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RESEARCH ARTICLE

A SURVEY OF GENE EXPRESSION DURING UREDOSPORE GERMINATION IN *PUCCINIA SORGHI*

Mehmet Ali SÜDÜPAK[1]

[1]Yozgat Bozok University, Faculty of Arts and Sciences, Department of Biology, Yozgat, mali.sudupak@yobu.edu.tr, ORCID:0000-0001-9439-0916

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ABSTRACT

Transcript profiling is commonly used to identify genes that are expressed under various conditions, in different tissues and developmental stages of organisms. Transcripts tags detected from germinating uredospores of *Puccinia sorghi* T09 isolate using a modified cDNA-AFLP approach were characterized in the current study. GenBank similarity searches and sequence mapping of 56 sequence tags to available genome sequence of Argentinian strain RO10H11247 revealed 38 *P. sorghi* similar sequences, corresponding to 31 individual Genbank records. With the obtained similarities and protein domain searches, presumed functions associated with the 27 *Ps* TDFs were inferred, the majority of which appear to encode products that appear to be important in host invasion, pathogen growth and proliferation. Among them, chitinase, oligopeptide transporter protein, peptidyl-tRNA hydrolase, signal peptide, small secreted proteins, velvet and cutinase domain containing proteins are the prominent ones. Expressions of four selected genes, three of which are newly identified, were verified in germinating spores and infected plant leaf material cDNA preparations with RT-sqPCR. Together with the newly identified and annotated *P. sorghi* genes, obtained profile, in general, represent a gene set whose products are conceivably involved in host invasion and pathogenesis along with the basic housekeeping functions.

Keywords*: P. sorghi, germinating uredospore, cDNA-AFLP, Expressed Sequence Tag.*

1. INTRODUCTION

The common maize rust is one of the important diseases of corn caused by the *Puccinia sorghi* Shew., which appears to inflict significant yield loses up to 40% depending on the severity of infection [1]. As an obligate biotrophic basidiomycete, *P. sorghi* (*Ps*) manifests its main pathogenicity during the uredospore cycle propagation on corn. Genes conditioning race-specific resistance to the *Ps* races have been identified from various sources of corn germplasms and employed to same extent in rust control [2,3]. However, the protection provided by these vertical resistance factors depends on the presence of their corresponding avirulance/effector gene products in the invading pathogen, and this race specific resistance often breaks down by the appearance of the new virulent races.

Various techniques, such as gel-based DDRT-PCR, cDNA-AFLP, hybridization based macroarray, microarray and sequence based SAGE, transcriptome sequencing have been used to detect expressed

sequences and study gene expression changes in cells/organisms during development and under different environmental conditions. Expressed Sequence Tag (EST) analysis is one of these techniques that has been used to generate EST databases, which are valuable source in gene characterizations, expression analysis and microarray designs. A number of EST studies have been conducted in fungi to examine gene expression at various developmental stages and structures. Hahn et al. [4] studied expressed genes during the *Uromyces faba* infection of broad bean at uredospore germination stage and haustorium. They detected dramatic expressional changes between these transcript profiles. EST analysis covering resting uredospore, germinating uredospore, appressorium, haustorium formation stages of *Puccinia triticina* infections of susceptible and resistant wheat genotypes with 13 different cDNA libraries generated 25.558 sequence tags [4]. Zhang et al. [6] obtained a set of 4798 EST sequences from germinating uredospores of *Puccinia striiformis*, and found that 23.9% and 13.3% of the identified 1118 unisequences are homologous to functionally characterized proteins and hypothetical proteins, respectively while 62.8% unisequences had no significant homologs. A number of other EST studies were conducted in fungi to obtain gene expression profiles under various conditions [7,8].

Elucidation of mechanisms and underlying genetic factors in the pathogen, such as virulence factors, effectors and other infection facilitating activities participating in host invasion and pathogenesis are essential to both understanding and manipulation of the pathosystems. Factors functioning in host resistance and pathogen avirulance/virulence are elucidated to some extent in a number of obligate host-parasite systems [9-11]. The maize-*Ps* pathosystem, however, has not been characterized sufficiently in this regard. Recent *Ps* genome sequencing and annotation [12] provide a valuable genomic tool to functional genomic studies and addressing to the specific aspects of the *Ps* growth and infections. Current study uses this genomic information [13] and other sequence databases and describes a survey of the messages expressed in germinating *Ps*. uredospores and infected plant material.

2. MATERIALS AND METHODS

2.1. Biological material preparation and total RNA isolation

Puccinia sorghi race T09 uredospores grown on A188 seedlings and infected A188 leaf material were used in the study. Total rust RNA isolations were carried out from freshly collected ~1 mg spores, which were germinated overnight on a 15 ml sterile dH_2O in (6x1) petri dishes in a dark cabin at room temperature (18-24 ^oC). Similarly, leaf materials were prepared as two parallel sets, one for control and one for inoculation sample as described in Südüpak [14] (2014). Control and inoculation seedlings were kept at 24-27 ^oC under natural daylight with a light/dark photoperiod of 16/8 h, and leaf materials were sampled at 24, 48, 72 and 96 post inoculation hour (pih) intervals as ~100 mg 2nd leaf segments. Both fungal mat formed by the germinated uredospores and leaf samples were individually collected and quickly placed into 1.5 ml. tubes individually and frozen in liquid nitrogen. Without thawing, the sampled materials were grounded individually into fine powders in their tubes, and RNAzol (Molecular Research Center, MRC) extraction buffer was added to powder at the scale of 100 µl per mg rust material and 1 ml per 100 mg plant material and mixed to obtain a homogenates. RNA extractions were carried out according to the manufacturer's instructions. At the final step, RNA pellets were washed with 400 µL of 75% ethanol (prepared with DEPC-treated water) twice and briefly dried to evaporate ethanol. Pellets were dissolved in 50 µL. DEPC-treated water and stored at -20. Nucleic acid concentrations were measured spectrophotometrically at 260 nm with A_{260}/A_{280} ratio of 1.8-1.9 and A_{260}/A_{230} ratio around 2.3.

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2.2. cDNA synthesis, TDF generation and sequencing

cDNA synthesis, other nucleic acid manipulations and cDNA-AFLP detection of mRNA tags were carried out as described in Südüpak [14]. Elution of TDF (Transcript Derived Fragment) bands, cloning and preparation to sequencing reactions are also given in Südüpak [14]. Only 9 TDFs were directly sequenced using either one of the cDNA-AFLP primers while colony PCR amplified products were sequencing template in others. TDF sequencing was conducted by the commercial service providers, and results were provided as sequencing files. 3'-RACE experiments for the isolation of AC/CC R2-4 transcript end were carried out using a forward gene specific primer and an oligo designed to bind to mRNA 3' ends.

2.3. Bioinformatics

Sequence data were first manually examined in SnapGene Viewer for the presence of adapter and primer tags. TDF sequences with trimmed adapter segments were compiled into a text file in FASTA format to be used in similarity searches and mapping to *Ps* scaffolds. Sequence data were first *Ps* genome blasted in NCBI (ncbi.nlm.nih.gov/BLAST) and TDF sequences displaying significant (<1e-4) similarities to *Ps* genome sequence (GCA_001263375) scaffolds were determined. These sequences were then submitted to blast/blat routine of the Ensembl Genomes [13] (fungi.ensembl.org/Puccinia_sorghi_gca_001263375/Tools/Blast) to confirm and visualize the TDF similarity regions on scaffolds with the annotated genes, if there is any. TDFs mapping (with very high similarity) very closely to (e.g. to presumed 5'-UTR or 3'-UTR) or as part of an annotated genes were considered as the annotated gene derivatives and designated accordingly. TDF mapping to regions with no annotated genes were studied in gene prediction programs in that the genomic sequences surrounding the TDF similarity region with the size of an average *Ps* gene were copied and submitted to FGENESH routine of Softberry (softberry.com) website with the organism=Puccinia (rusts) selection, probable genes, if there is any, were predicted. Search reveals structure of the predicted gene with its ORF and encoded polypeptide sequence. Predicted gene and its ORF sequence were then examined to position TDF sequence. Majority of the TDFs mapped to 3' ends of the annotated and predicted genes, which is consistent with the strategy used in their isolation. Protein products of previously annotated and newly predicted genes were examined to make inferences about their structures and functions using protein databases and tools such as UniProt (uniprot.org) InterProScan, iPSORT, SignaIP [15].

2.4. RT-sqPCR validations of selected TDF expressions

Three newly identified (MN199982, MT677935, MT677936) and two previously predicted, *PsOPT* (KNZ51092) and *PsChi* (KNZ44027) genes were selected for validation studies (Table 1). A pair of RT-PCR primers were designed for each gene using the web-based program, Primer3Plus (sourceforge.net/projects/primer3), with its modified parameters for amplifying products between 90 and 250 bp size range covering the TDF BLAST homology segments. Primers and other oligos used in the study were synthesized by Oligomer Biotechnology (Ankara) while enzymes and other reagents were purchased from Fermentas-Thermo, Sigma and Merck.

In expressional verification studies, material preparation, RNA isolation and cDNA synthesis for two-step RT-sqPCR experiments were carried out essentially as given in Südüpak [14]. RT-sqPCRs were prepared as individual 25 µl mixtures containing 2.5 µl 10x reaction buffer (Fermentas-Thermo), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 10 pmoles of each Forward and Reverse gene specific primers, 0.5 U *Taq* DNA polymerase (Fermentas-Thermo) and 2 µl of 10x diluted first strand cDNA template. Temperature and time profiles of the PCRs were setup as follows; an initial denaturation period of 3

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min at 95 °C, followed by 35 cycle-PCR, each cycle consisting of a denaturation step at 94 °C for 20 sec., an annealing at 59 °C for 30 sec. and extension at 72 °C for 50 sec, amplifications were finalized with a 5 min. extension period at 72 °C. Reaction mixtures were individually mixed with 5 µl of 6X loading dye and electrophoresed for 2 hours in 2% 0.5XTBE agarose gels containing 0.4 µg ml^{-1} ethidium bromide, and products were visualized and documented on a 312 nm UV transilluminator.

Table 1. List of genes with their RT-PCR primers used in expression verification studies.

Genes (GI, accession #)	**Primer sequences, 5'→3' F/R**	**Product (bp)**	**PCR annealing temperature (°C)**
HKG-U (GRMZM2G047204)	GCGTGCTCTTTCGTCAGATGTG CCTACTGTTGGCTGGAGACTGG	156	59
Ps1-2 (MN199982)	TGTAAGCAAGTTGGCGTTAG CTCCTGTTGAGGTCAATGTC	131	59
Ps2-4 (MT6777935)	CCGGTGTGATTGCTTCTCCT GTATGTTCCGCCGTATGGGT	161	59
PsChi (KNZ44027.1)	CACCTGAACATCTGGTCCCA TGGTTCTCAGTGTACGACCG	105	59
PsOPT (KNZ51092.1)	GATGGTGGCACCCAGATCAT GGGACAGACCTAGGAGGGTT	116	59

3. RESULTS

Rust TDFs generated in a previous cDNA-AFLP study were utilized to examine the expressed messages in the germinated *Ps* T09 uredospores and infected plant material. Sequence characterized 56 TDFs with a size range between 24 bp to 354 bp were divided into two groups as *Ps* similar and non-Ps similar according to the obtained blast similarity results. Among 49 *Ps* similar TDFs, 38 displayed significant similarities (<1e-6) to 31 *Ps* GenBank entries, the majority of which have annotated functions. Nine contigs (TDFs derived from the segments of same gene) were identified in the data set while many of the TDFs represented as singletons (Table 2). Obtained similarities for 11 TDFs were not significant (1e-4<), thus, they are not listed in the table. Five of the non *Ps* similar TDFs displayed similarities to sequences from other fungi, one of which displaying a significant similarity to the rust infecting RNA virus is listed in table. Remaining two TDFs found to be similar to organelle and bacterial DNA sequences are not listed in the Table 2 as well.

Table 2. Expressed Sequence Tags (EST) identified in the study.

TDF #	**Size***	**Accession #**	**GenBank record showing the closest similarity**	**+E-val.**
Newly identified and annotated gene TDFs				
[1]AC/GG R1-2	354	MN199982	*Ps* **uncharacterized (secreted) protein**	0.0
AC/CC R5-2	117	MN199982	Maps to 3' end of MN199982, revealing its 3'-UTR sequence.	0.0
[1]AC/CC R2-4	281	MT677935 MT677936	*Ps* **uncharacterized (secreted) protein** similar to MN199982 (identical two copies reside on a ~4 kb tandemly duplicated gDNA)	0.0
AC/CG R3	198	MT677935	Aligns to 5' end of the *Ps*. AC/CC R2-4	6e-57

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		MT677936	duplicated genes, revealing their 5'-UTR sequences.	
AC/GG R2-3	306	MN190718	*Ps* **cytochrome C oxidase subunit 1** (tag covers ORF 3'end and 3' UTR segments)	0.0
AC/CG R1	300	NC_044103.1	*P.tiriticina* mt **NADH-ubiquinone oxidoreductase subunit 1**, aligns to the ***Ps* mt scaffold_46**, annotated in this study.	5e-137
GG/TG R5-5	120	LAVV01008557.1	Maps to the scaffold_354, positioning ~1500 bp to 3' end of KNZ52507. A new ORF close to the tag position, whose aa sequence is similar (3e-62) to KNZ64419.1 was identified	7e-54

TDFs from genes with unknown and known functions or bearing a domain

GG/CA R9	95	KNZ54019.1	*Ps.* **uncharacterized protein containing cutinase domain**	3e-41
AC/GG R3-4	198	KNZ56820.1	*Ps* **uncharacterized protein containing velvet domain**	1e-97
[1]TG/GA R3	144	KNZ51092.1	*Ps*. **putative oligopeptide transporter** (OPT)	8e-39
GA R5	48	KNZ45441.1	*Ps*. **uncharacterized conserved protein**, putative **monocarboxylate transporter 2**	1.7e-7
GA R4	80	KNZ54866.1	*Ps*. **putative signal peptide**	9e-23
AC/CC R6-5	173	KNZ63665.1	*Ps* **protein (serine/threonine) kinase**	0.0
GG/TG R3, R4	170	KNZ54998.1	*Ps*. **Rab family protein GTPase**, **Rab4**	7e-80
AC/CC R8-3	115	KNZ50565.1	*Ps*. **nucleotide, nucleic acid binding hypothetical protein,**	0.0
[2]AC/GG A2, R2-1	228	KNZ64435.1	*Ps* **peptidyl tRNA hydrolase** mRNA	2e-124
[1]AC/CC-A11, R11, R4, R12-5	94	KNZ45394.1	*Ps*, putative **phosphatidylethanolamine binding protein**	8e-42
AC/CG R4	190	KNZ60925.1	*Ps*. **uncharacterized Zn binding protein** (may be a Zinc finger protein)	3e-34
AC/CC R16-5	57	KNZ50028.1	*Ps*. **hypothetic protein containing wax synthase domain**	0.0
[1]AC/GG R7-2	87	KNZ44027.1	*Ps* **chitinase**	4e-30
AC/GG R5-2	96	KNZ51711.1	*Ps* **hypothetical (secreted) protein**	3e-143
AC/CG R6	119	KNZ54287.1	*Ps* **hypothetical protein** similar to **syntaxin binding** protein	1e-36
GT R6	95	KNZ44173.1	*Ps.* **uncharacterized protein containing DUF3759 domain**	1e-4
AC/CC R13, R14	76	KNZ59498.1	*Ps*. **hypothetic protein similar to steroid reductase**	1e-33
AC/CC R9-3, R10	108	KNZ62659.1	*Ps*. predicted **exodeoxyribonuclease V**	4e-51
AC/GG R6-1	73	KNZ54883.1	*Ps* **hypothetical protein, dihydrolipoamide dehydrogenase**	2e-26
AC/GG R8-2	56	KNZ52430.1	*Ps* **histon 3.1** (similar to 3' UTR)	2e-34
AC/CC R3-4	240	LAVV01006726.1	*Ps* **28S rRNA 3'-end fragment**	8e-121

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AC/CC R5-5	98		*Ps* **28S rRNA sequence, derived from 5' end.**	8e-48
AC/CC R7-3	126	KNZ46759.1	*Ps* **hypothetical protein**	0.0
GG/CA R14	71	KNZ49933.1	*Ps.* **uncharacterized protein**	2e-15
TDFs displaying significant similarities to sequences from other fungi				
GG/TG R2	200	GW673618.1	*Melampsora larici-populina* mRNA sequence, derived from narnavirus RNA dependent RNA polymerase seq.	8e-30

[1]Expressed in both germinating *Ps* spores and infected plant.
[2]Expressed in infected plant.
[+]Nucleotide sequence similarity E-value.
[*]Fragment sizes are in bp. In cases where more than one TDF displayed closer similarity to the same GenBank record, only the largest product size is listed in the column.

ESTs, AC/CC R1-2, AC/CC R2-4, AC/CC R5-2 and AC/CG R3 were derived from transcripts of the genes that were not previously identified. GenBank similarity and coding sequence searches revealed that fragments correspond to ORF containing genomic segments. Gene prediction and sequence mapping of the AC/CC R1-2 TDF to the available Argentinian *P. sorghi* race RO10H11247 genome sequence, scaffold (LAVV01007405.1) has shown that fragment was derived from a transcript with a 327 bp ORF which resides on a minimum of 498 bp genomic segment containing three-exons intervened by two short introns (80 and 71 bp respectively), which encodes 108 aa polypeptide with an unknown function. This TDF contained all three exons along with 21 bp 5' UTR sequence. TDF AC/CC R5-2 corresponded to the 3' UTR of this gene. Sequence was GenBank accessioned as MN199982 (Table 2). Similarly, *Ps* genome blast searches revealed that TDFs, AC/CC R2-4 and AC/CG R3 were derived from the Scaffold_1712 (Sequence ID: LAVV01006518.1, Length: 7291 bp), which contained two tandemly duplicated genomic segments, each copy bearing a previously unknown gene whose first two exons and 3rd exon 5' end sequences are represented on AC/CC R2-4 while AC/CG R3 tag derived from the 5'-UTR sequence. RACE experiments were conducted to recover the transcript 3' end. A sequence data covering the partial 5'-UTR, coding sequence (CDS) and the (presumably) complete 3'-UTR were obtained. Aligning this sequence to the same scaffold indicated the presence of an extra intron residing in the 3' UTRs of the both copies. Sequences and duplicated gene annotations were GB accessioned as MT677935 and MT677936. All three genes (MN199982 MT677935 and MT677936) encode polypeptides that are similar in length and sequence containing a number of nearly perfect PYGGY repeats at their C-terminal ends and have typical signal peptides at the N-terminals (details are given in GenBank records)

Functional assessment of the identified ETSs provide information about the expression profile displayed during uredospore germination and host infection. The most abundantly expressed contig represented by four TDFs was phosphatidylethanolamine binding protein, which was detected in both germinating spores and infected leaf samples. Peptidyl tRNA hydrolase similar TDFs (contig) found in majority of the studies are similarly expressed in both germinating spores and infected plant tissues. Also, newly identified gene (MN199982, MT6777935 and MT6777936) contigs each represented by two TDFs are expressed in germinating spores and infected plant tissue (Figure 1). Similarly, three other gene contigs (Rab family protein GTPase, exodeoxyribonuclease V and hypothetic protein similar to steroid reductase) each represented by two TDFs expressed in germinating spores without information about their in planta expression. Genes identified with singleton similarities also bear functions that are known to be important in fungal growth and the process of host colonization:

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Chitinase, 28S rRNA encoding sequence, oligopeptide transporter, putative monocarboxylate transporter 2, hypothetic protein containing wax synthase domain, hypothetic protein similar to steroid reductase, cytochrome C oxidase subunit 1 appear to be examples of fungal growth promoting ones while cutinase domain containing protein, several small secreted protein encoding ones including three newly identified genes, signal peptide and velvet domain containing uncharacterized protein encoding messages presumably function in host invasion and pathogenesis. Protein (serine/threonine) kinase, zinc finger similar protein, nucleotide, nucleic acid binding hypothetical protein and syntaxin binding protein similar ones could have function in either or both of these processes.

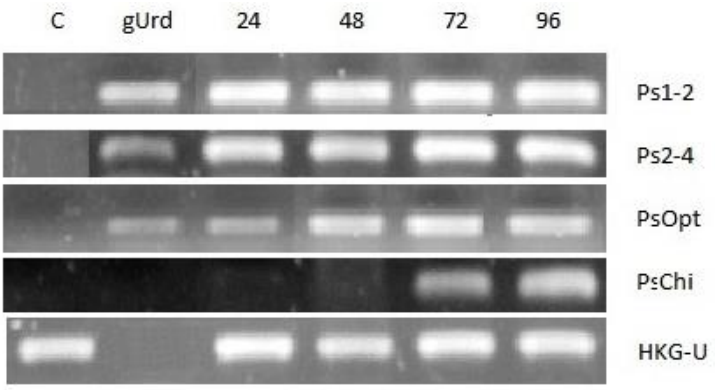

Figure 1. RT-sqPCR expression profiles obtained from the selected genes (*Ps1-2*, *Ps2*-4, *PsOPT*, *PsChi*) along with the maize housekeeping gene (*HKG-U*). Abbreviations and numbers indicate; C, uninfected plant control, gUrd, germinating uredospore, 24, 48, 72 and 96 hrs of post-inoculation plant material cDNA samples.

Expressions of four rust genes along with a maize housekeeping gene [16] were studied in germinating spore and infected plant material time course samples (Table 1). RT-sqPCR results revealed that *Ps1-2* (MN199982) *Ps2-4* (MT6777935 and MT6777936) and *PsOPT* are expressed relatively weakly in germinating spores whereas a marked expression increase was apparent in the infected plant material parallel to the post-infection hour increase as seen in Figure 1. Chitinase expression in germinating spores were found to be barely detectable and this was also the case at the initial infection hours while an apparent induction occurred at 72 and 96 post-infection hour infected plant materials.

4. DISCUSSION

Functional assessment of identified genes in transcriptome studies provides information about the functional spectrum and expressional modulations involved in the studied processes. Studies with the rust fungi have revealed that host penetration and colonization require functions involved in host defense suppression and acquiring nutrients from the host metabolism. Transcriptome analyses of spores and germinated urediniospores revealed that genes involved in cell proliferation, releasing energy from stored lipid reserves and processing them via glyoxylate/gluconeogenesis pathways are upregulated [17]. Haustoria, specialized infection structures formed inside the host cells, are the sites where energy production and biosynthetic process related functions are predominantly expressed

along with the haustorially expressed secreted proteins (HSPs) [11,17]. Many of these secreted proteins are also expressed in germinated spores [18].

Present survey reveals a function profile that appear to be important in fungal growth, host invasion and colonization. Four small-secreted proteins along with the oligopeptide and monocarboxylate transporters and several hydrolytic enzymes detected in the study (Table 2) corroborate the reported aspects of this type of studies. Up-regulation of transcripts coding for small secreted proteins, secreted hydrolytic enzymes, and transporters in germinating spores and in planta have been reported in a number of studies [11,18]. These functions, some of which are represented as the expanded gene families and up regulated during infection in rust fungi, presumably play important roles in host infection, nutrient acquisition and regarded as specific adaptations to the extreme parasitic lifestyle [19]. Small-secreted protein genes, many of which are known to be species-specific indication of rapid evolution, constitute an important component of the rust and mildew genomes and are presumed to be involved subverting host immune responses [11]. Chitinase expression during spore germination and host infection is also commonly detected message, which apparently functions in fungal morphogenesis and proliferation. A cutinase domain containing protein encoding message expression in germinating spores suggest that it participate in host invasion as well. Velvet domain containing proteins found in fungi are known to participate in fungal development, pathogenicity and virulence [20,21]. Detected message tag indicates that velvet proteins are also expressed during rust infections and may function in host invasion. ESTs individually displaying significant similarities to a serine/threonine kinase, a Rab family protein GTPase, a signal protein and a zinc finger protein presumably are utilized in signal transduction and transcriptional regulation during the early stages of the infection [22,23]. Lineage-specific family expansions in genes encoding zinc-finger proteins were observed in rusts. Nucleotide, nucleic acid binding proteins detected in the expression profile are also frequently reported and up-regulated messages in rust infections [18,19]. Phosphatidylethanolamine binding protein encoding transcript derived tags, detected as the most abundant EST in the expression profile, suggest that it may function in the processes of host penetration and colonization. Peptidyl-tRNA hydrolases, cleaving amino acid ester bond of the peptidyl-tRNAs, function as one of the rescue factors of the stalled ribosomes [24]. Accumulation of peptidyl-tRNAs is detrimental to cell, which could hinder fungal proliferation. These enzymes are regarded as target for antimicrobials. Two tags detected in the expression profile indicate that they are expressed and presumably function during *P. sorghi* infections.

In conclusion, as obligat biothrophic fungal pathogens, rusts are known to use intricate mechanisms in penetration and establishing intimate contacts to host cells and in manipulating host physiology and defense [25]. A number of genes that appear to be important in these mechanisms have been identified as ESTs in the present study. Several of them are presumably involved in signal transduction, such as, serine/threonine kinase, Rab4. Similar functions are being reported to be upregulated during urediniospore germination and plant infection [23]. Several other identified genes encode products, such as cutinase, secreted proteins, that are possibly involved in breaching host defenses and suppressing host immunity. A number other genes encodes functions that are involved in metabolite transfer, modification and fungal proliferation. The transcript levels of four selected genes in germinated urediniospores and infected maize leaves were studied by sqRT-PCR assays. All of them are expressed in germinated uredospores except for chitinase and induced in the infected material samples, which suggests that their products conceivably play some roles during spore germination and *P. sorghi* growth in plant tissues.

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