resistance to *Bgt* and HR-accompanied host resistance to *Bgh* (data not shown) confirming the earlier results on *HvRBOHF2* transcript accumulation in barley-*Bgh* interactions (Hückelhoven et al., 2001b). It is possible that NADPH oxidase activity is not regulated on the transcriptional level. Alternatively, one or more of the five additional *HvRBOH* (NADPH oxidase) genes described in barley (Lightfoot et al., 2008) could be responsible for the elevated NADPH oxidase activity during symptomless nonhost resistance and HR-accompanied host resistance to powdery mildews.

Our experiments demonstrated gene expression changes in barley specific to the NADPH oxidase-associated, relatively early $O_2^{\cdot-}$ accumulation and symptomless nonhost resistance to *Bgt*. We found a transient increase in expression of genes encoding superoxide dismutase and the cell death regulator BAX inhibitor-1 (*HvSOD1* and *HvBI-1*) in nonhost-

resistant leaves 24 hours after inoculation (HAI), when $O_2^{\cdot-}$ accumulation and elevated NADPH oxidase-activity were also detectable. The quick, transient increases in expression of genes that down-regulate ROS and cell death during symptomless nonhost resistance to *Bgt* are a clear indication of fast, efficient defense responses that may rapidly inhibit (kill) the pathogen, consequently, no further expression of these genes would be needed. On the other hand, during HR-type host resistance to *Bgh*, elevated expression of *HvSOD1* and *HvBI-1* was retained at later time points (24, 48 and 72 HAI), likely mirroring the slower defense responses characteristic of an HR, allowing limited pathogen spread before the final development of resistance. In case of *HvBI-1* the above-mentioned gene expression changes have been previously described in different barley cultivars by using another *Bgt* race and semiquantitative assays (Hückelhoven et al., 2001b; Eichmann et al., 2004). Here we could confirm these results in cv. Ingrid by the more sensitive RT-qPCR. On the other hand, our study is the first to describe the transiently induced expression of a SOD gene (*HvSOD1*) during symptomless nonhost resistance of barley to *Bgt*. Although silencing of *HvSOD1* had no significant influence on infection of barley by *Bgh* (Lightfoot et al., 2017), it enabled more intensive leaf necrotization following ROS-generating herbicide stress. This suggests a role for the CuZn-SOD protein encoded by *HvSOD1* in maintaining cytosolic redox status, a possible reason for sustaining elevated *HvSOD1* expression during HR-associated host resistance to *Bgh*, as opposed to symptomless nonhost resistance to *Bgt*.

Importantly, our investigations have shown that $O_2^{\cdot-}$ may have a functional role in symptomless (type I) nonhost resistance. First, we demonstrated that a heat shock (49 C° for 45 seconds) partially suppresses symptomless and HR-accompanied host resistance of barley to *Bgh* (Barna et al., 2014) parallel to a concomitant decline of $O_2^{\cdot-}$ accumulation. Next we showed that the same heat shock can partially suppress symptomless nonhost resistance to *Bgt* in three near isogenic lines of barley cv. Ingrid (*Mlo*, *Mla12* and *mlo5*). A combination of heat

shock and antioxidant (SOD and CAT) treatments further enhanced fungal growth in *Bgt*-inoculated barley, while $O_2^{\cdot-}$ levels declined. Our results also imply that heat shock may suppress ROS, e.g. $O_2^{\cdot-}$, by inducing antioxidant (ROS-scavenging) processes. In fact, Barna et al. (2014) showed that heat shock-exposed barley displays a slight decline in H_2O_2 concomitant with an increase in CAT activity. When plants are exposed to heat, excessive ROS production activates heat shock factors which may induce the expression of antioxidant (ROS-scavenging) genes, a process associated with heat stress tolerance (Driedonks et al., 2015 and references herein). Importantly, the effect of heat exposure on suppressing disease resistance of e.g. tobacco to *Tobacco mosaic virus* has been shown to be due in part to a stimulation of antioxidant enzymes like dehydroascorbate reductase and down-regulation of $O_2^{\cdot-}$ accumulation (Király et al., 2008). Taken together, it seems that heat exposure (heat shock) of plants may suppress disease resistance, including symptomless nonhost resistance, by mechanisms including a simultaneous down-regulation of ROS ($O_2^{\cdot-}$) production and suppression of ROS accumulation (antioxidant induction).

However, besides ROS ($O_2^{\cdot-}$), other factors may also contribute to symptomless (type I) nonhost resistance. For example, overexpression of a cell death suppressor gene (*HvBI-1*) in barley epidermal cells could partially suppress symptomless nonhost resistance to *Bgt* at the penetration stage (Eichmann et al., 2004). *Arabidopsis* mutants deficient in synthesis of glucosinolates also display a partially suppressed nonhost resistance to different powdery mildew pathogens (Bednarek et al., 2009). Recently, the central role of a transmembrane receptor-like kinase (HvLEMK1) in mediating symptomless nonhost resistance of barley to *Bgt* has been demonstrated (Rajaraman et al., 2016); silencing of HvLEMK1 led to limited colonization and sporulation of the pathogen to a similar extent as shown in the present study by exposing *Bgt*-inoculated barley to heat shock and antioxidant (SOD and CAT) treatments.

In conclusion, our results suggest a relatively early vs. late $O_2^{\cdot-}$ accumulation to be a pivotal factor governing the development of symptomless (type I) nonhost resistance vs. the slower HR-type host resistance in various plant-pathogen interactions (infections by [hemi]biotrophic pathogens). In barley, the relatively early (1 DAI) $O_2^{\cdot-}$ accumulation during symptomless nonhost resistance to wheat powdery mildew (*B. graminis* f. sp. *tritici*) is localized to mesophyll chloroplasts of inoculated leaves and coupled to enhanced NADPH oxidase activity and transient increases in expression of genes regulating $O_2^{\cdot-}$ levels and cell death. Finally, the suppression of symptomless nonhost resistance of barley to wheat powdery mildew (*Bgt*) by heat shock and antioxidant treatments (i.e. achieving partial susceptibility) points to a functional role of $O_2^{\cdot-}$ in symptomless (type I) nonhost resistance.

Contributions

All authors conceived and designed laboratory experiments. AK, RB, BB, YMH and LK performed powdery mildew infection experiments including superoxide detection in barley and wheat. AK, RB, RA, YMH and IS carried out additional similar experiments involving infections of various hosts with biotrophic pathogens. AK, RB, RA, YMH and BB were responsible for carrying out heat shock and antioxidant treatments. AK, RB, RA, JF and LK were responsible for NADPH oxidase activity and gene expression assays. AK, BB, ZK and LK wrote the paper.

Acknowledgments

The help of Dr. Miklós Pogány (Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest, Hungary) and Dr. László Vajna (NARIC

Fruitculture Research Institute, Érd, Hungary) in light microscopy is gratefully acknowledged. This research was supported by grants of the Hungarian National Research, Development and Innovation Office (K77705, PD83831 and K111995).

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Figure legends

Figure 1 (a) Symptomless nonhost resistance of barley (*Hordeum vulgare* cv. Ingrid *Mla12*) to wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*, *Bgt*) is associated with a relatively early accumulation of superoxide ($O_2^{\cdot-}$) in inoculated leaves. $O_2^{\cdot-}$is visualized by nitro blue tetrazolium chloride (NBT) staining. "*Bgh* host resistance (HR)" = host resistance with a hypersensitive response (HR, local necrotic lesions) of barley cv. Ingrid *Mla12* to barley powdery mildew (*Blumeria graminis* f. sp. *hordei*, *Bgh*). "*Bgh* susceptibility" = susceptibility of barley cv. Ingrid (wild type, *Mlo*) to *Bgh*. DAI = days after inoculation. Repeated experiments lead to similar results. **(b)** Symptomless nonhost resistance to *Bgt* in different near isogenic lines of barley cv. Ingrid (*Mla12, Mlo* and *mlo5*) is associated with $O_2^{\cdot-}$ accumulation, as visualized by NBT staining. Simultaneous infiltration of superoxide dismutase and catalase (SOD and CAT, 2500 and 5000 units ml^{-1}, respectively) immediately after inoculation with *Bgt* suppresses $O_2^{\cdot-}$accumulation, indicating the specificity of NBT for $O_2^{\cdot-}$. Percentage of NBT-stained leaf area was quantified by the ImageJ program. Numbers represent means ± SD from three independent biological experiments.

Figure 2 Symptomless nonhost resistance of barley (*Hordeum vulgare* cv. Ingrid *Mla12*) to wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*, *Bgt*) **(a)** is associated with a relatively early accumulation of superoxide ($O_2^{\cdot-}$) in mesophyll cells (chloroplasts) of inoculated leaves [**(b)** and **(c)]**. $O_2^{\cdot-}$is visualized by nitro blue tetrazolium chloride staining. "*Bgh* host resistance (HR)" = host resistance with a hypersensitive response (HR, local necrotic lesions) of barley cv. Ingrid *Mla12* to barley powdery mildew (*Blumeria graminis* f. sp. *hordei*, *Bgh*). DAI = days after inoculation. Bar in **(b)** = 40 µm; Bar in **(c)** = 20 µm. Repeated experiments lead to similar results.

872

873 **Figure 3** A relatively early elevation of the activity of NADPH oxidase, an enzyme
874 responsible for pathogenesis-related superoxide production, as a marker of symptomless
875 nonhost resistance of barley (*Hordeum vulgare* cv. Ingrid *Mla12*) to wheat powdery mildew
876 (*Blumeria graminis* f. sp. *tritici*). "host resistance" = host resistance with a hypersensitive
877 response (HR, local necrotic lesions) of barley cv. Ingrid *Mla12* to barley powdery mildew
878 (*Blumeria graminis* f. sp. *hordei*). DAI = days after inoculation. Columns represent means ±
879 SD from three independent biological experiments. * and ** indicate statistically significant
880 differences from un-inoculated control plants at $p \leq 0.05$ and $p \leq 0.01$, respectively (Student's
881 *t*-test).

882

883 **Figure 4** A relatively early, transient increase in expression of genes (assayed by RT-qPCR)
884 that regulate superoxide ($O_2^{\cdot-}$) accumulation and cell death (superoxide dismutase, *HvSOD1*
885 and BAX inhibitor-1, *HvBI-1*, respectively) during symptomless nonhost resistance of barley
886 (*Hordeum vulgare* cv. Ingrid *Mla12*) to wheat powdery mildew (*Blumeria graminis* f. sp.
887 *tritici*). "host resistance" = host resistance with a hypersensitive response (HR, local necrotic
888 lesions) of barley cv. Ingrid *Mla12* to barley powdery mildew (*Blumeria graminis* f. sp.
889 *hordei*). HAI = hours after inoculation. A relative value of 1 represents gene expression in un-
890 inoculated control plants at 0 HAI. Columns represent means ± SD from three independent
891 biological experiments. * indicate statistically significant differences from un-inoculated
892 controls at $p \leq 0.05$ (Student's *t*-test).

893

894 **Figure 5** Heat shock-conferred partial suppression of barley (*Hordeum vulgare* cv. Ingrid)
895 host resistance to barley powdery mildew (*Blumeria graminis* f. sp. *hordei*, *Bgh*) is coupled to
896 reduced accumulation of superoxide ($O_2^{\cdot-}$) (visualized by nitro blue tetrazolium chloride

staining of inoculated leaves). *Mlo* = wild type barley cv. Ingrid, susceptible to *Bgh*. *mlo5* and *Mla12* = two near isogenic barley cv. Ingrid lines normally exhibiting symptomless (*mlo5*) or hypersensitive-type (*Mla12*) host resistance to *Bgh*. DAI = days after inoculation. "heat" = heat shock (immersing leaves in 49 °C water for 45 sec), 30 min before inoculation. Percentage of NBT-stained leaf area was quantified by the ImageJ program. Numbers represent means ± SD from three independent biological experiments.

Figure 6 Partial suppression of symptomless nonhost resistance of barley (*Hordeum vulgare* cv. Ingrid *Mla12*) to wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*, *Bgt*) by a combination of heat shock and infiltration of antioxidant enzymes (superoxide dismutase and catalase, SOD and CAT). Development of weak powdery mildew symptoms (fungal colonies) and HR-type local necrotic lesions 7 days after inoculation (DAI). The area marked by a rectangle in the left panel is shown as a microscopic image on the right. Bar = 100 µm. Heat shock (immersing leaves in 49 °C water for 45 sec) was applied 30 min before inoculation. Simultaneous infiltration of SOD and CAT (2500 and 5000 units ml^{-1}, respectively) was conducted immediately after inoculation. Repeated experiments lead to similar results.

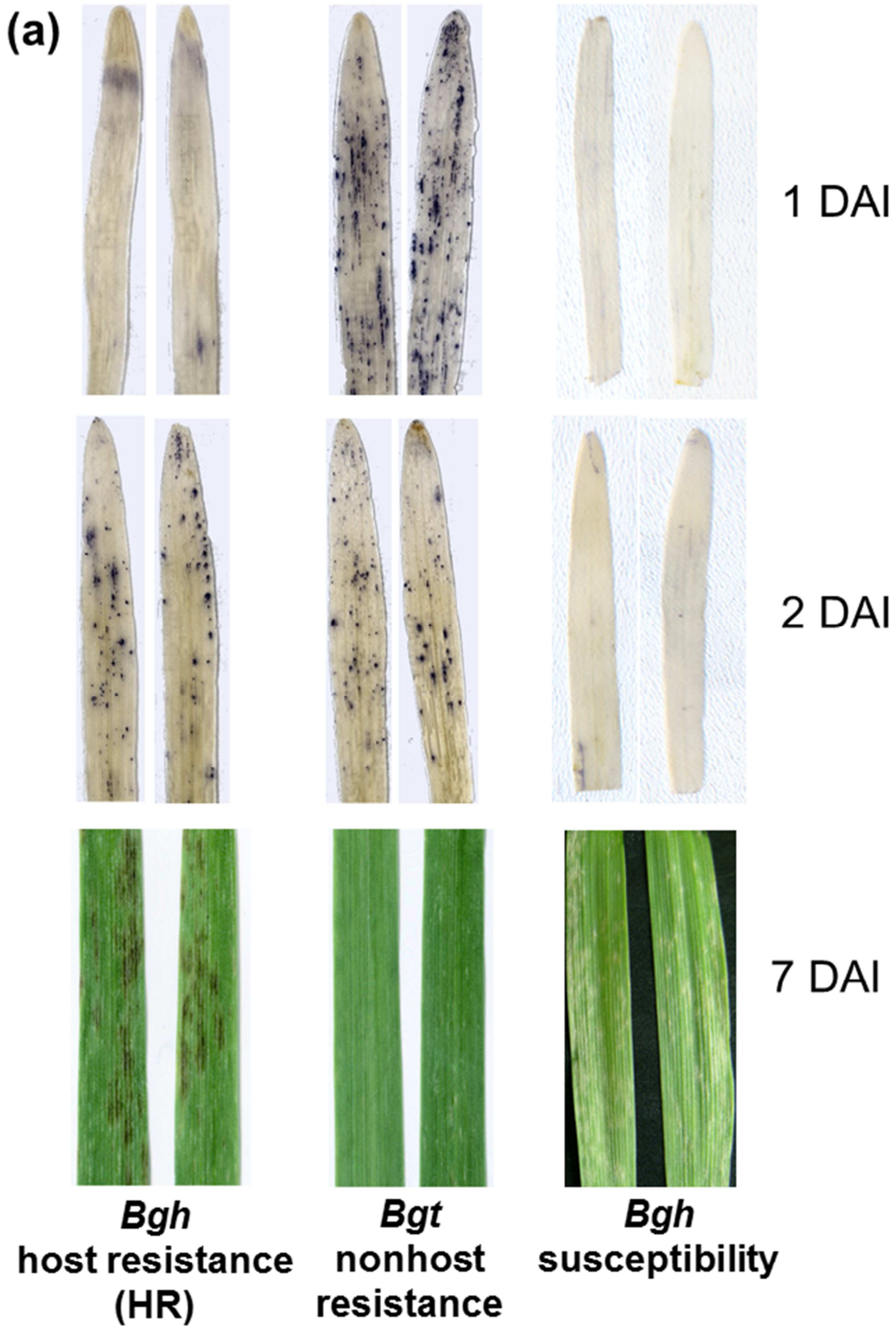
Figure 7 (a) Heat shock-conferred suppression of symptomless nonhost resistance of barley (*Hordeum vulgare* cv. Ingrid) to wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*, *Bgt*) is enhanced by infiltration of antioxidant enzymes (superoxide dismutase and catalase, SOD and CAT). Growth of *Bgt* in infected and treated leaves 3 days after inoculation (DAI) in different near isogenic lines of barley cv. Ingrid (*Mlo, Mla12, mlo5*). In the upper left panel, growth of barley powdery mildew (*Blumeria graminis* f. sp. *hordei*, *Bgh*) in wild type barley cv. Ingrid (*Mlo*) is shown as a positive control of host susceptibility. Repeated experiments lead to similar results. **(b)** Suppression of symptomless nonhost resistance to *Bgt* by heat

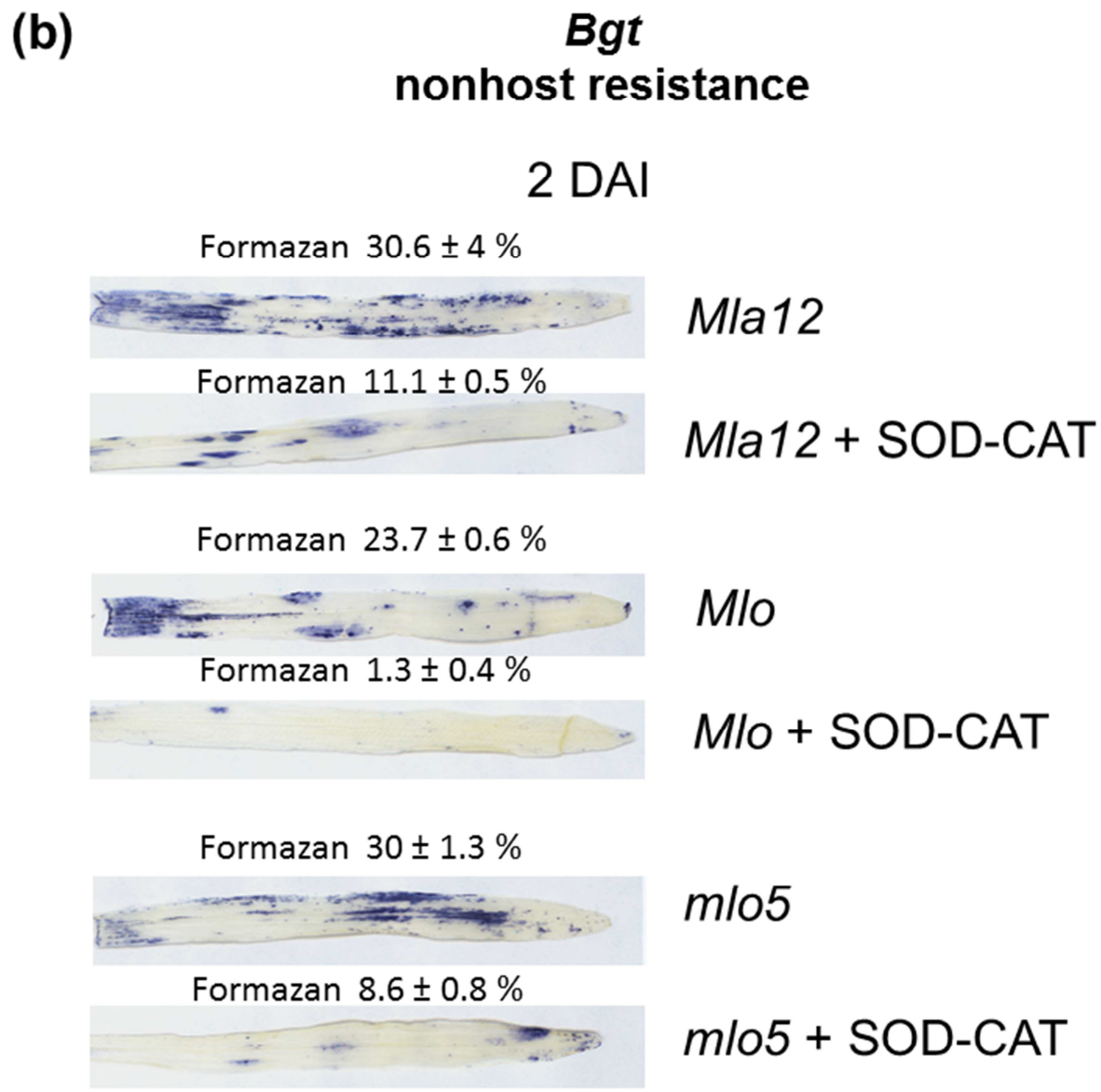
shock and antioxidants in different near isogenic lines of barley cv. Ingrid (*Mlo, Mla12, mlo5*) is coupled to a reduced accumulation of superoxide (O_2^-) (visualized by nitro blue tetrazolium chloride staining of inoculated leaves). Percentage of NBT-stained leaf area was quantified by the ImageJ program. Numbers represent means ± SD from three independent biological experiments. Heat shock (immersing leaves in 49 °C water for 45 sec) was applied 30 min before inoculation. Simultaneous infiltration of SOD and CAT (2500 and 5000 units ml^{-1}, respectively) was conducted immediately after inoculation. Fungal structures were visualized by Pelikan blue staining as described in Materials and Methods.

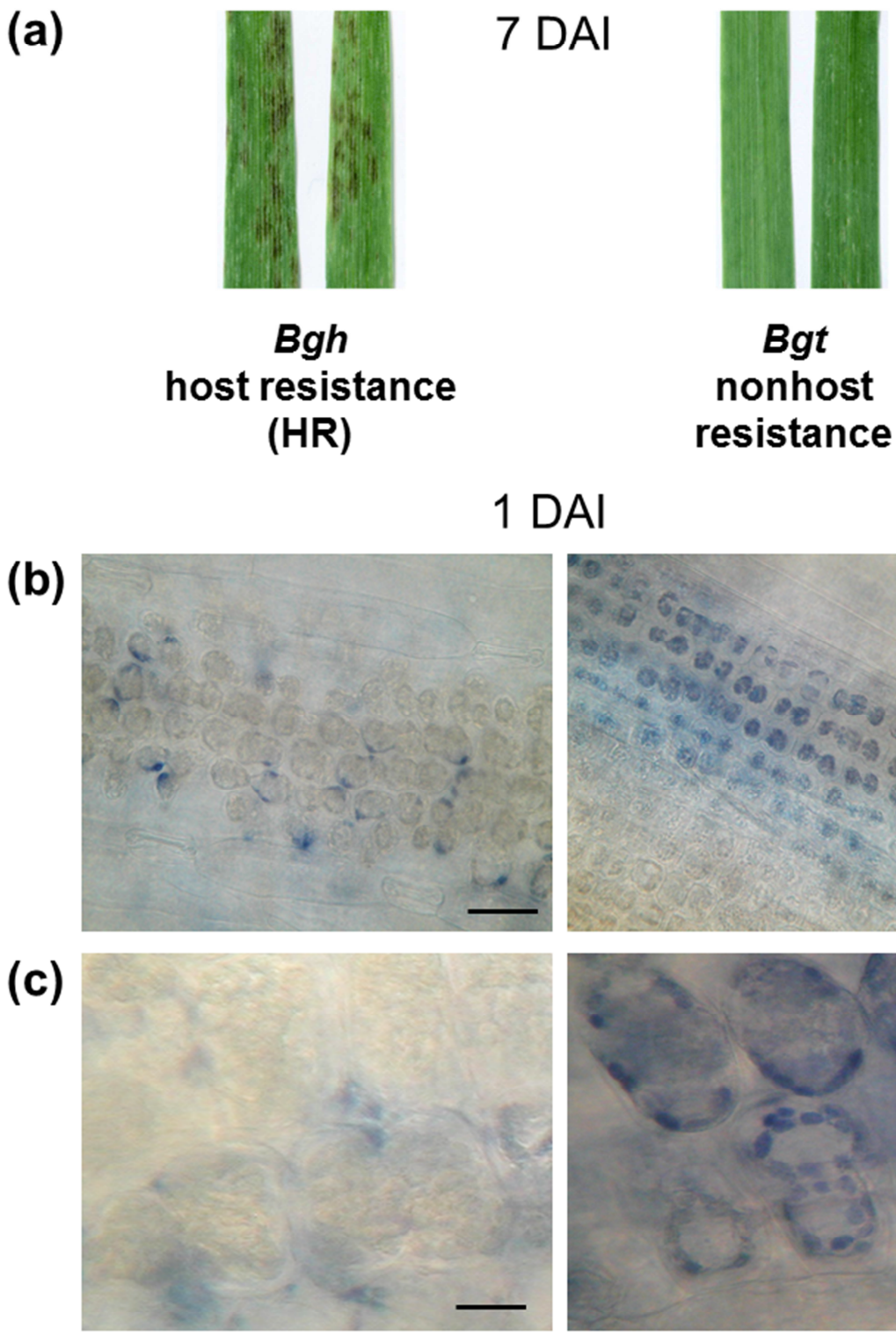
Table 1 Differential patterns of superoxide ($O_2^{\cdot-}$) accumulation in infected leaf tissues during plant-pathogen interactions that result in susceptibility, HR-type host resistance or symptomless (no visible HR) nonhost resistance

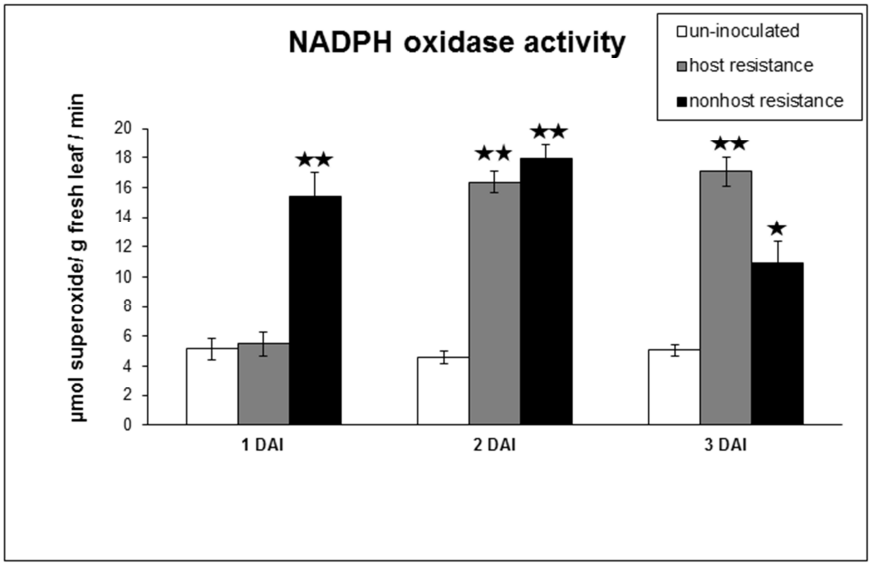
Plant – pathogen interaction	Plant response	Superoxide ($O_2^{\cdot-}$)[1]
Hordeum vulgare – *Blumeria graminis* f.sp. *hordei*, A6 cv. Ingrid *Mlo* (wt)	**susceptibility**	- (up to 48 HAI)
H. vulgare – *B. graminis* f. sp. *hordei*, A6 cv. Ingrid *Mla12*	**host resistance (HR)**[2]	+ (44-48 HAI)
H. vulgare – *Blumeria graminis* f. sp. *tritici*, cv. Ingrid *Mla12* Hungarian isolate	**nonhost resistance**[3]	+ (22-24 HAI)
H. vulgare, – *Puccinia hordei*, Hungarian isolate cv. Ingrid *Mlo* (wt)	**susceptibility**	- (up to 48 HAI)
H. vulgare – *Puccinia hordei*, Hungarian isolate cv. Botond	**host resistance (HR)**	+ (44-48 HAI)
H. vulgare – *Puccinia triticina*, race 77 cv. Botond	**nonhost resistance**	+ (22-24 HAI)
Triticum aestivum – *Puccinia triticina*, race 77 cv. Buzogány	**susceptibility**	- (up to 96 HAI)
T. aestivum – *Puccinia triticina*, race 77 cv. MV-Emma	**host resistance (HR)**	+ (92-96 HAI)
T. aestivum – *Puccinia hordei*, Hungarian isolate cv. MV-Emma	**nonhost resistance**	+ (68-72 HAI)
Vitis vinifera – *Erysiphe necator* cv. Nimrang Hungarian isolate	**susceptibility**	- (up to 48 HAI)
V. vinifera – *Erysiphe necator* cv. Kishmish vatkana Hungarian isolate	**host resistance**[4]	+ (44-48 HAI)
V. vinifera – *B. graminis* f. sp. *hordei*, A6 cv. Kishmish vatkana	**nonhost resistance**	+ (22-24 HAI)
Solanum tuberosum – *Phytophthora infestans*, K-39 cv. Hópehely	**susceptibility**	- (up to 48 HAI)
Solanum tuberosum – *Phytophthora infestans*, K-39 cv. White Lady	**host resistance (HR)**	+ (44-48 HAI)
Solanum tuberosum – *B. graminis* f.sp. *hordei*, A6 cv. White Lady	**nonhost resistance**	+ (22-24 HAI)

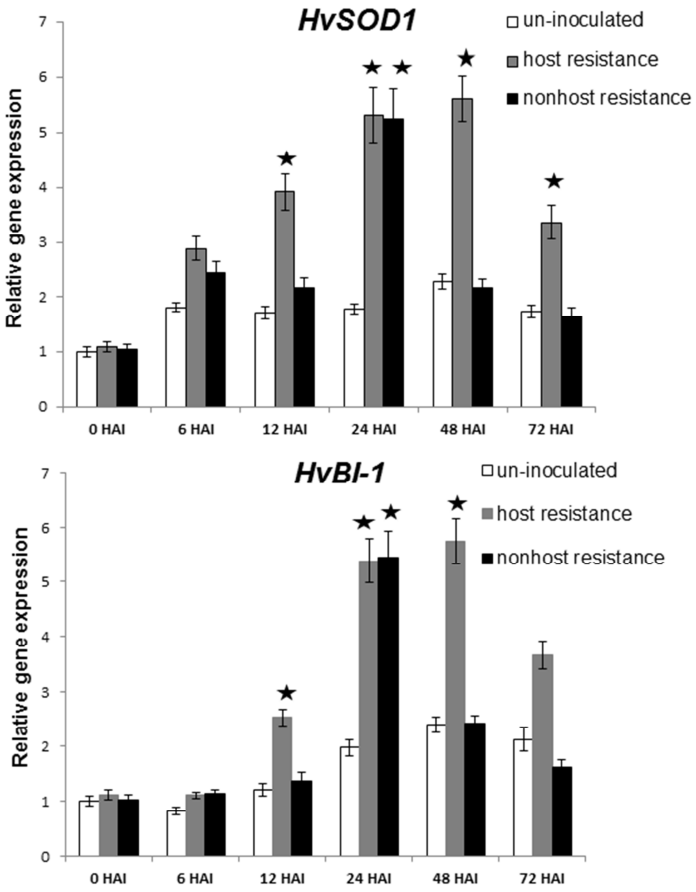
[1]Detection of superoxide ($O_2^{\cdot-}$) in infected leaves by nitro blue tetrazolium chloride (NBT) staining at indicated time points (HAI = hours after inoculation). Samples are considered positive ("+") for $O_2^{\cdot-}$ when the percentage of NBT-stained area per leaf is more than 5 % (ImageJ quantification, see Materials and Methods). Repeated experiments led to similar results. [2]HR= hypersensitive response, localized tissue necrosis. [3]"nonhost resistance" = Type I, without visible HR symptoms. [4]without a macroscopically visible HR.

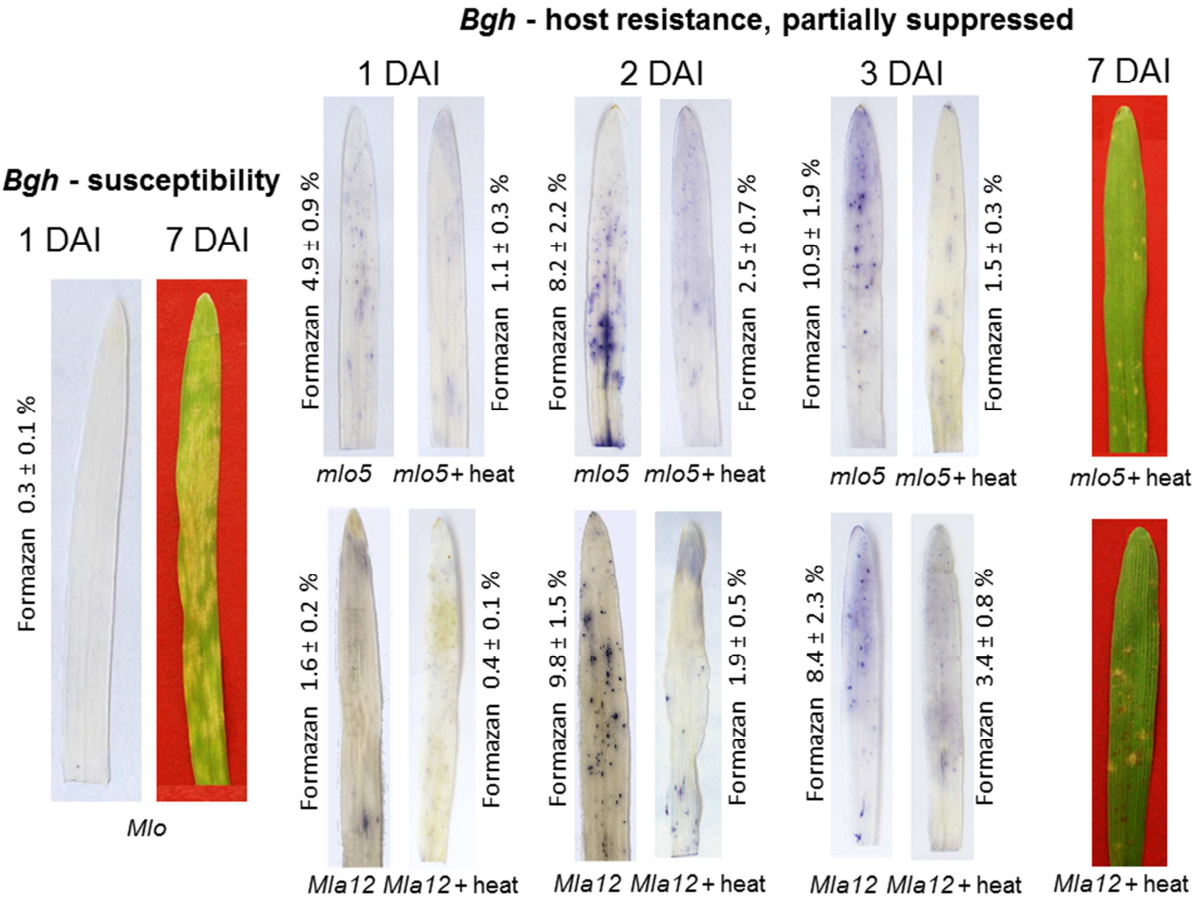

(a)
1 DAI
2 DAI
7 DAI
Bgh
host resistance
(HR)
Bgt
nonhost
resistance
Bgh
susceptibility


(b)
Bgt
nonhost resistance
2 DAI
Formazan 30.6 ± 4 %
Mla12
Formazan 11.1 ± 0.5 %
Mla12 + SOD-CAT
Formazan 23.7 ± 0.6 %
Mlo
Formazan 1.3 ± 0.4 %
Mlo + SOD-CAT
Formazan 30 ± 1.3 %
mlo5
Formazan 8.6 ± 0.8 %
mlo5 + SOD-CAT

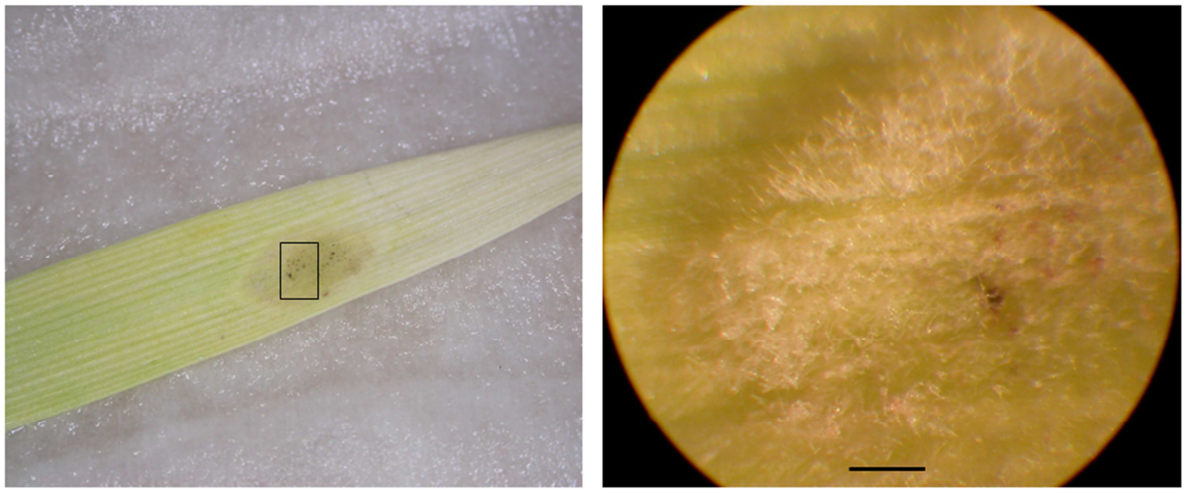

(a)
7 DAI
Bgh
host resistance
(HR)
Bgt
nonhost
resistance
1 DAI
(b)
(c)



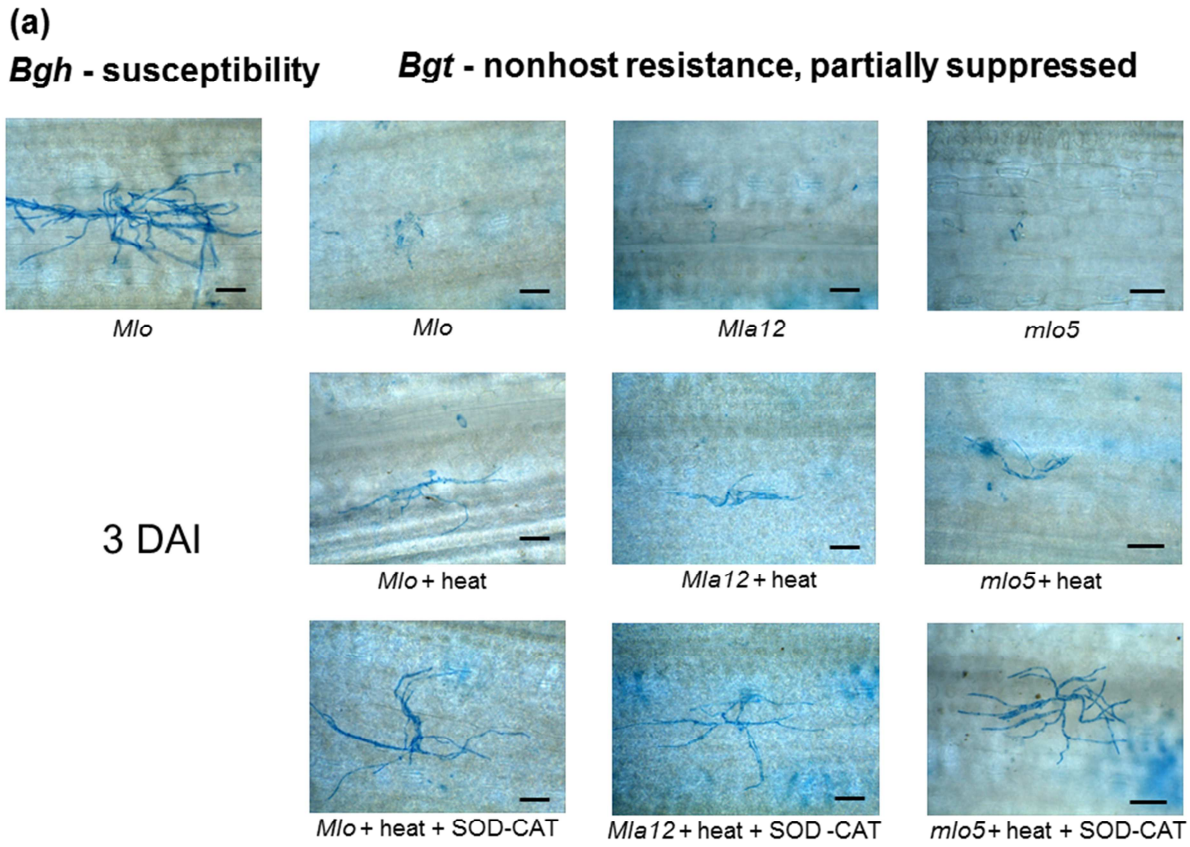


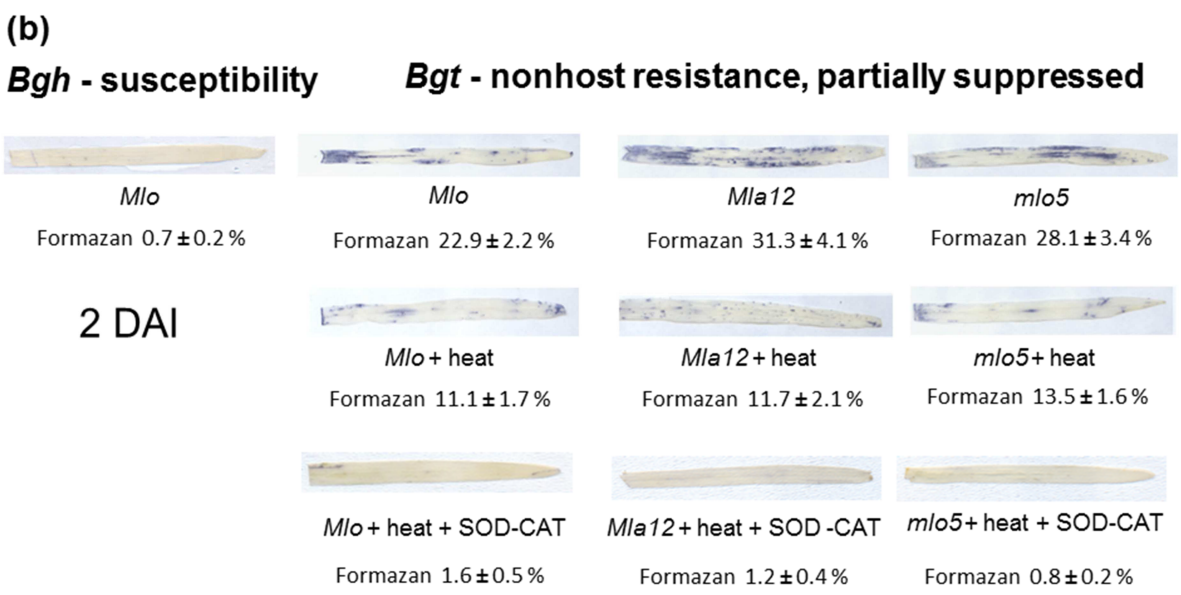


***Bgt* - nonhost resistance, partially suppressed**



7 DAI





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Superoxide ($O_2^{\cdot-}$) accumulation contributes to symptomless (type I) nonhost resistance of plants to biotrophic pathogens

HIGHLIGHTS

- Early superoxide ($O_2^{\cdot-}$) accumulation in symptomless (type I) nonhost resistance
- Barley-powdery mildew type I nonhost resistance: $O_2^{\cdot-}$ in mesophyll chloroplasts
- Type I nonhost resistance: NADPH oxidase activity, related gene expression changes
- Heat shock/antioxidants suppress barley-powdery mildew type I nonhost resistance
- $O_2^{\cdot-}$ may have a functional role in symptomless (type I) nonhost resistance

Contributions

All authors conceived and designed laboratory experiments. AK, RB, BB, YMH and LK performed powdery mildew infection experiments including superoxide detection in barley and wheat. AK, RB, RA, YMH and IS carried out additional similar experiments involving infections of various hosts with biotrophic pathogens. AK, RB, RA, YMH and BB were responsible for carrying out heat shock and antioxidant treatments. AK, RB, RA, JF and LK were responsible for NADPH oxidase activity and gene expression assays. AK, BB, ZK and LK wrote the paper.