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Nonhost resistance EST profiling of wheat interacting with *Blumeria graminis* f. sp. *hordei* identifies genes for durable resistance to powdery mildew

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Abstract Microbial pathogens cause great losses in many crops, including wheat. Plants overcome pathogen attack by triggering defense mechanisms relying on host resistance (HR) and non-host resistance (NHR). NHR can be a multi-gene driven, durable response, but the specific molecular mechanisms and genes involved remain elusive. The characterization of differentially expressed gene profiles between inoculated and non-inoculated plants can be of importance to elucidate the mechanisms behind NHR in wheat. In this study, common wheat (*Triticum aestivum* L.) was inoculated with the barley pathogen, *Blumeria graminis* f. sp. *hordei* (*Bgh*). Expressed sequence tags (ESTs) were identified by the cDNA-AFLP technique at different time points up to 96 h post inoculation. Twenty-one ESTs were identified and annotated by comparison with the *Arabidopsis thaliana* genome, proteome, and protein–protein interaction network. The ESTs were categorized based on gene ontology and functional assignment to categories related to lipid, hormone, secondary metabolism, redox, signaling and biotic stress. Expression of identified ESTs was verified by RT-qPCR. Expression of RPP13-like protein 3, F-box/FBD/LRR-repeat protein, wall-associated receptor kinase 1, DNA polymerase delta small subunit protein 2 was significantly induced, while expression of lanosterol synthase and zinc-binding ribosomal protein family was significantly repressed, indicating that they may play roles during *Bgh* attempted penetration of wheat leaves. This study revealed that expression of a diverse set of genes was affected during NHR of wheat, including possible signaling genes initiating effector triggered immunity (ETI) and hypersensitive response, and the overlap between NHR-induced genes and gene for host resistance is considerable.

Keywords PTI · ETI · Biotrophic pathogen · Hypersensitive response · *Bgh*

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Background

Wheat (*Triticum aestivum* L.*; 2n=42; AABBDD*) is one of the most important crops, providing a substantial amount of nutrients for approximately one-third of the world population (Consortium, 2014; Dubcovsky & Dvorak, 2007). As human populations continue

to increase, the importance of wheat will only become greater (Foley et al., 2011; Tilman et al., 2002). One of the major limitations to wheat production is the occurrence of a variety of microbial diseases.

Wheat powdery mildew pathogen, *Blumeria graminis* f. sp. *tritici* (*Bgt*), is a biotrophic Ascomycete fungus (Erysiphales) reproducing exclusively on living wheat plants (Panstruga & Kuhn, 2019; Wicker et al., 2013). In contrast to the necrotrophic pathogens, which first kill tissues and then feed on dead plant cells, biotrophs feed on living cells, and thus must manipulate the host for a relatively prolonged period of time (Laluk & Mengiste, 2010). *Bgt* can be potentially devastating, typically causing yield losses of 13–34%, which may go up to 70% when conditions are optimal for the pathogen (Li et al., 2011; Mwale et al., 2014). Closely related to *Bgt* is *Bgh* (*Blumeria graminis* f. sp. *hordei*), but there are many differences as they likely diverged approximately 6.3 (± 1.1) million years ago, co-evolving with their hosts, wheat and barley, respectively (Stukenbrock & McDonald, 2008; Wicker et al., 2013). Resistance of wheat against *Bgh* is a form of non-host resistance (NHR). NHR is thought to be highly durable as cross infections between hosts are rare, even though they have been reported (Aime et al., 2018; De Vienne et al., 2013). NHR is not fully understood, but there appears to be a considerable overlap between NHR and host resistance (Lee et al., 2016).

Based on visual symptoms, triggered NHR against bacteria, fungi and oomycetes has been divided into two types. Type I with no visible symptoms is due to PAMP recognition by plant pattern recognition receptors (PRRs) resulting in PAMP-triggered immunity (PTI), and type II with a hypersensitive response (HR) is due to pathogen effectors that are directly or indirectly recognized by host proteins possessing nucleotide-binding site, leucine-rich repeat domains (NBS-LRR) resulting in effector-triggered immunity (ETI). For powdery mildews, resistance has been divided into pre- and post-haustorial resistance, which are most often associated with papillae formation and HR, respectively (Pérez-García et al., 2009). The induction of genes encoding pathogenesis-related (PR) proteins occurs both during type I NHR, e.g. wheat stripe rust on broad bean (Cheng et al., 2012), and type II NHR, e.g. barley powdery mildew on wheat (Rezaei et al., 2019) against biotrophic pathogens.

In type II NHR, ETI is triggered by intracellular NBS-LRRs (Chen et al., 2019; Dempsey & Klessig, 2017). Hence, the wheat-*Bgh* interaction producing HR may be an ideal NHR system to study type II NHR as a form of durable resistance against biotrophs, and possibly identify genes that could be potential candidate for resistance against powdery mildews.

One approach to identifying genes involved in type II NHR is to use the cDNA-AFLP technique. It is a powerful, reliable, and reproducible method for gene expression profiling in response to stresses, such as invasion by pathogens or plant growth-promoting microorganisms, as it can detect low-abundance transcripts independently without prior knowledge of gene sequence (Abd El-Daim et al., 2018; Guo et al., 2014). In combination with validation techniques (e.g., real-time quantitative-PCR (RT-qPCR) and northern blotting), this technique has been extensively used to identify expressed sequence tags (ESTs) associated with plant diseases (Xiao et al., 2016). For example, applying cDNA-AFLP, Gao et al. (2014) identified three acetolactate synthase homologs in tomato among transcripts up-regulated in resistant cultivars but not in susceptible cultivars to powdery mildew (*Oidium neolycopersici*). Other examples of cDNA-AFLP studies in plant-pathogen studies are the discovery of a putative *LRR-RLK* gene up-regulated in pepper during resistance to *R. solanacearum* (Mou et al., 2019), 11 different ESTs up-regulated in wheat during an incompatible interaction with *Puccinia striiformis f. sp. tritici (Pst)* (Wang et al., 2010), and two LRR-RLKs, *TaRLK1* and *TaRLK2* up-regulated in *T. aestivum* during resistance against *Bgt* (Chen et al., 2016).

In this study, EST profiling by cDNA-AFLP technique was performed in time course manner up to 96 h post inoculation (hpi) to identify ESTs associated with NHR of wheat to the *Bgh* isolate Arsari-2015–1, and histochemical analysis showed papilla formation and HR involvement.

Materials and methods

Plant material

In this study, the wheat (*Triticum aestivum* L.) cv. "Darya", *SHA4/CHILCM91099-25Y-OM-3 N-1Y-OYZ-O10M-OY-3 M-O10* was used, which were obtained from the Seed and Plant Improvement

Institute Karaj, Iran (the SPII Institute). Seeds were disinfected and germinated in the dark on sterilized filter papers at 22 °C, and 10 seeds were sown per 30 cm diameter pot containing sterilized peat moss, with three replicates per sample. The pots were placed in a growth chamber, with 16 h light/ 22 °C and 8 h dark/ 16 °C, with 60% relative humidity. Seven-day-old seedlings were inoculated with *Bgh*.

Bgh isolation, identity and inoculation

Isolate AR-sari-2015–1 of *Bgh* was isolated from naturally infected leaves of barley, using the leaf symptom method (Walker et al., 2011). The inoculum was maintained by weekly transferring to uninfected barley cv. 'Afzal' in a growth chamber. In addition to visual evaluation of the disease symptoms, PCR amplification of internal transcribed spacer (ITS) of ribosomal genes was performed to identify the isolate according to Walker et al., 2011 (Supplementary Table 1). The ITS fragment was cloned, sequenced and analyzed against non-redundant nucleotide NCBI database and was deposited in GenBank with the accession number of MF661901 (https://www.ncbi. nlm.nih.gov/ nuccore/ MF661901.1). Furthermore, BLASTN searches of the NCBI database were used to retrieve additional ITS sequences of *Bgh* and *Bgt* isolates that were highly similar to that of *Bgh* isolate AR-sari-2015–1.

Seven-day-old seedlings of wheat were inoculated with *Bgh* as per Aghnoum et al. (2010), and Romero (2018) with the conidia concentration adjusted with a hemocytometer to give ~ 50 conidia/mm^2 leaf. Each replicate was ten plants harvested at 0 (as control), 6, 12, 24, 36, 48, 72 and 96 hpi. Leaf samples were immediately frozen in liquid nitrogen and stored at -80 for RNA extraction.

Histochemistry

Inoculated leaves were stained with DAB-staining (3,3'-diaminobenzidine) for detection of H_2O_2 (Thordal-Christensen, 2003), and Evans blue for fungal structure visualization. The tissues were observed under an inverted microscope.

Total RNA extraction and conduct of cDNA-AFLP

Total RNA was isolated from 100 mg frozen leaf tissues with each sample composed of three plants, using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and dissolved in DEPC-treated ddH_2O. Purity and concentration of the RNA were determined using a 1% agarose gel and spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA). For elimination of the potential genomic DNA contamination, RNA samples were subjected to DNase treatment (DNase I RNase-free, Thermo Scientific, USA). There were three biological replications per time point.

cDNA-AFLP was done with modifications as described by Feron et al. (2004). For the first- and double-strand cDNA synthesis, 5 µg of total RNA was added to streptavidin-coated PCR tubes (mRNA Capture kit, Roche, Switzerland), and then synthesis conducted according to the manufacturer's instructions (Fatemi et al., 2019; Martin et al., 2011). 3´-captured double-strand cDNA was digested in two separated consecutive reactions, using *EcoR*I and *Mse*I restriction enzymes, respectively (Thermo Scientific, Karlsruhe, Germany). The fragments were ligated to *EcoR*I and *Mse*I adaptors (Supplementary Table 1). The ligation reactions were diluted five times and used as the template for pre-amplification reactions with primers for each adaptor. PCR contents and amplification profiles for pre-amplification were conducted according to Fatemi et al., 2019. Each pre-amplification product was separated in 1% agarose in 0.5X TAE buffer to check for amplification based on a smear of 50–500 bp (Vuylsteke et al., 2007). The PCR products were heat-denatured at 95 °C for 5 min and separated on 6% denaturing polyacrylamide in a Sequi-Gen GMTM sequencing acrylamide electrophoresis instrument (Bio-Rad, Hercules, CA, USA). The amplicons were stained with silver nitrate (Bassam et al., 1991). The gels were scanned with an imaging densitometer (GS-800, BioRad).

Isolation, cloning, sequencing, and analysis of the ESTs

Based on the presence, absence and/or differential intensity of the bands visualized on gels, bands showing interesting pattern of alterations were selected using the profiles created by Quantity One gel image analysis software (version 4.4.1, Bio-Rad). The bands were cut with sharp razor blade from the gels. Then, they were eluted in 50 µl of sterile distilled water

overnight (4 °C). The isolated bands were re-amplified applying the same PCR conditions as used for pre-amplification, which was: 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min, 30 cycles; 4 °C hold. An aliquot of the PCR products was run on 1% agarose in 0.5X TAE buffer and compared with the band size on the original gels. After size validation, the single band PCR products were selected and ligated into pTZ57R/T T/A cloning vector (InsTAclone PCR Cloning Kit, Thermo Scientific, USA). Then, the electro-transformation of the vector carrying the PCR products into *E. coli* strain DH5a competent cells was performed. After PCR with M13 F/R primers and re-confirmation on the agarose gel, final products were sequenced.

Bioinformatics analysis

The sequences of the 21 ESTs were subjected to VecScreen software available at www.ncbi.nlm.nih.gov/VecScreen to remove the vector and universal M13 primer sequences. The EST sequences were deposited in the GenBank dbEST database under BioSample number SAMN07359425; library number: LIBEST_028812 (accession numbers JZ971236-JZ971258).

The sequences were used as queries in a BLASTn search of the NCBI non-redundant nucleotide database and the wheat genome database: https://phytozome.jgi.doe.gov/pz/portal.html. They were further analyzed for annotation and functional categorization using the Mercator pipeline: https://www.plabipd.de/portal/web/guest/mercator-sequence-annotation. A unique BIN code was assigned to each predicted protein, utilizing MapMan BIN ontology. The predicted protein sequences were used as queries in a BLASTP search of *Arabidopsis thaliana* and used for localization prediction using different programs including AdaBoost, EpiLoc, Plant-mPloc, SLPFA, SLP-Loca. The most repeatable predictions for each protein homolog of *A. thaliana* to the corresponding wheat sequences were used. Protein–protein interaction prediction was conducted via STRING v11.0.

RT-qPCR

RT-qPCR validations were performed using RNA obtained from inoculated seedlings of wheat that were grown under the same conditions as in the cDNA-AFLP experiment. Seven-day-old seedlings were inoculated (~50 conidia per mm^2 leaf) with the *Bgh* spores maintained on susceptible barley cv. Afzal. Isolation of total RNA was performed for inoculated seedlings at 6, 12, 24, 36, 48, 72 and 96 hpi as well as non-inoculated samples (0 hpi) for three independent biological replications. RNA was obtained from frozen wheat leaf tissue (100 mg) ground and mixed with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), which were extracted following the manufacturer's instructions, and DEPC-treated ddH2O. RNA samples were treated with *DNase I* at 37 ºC for 15 min and used in PCR. Afterwards, RNA samples were subjected to PCR with three rDNA-based primers for detection of DNA contaminations (Hashemipetroudi et al., 2018). cDNA was generated using the QuantiTect reverse transcription kit (Qiagen) following the manufacturer's instructions, and the cDNA was assessed electrophoretically and spectrophotometrically. The primers for RT-qPCR were designed based on the EST sequences and the wheat actin sequence, which was used as the reference gene, using Primer3Plus (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi) (Supplementary Table 2). The RT-qPCR was performed in three technical replications on CFX96™ Touch Real-Time PCR Detection System (C1000 Thermal Cycler, Bio-Rad) in 12.5 µl reactions containing 25 ng template, 1X hot start SYBR Green PCR Master Mix (Thermo Scientific), 0.3 mM each primer using a thermal program according to manufacturer instructions with an annealing temperature of 60 °C and 40 cycles. Dissociation curves of the PCR products were recorded between 55 and 95 °C. Non-template control (NTC) was featured in reaction for each primer. To analyze RT-qPCR data, $2^{-\Delta\Delta cT}$ method was employed, and cycle threshold (CT) value for the wheat actin gene was used for normalization (Livak & Schmittgen, 2001). The results were statistically analyzed using T-test in SAS software version 9.7.

Results

Identification of isolate and histochemistry of the interaction

Bgh isolate "AR-sari-2015–1" (https://www.ncbi.nlm.nih.gov/nuccore/MF661901.1) was obtained from leaves of *Hordeum vulgare* cv. Afzal. The sequence

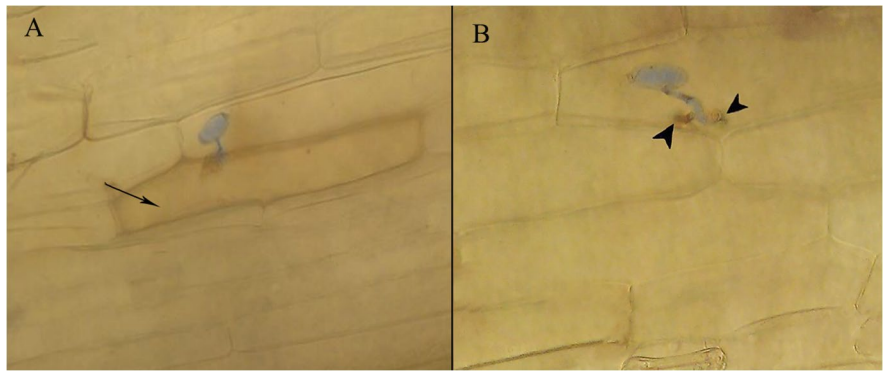

Fig. 1 Light microscopy of 3, 3'-diaminobenzidine (DAB)-stained tissues for H_2O_2 production for fungal structure visualisation at the sites of attempted penetration on wheat leaves infected by *Blumeria graminis* f.sp *hordei*. Localized host cell death in the form of hypersensitive response (HR) shown by arrow in **A,** and papillae formation at the site of attempted penetration shown by pointed arrow in **B**

of its ITS rDNA region had 99% nt match with that of *Bgh* isolate 6 (HM484333.1) with query cover of 100% and E value of 2e-162. A sequence-based phylogenetic tree showed that the ITS sequences of *Bgh* isolate AR-sari-2015–1 and *Bgh* isolate 6 clustered together (Supplementary Fig. 1). Histochemical analysis showed that penetration of wheat leaves with *Bgh* isolate AR-sari-2015–1 occurred, but growth in the leaf was stopped by papilla formation, host cell death (HR) and H_2O_2 production at approx. 48 hpi (Fig. 1).

Identification of 21 ESTs during the NHR wheat-*Bgh* interaction

To identify wheat ESTs differentially regulated during the *Bgh*-wheat interaction, cDNA-AFLP transcriptome profiling was performed at 0, 6, 12, 24, 36, 48, 72 and 96 hpi (Fig. 2). Approximately 2300 bands were visualized, and 21 bands showing the greatest differences from 0 hpi that were unique to *Bgh*-inoculated samples were sequenced. The majority of 21 ESTs showed homology to genes of *A. thaliana* (Table 1). Among them, there were sequences matching a putative disease resistance RPP13-like protein 3 (*RPP13L3*; JZ971237) that is a coiled-coil (CC)-NB-LRR protein (CNL) functioning against biotrophs, a F-box/FBD/LRR-repeat protein (*FBL*; JZ971249) with leucine-rich repeat receptor that can act as a regulator of programmed cell death, a laccase-9 (*LAC9*; JZ971247) involved in lignin production, a cell wall-associated receptor kinase 1 (*WAK1*; JZ971258) that could serve as an important signaling gene, a

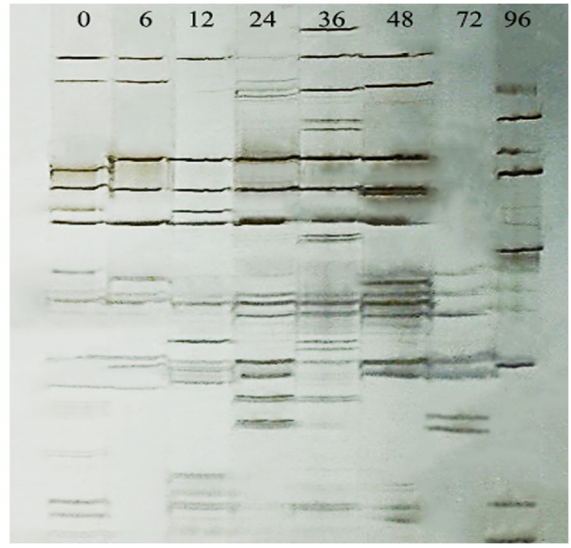

Fig. 2 Examples of acrylamide gels used for isolation of the ESTs differentially up/down-regulated during infection with *Blumeria graminis* f.sp *hordei*

zinc-binding ribosomal protein (*RPL37AB*; JZ971246) that is a large subunit ribosomal protein (RPLs) reported to act in NHR against bacteria in other plants, a lanosterol synthase (*LAS1*; JZ971239) in the terpene cyclase family, a transducin/WD40 repeat-like superfamily protein (*Transducin*; JZ971248) that is RNA-binding protein (RBP) that binds pre-mRNA molecules involved in several functions, and a WRKY transcription factor 18 (*WRKY18*; JZ971243) that is regulated by the defense hormone, SA.

Functional annotation of the 21 ESTs

The 21 ESTs were assigned functional annotation using the *T. aestivum* genome and hierarchical term assignation mapping (BINs) with MapMan and Mercator pipeline (Supplementary Table. 3). The proteins were categorized as lipid, hormone, and secondary metabolism, redox, DNA, RNA, protein, stress and signaling, and some were not assigned as unknown proteins (Fig. 3).

Confirmation of expression pattern of selected ESTs

The expression patterns of the ESTs *RPP13L3, LAS1*, *RPL37AB*, *FBL, POLD2*, *WAK1* during NHR were determined by RT-qPCR (Fig. 4). Compared to the control (0 hpi), significant up-regulation was observed for both *FBL* (3.9-fold, p-value < 0.01) and *POLD2* (2.6-fold, p-value < 0.01) at 36 hpi, which was after penetration but prior to the visible HR, and *WAK1* (2.1-fold, p-value < 0.01) at 12 hpi, which was when normally *Bgh* starts forming haustoria. Compared to the control (0 hpi), significant down-regulation was observed for *LAS1* (at all time points except 72 hpi), *RPL37AB* (at all time-points) and *WAK1* (only at 96 hpi). The largest down-regulation for both *RPL37AB* and *LAS1* was -sevenfold at 48 hpi (p-value < 0.01), which was at the typical time of the HR, whereas the down-regulation of *WAK1* was only -3.4-fold (p-value < 0.05) at 96 hpi, which was well after the HR. Co-regulation of *LAS1* and *RPL37AB* suggests that they may be affected through a related pathway. The heat map based on the expression profiles showed that most down-regulation was at the first and last time points (6 and 96 hpi), while most up-regulations occurred between those time points (Fig. 5).

Protein–protein interactions of RPP13L3 in Arabidopsis

Employing the Arabidopsis protein–protein interaction in STRING v11.0, the RPP13L3 homolog was further examined. In the network, SNC4 (suppressor of npr1-1, constitutive 4), and a transcription factor jumonji (jmjC) domain-containing protein (AT1G62310) with zinc-binding activity showed direct interaction with RPP13L3 (Fig. 6). RPP13L3 through a resistance signaling protein of the Toll-interleukin 1-like receptor (TNL) type (AT4G23440) interacted with two CNL proteins, RPS5, which is a well-studied protein functioning against downy mildew *Hyaloperonospora parasitica*, and a pentatricopeptide (PPR) repeat-containing protein. An NHR EST for *PPR* was also isolated in this study (Table 1). RPP13L3 also has a functional partner (AT4G09360) possessing the same domain type (NB-ARC) that have nucleoside-triphosphatase activity. In Arabidopsis, this gene is heavily methylated in the exon rather than the promotor, like most NBS-LRR proteins (Kong et al., 2018). However, the distribution of methylated regions of *RPP3L3* gene appears in both the exon and promotor regions, which were clustered together (Supplementary Fig. 2 A). AT4G09360 is an atypical TNL connecting the interaction of RPP13L3 to RPP8L1 (a disease susceptibility protein). TNL proteins, like CNLs, can recognize pathogens via their LRR domains (Shao et al., 2019). It is notable that the vast majority of protein interactions of RPP13L3 at the first shell of interaction occurred with TNL type proteins (Supplementary Fig. 2 B).

Discussion

Histochemical analysis showed host cell death along with H_2O_2 production at 48 hpi, indicative of type II NHR with possible ETI activation in post-haustorial resistance. This indicates that the pathogen has avoided PTI at the pre-haustorial phase. To date, many NBS-LRR genes have been linked to ETI for host resistance (Lee & Yeom, 2015), but they remain elusive for NHR. Regulation of NBS-LRR proteins is related to hetero- or homo-dimerization at their N terminus domain and NBS-LRR functions either directly or indirectly against effectors (Reddy et al., 2019). For example, the NBS-LRR gene against *Bgh* in host resistance of barley, *Mla*, encodes for a CC domain that establishes homodimerization, which is vital for initiation of NBS-LRR genes function, leading to the HR (Lee & Yeom, 2015; Maekawa et al., 2011). In this study, a number of putative ESTs involved in the NHR to *Bgh* were identified and RT-PCR analysis confirmed their differential regulation during the interaction.

For host resistance to powdery mildew, *Pm3* in wheat and *Mla* in barley have been shown to confer

Table 1 List of ESTs isolated from wheat interacting with *Blumeria graminis* f.sp *hordei* during different time-points (0–96 hpi)

EST NO	cDNA fragments		*T. aestivum*(nt)		*A. thaliana* (Protein)		Biological process	Molecular function	Predicted location
	ACNO	Size	ACNO	E-value	symbol	ACNO[b]			
1	JZ971236	193	3D02G410000.1	7e-012	*PPR*	AT2G36240	Regulator (organelle)		Mitochondria
2	JZ971237 [c]	183	6D02G017600.1	1e-099	*RPP13L3*	AT3G46730.1	Igniting HR	ATP/ADP binding	Cytoplasm
3	JZ971238	178	2B02G217800.1	9e-073	*PAB1*	AT1G16470	Response to zinc ion	Peptidase activity	Cytoplasm
4	JZ971239 [c]	220	7D02G233100.3	e-121	*LAS1*	AT3G45130.1	Triterpenoid biosynthetic	Amyrin synthase	Nucleus
5	JZ971240	140	6B02G451000.1	3e-094	-	No similarity	-	-	-
6	JZ971241	106	3D02G195400.2	0.018	-	AT1G74150.1			Nucleus
7	JZ971242	73	7D02G342700.1	6e-054	-	AT1G18090	DNA repair	Nuclease activity	Nucleus
8	JZ971243	140	5A02G225500.1	2.0	*WRKY18*	AT4G31800	Response against fungus	Transcription factor	Nucleus
9	JZ971245	60	4B02G097800.1	0.38	*CSI3*	AT1G77460.1	Cellulose biosynthetic		Chloroplast
10	JZ971246 [c]	214	3A02G267900.1	2e-027	*RPL37AB*	AT3G10950	Ribosome biogenesis	Structural constituent of ribosome	Mitochondria
11	JZ971247	202	2B02G287000.1	0.027	*LAC9*	AT5G01050.1	Lignin catabolic		apoplast
12	JZ971248 [a]	176	AK450379.1	2e-66	*WD-40*	AT1G10580	RNA-splicing	Nucleotide-binding	Nucleus
13	JZ971249 [c]	227	U02G026000.1	8e-040	*FBL*	AT1G78760	Regulation of HR		Nucleus
14	JZ971251	234	4B02G253500.1	2e-093	*FUC95A*	AT4G34260.1	Cell wall polysaccharide metabolic	1,2-Alpha-L-fucosidase	Nucleus
15	JZ971252	271	7D02G531100.1	4e-084	*PIE1*	AT3G12810.1	Response against bacterium	ATP binding	Cell Wall
16	JZ971253	182	6B02G409800.1	6e-072	*CDSP32*	AT1G76080	Response to oxidative stress	Protein binding	Plastid
17	JZ971254	202	4B02G101800.1	4e-024	*PDIL1-1*	AT1G21750	Regulation of HR	Disulfide Isomerase activity	Membrane
18	JZ971255	229	7B02G468100.2	e-123	*PIE1*	AT3G12810	Defense against bacteria	DNA binding	Nucleus
19	JZ971256	98	5B02G354900.1	2e-025	*RAD3*	AT1G20720			Membrane
20	JZ971257 [c]	150	4B02G321500.1	8e-079	*POLD2*	AT2G42120		DNA repair	Nucleus
21	JZ971258 [c]	197	5D02G520800.1	1.6	*WAK1*	AT1G21250.1	DAMP receptor	Kinase Activity	Membrane

[a]EST did not hit local BLAST, but it was found in NCBI

[b]The proteins of *Arabidopsis thaliana* homolog with wheat sequences according to NCBI and Mercator automated annotation pipeline

[c]The ESTs validated using RT-qPCR

[d]The full accession number (ACNO) of the *Triticum aestivum* sequences without prefixed; TraesCS

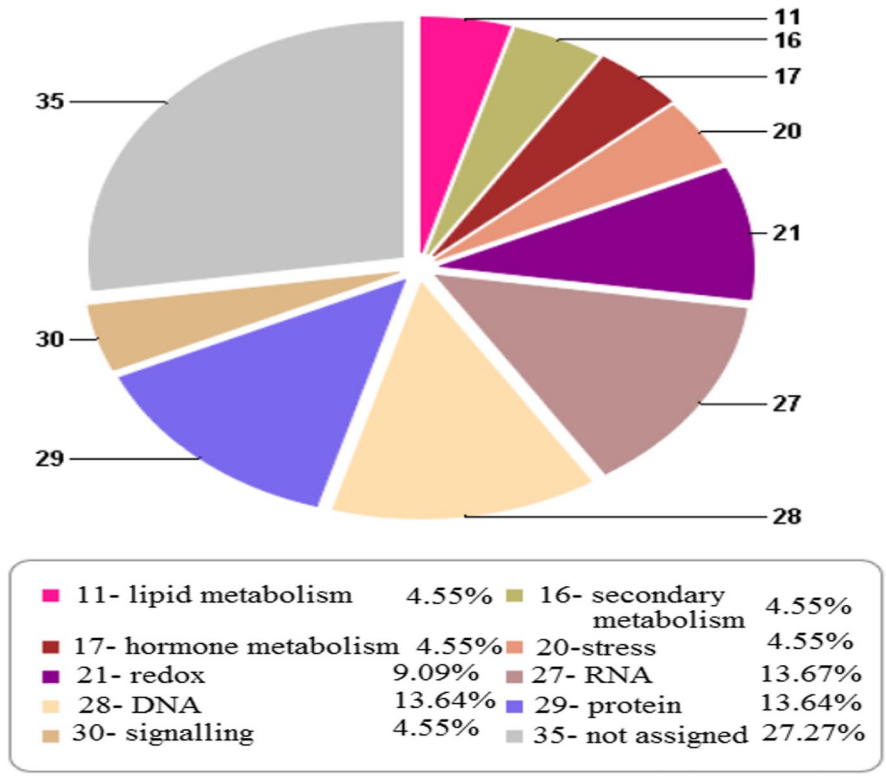

Fig. 3 Classification of the sequenced ESTs based on local BLAST results and annotation analyses using Mercator comprehensive pipeline exploiting the MapMan BIN ontology. Each bine code represents a unique function

resistance against *Bgt* and *Bgh,* respectively (Seeholzer et al., 2010; Yahiaoui et al., 2004). They both encode CNL type proteins. In this study, a homolog of RPP13L3 was identified, which is also a CNL, encoding a protein that interacts with SNC4, a suppressor of *NPR1*. *NPR1* suppression when *PR5* and *PR1* were up-regulated at 24 hpi and *PAL* up-regulation at 12 hpi has been reported (Shah, 2003). Thus, *RPP13L3* may also play a role in defense gene expression during type II NHR to *Bgh*.

In this study, the NHR-induced EST for *WAK1*, that encodes plant cell wall-associated kinase 1, peaked at 12 hpi indicating a role in perception of the non-pathogen attempting to penetrate the plant cell wall. WAKs send signals between cell wall and plasma membrane during pathogen attack (Uma et al., 2011). They maintain integration between the extracellular matrix and plasma membrane, and coupled with gly-rich proteins, they can monitor pectin integrity (Afzal et al., 2008; Sopory, 2019). They also possess cytoplasmic ser/thr-protein kinase activity acting as a central processor perceiving external information through their extracellular region, which holds two EGF-like repeats. That might indicate a role in fine-tuning gene expression and the oxidative burst after the *Bgh* penetration attempt. *WAK1* induction in this study could be a signal of disturbance by *Bgh* to cytoplasm, leading to activation of downstream pathways (Saxena, 2019).

Another NHR-induced EST identified was *FBL* which was up-regulated at 12 hpi and peaked at 36 hpi indicating an early role in the interaction. FBL protein contains F-box, fibrin binding (FBD), and LRR domains. It is a regulator of cell death in tomato and tobacco (van den Burg et al., 2008). Thus, *FBL* may function as an early regulator of HR in wheat NHR interaction.

The NHR-induced EST, *POLD2* expression was detected between 12 hpi up to 72 hpi, consistent with an involvement in the host response during haustoria formation and HR (Givechian et al., 2018). *POLD2* encodes for DNA polymerase delta subunit 2 that is involved in DNA repair. Direct involvement of DNA repair in regulation of gene expression during plant immune responses has previously been reported (Song et al., 2011), and this result suggests involvement in NHR to *Bgh*.

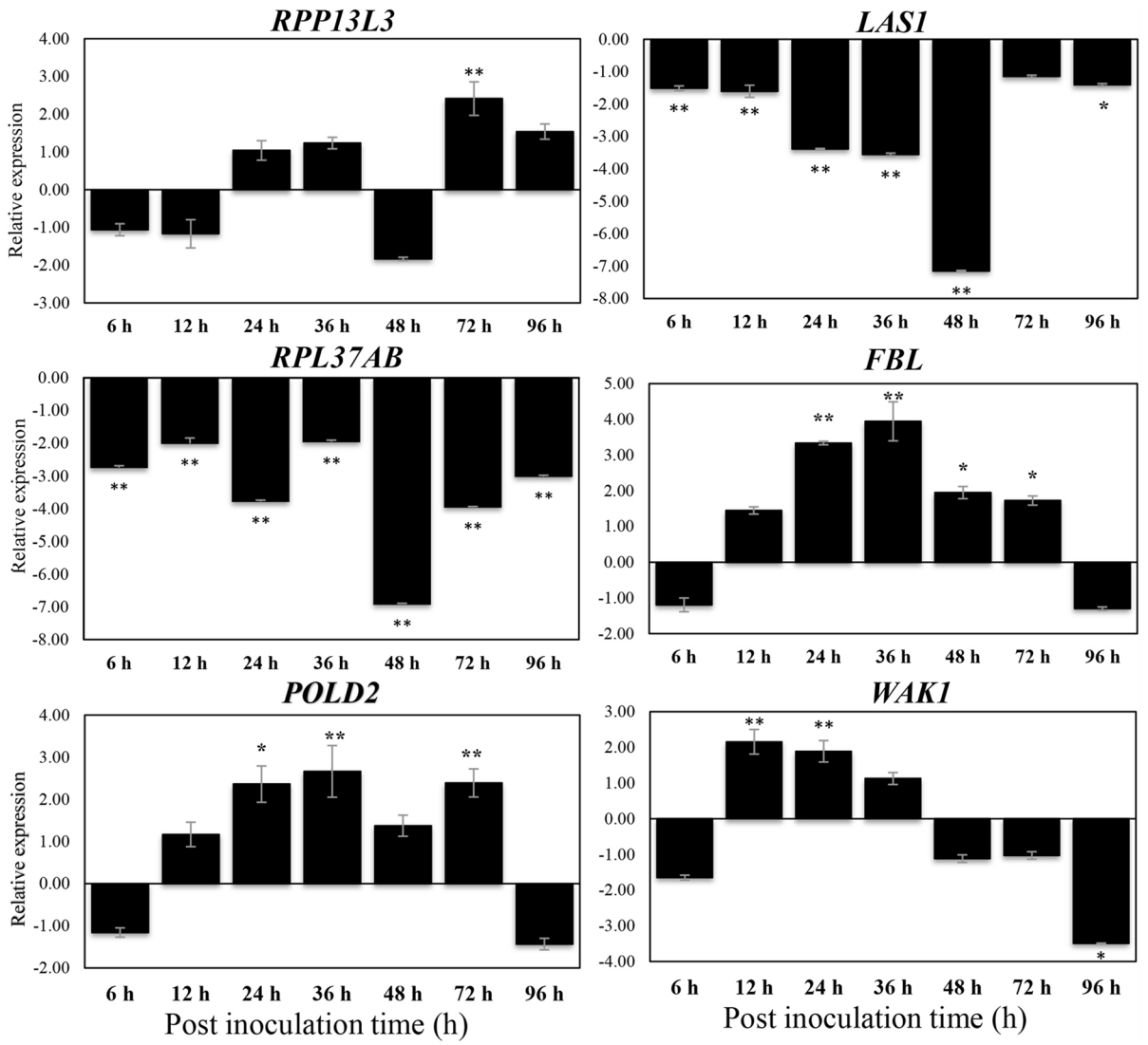

Fig. 4 Relative transcript levels of six genes differentially regulated during NHR of wheat-*Bgh*, namely *RPP13L3*, *LAS1*, *RPL37AB*, *FBL*, *POLD2*, *WAK1*, were monitored by RT-qPCR. Relative gene expression was quantified employing $2^{-\Delta\Delta CT}$ method where control (0 h) is 1. The mean expression value was calculated from three independent replicates. Vertical bars represent the standard errors. A single asterisk (* $P<0.05$, $n=3$) and double asterisks (** $P<0.01$, $n=3$) represent significant difference

There were also several other NHR-induced ESTs identified with potential roles in the NHR, among which an EST up-regulated early in the interaction at 6 hpi was *WRKY18*. WRKY transcription factor 18, SA responsive, interacts with *NPR1* and modulates its expression (Chen et al., 2019) indicating the importance of SA in the NHR of the wheat-*Bgh* interaction. An EST for transducin was induced at 6 hpi that is a WD40 family encoding for RNA-binding proteins (RBP). It binds pre-mRNA molecules involved in several functions including RNA-splicing, mRNA 3'-end processing and export from the nucleus, and termination of RNA-pol II transcription. Major defense-related roles have been found for RBP-mediated RNA-splicing and processing small interfering RNAs that can be involved in post-transcriptional regulation of NBS-LRRs (Dutta et al., 2017). Overexpression of Arabidopsis RBP-defense related 1

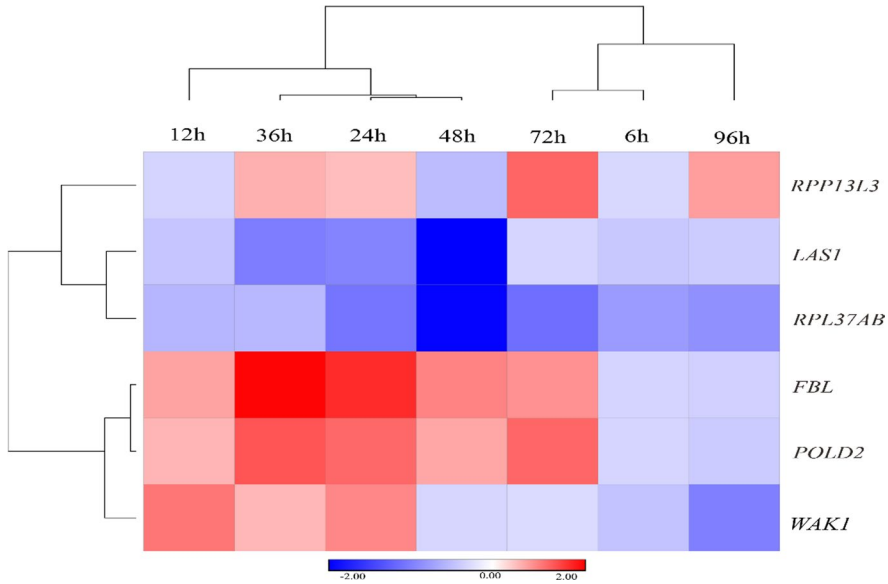

Fig. 5 Clustered heat map of the expression pattern of the genes regulated at different time course during NHR of wheat-*Bgh.* The red color represents the highest value, and the blue shows the lowest value of expression, clustered according to Euclidean distance

(AtRBP-DR1) led to enhance in SA level and *PR1* expression and subsequent resistance against *P. syringae* (Qi et al., 2010). In addition, an EST for *PAB1* was identified that is a zinc-responsive protein that could play a role in homeostatic mechanisms during stress in plants. It is also involved in the degradation of ubiquitinated protein, and inhibition of proteasome activity that activates programmed cell death in plants (Tsunezuka et al., 2005). Another EST that appeared to be up-regulated at 12 and 24 hpi was *Lac9,* which is a laccase. There is report that *Lac5* is required in lignin polymerization and deposition in cell wall during pathogen attack (Wang et al., 2015). Consistent with observation of papilla, *Lac9* could also be producing papilla, or cell wall apposition, at PAMP-recognition phase for maintenance of first line of NHR. EST induced at 12 hpi for *PPR* (pentatricopeptide repeat-containing protein) that commonly regulate organelle gene expression at the post-transcriptional level through sequence-specific binding with RNA, leading to altered expression following the change in RNA sequence, translation and turnover which consequently results in effects on plant environmental responses (Barkan & Small, 2014). Based on the protein–protein network of RPP13L3 in Arabidopsis, a PPR with one mediator (AT4G23440) interacted with RPP13L3 and directly interacted with RPS5 (also recognizes the avrPphB type III effector avirulence protein from *P. syringae*). Also, an EST induced at 24 hpi was identified as a thioredoxin-like protein *CDSP32*. Thioredoxin-like proteins play key roles during oxidative stresses (Vieira Dos Santos & Rey, 2006) and ROS accumulation that is required for production of HR against non-pathogens (Uma et al., 2011). Thus, in this NHR system, *CDSP32* could be involved in ROS production pathways. Other ESTs identified at 24 and 48 hpi, respectively, were *PIE1* and *PDIL1-1*. *PIE1* loss of function leads to a decline in basal resistance leading to impaired ETI (Berriri et al., 2016), and *PDIL1-1*, similar to *FBL1,* is a regulator of the HR (Stolf et al., 2011).

In addition, several NHR-suppressed ESTs could play a role in the interaction. The NHR-suppressed EST, *LAS1,* a lanosterol synthase belonging to the terpene cyclase family (Christianson, 2017), was suppressed significantly at almost all time-points. In plants, oxidosqualene cyclases converts 2,3-oxidosqualene to lanosterol (Xu et al., 2017). Terpenoid cyclases are responsible for the catalysis of the most involved chemical reactions, in which more than half of the substrate carbon atoms undergo changes in bonding and hybridization during a single enzyme cyclization reaction (Sawai et al., 2006). Lanosterol synthase is involved in the production of steroidal glycoalkaloids in potato (Abd El-Daim et al., 2018), and some saponins are steroidal glycoalkaloids acting as plant defense compounds (Osbourn, 1996) Thus, changes in *LAS1* expression could indicate a

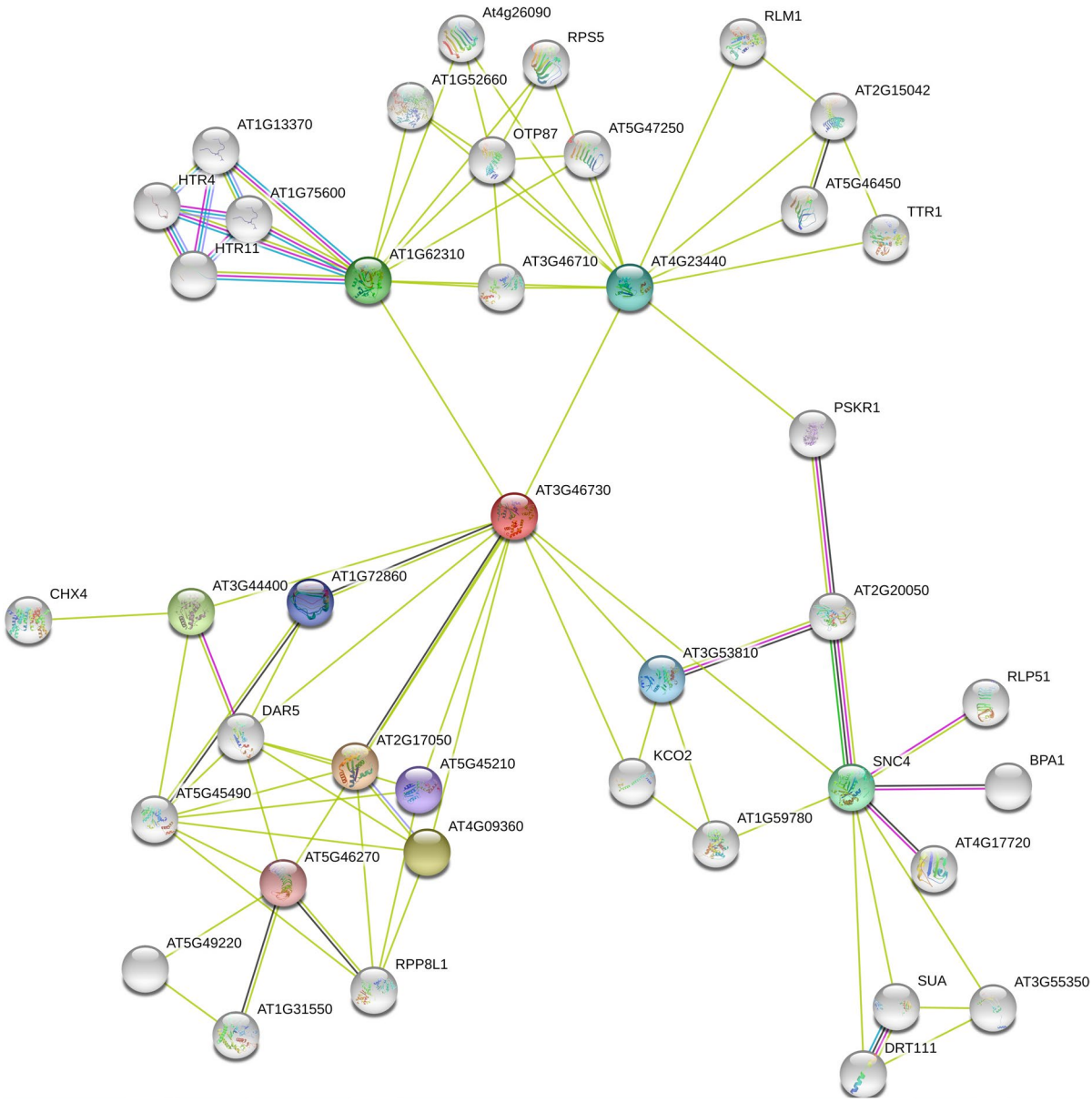

Fig. 6 The protein–protein interaction network of RPP13L3 constructed in STRING v11. The network was extended via an additional 30 proteins, and the confidence cutoff for representing interaction links was set to 0.400. Color of lines show the type of interaction, network nodes show proteins. The colored nodes show query proteins of the first shell. White nodes represent the second shell of interactors

shift in antimicrobial compound production during NHR.

Another NHR-suppressed EST was *RPL37AB* that showed significant suppression at all time-points. *RPL37AB* is a RPL mainly functioning in translation. In addition, RPLs exhibit extra-ribosomal functions, including regulation of protein synthesis and stress signaling (Warner & McIntosh, 2009). A positive role of RPLs in NHR of *Nicotiana benthamiana* to *P. syringae* was shown by silencing of the ribosomal proteins *RPL12* and *RPL19* that was demonstrated as a delay in non-host response (HR) induced by bacteria (Nagaraj et al., 2016). Thus, they may act similarly in the wheat NHR to *Bgh*. The timing of changes in

expression of *LAS1* and *RPL37AB* in this study indicates possible co-regulation, but that requires further studies.

Conclusions

This study identified putative ESTs in the wheat-*Bgh* type II NHR system. Histochemical analysis indicated that papilla formation and HR stopped *Bgh* spores from penetration at 48 hpi. The identified ESTs showed a diverse array of functions, revealing a considerable overlap between this type II NHR and ETI in host resistance. This work highlighted the role of several EST with known functions in host resistance interaction which can be candidates for breeding program against *Bgh* in cereal.

Abbreviations AFLP: Amplified fragment length polymorphism; *Bgh*: *Blumeria graminis* f. sp. *hordei*; *Bgt*: *Blumeria graminis* f. sp. *tritici*; CIMMYT: International Maize and Wheat Improvement Center; PTI: Pathogen-associated molecular pattern immunity; ETI: Effector triggered immunity; R gene: Resistance gene; TNL: TIR-NBS-LRR; CNL: CC-NBS-LRR; *RPP13L3*: Disease resistance protein RPP13-like protein 3; *LAS1*: Lanosterol synthase; *RPL37AB*: Zinc-binding ribosomal protein family; *FBL*: FBD/LRR-repeat protein; *POLD2*: DNA polymerase delta small subunit protein; *WAK1*: Wall-associated kinase1; ACNO: Accession number; BIN: hierarchical term assignation mapping

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Authors' contributions AR conducted the experiments, lab work, data analysis and wrote the manuscript. SHH and VB helped in data analyses. VB, PHG and HR conceived the work and SM, VB and SHH provided advice on analyzing and/or interpreting the experiments. PHG helped with writing and revisions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets measured and analyzed during the study are available from the corresponding author upon reasonable request.

Declarations

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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