Research Article

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Cold hardening protects cereals from oxidative stress and necrotrophic fungal pathogenesis

Abstract: The effects of cold hardening of cereals on their cross-tolerance to treatments leading to oxidative stress were investigated. Long-term exposure to low non-freezing temperatures provided partial protection to wheat and barley plants from the damage caused by paraquat and hydrogen peroxide treatments. It also conferred resistance in two barley cultivars to the necrotic symptoms and growth of the fungal phytopathogen *Pyrenophora teres* f. *teres*. Pathogen-induced oxidative burst was also reduced in cold hardened plants. The possible roles of host-derived redox factors and other signaling components in the observed forms of cereal cross-tolerance are discussed.

Necrotic symptoms of *Pyrenophora teres* f. *teres* on leaves of cold hardened and control barley leaves (5 dpi)

Accumulation of hydrogen peroxide in *P. teres* f. *teres*-infected cold hardened and control barley leaves

| cold hardened | control |

Hordeum vulgare cv. Himalaya

1 Introduction

Plants as sessile organisms are especially dependent on efficient cellular pathways that provide protection from extreme temperatures. Cold hardening or cold acclimation describes the capability of organisms to be adapted to freezing temperatures by previous exposure to inductive conditions such as incubation at moderately cold (but non-freezing)

temperatures [1]. The ability to acquire cold hardiness varies greatly between plant species and even varieties.

Cold hardening induces a physiological state in plants that confers tolerance not only to subsequent low temperature treatments but also to impacts of other unfavorable environmental conditions (e.g. sulphur dioxide treatment [2], drought and photooxidative damage [3], heavy metal stress [4] or pathogens [5]). The protection from other potentially harmful environmental factors provided by cold acclimation is perceived as a form of cross-tolerance. Reactive oxygen intermediates (ROI) are crucial components of redox-mediated signaling networks in stress-affected plants and their precisely controlled accumulation may integrate environmental and metabolic cues. Thus, ROI have been viewed as key regulators during the process of cross-tolerance [6-8].

Exposure to low temperatures results in an increased formation of ROI in cereals and other plant species [9-13]. Incubation of plants at low temperatures also affects the energy source and sink balance of chloroplasts which leads to the production of excess ROI [14,15]. However, aerobic

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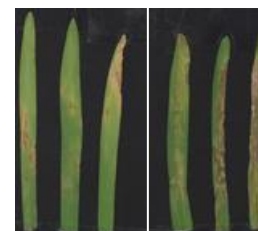**Borbála D. Harrach, Zoltán Bozsó, András Künstler,** Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences
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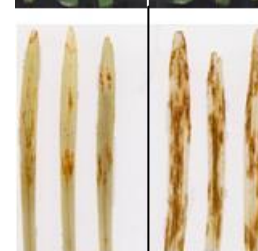Uta von Rad, Institute of Biochemical Plant Pathology, Helmholtz
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organisms including plants also possess various enzymatic and non-enzymatic antioxidant systems that scavenge excessively accumulating ROI and maintain cellular redox homeostasis. Cereals subjected to low temperature have also been reported to respond with an increased activity of antioxidants [3,15,16-20].

Pyrenophora teres (anamorph *Drechslera teres*) is a fungal pathogen of barley and is the cause of the net blotch disease. The spot form of the disease caused by *Pyrenophora teres* f. *maculata* is characterized by circular brown necrotic leaf lesions, whereas the net form caused by *Pyrenophora teres* f. *teres* produces longitudinal necrotic lesions with netted patterns on the leaves of infected plants. Even the lifestyles of the two fungi appear different: *P. teres* f. *maculata* is a typical hemibiotroph with a marked initial biotrophic stage in its lifecycle while *P. teres* f. *teres* appears to avoid this and quickly establishes itself as a necrotroph in the tissue of infected barley leaves [21].

We present the protective effects of cold hardening from chemical treatments that cause oxidative stress and from the pathogenesis of the necrotrophic fungus *P. teres* f. *teres* in the leaves of various cereal cultivars. The possibility of the involvement of redox factors and additional signaling components is then discussed.

2 Methods

2.1 Plant material and growth conditions

Seeds of winter wheat (*Triticum aestivum* cv. Emese), winter barley (*Hordeum vulgare* cv. Hardy) and spring barley (*Hordeum vulgare* cv. Himalaya) were sown in a Pindstrup Plus Orange moderate nutrient level peat medium and grown in growth chambers programmed for a 16h light/8h dark cycle with 200 μmol m^{-2} s^{-1} irradiation. Control plants were grown at 20°C temperature for 7 days. Plants subjected to low temperature (cold hardening) were initially germinated and kept at 20°C for 4 days before being transferred to 5°C temperature for 4 weeks. This procedure of cold hardening induction has been routinely used by others [4,22-26]. Before experiments cold-treated plants were removed and incubated at the control temperature for 2 hours.

2.2 Treatments causing oxidative stress in leaves of cereals

Twenty leaf segments (0.25 g) from the cereals (approx. 1 cm each) were weighed and subsequently floated in either 35 ml of double distilled water (to monitor background leakage from the samples) or 35 ml solutions of 10 μM methyl viologen (paraquat) or 100 mM hydrogen peroxide dissolved in double distilled water. Conductivity of the bathing solution was measured by an Oakton pH/CON 510 meter at the beginning of the experiments and again after 48 hours. Time zero conductivity results varied on average between 50 and 60 μS/g fresh plant material for all treatments.

2.3 Cultivation of *Pyrenophora teres* and pathogen assays

Strain 289 of *Pyrenophora teres* f. *teres* used throughout this work was collected in Martonvásár, Hungary. To confirm species identity of this *P. teres* strain, DNA extracted from the culture was subjected to specific PCR assays as described by Taylor et al. [27], Williams et al. [28] and Ficsor et al. [29]. The fungus was cultivated on PDA medium. Inoculum for infecting plants was prepared by growing the fungus in PDA liquid medium at room temperature under constant shaking for 4 days. The fungal biomass was then harvested by filtering the culture through two layers of cheesecloth before washing the yield extensively in tap water and removing the excess water by squeezing. The harvested fungal material was then weighed before being thoroughly homogenized in tap water using a blade mixer and diluted to a 0.2 g fungal material/ml tap water concentration. The inoculum was administered onto the primary leaves of barley plants using a fine paintbrush. The plants were then covered by transparent plastic bags to promote the humidity essential for the establishment of the infection. Inoculated plants were incubated at a 16h light (23°C)/8h dark (20°C) cycle and the plastic covering was removed after 24 hours. Disease symptoms were evaluated 5 days after inoculation for the susceptible Himalaya cultivar and 7 days after inoculation for the moderately susceptible Hardy cultivar. Hydrogen peroxide accumulation in *P. teres*-infected leaves was evaluated by DAB (3,3'-diaminobenzidine tetrahydrochloride dihydrate) staining [30]. Biomass of *P. teres* developed in the leaves was assessed by semiquantitative PCR using a primer pair (FW 5'- TAC CGC TGC TCA GAA CAT CA -3'; REV 5'- GGA TAC CGT TCA GTT TGC CC-3') designed for a putative *P. teres* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence (GenBank accession number: XM_003302119) and another primer pair (FW 5'-ACC CTC GCC GAC TAC AAC AT-3'; REV: 5'-CAG TAG TGG CGG TCG AAG TG-3') for a barley ubiquitin sequence (GenBank accession number: M60175). Amplification of this barley ubiquitin sequence served as a reference. PCR products amplified by the two

primer pairs are 221 and 263 nucleotides long. Barley leaves (5-6 primary leaves/treatment) were harvested and ground in liquid nitrogen 5 days after the inoculation and subjected to DNA extraction using a Viogene Plant Genomic DNA Mini kit (Viogene BioTek Corporation, Taipei, Taiwan) according to the manufacturer's instructions. Polymerase chain reactions were performed by a KAPA Taq PCR system (KAPA Biosystems, Woburn, Maryland, USA) with 60°C annealing temperature for 27 cycles. PCR products were analysed in a 1 % agarose gel by an AlphaImager EP system.

2.4 Microarray analyses

RNA extraction and first strand cDNA synthesis were performed according to Pogány et al. [31]. Transcriptional profiling of cold hardened and control barley leaves (cultivar Hardy) were carried out using a custom-designed microarray platform representing approximately 1500 barley EST clones. The fold change result for each transcript was generated from signals of eight hybridization spots: two independent sets of cold hardened and unhardened leaf samples were collected, two technical replicates per sample were performed and each clone was spotted twice onto one slide. Labelling with Cy dyes, hybridization, scanning and handling of raw microarray data were performed as described earlier [31]. False discovery rate (FDR) values revealing significant differences between the two treatments were calculated using the Rank Products method [32,33]. A list of barley EST clones printed onto the surface of microarray slides is provided as Supplemental Information.

3 Results

3.1 Cold hardening increases tolerance to treatments with paraquat and hydrogen peroxide in cereals

Cold hardened and control leaf segments of both wheat and barley were floated in solutions of methyl-viologen (paraquat) and hydrogen peroxide. Following 48 hours of incubation the leakage of electrolytes was measured in the bathing solution. This parameter is an indicator of oxidative stress-derived damage of cellular plasma membranes. The leaf samples subjected to long-term cold temperatures consistently exhibited tolerance to the treatments of paraquat and H_2O_2 confirming that cold hardening of cereals develops an increased protective

physiological status against treatments that cause oxidative stress (Fig. 1).
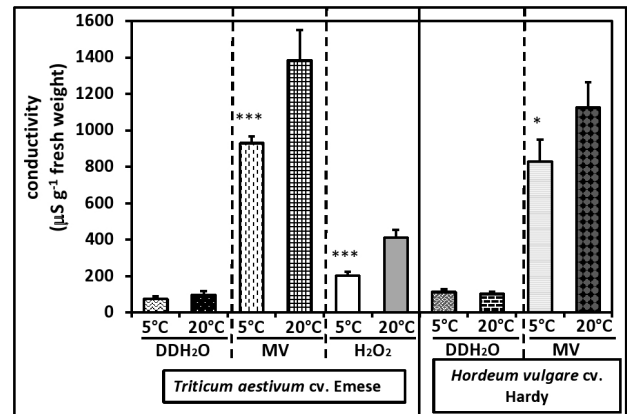

Figure 1. Effect of cold hardening on the tolerance of wheat and barley leaves to exposure to solutions of paraquat/methyl viologen (MV) and hydrogen peroxide (H_2O_2). Treatment abbreviations: 5°C DDH_2O – cold hardened samples floated in double distilled water; 20°C DDH_2O – control samples floated in double distilled water; 5°C MV – paraquat-treated, cold hardened samples; 20°C MV – paraquat-treated, control samples; 5°C H_2O_2 – hydrogen peroxide-treated, cold hardened samples; 20°C H_2O_2 – hydrogen peroxide-treated, control samples. Twenty leaf segments were floated in double distilled water or in solutions of 10 μM paraquat or 100 mM H_2O_2 before conductivity of the bathing solution was measured after 48 hours. Bars show means ± SD of two independent experiments (with 3 biological replicates in each). Statistical differences between means were calculated by Student's t-test (***P ≤ 0.001, *P ≤ 0.05).

3.2 Cold hardening confers resistance in two barley cultivars to infections of the fungal pathogen *Pyrenophora teres*

Necrotic symptoms caused by the advancement of *P. teres* f. *teres* colonization on leaves of two barley cultivars were macroscopically evaluated. The accumulation of fungal biomass in the plants was also assessed using semiquantitative PCR detection. It was observed that cold hardening conferred remarkable defense to both barley cultivars against the net blotch disease. Low temperature-treated Himalaya and Hardy barley plants not only developed reduced disease symptoms (Fig. 2/A and B) but the accumulation of *P. teres* biomass within their tissues was also restricted (Fig. 2/C). These results reveal that cold hardening of barley inhibits the symptom development of infection by *P. teres* f. *teres* but in fact also provides resistance to tissue colonization by the fungus.

A potential role of the host's antioxidative system in the observed fungal resistance was indirectly suggested
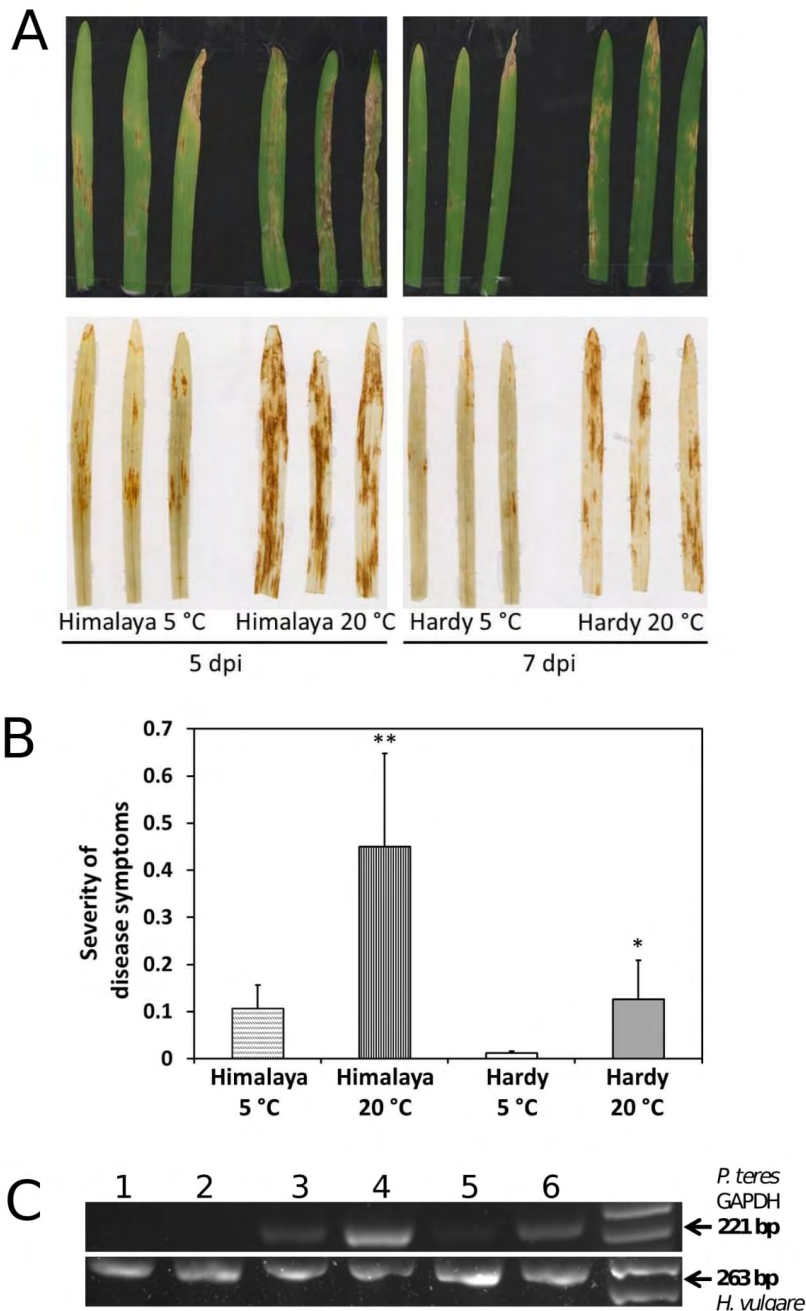
Figure 2. Cold hardening confers resistance in two barley cultivars to the colonization of necrotrophic fungal plant pathogen *P. teres* f. *teres*. A) Leaves of cold hardened plants (5°C) exhibit reduced *P. teres* symptoms (upper two images) and diminished accumulation of hydrogen peroxide (lower two images) than control plants (20°C). Two barley cultivars were used: Himalaya is highly susceptible (symptoms were evaluated at 5 dpi) and Hardy is moderately susceptible to *P. teres* f. *teres* (symptoms were evaluated at 7 dpi). Hydrogen peroxide accumulation was visualized by DAB staining (see Methods), with brown precipitates indicating the presence of H_2O_2. B) Assessment of *P. teres* symptom severity was performed by macroscopic evaluation of diseased leaves. Ten inoculated primary leaves were assessed for each treatment. Bars show means ± SD of ratios between necrotized and total leaf areas. The experiment was repeated twice each giving similar results. Statistical differences between means were calculated by Student's t-test (**$P \leq 0.01$, *$P \leq 0.05$). C) Results of semiquantitative PCR amplifications showing *P. teres* f. *teres* fungal biomass is reduced in cold acclimated barley leaves. *P. teres* biomass in diseased leaves was evaluated using a primer pair designed for a *P. teres* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequence. Evenly amplified fungal DNA amounts are compared to reference barley DNA levels in the same samples amplified by a primer pair designed for a barley ubiquitin sequence. Lanes: 1) uninfected *H. vulgare* cv. Himalaya plants 2) uninfected *H. vulgare* cv. Hardy plants 3) cold hardened *H. vulgare* cv. Himalaya plants inoculated with *P. teres*. 4) control, inoculated *H. vulgare* cv. Himalaya plants 5) cold hardened, inoculated *H. vulgare* cv. Hardy plants 6) control, inoculated *H. vulgare* cv. Hardy plants.

by ROS staining experiments with an accumulation of H_2O_2 detected in net blotch-infected tissues. In accordance with the observed symptoms, cold hardened leaves showed greatly diminished H_2O_2 accumulation around the longitudinal lesions resulting from fungal infection (Fig. 2/A).

3.3 Microarray results

A custom-designed barley microarray platform was used to detect transcriptomic changes in cold-hardened barley (*H. vulgare* cv. Hardy) plants in comparison with unhardened samples. Considering the increased oxidative stress tolerance observed in the cold hardened plants, gene activity associated with cellular redox balance were of primary interest. We were only able to find a small number of differentially regulated genes in those plants subjected to long-term cold temperature and importantly did not detect the induction of genes belonging to the cellular antioxidative machinery.

Two of the differentially expressed transcripts corresponded to photosynthetic genes: Ribulose bisphosphate carboxylase (RuBisCO) large chain (GenBank accession: HD11G07) was upregulated, whereas the photosystem II CP43 reaction center protein represented by two EST clones (HO15K23, HB25K03) was downregulated in leaves of cold hardened barley plants. Additionally, two housekeeping genes encoding glyceraldehyde 3-phosphate dehydrogenase (HO29I20) and polyubiquitin (HW05D19) showed increased transcriptional activity, while a glutathione S-tranferase transcript (HQ01C03) appeared repressed. Interestingly, transcripts of two antimicrobial proteins also exhibited altered abundance in cold hardened barley leaves but in opposite directions: gene activity for a glucan endo-1,3-beta-glucosidase (HP08M16) was upregulated while a thionin with two corresponding EST clones (HC14G11, HC11E06) was repressed. Finally, a putative acid phosphatase gene (*bci3*) represented by two clones (HW01P13, HX10I11) was induced in leaves of cold hardened plants (Fig. 3). Each of these fold change results were associated with FDR values below 0.1.
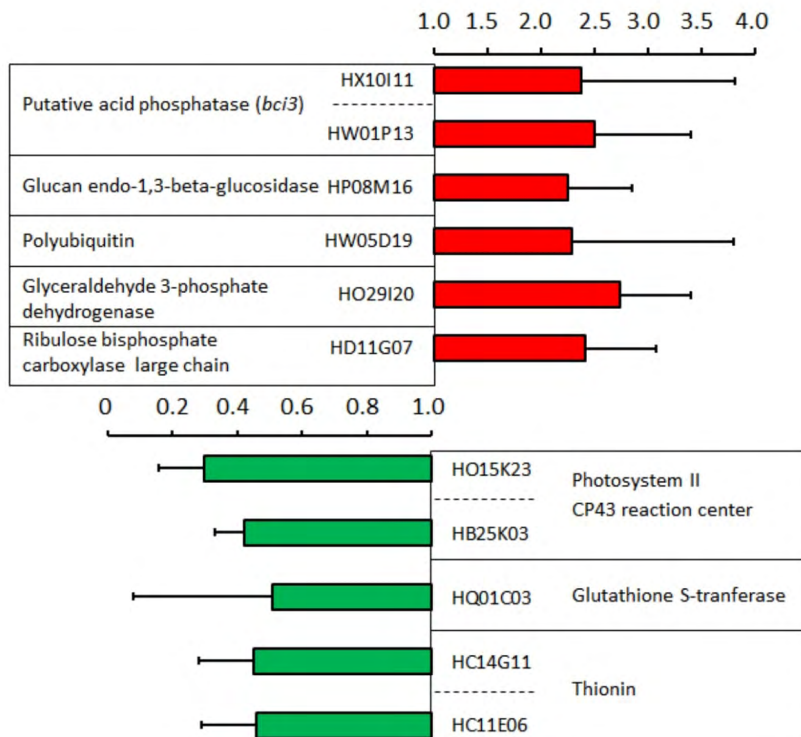

Figure 3. Results of microarray analysis showing transcripts with altered expression in cold hardened barley plants (*Hordeum vulgare* cv. Hardy) in comparison with mRNA expression of unhardened plants. Transcripts are labelled with corresponding EST GenBank accession numbers and putative gene functions. Fold change results were associated with FDR values below 0.1 calculated by the Rank Products method. Red bars show upregulated transcript levels and green bars indicate repressed transcripts in leaves of cold hardened barley plants. Fold change results were generated from signals of eight hybridization spots: two independent sets of cold hardened and unhardened leaf samples were collected, two technical replicates per sample were performed, and each clone was spotted twice onto one slide. Error bars show standard deviations.

4 Discussion

We found that cold hardening of the leaves of two wheat and barley cultivars alleviated tissue damage of paraquat and hydrogen peroxide treatments. Cold hardening-induced paraquat tolerance in plants has been reported by Bridger et al. [34] and Streb et al. [26]. The herbicide active ingredient paraquat is a strong electron acceptor in photosystem I that produces the ROI superoxide in chloroplasts even under low light conditions, leading to the destruction of chloroplast membranes and eventually to cell death [35,36]. Similarly, the exposure of cells to even millimolar concentrations of hydrogen peroxide also triggers severe oxidative stress and necrotic cell death [37]. Both of these treatments are detrimental to the cellular redox equilibrium. Interestingly, paraquat tolerant tobacco plants that were selected in tissue cultures using paraquat-containing media showed marked tolerance to low-temperature stress [38].

Since disease symptoms of necrotrophic pathogens are usually associated with oxidative stress in infected host tissues [39], the susceptibilty of two barley cultivars to *Pyrenophora teres* f. *teres* following cold hardening was assessed. Curiously, cold acclimation conferred resistance to the fungus in both cultivars and was manifested not only in diminished disease symptoms but also in reduced fungal growth. Similiar results have been observed in studies using different pathosystems, where increased pathogen resistance was reported in cold-pretreated plants [23-25,40,41]. Cold hardening seemingly protected infected barley leaves from the accumulation of hydrogen peroxide also, indicating a decreased level of oxidative stress in cold acclimated barley tissues following *P. teres* f. *teres* infection. This particular result raised the possibilty of the involvement of a more active antioxidant system in the cold hardened leaves of cereals.

Although the transcriptome study of cold acclimated barley leaves in our cereal samples did not confirm the induction of the antioxidant machinery, cold hardening is known to be accompanied by increased antioxidant activity as it was presented at gene expression, protein and enzyme activity/metabolite levels [12,18,19,42]. We may have failed to detect transcriptional change in the expression of genes encoding antioxidants because of insufficient hybridization efficiency – approximately 3 percent of the EST clones represented in our microarray encoded putative antioxidants such as superoxide dismutases, catalases or ascorbate and glutathione metabolism-related proteins. All transcripts detected in our samples belonged to corresponding genes with known high basal expression levels according to eFP browser. Consequently, transcripts represented by lower mRNA expression levels probably remained unnoticed.

Functional predictions of transcript sequences suggested that cold hardening affected the activity of genes involved in photosynthesis, which is characteristic of cold acclimated plant tissues [14,43,44]. One glucan endo-1,3-beta-glucosidase transcript was also found to be upregulated. This group of enzymes (EC 3.2.1.39) is known to function in overwintering but their role in protection from fungal pathogenesis cannot be ruled out [45]. The induction of the mRNA expression of an acid phosphatase (*bci3*) which shows sequence similarity with plant vegetative storage proteins was also detected [46].

It is apparent that a previous long-term cold exposure in cereals may modulate a broad spectrum of host responses given to a subsequent fungal pathogen attack. Notably, cold acclimation in plants is a potent inducer of salicylic acid and ethylene accumulation [47-50], transcription factors [40], calcium signaling [51,52], pathogenesis-related and antifungal proteins [5] along with anatomical changes, and cell membrane or cytoskeleton restructuring [53]. Consequently, besides plant antioxidative systems several other host factors should be considered as potential mechanisms behind the observed resistance response in cold hardened barley challenged by infection with *P. teres* f. *teres*.

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