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# Expression Analysis of *Pyrenophora teres* f. *maculata*-Responsive Loci in *Hordeum vulgare*

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## HIGHLIGHTS

- We revealed the involvement of three barley loci in the defense response against *Pyrenophora teres* f. *maculata*.
- These genes probably act as components of complex signaling pathways and would have cumulative effects.
- The studied genes were mapped on chromosomes *5H* and *7H* and phylogenetically characterized.
- These pathogen-responsive genes are promising for pathogen resistance engineering in barley and its relatives.

**Abstract:** *Pyrenophora teres* f. *maculata* is the causal agent of barley spot form net blotch (SFNB), a major stubble-borne disease in many barley-growing areas worldwide. In plants, the Nucleotide-Binding Site-Leucine-Rich Repeat (NBS-LRR) gene family functions in immunity against a variety of pathogens and pests. From a pre-established set of NBS-type resistance gene candidates, we have selected three candidate genes, HvNBS10, HvNBS72 and HvNBS85, to analyze their possible involvement in *P. teres* f. *maculata* resistance. The studied genes were mapped on chromosomes 5H and 7H. Expression profiles using qRT-PCR, 48 hours after infection by *P. teres* f. *maculata*, revealed that the transcription of all genes acted in the same direction (down-regulation) in both resistant and susceptible cultivars, although they showed a variation in transcript dosage. This result suggests that coordinated transcriptional responses of multiple barley NBS genes would be required to an efficient response against *P. teres* f. *maculata*. Moreover, the phylogenetic analysis revealed that the studied barley candidate *R* genes were characterized by a high homology with the barley *Nbs2-Rdg2a* gene conferring resistance to the fungus *Pyrenophora graminea*, suggesting a common origin of *P. graminea* and *P. teres* resistance genes in barley, following pathogens evolution. The genes characterized in the present study hold potential in elucidating the molecular pathways and developing novel markers associated with SFNB resistance in barley.

**Keywords:** Spot form net blotch (SFNB); Nucleotide-binding site (NBS); Leucine-rich repeat (LRR); qRT-PCR; *Hordeum vulgare*.

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## INTRODUCTION

Plants are surrounded by a diverse range of pathogens. Against them, the first line of plant defense is non-host resistance, based on the recognition of a broad range of invariant pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) by dedicated membrane-bound receptors, called pattern recognition receptors (PRRs), leading to the activation of pattern-triggered immunity (PTI) [1]. In the second phase, pathogen invasion may lead to the activation of effector-triggered immunity (ETI) that acts through cytoplasmic immune receptors responsible for the recognition of factors secreted by pathogens, which, in turn, activate downstream signaling pathways leading to a rapid local programmed cell death called hypersensitive response (HR). The defense response using cytoplasmic immune receptors is a well-known strategy characterized by specific interaction between disease resistance (*R*) protein receptors of plants and corresponding avirulence (*avr*) gene products.

The most common *R* gene class is represented genes encoding proteins containing a nucleotide-binding site (NBS) that is often associated with a leucine-rich repeats (LRR) domain [2]. The NBS domain is involved in signaling through the binding and hydrolysis of ATP and GTP and includes several highly conserved and strictly ordered motifs such as P-loop, kinase-2, Kinase-3a and GLPL [3]. The LRR domain typically consists of 20–30 amino acid repeats that are often involved in protein-protein interaction and, more precisely, binding to pathogen-derived molecules [4]. The LRR domain is thought to be the primary determinant of pathogen recognition specificity [5]. The NBS-LRR class of *R* genes can be divided into two subfamilies: the TIR-NBS-LRR (TNL) proteins that contain an N-terminal Toll/Interleukin1 receptor domain (TIR) and the non-TIR-NBS-LRR, where a coiled-coil (CC) domain can be substituted to the TIR one, defining CC-NBS-LRR (CNL) proteins [2]. Transcriptome analysis is a powerful tool for studying the role of *R* genes in disease resistance. Recently, the availability of draft or reference genomes for several plant species has enhanced the evaluation of expression levels of plant predicted genes. Resistance gene candidates (RGCs) encoding NBS-LRR proteins have been cloned from several plant species and many have co-localized with known resistance gene loci for both qualitative and quantitative resistance [6,7].

Barley, *Hordeum vulgare* L., is one of the world's oldest cultivated crops, spreading from its origin center in the 'fertile crescent' in the Middle East [8]. Since ancient times, barley has been used worldwide for animal feed and human food. The necrotrophic fungus *Pyrenophora teres* Drechs. is the causal agent of net blotch of barley, and exists in two forms, which, although morphologically indistinguishable under the microscope, can be differentiated by the symptoms observed on susceptible barley genotypes [9]. In fact, net form net blotch (NFNB), caused by *P. teres* f. *teres*, is manifested by necrotic lesions with transverse and longitudinal striations, forming a net-like pattern of necrosis often accompanied by chlorosis [10], while spot form net blotch (SFNB) produces elliptical necrotic lesions surrounded by chlorosis [9]. In Tunisia, Cherif et al. [11] reported that net blotch is the most common disease of barley, occurring at high severity rates (70–80%) in some regions of the country. Several studies have reported that resistance to *P. teres* could be controlled by either one or several genes, depending on the source of resistance, the plant development stage, and the pathotype used for testing [12]. From the six different genes conditioning resistance to net blotch [13], four have been mapped [14-16]. QTLs conferring *P. teres* f. *maculata* resistance in seedling have been reported on chromosomes 2H, 3H, 4H and 7H [16,17]; and those conferring resistance in adult stage on chromosomes 4H, 5H and 7H [16]. Wang et al. [18] reported that net blotch-resistance QTLs varied in effect, with large effects QTLs located on chromosome 7H. Despite these findings, there is little knowledge about the expression of defense responses against *P. teres* f. *maculata*, after infection of barley and none of the genes conferring barley resistance to *P. teres* has been identified at molecular level. Therefore, the aim of the present study was to investigate the potential to *P. teres* f. *maculata* resistance among three *H. vulgare* candidate *R* genes, through the analysis of their transcriptional activity.

## MATERIAL AND METHODS

### Biological material and culture conditions

Isolates of *Pyrenophora teres* f. *maculata* were collected from different agroecological regions in Tunisia, between January 2015 and January 2016. From infected barley leaves, spore samples were isolated with an eyelash under a binocular loupe. Single-spore cultures were prepared by transferring single spores to plates containing 200 ml of V8 agar, 16 g of agar, and 3 g CaCO<sub>3</sub> per liter of H<sub>2</sub>O. These plates were incubated at 21°C and 12:12 h photoperiod under cool white light. Ten days later, conidia were harvested by adding 2 ml of water to the plates and scraping the culture with a rubber spatula. This suspension was filtered through a double layer of cheesecloth to separate large mycelia fragments from conidia, and then the spore concentration was adjusted to 5.10<sup>4</sup> conidia/ml, for inoculation.

### Inoculation of plants and resistance rating

Five barley cultivars, 'Bowman', 'Rihane', 'HD35', 'Martin', 'Roho', were tested in order to evaluate their responses against Strain "Pt 01/2016" obtained, in January 2016, from the region of El-Fahs located in North Tunisia (36.37 N, 9.91 E). Cultivar HD29 was used as uninfected control. Initially, barley genotypes were sown (five to seven seeds) in plastic pots (25 cm diameter) filled with loamy-claysoil and grown in a greenhouse at 15-22°C at INAT, and inoculated 3 weeks later. The conidial suspension was sprayed onto the plants using a DeVilbiss atomizer. Treatments were replicated three times and were arranged in a randomized complete block design. Next, the plants were placed in a mist chamber at 18-22°C and 12:12 photoperiod, where the humidity was maintained near saturation. After a 72 h moist period, the plants were transferred to a growth chamber at 21-24°C and 12:12 h photoperiod. At the 14th day following inoculation, the mycelia development was observed visually and plant responses to infection were rated on 1–10 disease reaction scales developed by Tekauz [19]. Genotypes showing lesions rated 5 or lower, which are restricted in size, are considered as resistant, while those with lesions rated above 5, which continue to expand over time, are considered as susceptible.

## Selection of barley candidate genes

From the Whole Genome Shotgun Sequence assembly of barley cv. Bowman (2,077,901 contigs) (<http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=CAJX01>), the NBS domain was tracked in translated contigs, using a Hidden Markov Model (HMM) procedure [20,21]. *Ab initio* gene prediction, using Fgenesh [22], followed by multiple computational analyses, enabled predicting 96 candidate genes, designated HvNBS1 through HvNBS96 [23]. Among these, three genes, namely HvNBS10, HvNBS72 and HvNBS85, are analyzed in the current study, for their possible involvement in *P. teres* resistance.

## Phylogenetic characterization of barley candidate *P. teres* resistance genes

Five reference barley resistance genes have been used for comparison. These include: (a) two non CNL barley *R* genes; *Rpg1* that confers resistance to stem rust, *Puccinia graminis* (GenBank accession ABK51311.1) [24], and the recessive barley *mlo* mutant allele conferring broad-spectrum resistance to powdery mildew, *Erysiphe graminis* f. sp. *hordei* (GenBank accession: CAB06083.1) [25]; (b) three CNL genes of the mildew locus A (*MLA*), namely *MLA10* (GenBank accession: AAQ55541.1) [26], *MLA1* (GenBank accession: ACZ65507.1) [27] and *MLA13* (GenBank: AAO16014.1) [28]. These three genes play a role in resistance to powdery mildew and it has been hypothesized that many variants of *MLA* are different alleles rather than separate genes [29]. Further, we have included in the phylogeny: (c) the sequence of the Nbs2-Rdg2a gene responsive to inoculation by the leaf stripe disease on barley, caused by the seed-transmitted hemi-biotrophic fungus *Pyrenophora graminea* (GenBank ADK47522.1) [30]. The *Arabidopsis thaliana* *RPM1* protein (GenBank accession CAA61131.1) [31] was used as outgroup.

Selected sequences were aligned using the program Muscle [32]. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) Version 5.0 [33] by employing the neighbor-joining (NJ) method with 1000 replicates for statistical reliability. All the other parameters were taken to the default settings.

## Total RNA extraction and cDNA synthesis

Total RNA was extracted from the leaves of control, susceptible and resistant cultivars, 48 h after inoculation by strain "Pt 01/2016" of *Pyrenophora teres* f. *maculata*, with a Trizol reagent (Invitrogen, Carlsbad, CA, USA). The following reagents were added into a sterile, nuclease-free tube on ice in the indicated order: 2.5 µg total RNA, 2 µl oligo (dT) primer, 11.5 µl nuclease-free water gently mixed, centrifuged at 2500 rpm and incubated at 95°C for 2 min. The tube was then chilled on ice, spun down, and placed back on ice. 4 µl reaction buffer (5X), 10 mM dNTP mix, 0.5 µl M-MLV Reverse Transcriptase, were mixed gently and centrifuged at 2500 rpm for 5 min. For gene-specific primed cDNA synthesis, samples were incubated for 60 min at 37°C. Reaction was terminated by heating at 70°C for 5 min. The synthesized cDNA was stored at -20°C.

## Quantitative Real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was employed to validate the relative change in expression of genes in the resistant and susceptible cultivars, separately. Quantitative RT-PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems, USA). The conditions for qRT-PCR were as follows: initial denaturation at 95°C (10 min) followed by 40 cycles of 95°C (15 s) and 60°C (1 min). After the cycle was complete, melting curves analysis was performed at 60–95°C to verify the specificity of the amplicon for each primer pair. The barley actin gene [30] was used as a housekeeping gene. The specific primers used for qRT-PCR, listed in Table 1, were designed by Primer 3 software (<http://simgene.com/Primer3>). The 25 µl reaction mixture contained 1 µl of template cDNA, 10 µl of IqGreen QPCR master Mix-Rox (Biomatik, USA), and 10 µM of forward and reverse primers.

**Table 1.** Primers used in qRT-PCR analyses.

Gene	Reference	Forward (5'-3')	Reverse (5'-3')
HvNBS10		CAAGGGGTTTCAGCAAC ATTT	GCTTTCCGTGCAAACAATTT
HvNBS72	Primers designed in the present study	TGGAAGGCCATTGAAG AAAC	CTATGGTGGCACATTTCGTTG
HvNBS85		CTCAGATGTGCCAGTG CCTA	CATTCCATTATCCGCAGCTT
Actin	Bulgarelli et al. [30]	ATGTGGCCATCCAGGC AGTGCTTT	TGGTCTCATGGATTCCAGCA GCTTCC

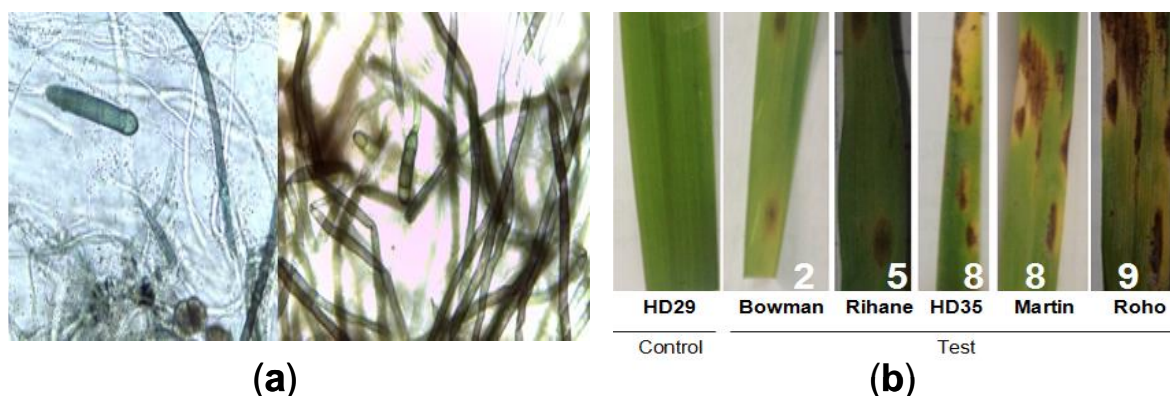
### Expression data analysis

Relative expression of mRNA was calculated by the comparative CT method (Relative quantification,  $RQ = 2^{-\Delta\Delta CT}$ ) described by Livak and Schmittgen [34]. The relative expression level of each investigated gene was normalized to that of the actin control. Expression analysis was based on the  $\log_2$  fold change of inoculated vs. control samples. Genes in resistant and susceptible plants were considered up or down-regulated if their  $\log_2$  gene expression ratios were higher than 1 or smaller than -1. DataAssist™ Software v3.0 (Applied Biosystems, USA) was used to generate a heatmap, reflecting the expression profiles. AnyGenes software (<http://www.anygenes.com/anygenes/excel.php>) was used to evaluate the  $2^{-\Delta\Delta CT}$  value of the candidate genes under experimental conditions.

## RESULTS

### Assessment of resistance to *P. teres* among barley genotypes

Strain "Pt 01/2016" of *P. teres* f. *maculata* used in this study produced abundant conidia in culture (Fig. 1a). Two weeks after inoculation, the tested varieties showed variable reactions to infection; the highest level of resistance was recorded in 'Bowman', while 'Rihane' was moderately resistant. 'HD35', 'Martin' and 'Roho' varieties were all susceptible (Fig. 1b).



**Figure 1.** Reactions of five barley cultivars tested against *Pyrenophora teres* f. *maculata*. (a): Conidia and filaments of *P. teres* f. *maculata*; (b): Typical spot symptoms elicited on barley leaves by *P. teres* f. *maculata* and rating of cultivars using the system of Tekauz [19].

### Structural characterization and chromosomal mapping of barley candidate genes

Three NBS-type *R* gene candidates in barley, HvNBS10, HvNBS72 and HvNBS85, were selected for the present study, from a larger pool containing 96 NBS-type gene candidates previously predicted in barley cv. Bowman [23]. The candidate genes structures are illustrated in Table 2 (Part A). Comparing HvNBS10, HvNBS72, HvNBS85 genes, against the barley genomic high confidence genes (May, 2016) of the International barley sequencing consortium (IBSC) ([https://webblast.ipk-gatersleben.de/barley\\_ibsc/](https://webblast.ipk-gatersleben.de/barley_ibsc/)), provided the results shown in Table 2 (Part B): HvNBS10 and HvNBS72 were mapped chromosome 7H, while HvNBS85 was located on chromosome 5H.

**Table 2.** Structural features and physical mapping of three barley candidate genes used in this study.

<b>A. Gene structure</b>									
Candidate gene	Contig accession number (Genbank)	Chain (+/-)	Gene		Coding region		DNA size	Exons	Protein length
			From	To	From	To			
HvNBS10	CAJX01007550 8.1	-	1576	4253	2263	3888	1626	1	541 aa
HvNBS72	CAJX01086387 9.1	-	129	3916	222	3428	3207	1	1068 aa
HvNBS85	CAJX01028650 1.1	-	1696	5590	1916	5449	3534	1	1177 aa

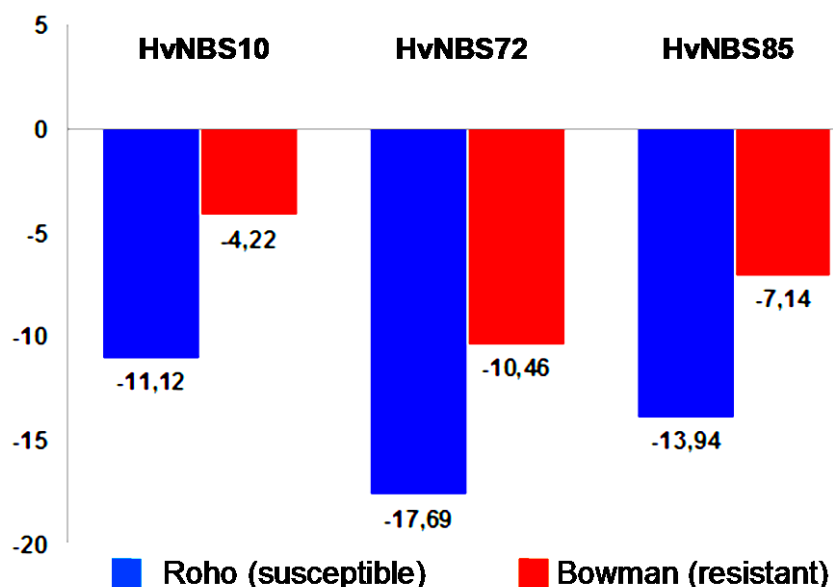
  

<b>B. Physical mapping</b>			
Candidate gene	Best matching gene (IBSC survey)	TBLASTN data (% identity; identities; e-value; score)	Chromosomal location
HvNBS72	HORVU7Hr1G0 26360	99; 1066/1068; 0.0; 2157	chr7H: 45224923-45229866
HvNBS85	HORVU5Hr1G0 06710	98; 1161/1179; 0.0; 2313	chr5H: 12035416-12041558

### Expression profiles of *H. vulgare* candidate genes upon *P. teres* infection

The transcriptional activity of HvNBS10, HvNBS72, and HvNBS85, upon infection by *P. teres* f. *maculata*, was examined by qRT-PCR. The most contrasted *H. vulgare* genotypes, namely resistant 'Bowman' and susceptible 'Roho', were challenged with the pathogen, and the expression of the four studied genes, following inoculation, was compared with 'HD29' uninoculated leaves.

For all genes, a differential expression (DE) was evident under infection by *P. teres* (Fig. 2). HvNBS10, HvNBS72 and HvNBS85 were all significantly downregulated, either in susceptible 'Roho' or in resistant 'Bowman', in comparison to the control. Nevertheless, a difference in transcript abundance was noted when comparing resistant and susceptible cultivars (Fig. 2), suggesting that these genes contribute to resistance through a transcript dose-sensitive mode. We speculate that these genes would possess common regulatory variants, such as miRNAs, that could potentially function as genetic expression modifying factors by modulating transcript stability. These data provide important insights into how barley may express resistance to infection by *P. teres* f. *maculata*.

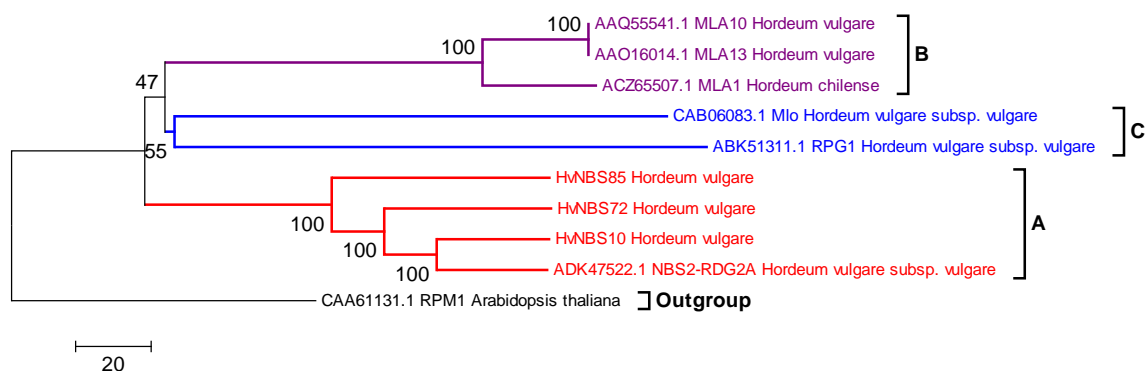


**Figure 2.** Relative transcript level of three HvNBSs obtained from quantitative real time PCR analysis in different cultivars of barley, following infection by *Pyrenophora teres* f. *maculata*.

Bar charts represent the values of  $\text{Log}_2(2^{-\Delta\Delta\text{CT}})$  for selected genes in resistant and susceptible cultivars. The barley actin gene was used as internal reference gene for data normalization.

### Comparative phylogeny

Phylogenetic analysis by the NJ method was conducted in order to determine the relationships among the studied *Pyrenophora teres* f. *maculata* resistance gene candidates (RGCs) and a set of *R* genes from barley. Based on relationships in the tree (Fig. 3), we could distinguish three clusters: Cluster (A) grouped the studied RGCs, HvNBS10, HvNBS72 and HvNBS85, that were characterized by their highest homology with the barley gene NBS2-Rdg2a (GenBank accession: ADK47522.1), conferring resistance to the fungus *Pyrenophora graminea*, as reported by Bulgarelli et al. [30]; cluster (B) included the three NBS-class genes *MLA1*, *MLA10* and *MLA13*; and cluster (C) contained non NBS genes *mlo* and *Rpg1*. The barley gene *NBS2-Rdg2a* has been mapped on the short arm of chromosome 7HS [35]. Therefore, our results strongly suggest a common origin of *P. graminea* and *P. teres* resistance genes in barley, through duplication and diversifying evolution, following pathogen evolution.



**Figure 3.** Neighbor-joining phylogenetic tree including HvNBS10, HvNBS72, HvNBS85 and other resistance proteins from barley.

Numbers on the nodes indicate bootstrap percentages. The *A. thaliana* RPM1 protein (CAA61131.1) was used as outgroup. Shown are the barley (*H. vulgare*) powdery mildew resistance proteins MLA1, MLA10 and MLA13 (CC-NBS-LRRs), the barley powdery mildew resistant allele *mlo*, the barley stem rust resistance gene *Rpg1* (non NBS-LRRs) and the disease resistance protein NBS2-RDG2A encoded by the NBS2-RDG2A gene responsive to the leaf stripe disease, *Pyrenophora graminea*, in barley.

## DISCUSSION

The present study revealed the probable involvement of three barley loci in the host defense response against *P. teres* f. *maculata*. The transcription of HvNBS10, HvNBS72 and HvNBS85 acted in the same direction in both resistant and susceptible cultivars, with a variation in transcript dosage. A significant number of investigations have already reported the key role played by such a coordinated functioning of different NBS-LRR loci, for a successful biotic stress resistance. In fact, plant NBS-LRR proteins act through a network of signaling pathways and induce a series of plant defense responses, such as activation of an oxidative burst, calcium and ion flows, induction of pathogenesis-related genes, and the hypersensitive response [36]. At least three independent, genetically defined signaling pathways in *Arabidopsis* are induced by NBS-LRR proteins [37]. Therefore, a large number of host genes may take part in resistance signaling and defense processes, and they may interact in a complex manner. In addition to resistance genes with cumulative effects, gene expression is often modulated, at the transcriptional level, by gene repressor/activator proteins that bind transcription factors, and, at the post-transcriptional level, by non coding microRNAs (miRNAs) that are able to modulate the expression in both normal and pathological conditions, by inhibiting translation or inducing degradation of transcripts [38-40]. Therefore, in order to efficiently develop resistant varieties, it is imperative to have a deeper knowledge of changes in the mRNA, protein, cellular metabolites and regulatory miRNAs after barley infection by *P. teres* f. *maculata*, which will lead to a greater understanding of the plant-pathogen interaction and enhance the efficiency of the development of new resistant genotypes.

Importantly, pathogen-responsive NBS-LRR genes identified in present study are promising to be used as candidate genes for engineering pathogen resistance in barley and related crop species, such as wheat, where *P. teres*, the causal agent of barley net blotch, has been already detected [41]. Notably, several *R* genes have been already used as transgenes within related species from the same family, such as the pepper *Bs2* gene, an NBS-LRR gene providing resistance in susceptible pepper, tomato and *Nicotiana benthamiana* against the bacterial spot disease caused by the bacterium *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) [42,43]. Further, transient expression of *Bs2* induced plant defense mechanisms in lemon against *Xanthomonas citri* subsp. *citri* (*Xcc*), causing citrus canker [43].

In conclusion, our study reports candidate genes that may play a role in resistance of barley and eventually other Poaceae to *Pyrenophora* species and increases our



understanding of the molecular mechanisms involved in barley defense against necrotrophic fungi.

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