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Differential expression analysis of defense-related genes responsive to *Tilletia indica* infection in wheat

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Abstract: Karnal bunt caused by *Tilletia indica* is a quarantine disease responsible for qualitative and quantitative losses in wheat. The present study aimed to determine the expression of defense-related genes involved in early stages of infection in response to *T. indica* by using the differential display reverse transcriptase-PCR method (DDRT-PCR) in susceptible (WL 711) and resistant (HD 29) cultivars of wheat. The DDRT-PCR banding profiles generated by the random primers in combination with oligo-dT primers of resistant and susceptible wheat plants were evaluated at 0 h, 12 h, 24 h, and 48 h after inoculation. Out of 9 sets of primers, 7 sets of primers produced remarkable induced transcripts. The highest up-regulated transcripts were observed in the resistant cultivar (HD 29), whereas the down-regulated transcripts were found in the susceptible (WL 711) cultivar. Several sets of primers were designed from the defense-related gene sequences available in the GenBank database, and of these, 4 primer sets amplified the desired specific bands. Based on the putative functions, 3 defense-related genes, namely puroindoline protein PINB (AT_KB2), β-1,4-glucanase (AT_KB3), and chitinase (AT_KB7), and 1 housekeeping gene, actin (AT_KB1), were characterized.

Key words: Defense-related genes, expression, DDRT-PCR, *Tilletia indica*, wheat

1. Introduction

Wheat (*Triticum aestivum* L.) is the second most important cultivated crop in the world. India stands first in area and second in production next to China in the world. In India, wheat is cultivated in an area of 29.3×10^6 ha with 85.9×10^6 t of production and 2938 kg ha^{-1} productivity (Directorate of Economics and Statistics, 2010). Among the diseases that affect wheat, Karnal bunt caused by *Tilletia indica* Mitra is one of the internationally important diseases. Karnal bunt is native to the Asian subcontinent and is so named because of its discovery in 1931 on wheat grown near Karnal, India (Mitra, 1931). Since then, it has been found in all major wheat-growing states of India, as well as in other parts of the world. *T. indica*, a quarantine pathogen, has become a major sanitary and phytosanitary issue in the wake of the recent Agreement of Agriculture stipulated by the World Trade Organization. Because of its international quarantine status, it has the potential for causing economic losses. Presently, 77 countries have restrictions on importing wheat from the areas where the disease occurs (Bonde et al., 2004). Being a seed-, soil-, and airborne pathogen, it is very difficult to manage once introduced to an area. Therefore, wheat-importing countries have imposed strict quarantine measures with zero-tolerance limits on shipment of wheat from diseased areas. The disease poses an economic threat to the wheat industry due to reduction in grain quality rather than yield loss.

Several cascades of defense-response genes are involved in the resistance mechanism. There are several methods used to isolate genes that are differentially expressed during pathogen infection. Differential display reverse transcriptase-PCR (DDRT-PCR) (Liang et al., 1993), a powerful technique, has been used to compare expression patterns of differential transcripts. This technique has also been exploited to isolate the genes specifically induced during pathogenesis in different host–pathogen interactions, namely, tomato–*Botrytis cinerea* (Benito et al., 1996), pepper–*Phytophthora capsici* (Munoz and Bailey, 1998), and potato–*Phytophthora infestans* (Collinge and Boller, 2001). Two putative genes for resistance in wheat against *T. tritici* were identified and characterized along with other known resistance genes by employing subtractive hybridization (Lu et al., 2005). Similarly, in cases of wheat infected with *Fusarium graminarium*, defense-response genes have been isolated, cloned, and

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characterized (Goswami et al., 2006). Several defense-response genes representing a diverse group of resistance genes such as hypersensitivity- and pathogenesis-related genes have been mapped on 21 different chromosomes of the wheat cultivar Chinese Spring using 36 different probes (Li et al., 1999). Two wheat transcripts responsive to *Bipolaris* in wheat induced by *B. sorokiniana* infection using DDRT-PCR were identified (Aggarwal et al., 2011). The teliospores of *T. indica* germinate at or near the soil surface and produce basidiospores that form hyphae, allantoid sporidia, and successive generation of sporidia. The sporidia are released in the presence of high humidity between 1800 and 0800 hours, and their release is optimum between 0200 and 0300 hours. The sporidia deposited on spikes germinate to produce hyphae and penetrate stomata, and grow intercellularly onto the base of the developing kernel. The plants are susceptible only during specific periods within the boot swelling to anthesis stage. The infection from airborne inoculum can occur from the time at which florets begin to emerge from the boot up to the soft dough stage. The early period (12–48 h) is crucial for establishment of the pathogen in the host (Carris et al., 2006). Information is not available about the physiological and molecular events regulating gene expression in wheat under *T. indica*-infected conditions. Therefore, it is important to analyze pathogen-responsive gene expression in resistant and susceptible wheat genotypes, as it may increase the understanding of the molecular mechanism of *T. indica* infection and the role of differential gene expression in Karnal bunt resistance. Therefore, the present study aimed to determine the induction of defense genes in resistant and susceptible cultivars of wheat during early stages of infection by *T. indica*.

2. Materials and methods

2.1. Cultures of *Tilletia indica* and wheat cultivars

The cultures of *T. indica* were raised from the teliospores of infected wheat seeds collected from Delhi (KB1), using the technique described by Warham (1987). Bits from monoteliosporic cultures were placed on potato dextrose agar in petri plates and incubated at 18 °C under 12-h photoperiod conditions. After 7 days, the petri plates were flooded with distilled water. Basidiospores were scraped from the surface with a spatula and filtered through 2-layered muslin cloth before inoculation. The plants of resistant (HD 29) and susceptible (WL 711) cultivars of wheat grown in a growth chamber at 24 °C/18 °C (day/night cycle), 60% relative humidity, and a photoperiod of 16 h (212 μmol $m^{-2} s^{-1}$ photon flux density) were inoculated at the boot stage by using a sterile hypodermic syringe with spore suspension containing 10^5 spores mL^{-1} (Aggarwal et al., 2010). The plant samples were collected at 0 h, 12 h, 24 h, and 48 h after inoculation.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the *T. indica*-inoculated boot samples collected at 0 h, 12 h, 24 h, and 48 h after inoculation using an RNA extraction kit (QIAGEN, RNeasy Plant Mini Kit). RNA quantity and quality was measured at 260 and 280 nm by UV-spectrophotometer (UVIKON, Italy) and by dehydrated formaldehyde gel electrophoresis, respectively. An RT-PCR kit (QIAGEN, Omniscript RT) was used for the synthesis of cDNA following the manufacturer's instructions. The template RNA (2 μg/20 μL reaction) was added to tubes containing Master Mix 10X buffer RT, 5 mM dNTPs, 10 μm oligo-dT primer, 10 U/μL RNase inhibitor, and 1 μL Omniscript RT, and the final volume was maintained by adding RNase-free H_2O. The Eppendorf tubes were carefully vortexed for 5 s and incubated for 60 min at 37 °C. The aliquot of the finished reverse transcription reaction was added to the PCR mix.

2.3. DDRT-PCR

Differential display PCR was performed in a 20-μL reaction mixture containing 1 ng of first-strand cDNA, 1 μL of each anchored oligo-dT and random primers (20 μM), 0.2 μL dNTP (5 mM), and 5 units of Taq polymerase (Bangalore Genei, India). The PCR was performed using a thermal cycler (Bio-Rad, iCycler, USA) programmed to 94 °C for 2 min followed by annealing at 39 °C for 60 s, extension at 72 °C for 60 s, and denaturation at 94 °C for 30 s, repeated to annealing temperature for an additional 39 cycles. The DDRT-PCR mixture was denatured with an equal volume of gel loading buffer (95% formamide, 0.1% xylene cyanol FF, and 0.1% bromophenol blue) at 90 °C for 2 min. The denatured products (2 μL) were separated by electrophoresis at 70 W on 6% polyacrylamide/7 M urea DNA sequencing gel. The gel was stained with silver nitrate and some of the amplified products were resolved on 1.2% agarose gel. A total of 9 primer combinations were tested arbitrarily using 9 random primers (Operon Technologies, USA) and 4 anchored oligo-dT primers (Table 1). Changes in mRNA transcripts between susceptible and resistant samples were recorded for each set of primers. In order to reduce the number of false positives, each experiment was repeated twice and only reproducible bands were rescued. The differentially expressed transcripts were collectively named WTRT ("wheat transcripts responsive to *Tilletia*"). Based on the comparative intensity and presence or absence, the WTRTs expressed following *T. indica* infection were grouped into 2 classes, namely WTRT-1 (induced/up-regulated) and WTRT-2 (suppressed/down-regulated).

2.4. Designing of primers and cDNA amplification

In order to study induction of defense genes, 9 gene sequences (Table 2) available in GenBank were selected and designed for the specific primers using Primer 3 software (input 0.4.0). The molecular mechanism of *T.*

Table 1. Differentially expressed transcripts following *Tilletia indica* infection in wheat.

Primer combination	Induced transcript (DDRT product from resistant sample)	Suppressed transcript (DDRT product from susceptible sample)
OPA 3/$dT_{(21)}$	-	-
OPA 4/$dT_{(21)}$	3	1
OPA 5/$dT_{(21)}$	6	4
OPA 6/$dT_{(20)}$GC	4	3
OPA 13/$dT_{(20)}$A	-	-
OPA 19/$dT_{(20)}$A	13	12
OPB 6/$dT_{(20)}$C	2	0
OPB 7/$dT_{(20)}$GC	5	4
OPB 8/$dT_{(20)}$GC	4	2
Total	37	26

Table 2. Specific DNA primers designed for screening of resistant and susceptible wheat cultivars.

Gene	GenBank Accession No.	Primer (5'–3')	Amplicon size (bp)	Gene description
Actin	DN 551593	F-GGAAAAGTGCAGAGAGAACG	597	Actin
		R-TACAGTGTCTGGATCGGTGGT		
PINB	GH239328	F-CGTGTCCAAGATCAAGCTCA	678	Puroindoline protein (PINB)
		R- AACATGGCGTCGCTCTACTT		
G lu 2	Z22874	F-AGCAGAACTGGGGACTCTTCT	348	1,4-glucanase
		R- CACATACGTACCGCATACACG		
LTP 1	DN551584	F-ACGTAGGTACTCCTCTCGCTGT	-	Lipid transfer protein
		R- GTTGATCGACCACTTCTTCTCA		
LTP 2	DN551617	F-GGTCACACACACACACACACA	-	Lipid transfer protein
		R- CGGGAGAGAAGTAACAACCAA		
Lipase	DN551653	F-CACAAAATATCGACCCACCAC	-	Lipase
		R- ACTGGGTATTCGTCTGTCAGC		
Chi A	X 95000	F-CCCTACACATGGGGCTACTG	280	ChiA 0.1 basic chitinase
		R- CCTGCCCGTAGTTGTAGTTGT		
PR1.1	AJ007348	F-ACTACGACTACGGGTCCAACA	-	Basic PR-1
		R- TCGTAGTTGCAGGTGATGAAG		
PR1.2	AJ007349	F-CGTCTTCATCACCTGCAACTA	-	Neutral PR-1
		R- CAAACATAAACACACGCACGTA		

indica and wheat interaction and the information about the genes involved in defense are not available. Some of the specific genes reported to impart general defense in different host–pathogen interactions, such as glucanases, chitinases, lipid transfer proteins (Mauch et al., 1988; Ge et al., 2003), and the specific gene puroindoline, which is one of the functional components of the wheat grain hardness locus and controls wheat kernel texture (Giroux and Morris, 1998), were selected in the present study. The first-strand cDNAs obtained from postinoculated resistant and susceptible cultivars of wheat were amplified with the gene-specific primers designed in the present study. Each reaction mix consisted of 20 ng of cDNA, 25 ng each of forward and reverse primers, 0.5 mM dNTP, 1.5 U Taq polymerase, and 1X buffer in a volume of 20 µL. The PCR amplification was performed using the following thermal cycling conditions: initial denaturation for 2 min at 94 °C, followed by 39 cycles of denaturation at 94 °C for 30 s, specific annealing temperature for each primer for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The amplified products were electrophoresed and visualized under a UV transilluminator.

2.5. Cloning and sequencing

The amplified bands were eluted and subsequently purified using QIAGEN gel extraction and purification kits (Promega, USA). The cloning of the fragment was performed with the pGEM-T Easy Vector System (Promega) following standard procedures. The competent cells were prepared and recombinant plasmid DNA was isolated (Sambrook et al., 2001). Presence of an insert was confirmed by restricting the recombinant DNA with *Eco*R1 and colony PCR. Cloned inserts were sequenced and submitted to GenBank at the National Center for Biotechnology Information (NCBI). The multiple sequence alignment and pairwise alignment were made using BioEdit, version 5.09 (Hall, 1999). The BLAST program (Altschul et al., 1997) was used to identify and confirm the regions of the respective clones. The nucleotide sequences were subjected to BLAST analysis (http://www.ncbi.nih.gov/index.html).

3. Results

The amplified bands obtained from the amplification of cDNA synthesized from the total RNA extracted from both resistant (HD 29) and susceptible (WL 711) cultivars of wheat at different postinoculation periods up to 48 h using 9 sets of random primers clearly indicated differences in the profiles of susceptible and resistant cultivars. Some of the bands, however, were unique to either susceptible or resistant genotypes. The differences in the number of bands were observed with different primer combinations in susceptible and resistant cultivars. The number of bands ranged from 2 to 13 in the resistant genotype and 1 to 12 in the susceptible genotype. A total of 37 PCR products were obtained with 7 primer combinations from the resistant wheat cultivar. On the other hand, a total of 26 PCR products were obtained in the susceptible cultivar (Table 1). The molecular weight of WTRT-1 ranged from 700 bp to 1000 bp, while that of WTRT-2 ranged from 550 bp to 800 bp. Primer combinations OPA 6/$dT_{(20)}$GC and OPA 19/$dT_{(20)}$A were suitable for amplifying both induced (up-regulated) and suppressed (down-regulated) WTRTs (Figure 1), whereas primer combinations OPA 3/$dT_{(21)}$ and OPA 13/$dT_{(20)}$A could not amplify any of these WTRTs (Table 1). The primer OPB 6 produced 2 bands in the resistant genotype, whereas no amplification was observed in the susceptible genotype. The OPA 4 primer also produced a higher number of bands in the resistant cultivar in comparison to the susceptible cultivar (Figure 1). The up- and down-regulated WTRTs were targeted for their differential expression in the resistant (HD 29) and susceptible (WL 711) cultivars for up to 48 h following fungal infection. It was observed that in the susceptible cultivar at 48 h after inoculation, WTRT-2 was completely suppressed. On the other hand, WTRT-1 showed expression at up to 24 h and 48 h after inoculation in the resistant cultivar.

The results of the gel profile of the susceptible and resistant cultivars obtained with defense gene-specific primers (Table 2) indicated that actin, which is a housekeeping gene, and β-1-4 glucanase, chitinase, and puroindoline protein provided specific amplicons of 597 bp, 348 bp, 280 bp, and 678 bp, respectively, in both the genotypes at all stages of sampling starting from 0 h to 48

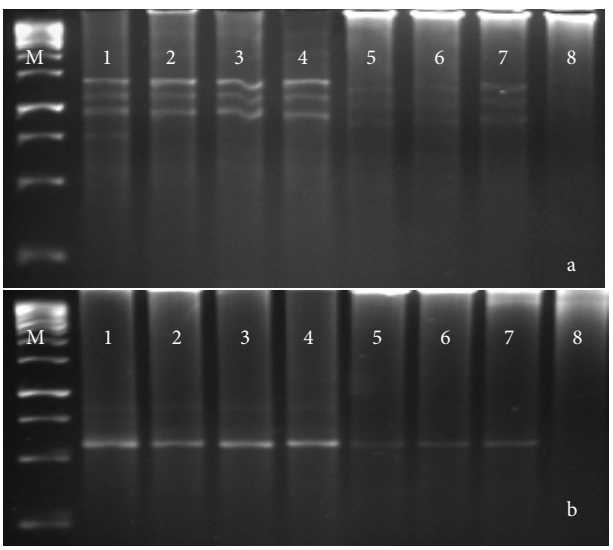

Figure 1. Amplification profile of differentially expressed transcripts obtained with primers a) OPA6 and b) OPA4 in wheat cultivars at different time intervals. Lanes 1: 0 h, 2: 12 h, 3: 24 h, 4: 48 h for resistant cultivar (HD 29); 5: 0 h, 6: 12 h, 7: 24 h, 8: 48 h for susceptible cultivar (WL 711); M: DNA ladder.

h after inoculation, but the expression level was higher for defense-related genes in the resistant cultivar (Figure 2). The densitometric analysis further supported a higher expression level of β-1,4-glucanase in the resistant cultivar. A 3-fold increase was observed at 48 h after inoculation (Figure 3). The expression level was gradually increased with increasing time intervals.

The gene-specific 4 PCR products generated in the present study were cloned and sequenced. The sequences were analyzed and classified as plant defense-responsive genes based on their putative functions observed in the NCBI BLAST search. The sizes of these defense-related genes varied from 280 bp (AT_KB7) to 678 bp (AT_KB2). The gene AT_KB1 (actin) matched the actin gene available in the NCBI database. It showed 100% homology with *Triticum aestivum* actin-like protein, 87% homology with *Brassica napus* actin, and 86% homology with *Zea mays* actin gene. Gene AT_KB2 (puroindoline protein PINB) matched the *Triticum aestivum* clone and uncultured bacterium partial 16S rRNA gene with 100% similarity, and AT_KB 3 (β-1,4-glucanase) also matched the sequences of β-glucanase available in GenBank. This transcript shared 95% homology with the sequence of *Triticum aestivum* β-1,3-glucanase and β-1,4-glucanase, and 94% with barley DNA for β-1,3-glucanase and β-1,4-glucanase. The transcript AT_KB 7 (basic chitinase) showed 95% sequence homology with the sequence of the *T. aestivum* ChiA gene during the BLAST search. These sequences were deposited to the NCBI GenBank database and accession numbers were obtained for AT_KB1 (JX470761), AT_KB 2 (JX470762), AT_KB3 (JX470763), and AT_KB7 (JX470764).

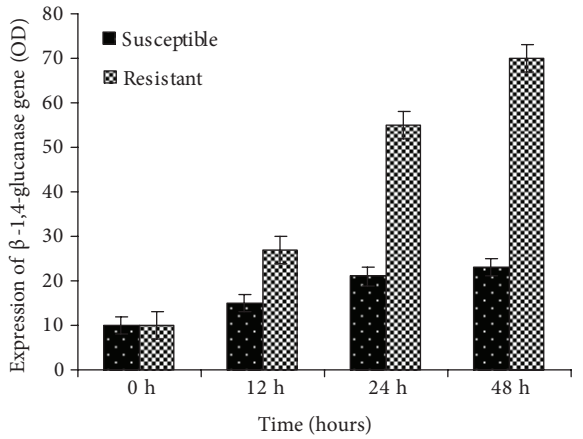

Figure 3. Expression level of β-1,4-glucanase in susceptible (WL 711) and resistant (HD 29) wheat genotypes against *Tilletia indica*.

4. Discussion

The sampling for RNA extraction from both the susceptible and resistant varieties was done up to 48 h after inoculation, keeping in mind earlier observations on the infection process, wherein there are reports that allantoid sporidia, on germination, penetrate through the ovary in the developing kernels and establish within 2 days for further colonization (Bonde et al., 1997). The transcripts generated in the present study could be further isolated and sequenced and the information can be used for investigating cDNA clones, isolating promoter sequences, etc., leading to better understanding of the molecular mechanism of Karnal bunt resistance. Some of the specific bands present only in resistant cultivars of

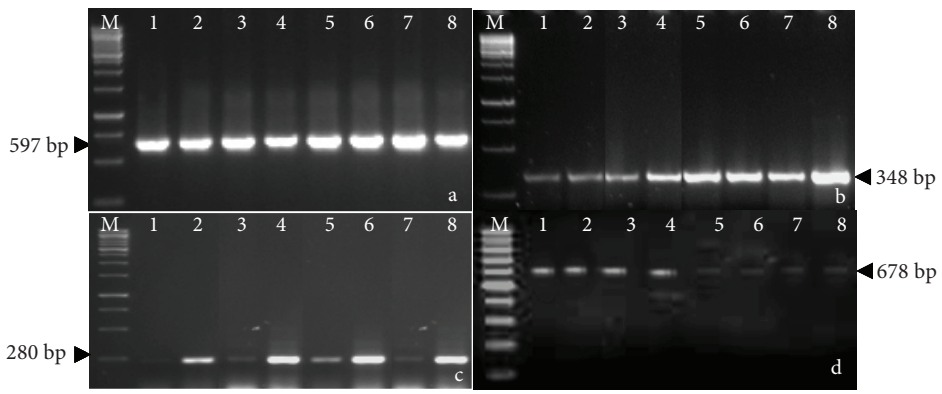

Figure 2. The amplification profiles obtained with specific primers for a) actin gene and b) β-1,4-glucanase gene at different time periods in wheat cultivars: lanes 1–4 for susceptible cultivar (WL 711) at 0 h, 12 h, 24 h, and 48 h; lanes 5–8 for resistant cultivar (HD 29) from at 0 h, 12 h, 24 h, and 48 h; M: DNA ladder. Amplification profiles for c) chitinase gene and d) puroindoline protein gene: lanes 1: 0 h, 3: 12 h, 5: 24 h, 7: 48 h for susceptible cultivar (WL 711); 2: 0 h, 4: 12 h, 6: 24 h, 8: 48 h for resistant cultivar (HD 29); M: DNA ladder.

wheat in response to inoculation by *T. indica* were cloned and sequenced. Their sequences were found to be similar to the sequences of defense-related genes available in the NCBI GenBank database. The up- and down-regulated WTRTs targeted for their differential expression in Karnal bunt resistant (HD 29) and susceptible (WL 711) cultivars indicated that the resistant line was capable of expressing certain transcripts for a longer period during infection, and these transcripts may be used as markers for screening genotypes with Karnal bunt resistant characteristics. These transcripts may be helpful as additional tools along with other molecular markers such as random amplification of polymorphic DNA (RAPD) (Kumar et al., 2004), as the RAPD techniques have shown limited polymorphism in wheat and have proven inadequate and incapable of identifying unique bands for use as molecular markers (Landjeva et al., 2007). As a result, the transcripts (WTRT-1 and WTRT-2) identified in the present study could be utilized in selection of Karnal bunt-resistant wheat genotypes.

Various defense-response genes in wheat, which include β-1,3-glucanase and chitinase, have been mapped using a hybridization technique (Li et al., 1999). Similarly, Kong et al. (2005) also observed induction of chitinase in wheat in response to *Fusarium graminearum*. High-level expression of chitinases seems promising against plant-pathogenic fungi (Okay and Özcengiz, 2011). These reported observations support our study related to glucanase and chitinase production. The defense-related genes β-1,4-glucanase, chitinase, and puroindoline protein, and the housekeeping gene actin, were characterized in the present study. Prior to this study, several researchers had also isolated and characterized the defense-related genes in various host–fungal pathogen interactions. Six differentially expressed cloned cDNAs obtained 24 h and 48 h after inoculation by *Colletotrichum gloeosporioides* in green fruits of pepper showed homology to aldehyde dehydrogenase, P23 protein, NP24 protein, cytochrome P450 protein, esterase, and MADS-box protein, respectively, which were involved in the resistance of ripe fruit to *C. gloeosporioides* infection (Oh et al., 2003). Four germin-like protein isoforms, a peroxidase, and a glutathione *S*-transferase, all implicated in oxidative processes including the oxidative burst, were identified. The chitinase isoforms implicated in fungal cell wall degradation and a nucleotide binding site–leucine-rich repeat disease-resistance protein homolog related to pathogen recognition were identified during DDRT-PCR of conifers and root rot pathogen *Ceratobasidium bicorne* interaction (Johnk et al., 2005). The change in expression levels of a leucine-rich repeat-containing protein kinase played an important role in the defense pathway against fungal infection in jute (Reza et al., 2013). Plant proteinase inhibitors also conferred resistance in plants against different competing organisms including fungi (Munir et al., 2013).

The expression level differences of 33 genes in DDRT-PCR of 2 yellow rust differential lines of wheat infected with the virulent and the avirulent *Puccinia striiformis* f. sp. *tritici* using quantitative real-time PCR were analyzed (Bozkurt et al., 2007). Based on the sequence similarity, 2 genes, namely cyclophilin-like protein (putative antifungal activity) and ubiquitin-conjugating enzyme, were identified. These are involved in programmed cell death, putative antifungal activities, disease resistance responses, and pathogenesis-related responses, including a few with unknown functions. The chitinase and stilbene synthase genes were more rapidly expressed in tolerant cultivars as compared to susceptible cultivars of grape within 1 day after inoculation with *Elsinoe ampelina* (Vasanthaiah et al., 2010). Induction of these antifungal genes appears to enable tolerant cultivars to withstand fungal infestation compared to susceptible cultivars. A number of defense-related transcripts were identified to be implicated in *Yr*1-mediated resistance, including classical pathogenesis-related (PR) transcripts and genes involved in plant cell defense responses, such as the oxidative burst and cell wall fortification, during microarray analysis of wheat during compatible and incompatible race-specific interactions with *P. striiformis* f. sp. *tritici*. A number of defense-related genes, including PR2, PR4, PR9, PR10, and WIR1 transcripts associated with the latter stages of Yr1-mediated resistance, were also identified (Bozkurt et al., 2010). In the present findings, the expression of β-1,4-glucanases was increased 3-fold at 48 h after inoculation with *T. indica*. β-1,3-Glucanases are capable of hydrolyzing β-1,3-glucans found in the cell wall of fungi. They can either degrade the cell wall of the pathogen or disrupt its deposition, leading to pathogen death (Mauch et al., 1988). They can also release cell wall fragments that act as elicitors of active host defense response (Yoshikawa et al., 1993). The expression of chitinase and puroindoline was also increased in the resistant cultivar (HD 29) in response to infection. Defense-response genes function in a variety of ways to inhibit fungal infection. Chitinases break bonds between the C1 and C4 of 2 consecutive N-acetylglucosamines of chitin, which is a main component of the cell wall in fungi (Collinge et al., 1993). Chitinase genes are up-regulated during early infection of wheat and barley spikes by *F. graminearum*. In particular, PR-1, PR-2 (β-1,3-glucanase), PR-3 (chitinase), PR-4, and PR-5 (tlp-1) transcripts accumulated in wheat spikes during *F. graminearum* infection. Puroindoline a and b (PINA and PINB) are the functional components of the wheat grain hardness locus and control wheat kernel texture (Giroux and Morris, 1998). The antifungal activity

of PINs has been demonstrated against several fungal plant pathogens and in transgenic crops (Zhang et al., 2011). In the present study, the expression of this gene has significance in providing hardness to the seeds to resist development of the disease. Plant lipid-transfer proteins (LTPs) must be considered as important peptides involved in plant defense against pathogens, since they present adequate inhibitory activities, suitable distribution, and concentration in plant tissues and an increase in their gene expression levels soon after infection. The role of LTPs in plant protection has also been investigated (Ge et al., 2003). However, in the present study its expression was not significant.

The findings of the present study indicated that the DDRT-PCR technique is suitable for determining the expression of defense-related genes in a resistant cultivar of wheat at early stages of infection by *T. indica*. Three defense-related genes, β-1,4-glucanase, chitinase, and puroindoline (PINB), and a housekeeping gene, actin, were characterized from both the resistant and susceptible cultivars of wheat in response to the infection by the pathogen. The levels of expression of these genes were greater in the resistant cultivar as compared to the susceptible cultivar of wheat, indicating their roles in imparting resistance in HD 29 against *T. indica*.

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