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Transcriptional profiling identifies the early responses to *Puccinia triticina* infection in the adult plant leaf rust resistant wheat variety Toropi

Alice Casassola[1,2] | Nelzo C. Ereful[1,3] | Camila M. Zanella[1] | Pooja Pandey[1] | Sandra P. Brammer[4] | Márcia S. Chaves[5] | José A. Martinelli[6] | Lesley A. Boyd[1]

[1]National Institute of Agricultural Botany, Cambridge, UK

[2]Faculdade de Agronomia, Instituto de Desenvolvimento Educacional de Passo Fundo (IDEAU), Passo Fundo, Brazil

[3]Philippine Genome Centre/Plant Physiology Lab., Institute of Plant Breeding, University of the Philippines, Los Baños, Philippines

[4]Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Embrapa Trigo, Passo Fundo, Brazil

[5]Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Embrapa Clima Temperado, Pelotas, Brazil

[6]Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

Correspondence
Lesley A. Boyd, National Institute of Agricultural Botany, Cambridge CB3 0LE, UK.
Email: lesley.boyd@niab.com

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Abstract

Leaf rust, caused by *Puccinia triticina* (Pt), is a major disease of wheat and a significant problem for wheat production in Brazil. The Brazilian variety Toropi, released in 1965, has maintained high levels of field, adult plant resistance (APR) to leaf rust across global locations, while microscopic studies have indicated prehaustorial resistance mechanisms. Analyses of gene expression in flag leaves of Toropi, during the early stages of Pt infection, were undertaken to explore the mechanisms behind the APR in Toropi. Differential expression of wheat genes was undertaken, comparing Pt- to mock-inoculated and Pt- to Pt-inoculated time points. Analysis of gene expression indicated a strong response to Pt, which was fully active by 6 h after inoculation (hai). More genes were downregulated than upregulated, particularly at 6 and 12 hai. Gene Ontology enrichment analysis indicated a shutting down of RNA and protein synthesis and an early effect on photosynthesis, with disruption of the electron transfer chain. Analyses of upregulated genes identified genes involved in ATP-binding and protein kinase activity at 6 hai, supporting a rapid metabolic response to Pt infection. A general upregulation of genes involved in transport and metabolism indicated the need to relocate protein and organic-based resources. Alignment of differentially expressed genes with the genomic regions defining four leaf rust APR quantitative trait loci (QTLs) in Toropi identified candidate resistance genes, including a sugar transporter, a receptor kinase and a seven-transmembrane MLO family protein. In addition, 60 Pt genes were identified, 11 being annotated as potential effector proteins.

KEYWORDS
adult plant resistance, leaf rust, *Puccinia triticina*, RNA sequencing, transcriptomics, wheat

Alice Casassola, Nelzo C. Ereful and Camila M. Zanella contributed equally to this work.

1 | INTRODUCTION

Leaf rust, caused by the fungal pathogen *Puccinia triticina* (Pt), is a major disease of wheat (*Triticum aestivum*; Chai et al., 2020), and a particular problem for wheat production in Brazil (CONAB, 2022). Annual wheat production in Brazil is around 5500 tonnes, but this only provides approximately 40% of the wheat consumed, the remainder being imported. Most wheat is produced in the southern states of Rio Grande do Sul, Santa Catarina and Paraná, accounting for about 90% of Brazilian wheat production. It has been estimated that growing a wheat variety susceptible to leaf rust, compared with a resistant variety, over the same area and season would result in an average annual loss of around $130,663,070 in the state of Rio Grande do Sul alone (Mallmann et al., 2011). Growing resistant wheat varieties is therefore the preferred option.

Many leaf rust (*Lr*) resistance genes have been reported, with the majority conferring all-stage, race-specific resistance. The cloned race-specific *Lr* genes *Lr1*, *Lr10*, *Lr21* and *Lr22a* all exhibit nucleotide-binding site (NBS) and leucine-rich repeat (LRR) coding regions, common in plant disease resistance (R) genes (Dinh et al., 2020). The exception is the race-specific gene *Lr14a*, which encodes a membrane-localized protein containing 12 ankyrin (ANK) repeats and structural similarities to Ca^{2+} permeable nonselective cation channels (Kolodziej et al., 2021). Race-nonspecific resistance genes are generally characterized by a partial resistance and expressed at post-seedling growth stages (Dinh et al., 2020; Pinto da Silva et al., 2018). *Lr34*, *Lr46* and *Lr67* are race-nonspecific, adult plant resistance (APR) genes that slow the development of the pathogen, delaying the appearance of pustules, which tend to be smaller and produce fewer urediniospores. Both *Lr34* and *Lr67* have been cloned and represent an ATP-binding cassette (ABC) transporter (Krattinger et al., 2009) and a hexose transporter (Moore et al., 2015), respectively.

Pt is a biotrophic fungal pathogen that requires living wheat tissues on which to complete its asexual lifecycle. Within 3 h of landing on the leaf surface, Pt urediniospores germinate and form an appressorium above a stomatal opening. By 6 h the fungus enters the stomatal cavity, forming a substomatal vesicle and primary infection hyphae. Upon contact with a plant mesophyll or epidermal cell, a septum develops across the hyphal tip, defining a haustorial mother cell (HMC). The first HMCs are seen approximately 12 h after inoculation (hai). An infection peg develops off the HMC, breaching the plant cell wall. The fungus develops a haustorium, invaginating the plant cell membrane to establish a close contact between the fungal haustorium and the plant cell plasma membrane, enabling the pathogen to feed from the living plant cell (Hu & Rijkenberg, 1998).

The interaction between the host plant and invading pathogen is a continuous, two-way communication process (Couto & Zipfel, 2016). Initial plant recognition of pathogen-associated molecular patterns (PAMPs) triggers PAMP-triggered immunity (PTI). The pathogen can suppress and/or avoid PTI through the delivery of pathogen effectors (effector-triggered suppression, ETS). Effectors are thought to suppress PTI either by preventing detection of the PAMPs by the host, or by affecting downstream PTI-signalling pathways. A second line of plant defence involves an effector recognition system, mediated by host resistance (R) genes that recognize effectors either directly or indirectly, leading to effector-triggered immunity (ETI). However, it is becoming increasingly apparent that the role of effectors goes beyond suppression of plant defence, having a strategic role in modifying the plant's environment to create conditions conducive to pathogen growth and reproduction. Therefore, many of the plant genes induced upon pathogen infection, once thought to be required for defence, are required for infection by the pathogen.

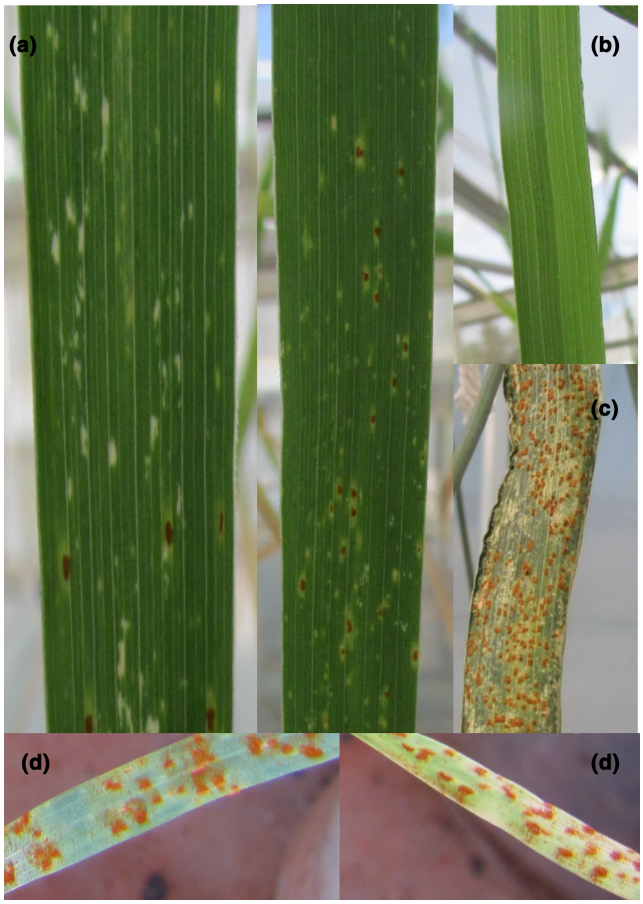

FIGURE 1 Leaf rust infection reactions seen on the wheat variety Toropi infected with *Puccinia triticina* (Pt) isolate MDT-MR. The leaf rust adult plant resistance in Toropi is characterized by a mixture of small, off-white to yellow flecks, with the occasional leaf rust pustule. (a) Flag leaves of Toropi infected with Pt. (b) Mock-inoculated flag leaf of Toropi. (c) Flag leaf of leaf rust-susceptible wheat variety Morocco inoculated with Pt. (d) Seedlings of Toropi inoculated with Pt. All leaf rust reactions are with isolate MDT-MR. [Colour figure can be viewed at wileyonlinelibrary.com]

The Brazilian wheat variety Toropi, released in 1965, was commercially cultivated for over 15 years. Throughout its cultivation it maintained a high level of field resistance to leaf rust (Figure 1), which is still effective, while being susceptible at the seedling stage to all known Brazilian Pt isolates (Barcellos et al., 2000). The leaf rust resistance in Toropi is defined as APR, that is, resistance that is

only effective post-seedling growth stages. Four quantitative trait loci (QTLs) that contribute to the leaf rust APR in Toropi have been identified (Rosa et al., 2019). They include a QTL on the long arm of chromosome 1B and *Lr78* on the short arm of 5D, also identified by Kolmer et al. (2018), and additional QTLs on 5AL and 3BS. The QTL on the long arm of chromosome 1B is in the same location as the *Lr46* locus (Rosa et al., 2019). The leaf rust APR in Toropi has been shown to display a unique, prehaustorial resistance (Rubiales & Niks, 2000), very different to that observed with R-gene-mediated resistance. In a study by Wesp-Guterres et al. (2013), the development of Pt was primarily halted at prehaustorial growth stages, namely appressoria and substomatal vesicles.

To investigate the prehaustorial resistance underlying the leaf rust APR in Toropi, we undertook a time-course analysis of gene expression in Toropi in response to Pt during the early stages of infection. Using RNA sequencing (RNA-Seq), we aimed to identify changes in wheat gene expression that corresponded to a strong PTI response. A read-depth analysis of gene expression in the primary flag leaf of Toropi inoculated with Pt, over the first 24 hai, was therefore undertaken. This enabled the identification of the biological and molecular responses that are involved in the leaf rust APR in Toropi.

2 | MATERIALS AND METHODS

2.1 | Plant material and Pt inoculations

The work undertaken in this study used the Brazilian wheat variety Toropi and the Brazilian Pt isolate race MDT-MR. Seed of Toropi was provided and verified by Márcia Soares Chaves, Embrapa-Trigo, Passo Fundo, Brazil. Professor Ana Christina Sagebin Albuquerque, Deputy Head of Research and Development at Embrapa-Trigo, provided permission to work with this Brazilian wheat variety as part of the project consortium agreement. Seed of Toropi has been deposited in the CIMMYT wheat germplasm collection (https://www.cimmyt.org/about/data/), accession BW804. Race MDT-MR was first detected in Brazil in 2005 and became a Pt race of concern due to its prevalence and virulence on many commercial varieties. Race MDT-MR is virulent on leaf rust resistance genes *Lr1*, *Lr3*, *Lr3ka*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr17*, *Lr20*, *Lr23*, *Lr24*, *Lr26* and *Lr30*, and avirulent on *Lr2a*, *Lr2c*, *Lr9*, *Lr16* and *Lr21*.

The Pt inoculation experiments were designed to reproduce the field conditions conducive to leaf rust infection in the southern region of Brazil, mainly when the flag leaf is fully expanded in October and November. Individual plants of Toropi were grown to ear emergence on the first tiller under a 14 h light/20°C and 10 h dark/16°C growing cycle, at 80% humidity. The first flag leaf was inoculated with Pt isolate MDT-MR when the ear was half to fully emerged (Zadoks growth stages 57 and 59; Zadoks et al., 1974). Plants were grown in a soil substrate (rice husk, peat and humus) and vermiculite mix, in 1-L pots. The flag leaf on the first tiller of each plant was inoculated with urediniospores of Pt race MDT-MR suspended in a light mineral oil (Soltrol 170) to a concentration of 5 mg/mL, or mineral oil alone (mock-inoculation). Pt- and mock-inoculated flag leaf samples for RNA were collected at 0, 6, 12 and 24 hai, and immediately placed into liquid nitrogen. The efficiency of the inoculation was verified 15 days after inoculation by the appearance of leaf rust pustules (Figure 1).

2.2 | Microscopic analysis of Pt development

Flag leaf samples for microscopy were collected at 1, 3, 6, 12, 24 and 48 hai. The leaf samples were cleared of chlorophyll and fixed in a solution of ethanol:chloroform (3:1, vol/vol) and 0.15% (vol/vol) trichloroacetic acid for 72 h. After 72 h the leaf samples were rinsed in this fixation solution, washed twice in 50% ethanol for 10 min and then placed in 0.1 M NaOH for 1.5 h to ensure optimal clearing. The leaf segments were then washed twice with deionized water. The samples were left in 0.1 M Tris–HCl (pH 5.8) for 30 min before staining with 0.1% (wt/vol) Uvitex 2B (dissolved in 0.1 M Tris–HCl) for 8 min, four washes with deionized water followed and one with 25% glycerol. The samples were left in deionized water overnight to remove excess Uvitex 2B stain. Leaf segments were stored in 50% glycerol. The leaf segments were mounted on microscope slides in 75% glycerol. The microscope slides were covered to ensure light did not reach the Uvitex 2B-stained leaf segments before microscopic observation.

Pt development was observed using epifluorescence microscopy on a Leica DM 2500 applying filter A4, at 10× and 20× magnification. The number of urediniospores observed at each of the following stages of Pt development were recorded: Pt0, ungerminated urediniospores; Pt1, germinated urediniospores with a single, unbranched germ tube; Pt2, germinated urediniospores with branched germ tubes; Pt3, appressorium developed at the end of the germ tube, above a stomatal opening; Pt4, infection hyphae grown from underside of the appressorium, through the stomatal opening, entering the substomatal cavity, forming a substomatal vesicle within the substomatal cavity; Pt5, infection hypha grown off the substomatal vesicle, a haustorial mother cell differentiated at the end of the infection hyphae; Pt6, the formation of additional hyphae off the initial infection hyphae; Pt7, abnormal growth of germ tubes on the surface of the leaf, appressorium developed at the end of a germ tube, but not above a stomata.

2.3 | RNA extraction, reverse transcription-quantitative PCR analysis and RNA sequencing

RNA was extracted from flag leaves using RNeasy Plant Mini kit (QIAGEN) and purified using the Turbo DNA-free kit (Invitrogen). RNA quality was determined using the Bioanalyser 2100 (Agilent Technologies) and quantified by NanoDrop 2000c (Thermo Scientific). All procedures were carried out according to the manufacturer's protocols.

For RNA-Seq analysis, RNA from three Pt- and three mock-inoculated flag leaves, at each time point, was sent to the Earlham Institute, Norwich, UK, for library construction and paired-end sequencing using Illumina HiSeq 2000. The Earlham Institute undertook quality control checks on the RNA, the construction of 24 TruSeq RNA libraries, data quality control, base calling and formatting, delivering approximately 100-bp paired-end reads. The datasets generated and analysed during the current study are available at ArrayExpress, accession number E-MTAB-9791 (https://www.ebi.ac.uk/fg/annotare/edit/11265/) (released 26th June 2022).

Reverse transcription-quantitative PCR (RT-qPCR) analysis was undertaken on a selection of genes differentially expressed between Pt- and mock-inoculated flag leaves of Toropi. Primers were designed for qPCR using Primer3Plus (Untergasser et al., 2012). Toropi RNA samples were converted to cDNA using the SuperScript IV First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. PCR amplification levels were normalized using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and the endogenous constitutive genes *ubiquitin* and *elongation factor-1a* (Casassola et al., 2015). All qPCRs were performed using SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich) at 95°C for 2 min; 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 15 s; and then 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. The specificity of the reaction was verified by melt curve analysis and the efficiency of each primer checked using the standard curve method. Primers with slopes between 3.1 and 3.6, and reaction efficiencies between 90% and 110%, were selected for further analysis. The qPCR of each gene, on each of three to four biological replicates, was repeated at least three times. Student's *t* test was performed to compare pairwise differences in expression, considering $p < 0.05$.

2.4 | Bioinformatic analysis pipeline: Quality checks of paired-end reads

Quality checks on the paired-end reads were performed using FastQC and adapter sequences and low-quality reads were trimmed using Trimmomatic (Table S1). After trimming, reads of less than 36 bases were removed from the dataset. Reads were then rechecked for quality and the presence of adapter sequences using FastQC, thereby ensuring the delivery of quality paired-end reads into the bioinformatic pipeline.

2.5 | Reciprocal mapping

As the Pt-inoculated libraries contain both wheat and Pt gene transcripts, we checked for sequence similarities between the wheat transcriptome of Toropi and Pt by mapping reads from the mock-inoculated wheat libraries against the Pt transcriptome reference EnsemblFungi release 35, Pt isolate 1–1, race BBBD (https://fungi.ensembl.org/Puccinia_triticina/Info/Index).

2.6 | Genome-guided assembly of paired-end reads

Paired-end reads from both Pt- and mock-inoculated libraries were aligned against the Chinese Spring IWGSC RefSeq v.1.0 (https://wheat-urgi.versailles.inra.fr/) transcriptome reference sequences using bowtie2 (Langmead & Salzberg, 2012) (parameter: --no-discordant), generating bam files (Table S1). Paired-end reads with low mapping quality and PCR duplicates were removed using SAMtools (Li et al., 2009) option: view –b –q 5 and option: rmdup, respectively. The unmapped paired-end reads were extracted using SAMtools (parameters: –b –f 4) and piped to PICARD to convert the sam to fastq files (parameter: SamToFastq). Extracted fastq files were then mapped against the Pt transcriptome reference sequence (EnsemblFungi release 35) using bowtie2. All scripts are stored in our github container with additional description: https://github.com/nelcaster7/Toropi.

Pearson's coefficient of correlation was used to create a correlation matrix between libraries using the normalized read counts. Normalization was performed using edgeR's trimmed means of M-values, as described below. Scatter plots between replicate libraries were generated using the 'pairs' function in R.

2.7 | Paired-end read count quantification and differential transcript expression analysis

Using the alignment bam files as input, read count quantification was performed using the alignment-based mode of Salmon and the wheat reference sequence fasta files from the mapping exercise using the IWGSC RefSeq v1.0 (URGI INRA [parameters: --numBootstraps --biasCorrect –useErrorModel]; Patro et al., 2017). The columns, names (ID) and the number of reads were extracted from the output generated by Salmon.

Differential expression of wheat transcripts, between treatments and time points, was analysed using edgeR. A count dataset, containing the read counts from each replicate library across all time points, was created for both Pt- and mock-inoculated samples using R (CRAN v. 3.4.2) in Linux. The count data frame was created describing the experimental factors: (a) treatments (Pt- and mock-inoculated) and (b) time points (0, 6, 12 and 24 hai). A design matrix was set up to combine these factors into a single factor called 'Group'.

Less-expressed isoforms were filtered out, the minimum requirement for isoform retention being at least two counts per million (CPM), in at least three samples. Normalization was performed with respect to library size to avoid bias due to variation in library sizes. A library scaling factor was calculated using trimmed means of M-values (TMM) implemented in edgeR (Robinson & Oshlack, 2010). TMM was used for library normalization as it is more robust than total count and RPKM procedures (Dillies et al., 2012).

We estimated the negative binomial dispersion using the 'estimateDisp' function, robustified against potential outlier isoforms. Quasi-likelihood (QL) dispersion was estimated around

the dispersion trend using a generalized linear model (GLM) via its 'glmQLFit' function. Using the 'makeContrasts' function, a contrast matrix was created to perform pairwise comparisons between Pt- and mock-inoculated treatments at 6, 12 and 24 hai, with the baseline expression differences being subtracted out (i.e., Pt.0hai–mock.0hai). The baseline adjustment was implemented to preclude observed expression differences at 0 hai, seen as a consequence of the lag time between Pt inoculation and leaf sample collection. For example, one of the components of the contrast matrix would be (Pt.6hai–mock.6hai)–(Pt.0hai–mock.0hai), which we call 'difference of difference'. To find genes that had responded differentially to the Pt treatments between two time points, contrasts were also added to the contrast matrix with baseline adjustment. For example, to find genes that are differentially expressed between 6 and 12 hai the contrast model would be (Pt.6hai – Pt.12hai)–(mock.6hai–mock.12hai).

A quasi-likelihood (QL) F-test was implemented to identify significant differential expression in each pairwise comparison. Mean difference (MD) plots were generated after calculating the QL F-test for each time point comparison between Pt- and mock-inoculated samples, and Pt- versus Pt-inoculated time samples. The MD plots provided a visual presentation of whether the normalization procedure effectively corrected for library size. Differentially expressed genes (DEGs) were called if transcript levels between pairwise comparisons exhibited a $\log_2$FC ≥ |1| (or fold change, FC ≥ 2) at a false discovery rate (FDR) *p*-value correction < 0.05 (Benjamini & Hochberg, 1995).

2.8 | Functional annotations of differentially expressed wheat genes

Functional annotations for the wheat DEGs were taken from the annotations provided with the Chinese Spring IWGSC RefSeq v2.0 (https://wheat-urgi.versailles.inra.fr/) transcriptome reference. Gene Ontology (GO) assignments were provided by RefSeq. Gene enrichment analyses were undertaken using the web-based tool AgriGO v. 2.0 (Tian et al., 2017) (http://systemsbiology.cau.edu.cn/agriGOv2/index.php). A singular enrichment analysis (SEA) for plant *Poaceae*, species *Triticum aestivum*, reference selected locus ID (IWGSC), using an enrichment significance threshold of FDR < 0.05, was applied. DEGs were defined by biological processes, cellular components and molecular function. The assignment of DEGs with the predicted pathways was further confirmed using published literature.

2.9 | Identification of Pt effector molecules

The paired-end reads from the Pt-inoculated libraries were mapped against the Pt transcriptome reference EnsemblFungi release 35; Pt isolate 1–1, race BBBD (https://fungi.ensembl.org/Puccinia_triticina/Info/Index). EBSeq–HMM (Leng et al., 2015; Leng & Kendziorski, 2021) was used to determine the expression of Pt genes across the time course. Read counts of Pt genes were extracted from each time point. Each column represents the four time points, 0, 6, 12 and 24 hai, in ascending order, each with three replicates. Rows with low read counts (row sums >4, where 4 corresponds to the number of column–time points) were removed to reduce object size, increase calculation speed and reduce noise. Normalization procedures were implemented using the function MedianNorm, a library size factor which reproduces the median normalization approach in DESeq (Anders & Huber, 2010). The function EBSeq–HMM test was used to estimate the posterior probability (PP). Genes were called as expressed if they exhibited PP ≥ 0.6 under a target FDR < 0.05. Pt genes were clustered based on their expression patterns over the 24-h time-course using R v. 3.4.2. Pt genes, identified as significantly expressed across time points by the EBSeq–HMM analysis, and were translated into protein sequences using the online utility tool Sequence Manipulation Suite (SMS; https://www.bioinformatics.org/sms2/). InterProScan (https://www.ebi.ac.uk/interpro/) was used to identify the potential function of these Pt genes. EffectorP was used to look for amino acid motifs defining potential effectors (http://effectorp.csiro.au/). Further characterization was undertaken using LOCALIZER (https://localizer.csiro.au) and EffectorP v. 3.0.

3 | RESULTS

The wheat variety Toropi expresses an APR to leaf rust that exhibits a leaf rust phenotype score of 3–5 (moderately resistant/moderately susceptible, MR/MS) to all Pt isolates tested (Figure 1). Microscopic studies of Pt development in flag leaves of Toropi had previously shown a prehaustorial phenotype, the development of Pt being primarily halted at prehaustorial growth stages, namely appressoria and substomatal vesicles (Wesp-Guterres et al., 2013). Therefore, we focused our transcriptome analyses on the first 24 h following inoculation with Pt. A single Pt isolate, race MDT-MR, suspended in mineral oil, was inoculated onto the primary flag leaf of Toropi adult plants and leaves sampled at 0, 6, 12 and 24 hai. Mock-inoculated (mineral oil) flag leaves were sampled at the same time points.

3.1 | Microscopic analysis of the development of Pt isolate MDT-MR

A microscopic study of MDT-MR development (Figure 2) indicated that this isolate progressed to a more advanced stage of Pt development than seen by Wesp-Guterres et al. (2013), where a different isolate of Pt was used, that is, race MFP. Isolate MDT-MR proved to be a very aggressive isolate, with the percentage of urediniospore germination being almost 100% by 3 hai. Germinated urediniospores were seen to have branched (Pt2, 5.7%), formed appressoria above stomata (Pt3, 7.5%) and even entered stomata (Pt4, 5.7%) by 3 hai. Hyphal growth off the initial substomatal vesicle,

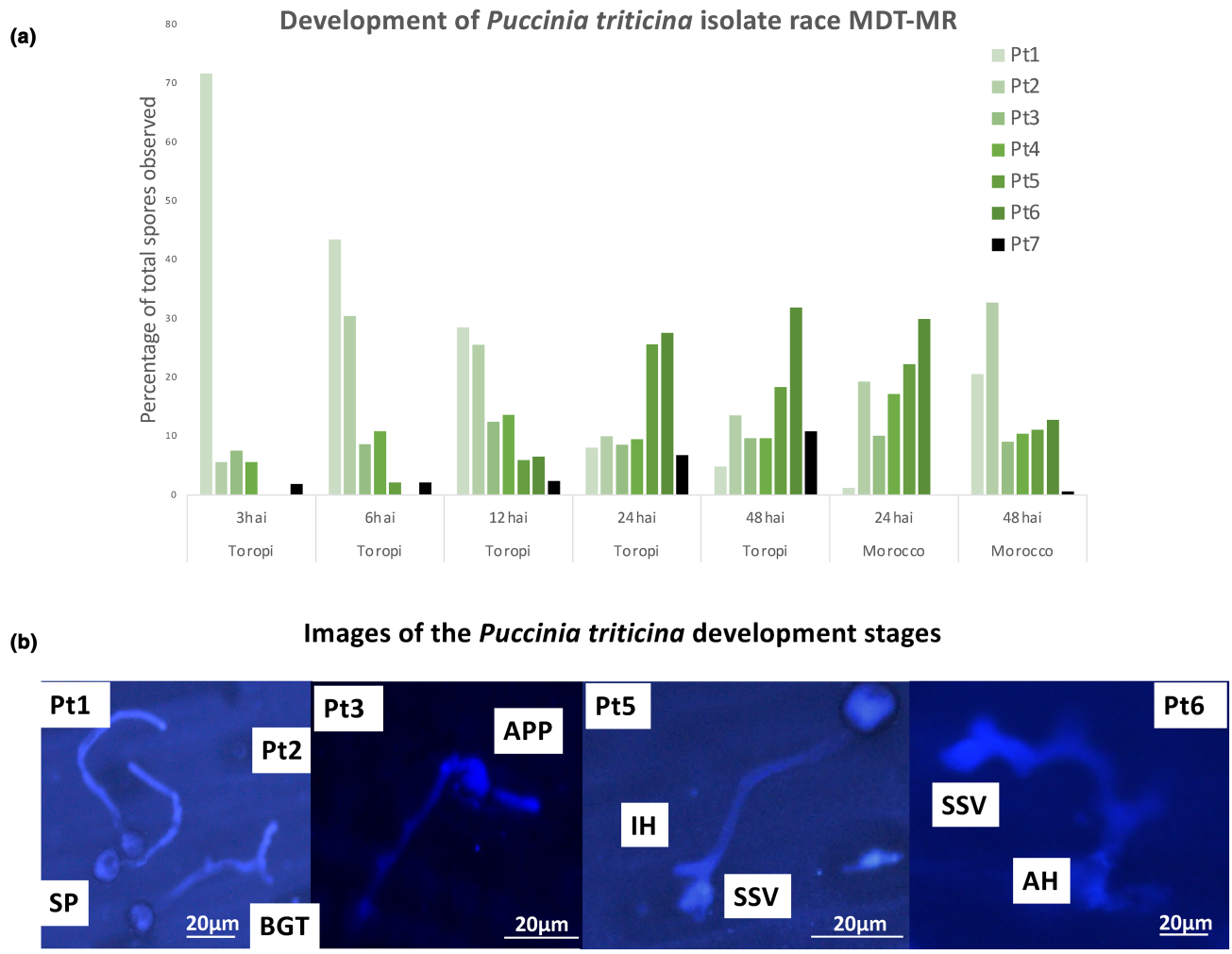

FIGURE 2 Development of *Puccinia triticina* (Pt) isolate race MDT-MR on flag leaves of the wheat variety Toropi. (a) The Pt race MDT-MR was inoculated onto flag leaves of Toropi and the susceptible control variety Morocco, and samples were taken for microscopic analysis of Pt development at 3, 6, 12, 24 and 48 h after inoculation (hai). The Pt development stages are Pt1, germinated urediniospores (SP) with a single, unbranched germ tube; Pt2, germinated urediniospores with branched germ tubes (BGT); Pt3, appressorium (APP) develops at the end of the germ tube, above a stomatal opening; Pt4, penetration hyphae grows from underside of the appressorium, through the stomatal opening, entering the substomatal cavity, a substomatal vesicle (SSV) develops within the substomatal cavity; Pt5, infection hyphae (IH) grow off the substomatal vesicle, a haustorial mother cell is differentiated at the end of the infection hyphae; Pt6, formation of additional hyphae (AH) of the initial infection hyphae; Pt7, abnormal growth of germ tubes on the surface of the leaf, appressorium develops at the end of a germ tube, but not above a stomata. (b) Images of the Pt development stages recorded. [Colour figure can be viewed at wileyonlinelibrary.com]

which had differentiated a haustorial mother cell, was observed at 6 hai (Pt5, 2.2%), while the formation of additional hyphae off the initial infection hypha was observed at 12 hai (Pt6, 6.5%). However, a high proportion of germinated urediniospores failed to locate stomata, forming appressoria on the leaf surface and not over stomata. These abnormal structures were seen as early as 3 hai (Pt7, 1.9%), increasing to 6.8% and 10.8% of all Pt urediniospores at 24 and 48 hai, respectively. This abnormal appressorium development was not observed for isolate MDT-MR on the leaf rust-susceptible variety Morocco (Figure 2). While it was not possible to visualize haustoria with the staining method used, no cell death response (i.e., autofluorescence indicative of cell death) was observed to be associated with Pt infection sites on either Toropi or Morocco. These observations further support a potential prehaustorial resistance operating in the wheat variety Toropi.

3.2 | Mapping of RNA-Seq paired-end reads from Toropi inoculated with Pt

After the removal of low-quality and short reads, a total of 1.3 billion paired-end reads remained for analysis (Table S1). Paired-end reads were mapped against the Chinese Spring IWGSC RefSeq v.1.0 reference, with library alignment rates ranging from 84.69% to 90.19%. Reads that did not map to the wheat reference were aligned against the Pt transcriptome reference. An average of

0.53% of the reads aligned with the Pt transcriptome, leaving approximately 10% of reads unmapped. Further analysis of these unmapped reads indicated possible artefacts and contaminant sequences from bacteria present on the wheat leaf surface. No reads from the wheat mock-inoculated libraries mapped to the Pt reference transcriptome.

3.3 | Differential gene expression in Toropi in response to Pt inoculation: Pt-inoculated versus mock-inoculated

Pearson's coefficient of correlation matrix indicated a high level of reproducibility between replicate libraries (Figure S1). *r* values ranged from 0.92 to 0.98, the exception being the 24 hai mock-inoculated libraries, where $r=0.89$ between mock rep 1 and rep 3. Composition bias between libraries was effectively removed by TMM normalization. MD plots between Pt- and mock-inoculated treatments (Figure S2) and between Pt-inoculated time point comparisons (Figure S3) were symmetrical at all time points, indicating that the normalization procedure was effective with respect to library size. However, multidimensional scaling (MDS) plots (Figure S4) indicated that at 0 hai the Pt- and mock-inoculated replicates clustered separately, below and above $\log_2FC=0$, respectively, suggesting that there was already a treatment effect at this time point. Although every effort was taken to ensure that the 0 hai Pt- and mock-inoculated samples were taken immediately after inoculation, it is inevitable that a small difference in time between inoculation and sampling will occur. As wheat genes were subsequently found to have been differentially expressed between the Pt- and mock-inoculated samples at 0 hai, the expression differences at this baseline time point were extracted out of the later time points as a 'difference of difference' adjustment.

Differential gene expression analysis using edgeR and the IWGSC Chinese Spring RefSeq cDNA reference, with statistical parameters set at $\log_2FC>|1|$ and $FDR<0.05$, identified 6737 wheat genes differentially expressed in response to inoculation with Pt compared with the mock-inoculation across the 6 to 24 h time points (Figure 3; Tables S2 and S3). More wheat genes were downregulated (Table S3) than upregulated (Table S2), especially at the early time points (Figure 3; 6 hai, 424 upregulated and 1080 downregulated; 12 hai, 856 upregulated and 1601 downregulated; 24 hai, 1374 upregulated and 1402 downregulated).

GO enrichment analysis (Table S4) indicated a shutting down of RNA and protein synthesis, particularly prevalent from the downregulation of genes involved in ribosome structure and function, and in protein phosphorylation. There was also an early effect on photosynthesis, with disruption of the electron transfer chain. Within the 386 genes downregulated in common, across all three time points, there was an enrichment for genes annotated as binding proteins for a range of substrates involved in RNA, DNA and ribosomal synthesis, and for genes related to photosynthesis. The downregulated genes in common between 6 and 12 hai (70 DEGs) and 6 and 24 hai (143 DEGs) had similar functions to the 386 DEGs downregulated in common across all time points, that is, genes involved in RNA, DNA and ribosomal synthesis, and photosynthesis. Early disruption was also observed for ion and P–P bond transmembrane transporter activity, in particular Zn transport. Ligase activity, in particular carbon–nitrogen ligase activity, was also disrupted at 6 and 24 hai.

There was a general upregulation of genes involved in carbohydrate metabolism and oxidoreductase activity (linked to defence through the generation of reactive oxygen species [ROS]) among the 71 genes upregulated in common across all time points (Table S4). In addition, genes linked to protein/peptide transport and localization, and nitrogen and organic compound transport, were upregulated, indicating the possible need to relocate protein and organic-based resources, either for the benefit of the host or the pathogen. In general, the upregulated DEGs in common between 6 and 12 hai (39 DEGs) and 6 and 24 hai (51 DEGs) showed similar annotations, with increased expression of genes related to biotic and/or abiotic stress, including transcription factors, transferases, protein transporters, kinases, NBS-LRR disease resistance proteins and hormone and senescence-associated proteins. An early induction of genes involved in peptide cleavage, specifically serine-type carboxypeptidase and serine-type endopeptidase inhibitor activity, was observed at 6 hai. At both 12 and 24 hai there was also an increase in the expression of transferases, including hexosyl and acyl transferases.

An analysis of the wheat genes differentially expressed between Pt- and mock-inoculated libraries at 0 hai (Table S5; 212 DEGs upregulated and 564 DEGs downregulated) indicated a rapid change in expression of genes involved in the cellular response to and regulation of active oxygen molecules, along with a downregulation in translation, but an upregulation in the biosynthesis of amino acid, lipid and carbohydrate molecules.

3.4 | Differential gene expression in Toropi in response to Pt inoculation: Pt-inoculated time point comparisons

We further examined differential gene expression in Toropi by comparing Pt-inoculated samples between time points, identifying genes as differentially expressed at a $\log_2FC\geq|1|$ (or $FC\geq2$), $FDR<0.05$ (Figure 3; Table S6). Comparing Pt-inoculated samples taken at 6 and 12 hai showed 909 genes to be significantly upregulated at 6 hai relative to 12 hai, while 919 genes were downregulated. Similarly, 571 genes were upregulated at 6 hai compared with 24 hai and 1286 downregulated. However, when comparing 12 and 24 hai time points there were far fewer genes differentially expressed (9 upregulated and 116 downregulated at 12 hai relative to 24 hai). This would indicate major changes in wheat gene transcription in Toropi at 6 hai, compared with 12 and 24 hai.

GO enrichment analyses (Table S7) identified an upregulation of genes involved in ATP-binding and protein kinase activity at 6 hai, suggesting a rapid metabolic response to Pt inoculation. Seven genes with chitinase activity and five with chitin and 23 with Ca^{2+}

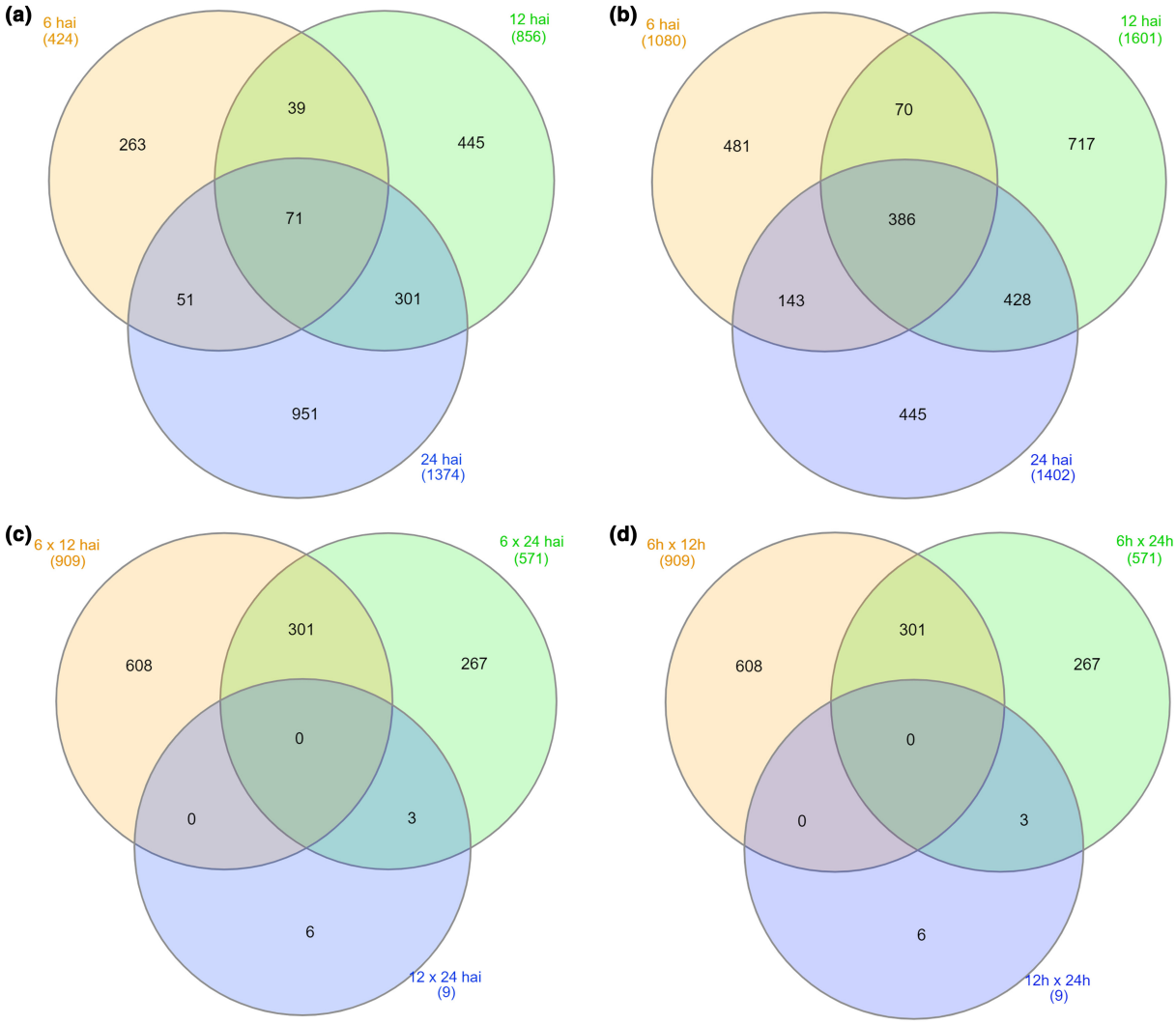

FIGURE 3 Venn diagrams showing the number of genes differentially expressed in the wheat variety Toropi following inoculation with *Puccinia triticina* (Pt). The number of (a) upregulated and (b) downregulated differentially expressed genes (DEGs) in pairwise comparisons between Pt- and mock-inoculated samples at 6, 12 and 24 h after inoculation (hai). (c) Upregulated and (d) downregulated DEGs in pairwise comparisons between Pt-inoculated samples at 6, 12 and 24 hai. [Colour figure can be viewed at wileyonlinelibrary.com]

ion binding properties were also enriched at 6 hai relative to 12 hai, while 13 genes involved in transmembrane transport and five involved in carbohydrate kinase activity were seen to be upregulated at 6 hai relative to 24 hai.

At 6 hai relative to 12 hai there was an enrichment among downregulated genes for lipid (10 DEGs) and RNA binding (19 DEGs), metal ion transmembrane activity (9 and 10 DEGs), aminoacyl-tRNA ligase activity (7 DEGs) linked to protein translation, UDP-glucosyltransferase activity (9 DEGs) linked to a downregulation of glucan biosynthesis and oxidoreductase activity (5 DEGs). At 6 hai relative to 24 hai, there was an enrichment for genes involved in biological processes related to DNA catabolic processes (5 DEGs) and regulation of RNA metabolic processes, including transcription (43 DEGs). Molecular function identified 26 downregulated genes involved in hexosyl group transfer and 19 in acyl group (nonamino acyl) transfer, along with eight DEGs with endonuclease activity.

As very few genes were identified as significantly up or downregulated at 12 hai relative to 24 hai, no GO enrichment groups were returned from the nine upregulated DEGs, while for the 116 DEGs downregulated, no molecular function enrichment could be established. Biological processes did indicate a possible downregulation of organonitrogen metabolic processes (five DEGs).

Looking at the overlap between the comparisons 6 hai versus 12 hai and 6 hai versus 24 hai, there were 301 genes in common that were significantly upregulated at 6 hai relative to both the 12 and 24 h time points. Again, these were related to protein modification, primarily protein phosphorylation, including an enhancement of ATP-binding and protein kinase activity at 6 hai. In addition, five DEGs with carboxypeptidase activity were upregulated at 6 hai. There were 391 DEGs that were downregulated at 6 hai relative to both the 12 and 24 h time points, with GO enrichment analyses suggesting a downregulation of peptidase activities at 6 hai associated with transmembrane function (11 DEGs with

peptidase and 7 DEGs with exopeptidase activity, and 17 DEGs with hydrolase activity).

3.5 | Validation of the differential gene expression seen in Toropi in response to Pt inoculation

Validation of differential gene expression was undertaken in repeat inoculations of Toropi with Pt race MDT-MR. Wheat genes were selected that had shown up- or downregulation in Toropi at 6, 12 and 24 hai. RT-qPCR was used to measure relative gene expression using two constitutively expressed wheat genes, *ubiquitin* and *elongation factor-1a*, for normalization. Of the DEGs selected, successful amplification was obtained for 10 genes. Of these 10 genes, eight confirmed the differential expression seen in the RNA-Seq analyses (Figure 4; Table S8). This included a glutaredoxin family protein upregulated at 6 hai, a sugar transporter upregulated at 12 hai and an oligopeptide and an amino acid transporter, as well as a putative kinase, upregulated at 24 hai.

3.6 | Alignment of DEGs with QTLs for leaf rust resistance in Toropi

Four genetic regions that contribute to the leaf rust APR in Toropi have been identified (Kolmer et al., 2018; Rosa et al., 2019). These include a QTL on the long arm of chromosome 1B located in the region of the APR *Lr46*, *Lr78* on the short arm of 5D and QTLs on 5AL and 3BS. Analysis of the genomic location of the DEGs was carried out (Table S9). The QTL on chromosome 1BL spanned an 8.4 cM region and contained 12 DEGs, three of which were upregulated. One of these upregulated genes was of potential interest, being annotated as a sugar transporter. On 3BS the leaf rust resistance QTL spanned a 13.7 cM region and contained 47 DEGs, of which 18 were upregulated. Of these upregulated genes, one was annotated as a receptor-like kinase, while another receptor-like kinase and a regulatory NPR1 protein were downregulated. The QTL region on 5AL (16.2 cM) contained 20 DEGs, 11 being upregulated, and the QTL region on 5DS (2.4 cM) contained 60 DEGs, of which 24 were upregulated. Within the QTL region on 5AL, a gene annotated as a seven-transmembrane MLO family protein was upregulated, while an NBS-LRR class of disease resistance gene was downregulated in the QTL region on 5DS.

3.7 | Expression of Pt transcripts during the first 24 h after inoculation

Using EBSeq–HMM, Pt genes with PP ≥ 0.6 (at FDR < 0.05) were considered as significantly expressed. This analysis identified 60 Pt genes as expressed during the first 24 h following inoculation (Table S10). Cluster analysis, based on common expression patterns, divided the 60 Pt genes into seven groups (Figure S5a–g). The levels of two Pt genes increased over the time course (Figure S5a). Annotations indicated that Pt gene PTTG_06795T0 resembled a DID3-like exonuclease 2, while PTTG_00715T0 may be a member of a solute carrier family 25, having transmembrane transporter activity. The gradual and consistent increase in these transcripts up to 24 hai would coincide with the formation of haustoria, the fungal feeding structures formed within plant cells.

Pt genes that were already present at 0 hai included PTTG_00864T0, PTTG_05501T0, PTTG_06512T0, PTTG_09867T0 (Figure S5c) and PTTG_10990T0, PTTG_07540T0, PTTG_02371T0 and PTTG_09215 (Figure S5d). Annotations for four of these Pt genes indicated a reverse transcriptase with a Tyl/copia-type domain, an isocitrate lyase, a UTP-glucose 1-phosphate uridylytransferase, and

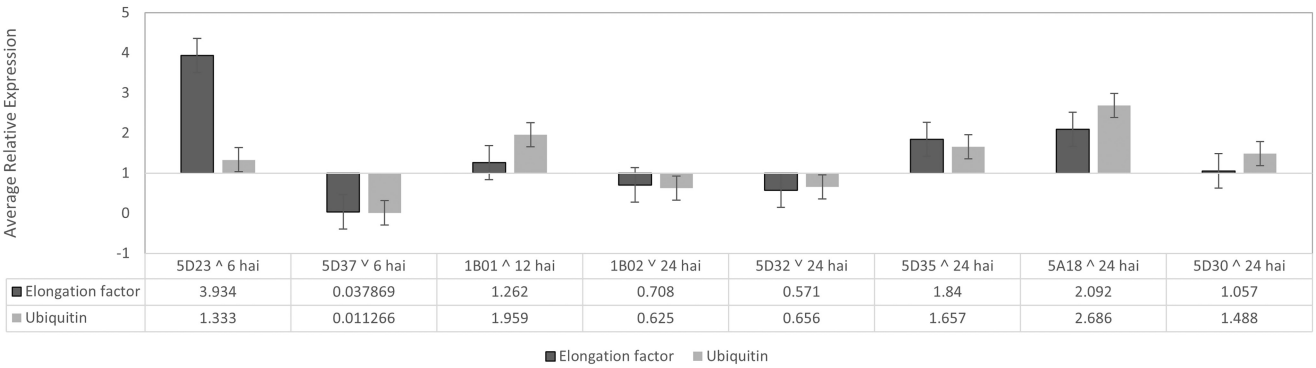

FIGURE 4 Validation of differential gene expression of selected differentially expressed genes (DEGs) from RNA-Seq analysis using reverse transcription-quantitative PCR. Gene expression was assessed in flag leaves of cv. Toropi inoculated with *Puccinia triticina* isolate MDT-MR in repeat inoculation experiments. 5D23, annotated as a glutaredoxin family protein, was upregulated at 6 h after inoculation (hai); 5D37, annotated as nodulin-like/major facilitator superfamily protein, was downregulated at 6 hai; 1B01, annotated as a sugar transporter, was upregulated at 12 hai; 1B02, annotated as RADIALIS-like transcription factor, was downregulated at 24 hai; 5D32, annotated as a response regulator, was downregulated at 24 hai; 5D35, annotated as a putative kinase, was upregulated at 24 hai; 5A18, annotated as an oligopeptide transporter, was upregulated at 24 hai; and 5D30, annotated as an amino acid transporter, was upregulated at 24 hai. Bars indicate standard errors.

a J-domain-containing protein. The remaining four Pt genes could not be functionally characterized.

Six Pt transcripts were identified as candidate effector molecules using EffectorP, with effector probabilities ranging from 0.570 to 0.914 (Figure 5, Table S11). EffectorP v. 3.0 defined five of these Pt transcripts as apoplastic effectors and the sixth as a cytoplasmic effector, with probabilities ranging from 0.734 to 0.981 (Table S10). EffectorP v. 3.0 went on to predict an effector function for an additional five of the Pt transcripts (three as cytoplasmic effectors and two as apoplastic) with probabilities from 0.782 to 0.828 (Table S10). Characterization of these candidate effectors using Localizer indicated a high probability ($p > 0.94$) of a chloroplast transit peptide in four of the effectors identified by EffectorP, PTTG_06512T0 (bp 9–37), PTTG_07921T0 (bp 19–59), PTTG_08794T0 (bp 53–81) and PTTG_07924T0 (bp 45–75) (Table S10). The remaining two candidate effectors showed no cellular localization signals. Of the additional five candidate effectors detected by EffectorP v. 3.0, three had nuclear domains and two had no domain, as defined by Localizer

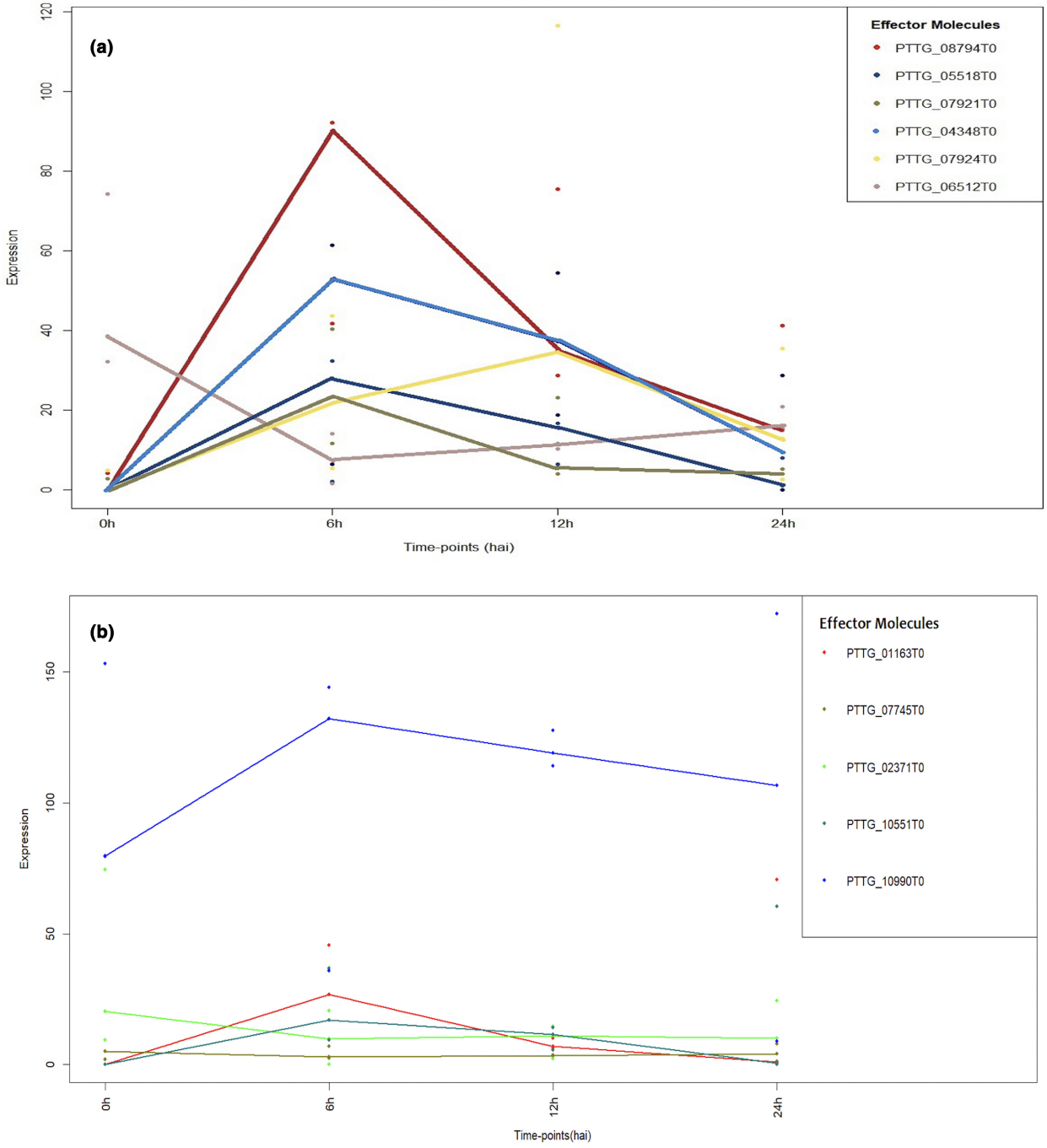

FIGURE 5 *Puccinia triticina* (Pt) genes annotated as candidate effector proteins. Following inoculation of the wheat cv. Toropi with the Pt isolate race MDT-MR (a) six Pt genes were identified as potential effectors using EffectorP. (b) An additional five genes were identified as potential effectors using EffectorP v. 3.0 (http://effectorp.csiro.au/). [Colour figure can be viewed at wileyonlinelibrary.com]

(Table S10). InterProScan and GO annotation analyses did not identify any conserved domains that would indicate a potential function for these candidate effectors.

Transcripts of the candidate effectors PTTG_06512T0, PTTG_10990T0 and PTTG_02371T0 (Figures 5 and S5c,d) were already present in Pt-inoculated libraries at 0 hai, with the levels of PTTG_06512T0 and PTTG_02371T0 falling at 6 hai. Candidate effectors PTTG_04348T0, PTTG_05518T0, PTTG_07921T0, PTTG_08794T0, PTTG_01163T0 and PTTG_10551T0 were all at near-zero levels at 0 hai, reaching maximum transcript levels at 6 hai (Figures 5 and S5e1,e2). PTTG_07924T0 also increased from near-zero levels at 0 hai, rising at 6 hai, but reaching maximum levels at 12 hai (Figures 5 and S5f1). Transcripts of PTTG_07745T0 remained at similar levels across the time points.

4 | DISCUSSION

Toropi is a wheat variety of considerable interest to pathologist and breeders. Resistance has been identified in Toropi not only to leaf rust, but also to stripe (Rosa et al., 2019) and stem rust, Fusarium head blight and powdery mildew (authors' unpublished data). While seedlings are susceptible to most isolates of Pt, Toropi has maintained field resistance since its commercial release in 1965 (Barcellos et al., 2000; Kolmer et al., 2018; Rosa et al., 2019). A microscopic study of Pt infection in Toropi indicated a prehaustorial resistance response, showing a significant reduction in the development of appressoria and substomatal vesicles compared with susceptible varieties (Wesp-Guterres et al., 2013). In this study, the Pt isolate race MDT-MR also exhibited a high level of failed infection attempts, with appressoria failing to form over stomata. Preliminary expression studies of selected wheat genes indicated transcription of defence-related genes, including two peroxidase genes and a β-1,3-glucanase, within 1 h following Pt inoculation (Casassola et al., 2015). Based on these findings we selected time points up to 24 hai to help unravel the biology underlying the unique leaf rust APR in Toropi, with RNA-Seq libraries being made from RNA taken from the primary flag leaves inoculated with the Pt race MDT-MR at 0, 6, 12 and 24 hai.

Changes in gene transcription were seen in Toropi at 6 hai, well before the formation of haustoria. The predicted functions of many genes upregulated at 6 hai were related to biological processes associated with early, PTI plant responses to pathogen attack (Couto & Zipfel, 2016). These included protein phosphorylation as indicated by the upregulation of genes with kinase activity and ATP-binding properties. Protein kinases play vital roles in plant defence during the early stages of pathogen infection. In *Arabidopsis*, the PAMP recognition receptors (PRR) FLS and EFR are both LRR-RLKs, while the chitin PRR encodes a lysin-motif receptor kinase (Couto & Zipfel, 2016). In addition, an increase in expression of proteins with Ca^{2+}-binding properties indicated activation of Ca^{2+} ion fluxes, while an increase in expression of genes with oxidoreductase activity, including peroxidases, indicated a rapid ROS reaction. These changes in gene expression would suggest that Toropi exhibits an early and strong PTI response following inoculation with Pt.

The upregulation of genes linked to peptide and amino acid transport and localization, mineral, nitrogen and organic compound transport, transmembrane transport and carbohydrate metabolism all indicate the need to relocate the plant's resources, either for the benefit of the host or the pathogen. The nonrace-specific rust resistance genes *Lr34* and *Lr67* encode an ATP-binding cassette (ABC) transporter (Krattinger et al., 2009) and a hexose transporter (Moore et al., 2015), respectively, highlighting the importance of transporters in wheat–rust interactions. In this study, ABC transporters were found to be upregulated at all time points, while a hexose transporter was upregulated at 6 hai. ABC transporters were also found that were downregulated, specifically at 6 and 24 hai, but the vast majority of downregulated transporters, at all time points, were zinc transporters.

Lignification of the plant cell wall has been shown to be a significant defence mechanism in wheat to the yellow rust pathogen *Puccinia striiformis* f. sp. *tritici*, the deposition of lignin provides a physical barrier against infection (Moldenhauer et al., 2008). Lignin is a phenolic-based polymer found covalently linked to cellulose and hemicellulose, having a pivotal role in cell wall structural integrity. In this study, 28 genes annotated as methyltransferases were upregulated in Toropi at 12 and 24 hai, including caffeic acid 3-O-methyltransferase and 5 hydroxyferulic acid methyltransferase, which are responsible for the methylation of lignin precursors (Ma & Xu, 2008). Therefore, lignification would also appear to be an important defence response in wheat towards the leaf rust pathogen.

Of the many transcription factors (TFs) differentially expressed in Toropi in response to Pt inoculation, NAC, basic helix–loop–helix (bHLH) and ethylene-responsive TFs were the most abundant. TFs have been implicated in many aspects of plant immunity, regulating gene expression during PTI and ETI, hormone signalling pathways and phytoalexin synthesis (Couto & Zipfel, 2016). NAC TFs have been shown to be regulated by microRNAs (miRNAs) through the Dicer-mediated RNA-induced silencing complex (RISC). In *Arabidopsis*, NAC TFs involved in plant cell death were found to be regulated by miR-164 (Kim et al., 2009). miR-164 has been associated with the leaf rust APR gene *Lr46*, levels of this miRNA being higher in wheat lines carrying *Lr46* compared with non-*Lr46* lines (Tomkowiak et al., 2020).

More wheat genes were downregulated than upregulated, especially at the early time points. Most notable were genes associated with photosynthesis, including photosystem I assembly proteins, photosystem II reaction centre proteins and cytochromes, including cytochrome b6. Many studies have shown that photosynthesis is disrupted in plants following infection, including infection with leaf and stem rust, and powdery mildew in wheat (Poretti et al., 2021). A haustorium-specific protein (Pst_12806) has been identified in *P. striiformis* f. sp. *tritici* that is translocated into chloroplasts, affecting chloroplast function and photosynthesis (Xu et al., 2019). Pst_12806 was shown to interact with the C-terminal Rieske domain of the wheat TaISP protein (a putative component of the cytochrome b6-f

complex), reducing the rate of electron transport and the production of chloroplast-derived ROS, thereby impairing photosynthesis, but also suppressing the host plant cell death response.

Many wheat genes involved in protein synthesis, including structural ribosome proteins, were also downregulated in Toropi following inoculation with Pt. This may represent a plant defence mechanism aimed at preventing the pathogen from reprogramming the host's cell metabolism in favour of the pathogen. In the barley powdery mildew pathogen, *Blumeria graminis* f. sp. *tritici*, the effector CSEP0064/BEC1054 has been shown to inhibit the degradation of ribosomal RNA induced by ribosome-inactivating proteins as a mechanism to prevent cell death and promote susceptibility (Pennington et al., 2019).

Alignment of the wheat DEGs with the four genetic regions that contribute to the leaf rust APR in Toropi (Rosa et al., 2019) identified potential candidate genes for each resistance QTL. These included a predicted sugar transporter in the *QLr.crc-1BL* region, near to the leaf rust APR gene *Lr46*. While the gene encoding *Lr46* has not yet been isolated, a similar leaf rust APR gene, *Lr67*, was found to encode a hexose transporter (Moore et al., 2015). This differentially expressed sugar transporter therefore represents a good candidate for the 1BL QTL.

Within the QTL region on the long arm of chromosome 5A, a gene annotated as a seven-transmembrane MLO family protein was upregulated. Although mutants within the *MLO* gene are widely known to confer resistance in cereals to powdery mildews, recent research has revealed a much broader role for MLO in plant sensing of external physical signals (Jacott et al., 2021).

Upregulation of a gene annotated as a receptor-like kinase within the QTL region on the chromosome arm 3BS supports a plant defence reaction. However, the downregulation of another receptor-like kinase and a NPR1-like protein, a regulator of pathogenesis-related (PR) gene expression, mediated through the salicylic acid signalling pathway (Glazebrook et al., 1996), may indicate a resistance mechanism not centred around R-gene resistance. That said, differential expression of PR genes was observed. A number of peroxidases were upregulated, primarily at 6 hai, in line with previous studies (Casassola et al., 2015), along with seven genes with chitinase activity and five with chitin-binding properties.

In addition to the wheat genes differentially expressed in response to Pt infection, we were able to identify 60 Pt genes expressed in the infected leaf tissue. Of the 60 Pt genes identified, 24 were expressed at high levels at 6 hai, well before the formation of haustoria. Eleven Pt genes annotated as candidate effectors, defined as small, cysteine- and serine-rich proteins, with signal peptides that enable delivery into plant cells and organelles (Figueroa et al., 2021). While the candidate effectors did not resemble any published fungal effectors, four did contain predicted chloroplast transit peptides. Three were present at high levels at 0 hai, while six were highly expressed at 6 hai, supporting a potential effector role in the Toropi–Pt interaction.

Breeding for leaf rust resistance has relied heavily on race-specific resistance genes, which have often not outlasted the commercial life of the wheat variety. More durable sources of resistance, such as *Lr34* and *Lr67*, are required. The leaf rust APR in Toropi has demonstrated longevity and could therefore provide a valuable source of leaf rust resistance. It had previously been demonstrated that Toropi confers a prehaustorial resistance. Here, we demonstrate that germinated urediniospores of the Pt race MDT-MR exhibit a reduced ability to locate and enter stomata, and that there is no hypersensitive, cell death phenotype associated with the early infection sites. Along with a rapid and strong differential gene expression response, these observations point to a leaf rust APR in Toropi that is more akin to a PTI rather than ETI response. The DEGs located within the four leaf rust resistance regions reported in Toropi (Rosa et al., 2019) represent potential candidate genes for each QTL. These candidate genes could provide useful targets for marker development, providing breeders with tools for the selection of the Toropi resistance during breeding. The identification of 11 candidate Pt effectors genes also provides additional resources to help unravel the biology of the unique leaf rust APR in Toropi.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available at ArrayExpress (https://www.ebi.ac.uk/biostudies/arrayexpress), accession number E-MTAB-9791 (https://www.ebi.ac.uk/fg/annotare/edit/11265/).

ORCID

Lesley A. Boyd https://orcid.org/0000-0001-6420-0990

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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