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Changes in gene expression profiles as they relate to the adult plant leaf rust resistance in the wheat cv. Toropi

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ABSTRACT

Leaf rust, caused by the foliar pathogen *Puccinia triticina* is a major disease of wheat in the southern region of Brazil and invariably impacts on production, being responsible for high yield losses. The Brazilian wheat cultivar Toropi has proven, durable adult plant resistance (APR) to leaf rust, which uniquely shows a pre-haustorial resistance phenotype. In this study we aimed to understand the interaction between *P. triticina* and the pre-haustorial APR in Toropi by quantitatively evaluating the temporal transcription profiles of selected genes known to be related to infection and defense in wheat. The expression profiles of 15 selected genes varied over time, grouping into six expression profile groups. The expression profiles indicated the induction of classical defence pathways in response to pathogen development, but also the potential modification of Toropi's cellular status for the benefit of the pathogen. Classical defence genes, including peroxidases, β-1,3-glucanases and an endochitinase were expressed both early (pre-haustorial) and late (post-haustorial) over the 72 h infection time course, while induction of transcription of other infection-related genes with a potential role in defence, although variable was maintained through-out. These genes directly or indirectly had a role in plant lignification, oxidative stress, the regulation of energy supply, water and lipid transport, and cell cycle regulation. The early induction of transcription of defence-related genes supports the pre-haustorial resistance phenotype in Toropi, providing a valuable source of genes controlling leaf rust resistance for wheat breeding.
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Introduction

In 2012/2013 Brazilian wheat production reached 5.5 million tons, of which 94.5% was produced in the southern region of the country [1]. Leaf rust is a major constraint to wheat production in this area, causing yield losses of up to 80% [2,3]. Chemical control of leaf rust in Brazil costs around US$ 30/ha and generally requires two sprays per crop [4]. In South America lost wheat production due to leaf rust is estimated to cost the industry 172 million dollars per year.

Breeding for wheat leaf rust resistance is complicated by the high level of genetic variation within the Brazilian *Puccinia triticina* population, exemplified by the pathogen's ability to rapidly overcome major resistance genes, resulting in new races [5].

Abbreviations: APR, adult plant resistance; qPCR, quantitative PCR; hai, hours after inoculation; PR, pathogenesis-related; MIP, major intrinsic proteins; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; G6DPH, glucose-6-phosphate dehydrogenase; ZIP5, putative zinc transporter; COMT1, caffeic acid O-methyltransferase; HSP80, heat shock protein 80; PRA2, class III peroxidase; LTP, type 1 non-specific lipid transfer protein precursor; WCAB, chlorophyll a/b-binding protein WCAB precursor; AQP1, aquaporin; FREX, fructan exohydrolase; RBR1, retinoblastoma related protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PAL, phenylalanine ammonia-lyase; LHC, light-harvesting complex; PTI, PAMP-Triggered-Immunity; ETI, Effector-Triggered-Immunity.

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Therefore, sources of durable APR, such as that found in the Brazilian cv. Toropi, have considerable value for Brazilian wheat breeding.

The bread wheat cv. Toropi (*Triticum aestivum* L.) was released as a commercial cultivar in 1965 with a partial level of APR to leaf rust (Fig. 1) which still remains effective despite 50 years of cultivation [6]. The APR in Toropi is a valuable "slow-rusting" type of resistance, being effective, while producing little or no selection pressure on the pathogen [7,8]. The APR in Toropi also displays a unique, pre-haustorial phenotype, restricting the formation of the primary infection structures; stomatal appressoria and sub-stomatal vesicles [9].

Although Toropi is derived from the cultivar Frontana there is no evidence that it contains the well characterized slow-rusting leaf rust APR gene *Lr34* [10]. Two recessive genes have been reported to be associated with the leaf rust APR in Toropi, temporary designated *Trp*-1 and *Trp*-2 [10]. *Trp*-1 and *Trp*-2 were located to chromosomes 1A and 4D, respectively [11]. In addition to leaf rust APR Toropi has a number of other agronomically important characteristics, including increased phosphorous absorption, translocation and distribution, tolerance to aluminum toxicity [12] and resistance to Fusarium head blight [13].

To identify the resistance mechanisms, genes and genetic pathways underlying the slow-rusting leaf rust APR in Toropi transcriptomics analyses were undertaken, looking at differential gene expression in Toropi flag leaf tissue at defined time points after *P. triticina* inoculation. Leaf tissue was sampled at eight time points after inoculation, including time points that represented the early stages of the pathogen's development and the pre-haustorial resistance in Toropi. The transcript profiles of 15 selected genes, previously identified as having a role in the cellular interactions between wheat, and both adapted and non-adapted isolates of the foliar pathogens causing leaf rust, powdery mildew and wheat blast (L. Boyd, unpublished data), were measured over the eight time points by quantitative PCR (qPCR). The putative function of these wheat infection-related genes in the colonization of wheat tissues by *P. triticina* and the pre-haustorial, leaf rust APR in Toropi is discussed.

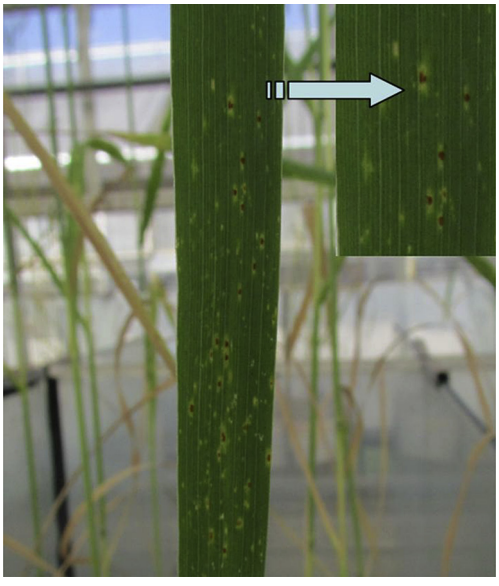

Fig. 1. Leaf rust phenotype on the wheat cv. Toropi. The adult plant leaf rust resistance in Toropi is characterized by a mixture of small, off-white to yellow flecks characteristic of necrotic and chlorotic plant reactions, and by the occasional leaf rust pustule. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Material and methods

Wheat-P. triticina inoculations and sampling

The wheat cv. Toropi was grown at 14 h light/10 h dark and 80% humidity until full expansion of the first flag leaf. Flag leaves were inoculated with urediniospores of *P. triticina*, race MDT-MR – Lr virulence: *Lr1*, *Lr3*, *Lr3ka*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr17*, *Lr20*, *Lr23*, *Lr24*, *Lr26*, *Lr30* [14] – in a mineral oil suspension. Mock inoculations were carried out using the same mineral oil without urediniospores. After inoculation plants were maintained at 80% humidity, in total darkness for 24 h, before being returned to normal growing conditions. Leaf rust infection was verified 15 days post inoculation (Fig. 1).

Twelve *P. triticina* inoculated flag leaves and six mock-inoculated flag leaves were collected from individual plants at each of the following time points; 0, 1, 3, 6, 12, 24, 48 and 72 h after inoculation (hai). Total RNA was extracted from all flag leaf samples using RNeasy Plant Mini Kit (Qiagen) and DNA removed using TURBO DNA-free™ Kit (Ambion), according to the manufacturer's protocols.

Selection of candidate infection-related genes and quantitative PCR analysis

Wheat genes were selected for analysis from a global wheat transcriptomics study involving inoculation with adapted and nonadapted isolates of the fungal pathogens *Blumeria graminis*, *Magnaporthe grisea*/*oryzae*, and *P. triticina*/*hordei* (L Boyd; unpublished data). Differentially expressed probe sets were selected from the Agilent wheat microarray (http://www.genomics.agilent.com) that represented unique wheat unigenes. Ten wheat genes (Table 1) were selected that showed differential transcript profiles across 4 time points (12, 24, 36 and 48 hai) following inoculation with *P. triticina* and/or *P. hordei* (data not shown).

Primers were designed for qPCR using Primer3Plus. Toropi RNA samples from each time point were converted to cDNA using the SuperScript™ III First-Strand Synthesis System (InvitrogenTM) according to the manufacturer's protocol. PCR amplification levels were normalized using geNorm (geNorm program v3.5) and three reference genes, ubiquitin [15], GAPDH and elongation factor-1α [16]. GeNorm calculates the normalization factor based on multiple control genes, resulting in more accurate and reliable normalization of gene expression data than is normally obtained using a single gene for normalization [17].

All qPCR 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 was checked using the standard curve method [16,18]. Primers with slopes between −3.1 and −3.6, and reaction efficiencies between 90 and 110% were selected for the analysis (Table 1). Primers previously designed by Tufan et al. [18] for standard defence-related genes were also assessed (Table 2). The qPCR for each gene, on each of 3 biological replicates, was repeated 3 times.

Transcript levels of all 15 infection-related wheat genes in *P. triticina* inoculated flag leaf tissue were compared to the levels of transcript in the mock-inoculated control samples, at each time point, providing relative transcript levels for each gene. Three biological replicates were analyzed at each time point. The expression value for each biological replicate was an average of the normalized technical replicates. Relative expression values of the three inoculated biological replicates, at each time point were obtained by dividing each biological replicate by the average of the mock expression values at each time point.

Table 1
Agilent probe sets selected for qPCR.

Probe	Gene annotation	Abbreviation	Sequence (forward and reverse)	GenBank accession no.
A_99_P156537	glucose-6-phosphate dehydrogenase	G6DPH	TCGTGTGCAGTTCAAGGATG CATGTACATGGCTTCTGATGGC	AB029454
A_99_P136820	putative zinc transporter ZIP 5	ZIP5	AGTTGGGTATTGTGGTGCAC ACATCTGGTGGAAGCTCAAGG	AK331366
A_99_P589522	caffeic acid O-methyltransferase	COMT1	ACGTCGACATGATCATGCTC ACTCGATGGCAAATGCGTTG	AY226581
A_99_P238786	heat shock protein 80	HSP80	TGATTGGCCAGTTTGGTGTC TGTGCTTGCTGGTCACAATG	U55859
A_99_P421267	class III peroxidase	PRA2	AACATCAACACTGCCTTCGC AGGTTGGTGTAGTAGGCGTTG	AY506496
A_99_P446157	type 1 non-specific lipid transfer protein precursor	LTP	TGCCATCGTTGTTGCTATCG TGCGTGTATGTGACCTCAAC	TC400994
A_99_P215566	chlorophyll a/b-binding protein WCAB precursor	WCAB	TTGTCCAAGCTATCGTCACG ACAAAGTTGGTGGCGAATGC	TC382127
A_99_P624287	aquaporin	AQP1	TGGTCAGACCACTGGATCTTC TGGCATCTTCTTTGCAGCAG	DQ867075
A_99_P112790	fructan exohydrolase	FREX	TTGACACCGAGAAGCATTGC TGCACAACAGTTTGCTCCTC	AB089271
A_99_P105865	retinoblastoma related protein 1	RBR1	TACCGTCAAGCCTTTGTTGG TGCATCGCCACCACTTTTTG	AY941772

Relative gene transcript levels were analyzed using ANOVA to identify significant differences between the expression levels of the 15 genes. The Tukey's test was used to determine which time points were significantly different, using the software InfoStat, version 2012 [19]. The relative abundance of gene transcripts at each time point was compared as a proportion of the total transcript abundance across all time points. This provides a time course transcript profile for each infection-related gene allowing genes with similar profiles to be placed in to expression groups.

Results and discussion

P. triticina enters wheat leaf tissues via stomata. Urediniospores germinate on the leaf surface forming an appressorial swelling above a stomatal opening within 3 hai. An appressorial hyphae grows through the stomatal opening, into the sub-stomatal cavity, where a sub-stomatal vesicle forms by 6 hai [20]. Infection hyphae develop from the sub-stomatal vesicle and contact with plant mesophyll cells results in the differentiation of haustorial mother cells around 12 hai, leading to the formation of haustorial feeding structures within living plant cells around 24 hai [20,21]. Wesp-Guterres et al. [9] demonstrated that in Toropi a large proportion of infection attempts were stopped before the formation of haustoria, i.e. pre-haustorial resistance. Significantly fewer appressoria developed on Toropi compared to the susceptible wheat genotype, with a significantly smaller number of these appressoria going on to form sub-stomatal vesicles, infection hyphae and haustorial mother cells. Plant cell death, measured by trypan blue staining, was associated with up to 37% of attempted infection sites in Toropi, but not until much later in the infection process (120 hai), after *P. triticina* ingress had been halted [9].

Transcript analysis of ten selected infection-related genes (Table 1), plus 5 common defence-related genes (Table 2) in Toropi at 8 time points after inoculation with *P. triticina* showed that the genes broadly grouped into six expression profiles (Fig. 2).

Profile 1 included a glucose-6-phosphate dehydrogenase (G6DPH), a putative zinc transporter (ZIP5), a heat shock protein 80 (HSP80), a fructan exohydrolase (FREX), a retinoblastoma related protein 1 (RBR1) and PR10 (Fig. 2). These six genes exhibited fairly constant expression throughout the 72 h time course, with no significant differences being found across time points (Table 3; Supp. Fig. 1). Although FREX appeared to exhibit slightly higher levels of gene transcription at 12 hai (Fig. 2), this was not significant (Table 3). The plant Lipid Transfer Protein (LTP) and caffeic acid O-methyltransferase (COMT1) genes were identified as significantly different by the ANOVA analysis, so were placed in separate groups, Profile groups 2 and 3, respectively (Fig. 2). LTP peaked at 1 and 12 hai and COMT1 peaked at 24 hai (Table 3, Fig. 2).

While expression of these eight genes (Profiles 1, 2 and 3) occurred throughout the *P. triticina* infection time course, these genes may still play an important role in plant defence (Supp. Fig. 1). The well characterized pathogenesis-related gene PR10 encodes a phenylalanine ammonia-lyase (PAL), the first enzyme in the phenyl propanoid pathway, being required for the biosynthesis of flavonoids, phenyl propanoids and lignin. LTPs transport lipids across membranes and have been implemented in plant defence, having antibiotic properties [22] and through the creation of mechanical barriers such as cutin [23]. Moreover, as pathogen infection damages the cell wall and associated membrane, lipid transport would be required for tissue repair. COMT1 catalyzes key steps in the biosynthesis of monolignols, precursors of plant lignin. Two distinct methyltransferases are responsible for the methylation of lignin precursors: caffeic acid 3-O-methyltransferase and 5 hydroxyferulic acid. Lignin is a phenolic cell wall polymer covalently linked to the cellulose and hemicellulose components of the plant cell wall, and has been shown to assist in the transport of

Table 2
Primer sequences used for qPCR of common defense-related genes.

Target	Gene annotation	Forward primer	Reverse primer
PR1	β-1,3-glucanase	CAATAACCTCGGCGTCTTCATCAC	TTATTTACTCGCTCGGTCCCTCTG
PR2	β-1,3-glucanase	AAGCACTTTGGGCTGTTCAATCCG	CCAGGCAGCTTATTCGAACGCAAA
PR4	Endochitinase	AAGTGCCTCCAGGTGACGAA	TGCACTGGTCGACGATCCT
PR9	Peroxidase	CAAGGTGAACTCGTGATGGA	TTGAGGATTCAACCGTCGTT
PR10	Phenylalanine ammonia-lyase	CAAGATGGTCGAGGCTTACC	CGAAGTCGATCATGAAGCAA

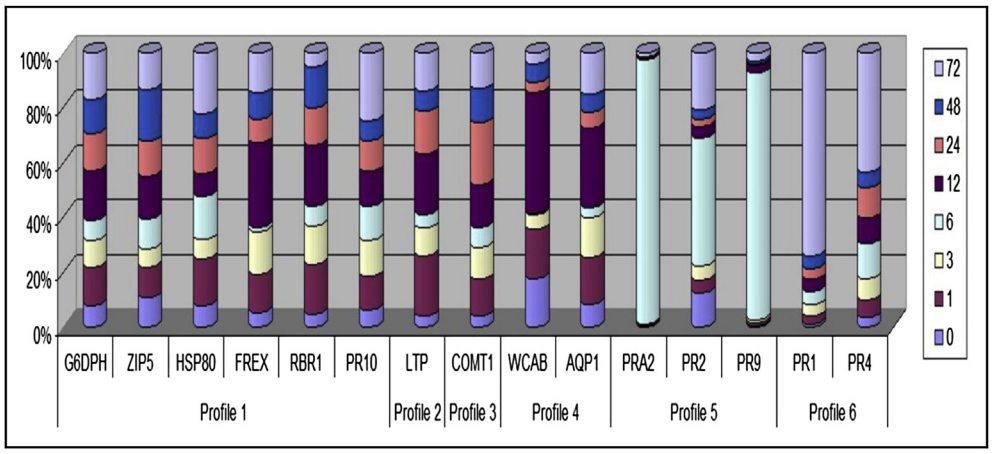

Fig. 2. Gene expression profiles in the wheat cv. Toropi in response to *Puccinia triticina* infection. Each bar represents one gene and the colors the relative transcript levels of that gene at each time point. Time points are hours after inoculation (hai). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

water and nutrients [24], as well as having a significant role in plant defence [25]. Lignification has been shown to be a significant defence mechanism in wheat to the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* [26], so may have a similar role in leaf rust resistance.

G6DPH helps maintain the levels of the co-enzyme NADPH, an important reducing agent that helps protect cells against oxidative damage through the maintenance of glutathione levels. ZIP5 is involved in zinc transport, zinc-superoxide dismutase being important in antioxidant defence [27–29]. Heat shock proteins, such as HSP80 function as molecular chaperones and play a critical role in protein folding and intracellular trafficking of proteins, in particular under heat and other stresses. FREX may release energy required to combat pathogen infection by removing terminal fructose molecules from fructans [15,30]. RBR1 controls the cell cycle transition from G1 to S and an increased expression of this protein in response to stress indicated that plants regulate the cytoskeleton, cell division and cell wall extension to survive [31].

Alternatively these genes could be up-regulated by *P. triticina* to aid the pathogen ingress and development, providing a preferred carbohydrate source in the release of fructose, a supply of reducing energy in the form of NADPH, and zinc for fungal growth and metabolism. While the pathogen may recruit plant LTPs for the production of fungal membranes associated with fungal structures such as haustoria, maintaining the nutrient supply to the fungus [32], or manipulate plant cell division for its own ends.

AQP1 and WCAB peaked at 12 hai (Fig. 2 – Profile 4; Supp. Fig. 1). Aquaporins (AQP1) are integral membrane proteins, part of a larger family of major intrinsic proteins (MIP) which form pores in cell membranes. The plasma membranes of both animal and plant cells contain aquaporin pores through which water can flow more rapidly than by diffusion through the phospholipid bilayer. Aquaporins selectively conduct water molecules in and out of the cell, while preventing the passage of ions and other solutes. *P. triticina* effectors may stimulate AQP1 expression to increase aquaporin pore formation, thereby allowing a greater flow of water within the plant cell in readiness for haustorial development. On the other hand, some isoforms of aquaporins are known to facilitate H_2O_2 transport across the cell [33], having a role in tolerance to abiotic stresses such as drought, salt and cold [34,35]. AQP1 may therefore have an indirect role in fungal defence, as H_2O_2 serves as a signal molecule for induction of pathogenesis-related (PR) proteins leading to the accumulation of phenyl propanoid compounds and ROS detoxifying enzymes [36,37].

WCAB is part of the photosystem II complex in chloroplast. The light-harvesting complex (LHC) in plants is formed by chlorophylls A and B, and these chlorophyll A–B binding proteins, being involved with adhesion of granal membranes and photo-regulation through reversible phosphorylation of its threonine residues. Molecular oxygen is released as a by-product of the energy conversion process, and this could be used as a precursor for ROS production [38].

PRA2, PR2 and PR9 belong to Profile 5 (Fig. 2; Supp. Fig. 1). PRA2, PR2 and PR9 transcript levels peaked at 6 hai, at which time sub-stomatal vesicles are generally visible in Toropi. The expression profiles of these genes were similar (Table 3), suggesting a complementary role in the Toropi-*P. triticina* interaction. Peroxidases (PRA2 and PR9) are a broad group of enzymes that catalyze the reduction of peroxides, being divided into three classes based on sequence comparisons. Class I peroxidases are intracellular enzymes. Class II and III contain the secretory fungal and plant peroxidases, respectively. Plant peroxidases are involved in many physiological and developmental processes, from germination to senescence, having roles in plant cell wall formation and lignification, and the production of ROS [39]. NCBI defines PRA2 (gene

Table 3
Expression levels of all 15 selected genes analyzed with ANOVA and Tukey's test.

Time points	Profile 1						Profile 2	Profile 3
	G6DPH	ZIP5	HSP80	FREX	RBR1	PR10	LTP	COMT1
0 hpi	0.45^{a}	0.60^{a}	0.52^{a}	0.46^{a}	0.31^{a}	0.45^{a}	0.26^{a}	0.18^{a}
1 hpi	0.77^{a}	0.61^{a}	1.13^{a}	1.19^{a}	1.19^{a}	0.86^{a}	1.47^{b}	0.60^{ab}
3 hpi	0.55^{a}	0.39^{a}	0.47^{a}	1.32^{a}	0.91^{a}	0.94^{a}	0.68^{ab}	0.52^{ab}
6 hpi	0.42^{a}	0.59^{a}	1.04^{a}	0.15^{a}	0.47^{a}	0.87^{a}	0.35^{a}	0.32^{ab}
12 hpi	1.00^{a}	0.87^{a}	0.55^{a}	2.64^{a}	1.44^{a}	0.91^{a}	1.47^{b}	0.70^{ab}
24 hpi	0.76^{a}	0.71^{a}	0.86^{a}	0.74^{a}	0.89^{a}	0.81^{a}	1.04^{ab}	1.00^{b}
48 hpi	0.69^{a}	1.06^{a}	0.59^{a}	0.82^{a}	0.99^{a}	0.50^{a}	0.51^{a}	0.56^{ab}
72 hpi	0.95^{a}	0.72^{a}	1.47^{a}	1.24^{a}	0.32^{a}	1.74^{a}	0.91^{ab}	0.57^{ab}

Time points	Profile 4		Profile 5			Profile 6	
	WCAB	AQP1	PRA2	PR2	PR9	PR1	PR4
0 hpi	2.73^{ab}	0.85^{a}	0.78^{a}	2.33^{a}	0.33^{a}	0.15^{a}	0.39^{a}
1 hpi	2.78^{ab}	1.66^{ab}	0.62^{a}	0.84^{a}	0.74^{a}	0.39^{a}	0.75^{a}
3 hpi	0.85^{a}	1.44^{ab}	0.39^{a}	0.98^{a}	0.69^{a}	0.56^{a}	0.91^{a}
6 hpi	0.05^{a}	0.37^{a}	146.47^{b}	8.72^{b}	57.35^{b}	0.67^{a}	1.47^{a}
12 hpi	6.82^{b}	2.88^{b}	0.96^{a}	0.79^{a}	1.75^{a}	0.62^{a}	1.06^{a}
24 hpi	0.57^{a}	0.57^{a}	0.28^{a}	0.47^{a}	0.40^{a}	0.51^{a}	1.29^{a}
48 hpi	1.01^{a}	0.64^{a}	0.59^{a}	0.64^{a}	0.78^{a}	0.64^{a}	0.69^{a}
72 hpi	0.62^{a}	1.48^{ab}	1.67^{a}	3.85^{a}	1.70^{a}	10.11^{b}	4.98^{b}

$^{a, b}$ Expression values within rows marked with different lower case letters are those that differ significantly according to Tukey's test ($\alpha < 0.05$).

accession number: AY506496) as a *T. aestivum* class III root peroxidase (http://www.ncbi.nlm.gov; [40]), but in this study was found to be expressed in wheat leaves. PR2 is an β-1,3-glucanase, a group of enzymes known to play a major role in plant defense and general stress responses through the regulation of callose deposition [18]. However, glucan is also found in fungal cell walls and plant β-1,3-glucanases are able to hydrolyze fungal glucans. Induction of PRA2, PR2 and PR9 transcription 6 hai with *P. triticina* may therefore be part of a ROS signaling defence pathway, and/or required for cell wall modifications, such as callose deposition [41], to restrict pathogen invasion before the formation of haustoria.

PR1 and PR4 had similar expression profiles, Profile 6 (Fig. 2; Supp. Fig. 1). PR1 being a β-1,3-glucanase and PR-4 an endochitinase [42]. These proteins are involved in the degradation of fungal cell walls, being required for the hydrolysis of glucan and chitin, respectively. PR1 and PR4 transcripts peaked at 72 hai in Toropi, well after the appearance of haustoria, and presumably are targeting secondary hyphal growth. However, PR2, also a β-1,3-glucanase belonged to Profile 5, with the highest transcript levels being seen at 6 hai, followed by 72 hai. This would suggest that expression of PR genes, with potentially similar functions is triggered by different stages of *P. triticina* development.

Conclusions

The pre-haustorial resistance seen in Toropi towards the leaf rust pathogen *P. triticina* and the early levels of expression of LTP, WCAB, AQP1, PRA2, PR2, PR9, before visible haustoria formation at 24 hai indicates a possible PAMP-Triggered-Immunity (PTI) resistance response. While the subsequent expression of genes involved in lignin formation (COMT1) at 24 hai, PR1 and PR4 at 72 hai, would suggest an Effector-Triggered-Immunity (ETI) resistance response, leading to the hypersensitive cell death seen in 37% of *P. triticina*-Toropi infection sites at 120 hai [9]. The pre-haustorial leaf rust resistance in adult plants of Toropi is unusual, and phenotypically resembles non-host resistance in *Arabidopsis* to barley powdery mildew [43,44] and in barley to non-adapted rust species [45], where infection is suppressed early by pre-haustorial mechanisms without cell necrosis, with the few haustoria that may establish eliciting a post-haustorial hypersensitive response. Consequently, leaf rust APR in Toropi is an interesting and valuable source of resistance for Brazilian wheat breeding programs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2014.12.004.

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