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Molecular Cloning of MAP Kinase Genes and *In silico* Identification of their Downstream Transcription Factors Involved in Pathogenesis of Karnal bunt (*Tilletia indica*) of Wheat

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Abstract

The mitogen-activated protein kinase (MAPK) pathways control diverse cellular functions in pathogenic fungi, including sexual differentiation, stress response, and maintenance of cell wall integrity. Here we identified and characterized *T indica* MAPK genes, which are homologues to the other fungi viz. Pmk1 (Pathogenicity MAP Kinase-1) and Fus3 that is known to play an important role in pathogenicity and mating pheromone response during fungal development. It was performed through gene specific genomic DNA amplification, cloning, sequencing, homology match, annotation, motif analysis, structure prediction and protein-protein interaction. Comparative genomics approaches were applied for studying the role of such genes involved in pathogenesis. These analyses give new insights into similarities and dissimilarities as well as specific characteristics of *T indica* genomes when compared with *Saccharomyces cerevisae* and *Ustilago maydis*. Multiple sequence alignment and motif analysis of these genes revealed high conserveness. The result of structure prediction showed that the models are authentic with 88.9% residues are found in most favored regions in TiFus3 while 83.1% residues in TiPmk1. Key functional regulations of these genes involved numerous biological processes including fungal development and pathogenesis can also be achieved through understanding of protein- protein interaction using a downstream target Ste12p transcription factor. Interaction between two different pairs of protein i.e. TiPmk1 with Ste12p and TiFus3 with Ste12p showed that TiPmk1 interact with Ste12p with -551.19 kcal total energy while TiFus3 interact with Ste12p -157.76 kcal total energy. There is currently no direct evidence for any direct activation of this transcription factor by MAP kinase in *T indica*, *in silico* interaction study provides ample opportunities to validate its role in the pathogenesis and fungal development. Thus, it will ultimately lead to help in the development of novel strategies for controlling the Karnal bunt (KB) disease of wheat, incited by *T indica*.

Keywords: *Tilletia indica*; Karnal bunt; TiPmk1; TiFus3; MAPK; Motif analysis; Protein-protein interaction

Introduction

The basidiomycete fungi *Tilletia indica* Mitra syn. *Neovossia indica* [1,2] causes Karnal bunt of wheat, an economically important disease which is prevalent in several countries. *Tilletia indica* has emerged as a useful model organism for studying the role of development in pathogenesis. Due to lack of an effective method for its control, the management of disease is only possible through understanding of the molecular mechanisms of disease progression. Management of the disease has become very much crucial due to the mode of dispersal of the pathogen, survival of teliospores in soil for a long period and unavailability of tolerant wheat cultivars. Most of the past investigations on development of fungal pathogenesis in other basidiomycete fungi have been directed on the role of pathogenesis related genes, which play important role in fungal development and infection [3]. However, mitogen activated signal transduction pathways play a crucial role in development of virulence levels in pathogens. Through mitogen activated protein kinase (MAPK) pathways, pathogens respond to external stimuli and alter their own features such as cell wall integrity, mating, morphological transition, adaptation to stress factors and this modification can leads to generate different virulence levels in phyto pathogens.

MAPK signaling pathways are ubiquitous and evolutionarily conserved in eukaryotic organisms connecting cell surface receptors to critical regulatory targets within cells that result in various morphogenetic processes [4]. MAP kinase activity is regulated through a tiered cascade composed of a MAP kinase (MAPK), a MAP kinase kinase (MAPKK), and a MAP kinase kinase kinase (MAPKKK). These enzymes are regulated by characteristic phosphorelay system in which a series of three protein kinases phosphorylate and activate one and another. In *S cerevisiae* MAP kinase controls the transduction of the pheromone signal in haploid cells while Kss1p, a gene in MAP kinase cascade regulates nitrogen starvation induced filamentous growth in diploid cells [5]. Homologues of *Fus3/Kss1* have been characterized in several pathogenic filamentous ascomycetes and play key roles in infection structure (appressorium) formation and host colonization [6]. A well-conserved MAP kinase gene Pmk1 is essential for fungal pathogenesis and for production of female reproductive structures in *Magnaporthe grisea* [7]. Unlike the situation in fungal-plant interaction, the Pmk1 like MAPK pathway is not required for virulence in the fungal-fungal interaction [8]. Identification of molecular mechanisms related to pathogenicity and development is very much crucial in framing biotechnological control measures against such pathogens. In these perspectives it is quite appropriate to study the MAPK genes in

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relation to pathogenesis and these studies will provide strong clues in-order to unveil the molecular mechanism associated with development and pathogenicity of *T indica*.

Within phytopathogenic and saprophytic fungi remarkable progress has been made in understanding which signal transduction pathways are required for controlling processes critical for disease. Three components of MAP kinase pathways have been identified by genome sequence analysis in the filamentous fungi *Neurosphora crassa* [9]. One of the predicted MAP kinases in *N crassa,* MAK-2, shows similarity to Fus3p and Kss1p of *Saccharomyces cereviceae*, which are involved in sexual reproduction and filamentation respectively [9]. Further studies have revealed how protein orthologous to Pmk1p/Fus3p/Kss1p are required for pathogenecity in many other phytopathogenic fungi [10]. The Fus3 MAP kinase pathway controls the transduction of the pheromone signal and is activated in response to the binding of a peptide-mating pheromone to cell type - specific pheromone receptors. In addition to activating transcription, transduction of the mating response results in reorientation of the cytoskeleton and secretary apparatus to polarize towards a mating partner [11]. Ultimately a complete understanding of the molecular basis of the ability of these diverse fungi to cause disease might be expected to reveal broad differences in the underlying mechanisms; however, it is also possible that conserved virulence factors common to all phytopathogenic fungi might be revealed. Over the past decade it has emerged that the signal transduction pathways which regulate key virulence functions are highly conserved across a wide range of plant pathogenic fungi [12].

Detection of mutant genes encoding components of the Pmk1p-dependent MAPK signaling pathway in *M grisea* (including the MAPK kinase kinase (MAPKKK) Mst11p, the MAPK kinase (MAPKK) Mst 7p and the adapter protein Mst50p) are a pathogenic in this species [13,14]. These components have been shown to physical interact which, together with their strong similarity to their yeast orthologues, supports the view that they act together within the same signaling pathway. Only in *S cerevisiae* has a clear link between the Ste12p and Pmk1p orthologue Fus3p been established and in this case modulation of Ste12p's activity is controlled indirectly via Fus3p's influence [15,16]. Further studies have revealed how proteins orthologues to Pmk1p/Fus3p/Kss1p are required for many other phytopathogenic fungi [10]. The product of MGSTE12 is a transcription factor in *M grisea* whose orthologue in *S cerevisiae* is activated by the Pmk1p orthologous Kss1p/Fus3p-dependent MAPK signal transduction cascades [17].

Availability of sufficient information about the understanding of the Pmk1p/Fus3/Kss1 signal transduction pathway and its role in fungal development and pathogenesis in diverse pathogenic fungi, however, nothing is known about the existence of such signaling pathway and its outputs in *T indica*. Attempts were made in present study to identify the MAP kinase genes from this economically important fungi through PCR amplification and designing of degenerate primers from other fungal systems. Taking advantages of evolutionarily conserved MAP kinase signal transduction pathways for regulating critical processes of disease development in diverse pathogenic fungi even distantly related with very different modes of infection, two homologues of MAP kinase (TiPmk1 and TiFus3) from KB were cloned from *T indica.* Highly reliable manual annotation of several complete fungal model genomes is a key resource for the fungal research community. With the advent of bioinformatics, which delivers the latest computational methodology and tools, it is possible to predict the role of each gene sequences during the life span and reproduction of a pathogen like *T indica*. In addition, they will also help to elucidate gene elements that have not been discovered so far or have been difficult to detect. In order to explore some potential outputs of Pmk1/Fus3 signaling pathway, an *in silico* interaction study were performed to identify the role of Pmk1/Fus3 regulated transcription factor Mst12p which impart its role in the activation of downstream proteins involved in the invasive growth, disease development, penetration and dimorphic switch/fungal differentiation.

Materials and Methods

Fungal culture and harvesting of mycelium

The fungi T *indica*, Pantnagar isolate (KBPN) was cultured in modified potato dextrose liquid media. All the constituents of the potato dextrose media were dissolved in distilled water. The liquid media (50 ml) was transferred to 250 ml conical flasks and autoclaved. Then these flasks were inoculated with mycelial discs or loop-full of inoculums from the slants prepared earlier. The cultures were incubated in BOD incubator at 22±2°C under light and dark conditions. The media containing the mycelial mat of *T indica* was filtered through a folded muslin cloth and washed several times in PBS (0.05 M, pH 7.2) and followed with sterilized distilled water. The wet mycelia were lyophilized for 5 hours to obtain the dry weight. Dry mycelial masses were stored in -80°C [18].

DNA extraction and quantification

In order to isolate DNA from the fungi *T indica*, Cetyltrimethyl ammonium bromide (CTAB) method was used [18,19]. The DNA was purified and quantified by the method as described by Sambrook et al. [20].

Designing of MAPK gene primers

The genes which are responsible in development of pathogenicity in various fungal systems were collected from the databases and alignment was done using CLUSTAL-W [21] Amino acid sequences of respective genes were aligned and primers were designed for the genes after identification of homologous regions by the back translation of homologous amino acid sequences. The primers for MAPKs were designed by using the *DNAStar* software (www.DNASTAR.com) [22] in such a manner to include the signature sequences of the MAPKs gene sub-family in the amplicons. In order to eliminate some mismatches ionosine nucleotides were inserted in the primers. The primers used were: TiPmk1 Fwd TGCGITTTTGGACTAGCGAGG & Rev CAGGTACGGGTGCTTGAGCGC and TiFus3 Fwd TGCGITTTTGGACTAGCGAGG & Rev GAAATACGGATGCTCCAGTGC which yield the product size of 600 and 500 bp respectively.

Genomic amplification and molecular cloning of MAP kinase genes (TiFus3 and TiPmk1)

PCR was carried out in 25 µl reaction mixture containing 10ng of genomic DNA, 200 µM each dNTPs, 0.3U Taq DNA polymerase enzyme,10mM Tris – HCl, 1.5mM MgCl2, 50mM KCl, 0.01% gelatin and 0.2 µM primer. Amplification was carried out three times in a Biometra T-gradient DNA thermocycler. PCR cycle was programmed as follow: initial denaturation of 5 min at 94°C; 35 cycles of denaturation at 94°C for 1 min, annealing at 38°C for 1 min and extension at 72°C for 2 min; followed by a final extension at 72°C for 7 min. the amplified products were stored at -20°C until they were subjected to electrophoresis. PCR products were electrophoresed in triplicare in 1.2% agarose gel in 0.5X TAE buffer along with DNA marker at the concentration of 250 ng/µl to identify respective bands.

The amplicons obtained by PCR were eluted from gel with the help of QIAquick Gel extraction kit as per manufacturer instructions (Qiagen GmbH, Hilden). The PCR amplicons were ligated in pGEM-T EASY vector system I (Promega Madison WI USA). For ligation, molar ratio of the vector and insert is very important. In the present study, standard 3:1 molar ratio (insert:vector) was used. Transformation was performed according to calcium chloride method. The competent cells of *E. coli* strain DH5α were prepared by $CaCl_2$ method [20]. Transformed bacterial cultures were spread over LB/ampicillin/IPTG/X-Gal plates and were incubated overnight at 37°C followed by screening for recombinants by blue white selection. The integrity of the cloned fragments was confirmed by analyzing the pGEMT-BMDL plasmid through colony PCR and restriction digestion. Nucleotide sequence of independent clones were determined with the dye terminator kit (ABI Prism, Perkin Elmer, NJ) and analyzed on Applied Biosystems 370 at University of Delhi.

In silico sequence analysis

The non-redundant database at NCBI (http://www.ncbi.nlm.nih.gov) was used to search for homologs of Pmk1 and Fus3 of using Blastp and blastx algorithm. All sequences (Figures 1 and 2) from different fungi were aligned using ClustalW method and phylogenetic and molecular evolutionary analysis was done using UPGMA (**U**nweighted **P**air **G**roup **M**ethod with **A**rithmetic Mean) method based on hierarchical clustering of MEGA version 4.0.02 [23]. Each node was tested using the bootstrap approach by taking 1,000 replications and a random seeding of 64,238 to ascertain the reliability of nodes. The number indicated in percentages against each node. The branch lengths are drawn to scale indicated.

Motif analysis

To identify the conserved motifs within the protein sequences of Pmk1 and Fus3 in *T indica* and other fungi, the deduced protein sequences of all the members of different fungi were analyzed by means of the online MEME (Multiple Expectation Maximization for Motif Elicitation) tool version 3.5.7 [24]. To identify conserved motifs in these sequences, the selection of maximum number of motif was set to 10 with minimum and maximum width was set 20 and 50 respectively while other factors were of default selections.

Three dimensional Structure prediction and evaluation

Complete coding region of *U maydis* and *S cereviseae* was taken respectively through homology search of TiPmk1 and TiFus3 genes due to only the partial sequence of TiPmk1 and TiFus3 genes of *Tillitia indica* are available. For constructing the structures of Pmk1, Fus3 and Ste12p, the template for homology modeling was searched with BLAST program on the Protein Data Bank (www.rcsb.org/pdb/) [25]. The sequence that showed maximum identity with high score and less e-value with the related query was used as a reference structure to build a 3D model. Three dimensional structures of Pmk1 and Fus3 were modeled using MOE software version 2010.10 (Chemical Computing Group's Molecular Operating Environment) [26] and energies were also minimized using parameters such as Force field= MMFF94X, Gradient= 0.05, Cutoff: On=8, off=10, Solvation: Dielectric=1, Exterior=80. Secondary structure components of the all three sequences were analyzed using SWISS-PDB viewer (http://spdbv.vital-it.ch) and Rasmol tools. The refined model was subjected to a series of tests for validation of consistency and reliability using PROCHECK [27]. Protein-Protein interaction between Pmk1 and Fus3 with their downstream partner Ste12p was performed by HEX docking software.

Results and Discussion

Mitogen-activated protein kinase (MAPK) cascades are the most conserved signal transduction systems in eukaryotes. Activated MAPKs (phosphorylated MAPKs) are often imported into the nucleus, where they phosphorylate and activate specific downstream signaling components such as transcription factors like Ste12p [28]. To understand the mechanism of pathogenesis of Pmk1 & Fus3 were cloned and their structural and interaction analysis was also performed using various bioinformatics tools.

MAP kinase genes (TiFus3 and TiPmk1) and its cloning

In the present study, attempts were made to study the role of MAP Kinase signaling in *T indica*. The primer sets used were efficient in reproducible amplification of expected sizes of amplicons 400 (TiPmk1) and 600bp (TiFus3) showed in Figure 1. The amplicons were cloned, nucleotide sequence of independent clones were determined with the dye terminator kit (ABI Prism, Perkin Elmer, NJ) and analyzed on Applied Biosystems 370 at University of Delhi, New Delhi. The obtained sequences 300bp (TiPmk1) and 223bp (TiFus3) were shorter than the size estimated by gel electrophoresis as the terminal sequences could not be read with confidence by using this technique. The cloned nucleotide sequences of TiPmk1 and TiFus3 were submitted as partial DNA to NCBI database and have received the accession numbers as FJ571362.1 and HQ268553 respectively and Protein id ACL98079.1 and AEB34779.1 for *T indica*.

Sequence analysis

Multiple sequence alignment of a representative set of 25 Pmk1 genes was done by Clustal-W method. Conserved region of all protein shown in Figure 2a. Serine/Threonine protein kinases active-site signature & protein kinase domain enclosed in black colored rectangle which are also present in TiPmk1. Phylogenetic tree of Pmk1 was constructed by UPGMA method of MEGA version 4.0.02. Phylogenetic tree of Pmk1 showed four major clusters A, B, C and D as shown in Figure 2b. *T indica* & *U maydis* are present in same cluster D. Through

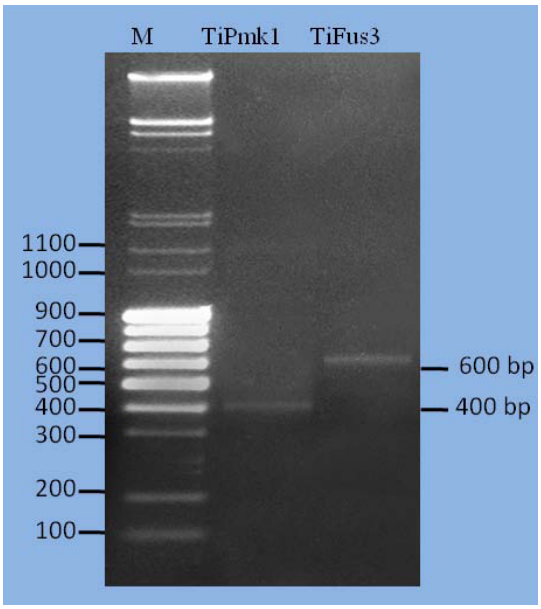

Figure 1: *Tilletia indica* genomic DNA amplification with MAPK gene specific primers and its cloning. Lane 1: M- DNA ladder, Lane 2: TiPmk1 and Lane 3: TiFus3.

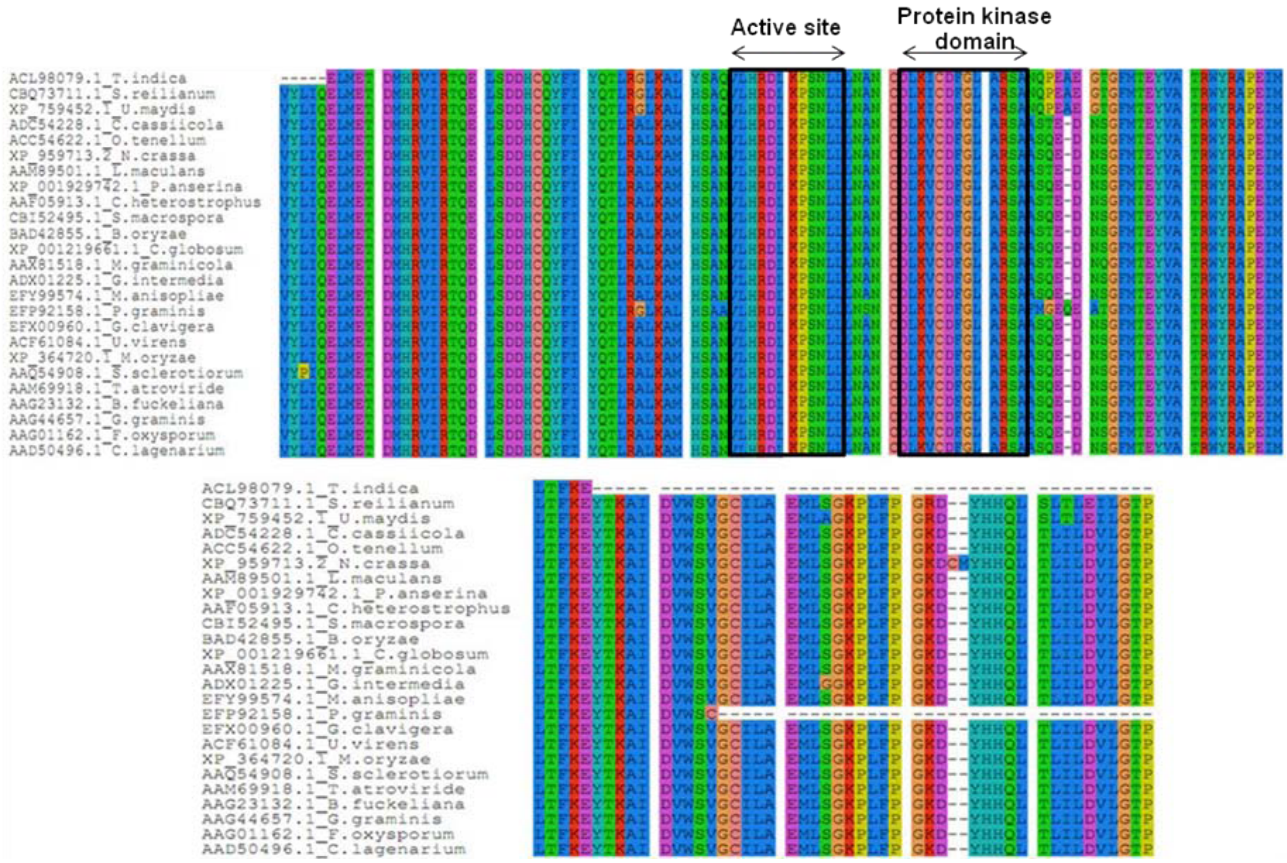

Figure 2a: Multiple Sequence alignment of amino acid sequences of Pmk1 genes. The conserved signature motifs of the Pmk1 are highlighted by enclosing in black colored rectangle.

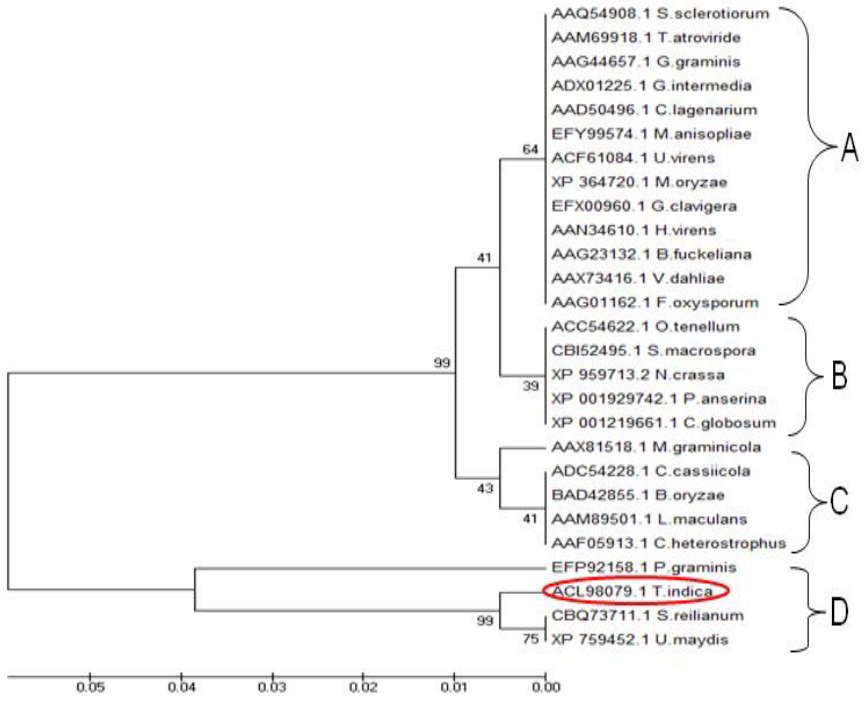

Figure 2b: Phylogenetic Tree of Pmk1 constructed by UPGMA method of MEGA version 4.0.02. Pmk1 of *T. indica* is highlighted by red color circle.

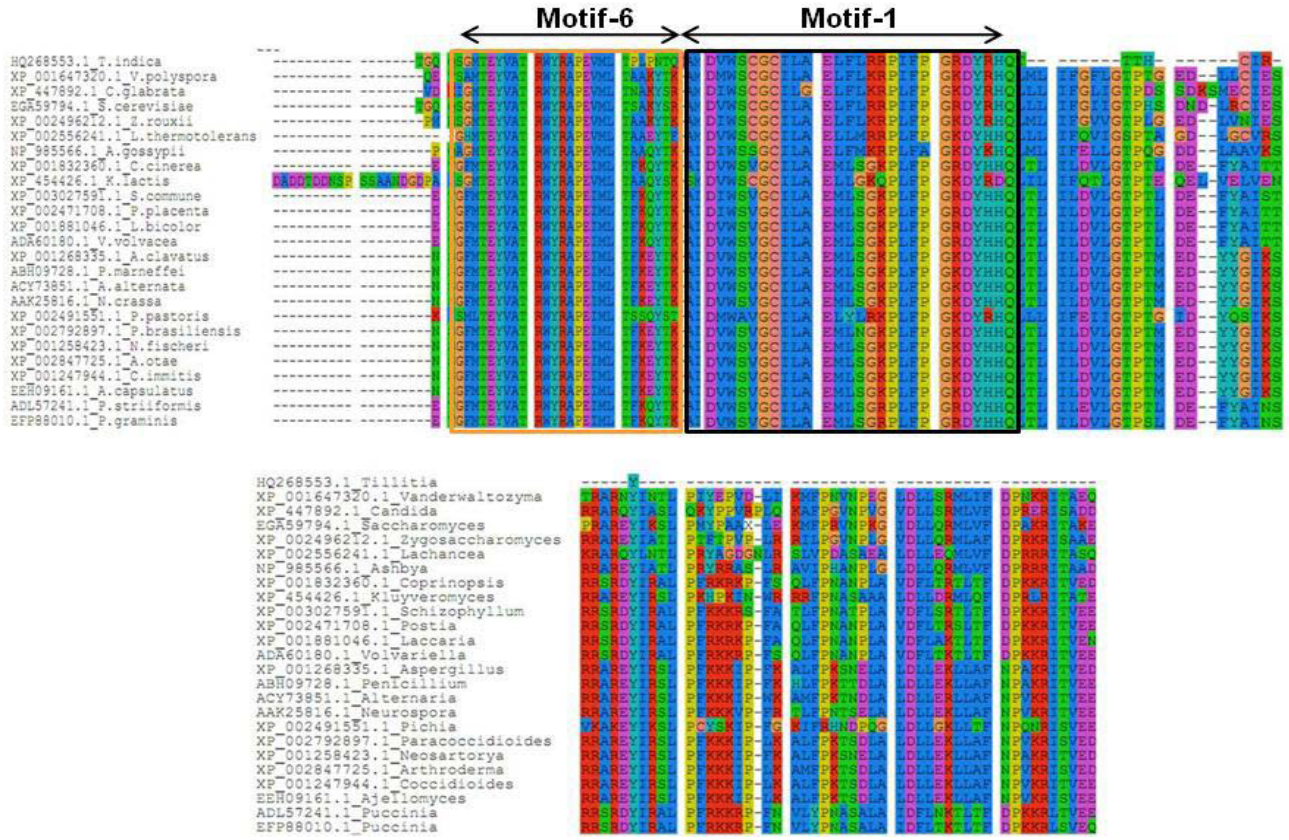

Figure 3a: Multiple Sequence alignment of amino acid sequences of Fus3 genes from different fungi. The conserved signature motifs of the Fus3 are highlighted by enclosing in orange and black colored rectangle.

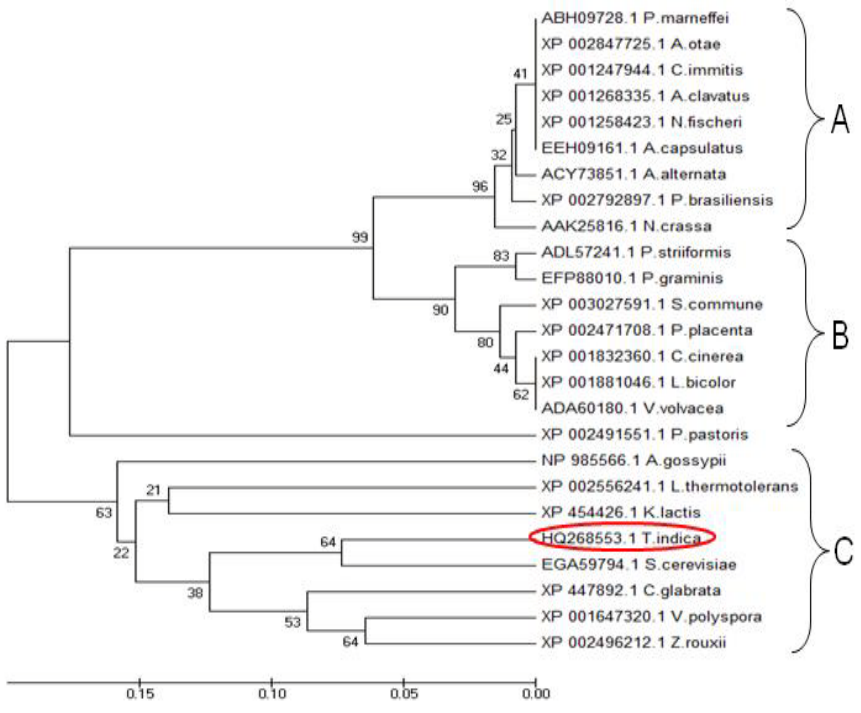

Figure 3b: Phylogenetic Tree of Fus3 constructed by UPGMA method of MEGA version 4.0.02. Fus3 of *T. indica* is highlighted by red color circle.

phylogenetic analysis it could be interpreted that many of the genes involved in fungal growth and pathogenesis are conserved throughout the evolutionary process.

In case of Fus3, Multiple sequence alignment of 25 Fus3 protein sequences from different fungi was done. Conserved region shown in orange and black colored rectangle which are showing motif 6 and motif 1 respectively (Figure 3a). Both motifs contain protein kinase domain which present in all MAP kinase. Three major clusters A, B and C were shown in phylogenetic tree (Figure 3b). *T indica* and *S cerevisiae* are present in same cluster C indicating that TiPmk1 and TiFus3 are two homologues present in *T indica* and might be imparting different roles through activation of different MAPK signaling pathways in response to different stimuli at upstream levels.

Motif analysis

All protein sequences of Pmk1 genes contain all 10 motifs except *M anisopliae, P graminis* and *T indica. M anisopliae* which contains only nine motifs because motif 2 is absent while *P graminis* contain eight motifs, motif 5 and 8 are absent. Due to partial sequence of *T indica*, which has only two motifs 1 and motif 7 representing Serine/Threonine protein kinase active-site and protein kinase domain respectively. The residues of these motifs are also involved in interaction with Ste12p, downstream partner of Pmk1.These motifs also shown in multiple sequence alignment of Pmk1 (Figure 2a). In all Pmk1, motif-1 is most commonly observed and contain Serine/Threonine protein kinases active-site signature. Motif-2 is also present most frequently in all Pmk1 with n-glycosylation site, protein kinase phosphorylation site, casein kinase II phosphorylation site and n- myristoylation site. Motif-3 also contain casein kinase II phosphorylation site. Motif 4 contain n-glycosylation site beside the protein kinase domain. Motif 5 and motif 6 both contain casein kinase II phosphorylation site. Like other fungi, motif 7 is also present in the *T indica*, which is having n- myristoylation site and protein kinase domain. Motif 8, 9 and 10 contain protein prenyltransferases alpha subunit repeat, protein kinase C phosphorylation site and casein kinase II phosphorylation site respectively. Block diagram of multilevel consensus sequences of Pmk1 gene for the MEME defined motifs is shown in Figure 4 and Table 1.

In case of Fus3 all protein sequences contain all 10 motifs except *S cerevisiae* and *T indica* which contain 7 and 2 number of motifs respectively. Motif 3, motif 5 and motif 8 are absent in *S cerevisiae*, *T indica*, contain only two motifs 1 and motif 6 due to partial sequence, which both having protein kinase domain and protein kinase C phosphorylation site. These motifs also shown in figure 3 of multiple sequence alignment of Fus3. Motif 2 having Serine/Threonine protein kinases active-site signature and also protein kinase c phosphorylation site. Motif-3 was also most commonly observed which contain protein kinase phosphorylation site, casein kinase II phosphorylation site and n- myristoylation site. Motif 4 is most commonly observed in all sequences which contain protein kinase phosphorylation site, casein kinase II phosphorylation site and protein kinase domain. Motif 5 and motif 7 contain casein kinase II phosphorylation site and protein kinase C phosphorylation site respectively. Motif-8 which is most commonly observed in all Fus3 sequences, contain n-glycosylation site. Motif 9 & 10 do not show significant function. Multilevel consensus sequences

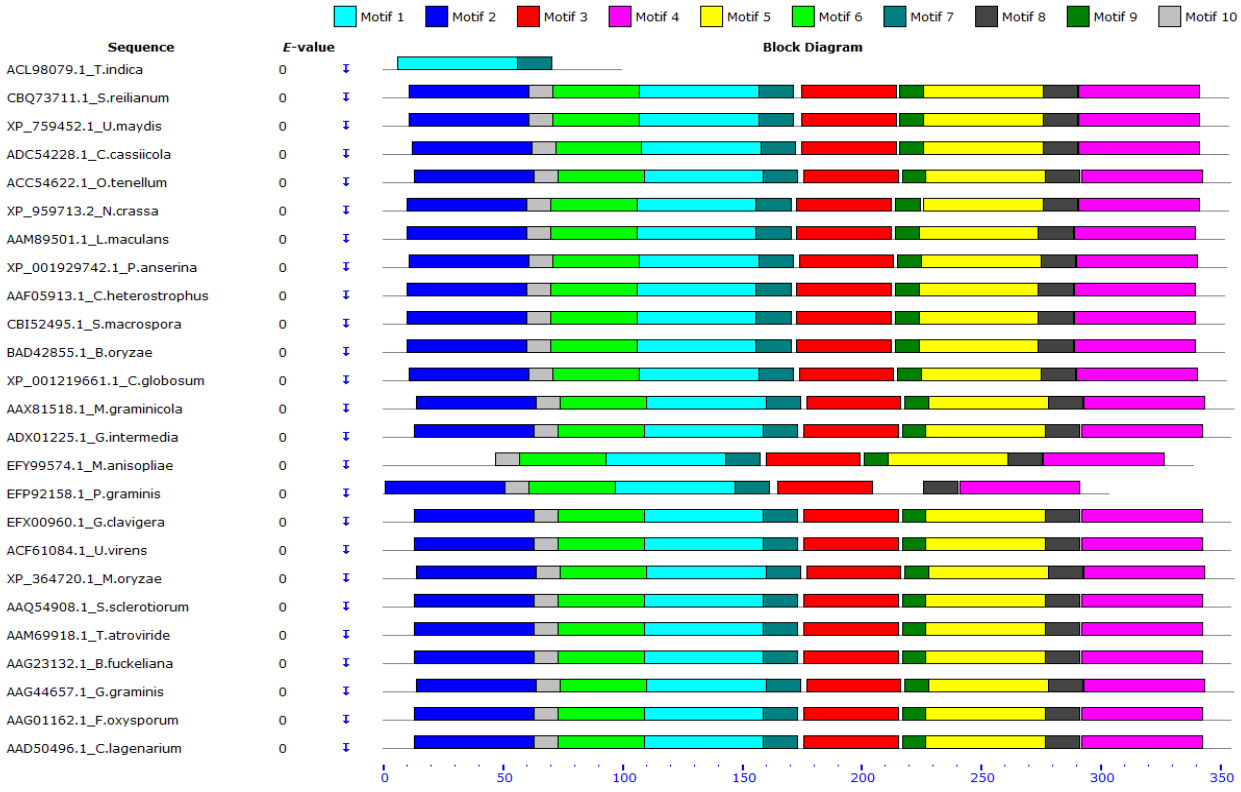

Figure 4: **Block Diagram of Multilevel consensus sequences for the MEME defined motifs of Pmk1 proteins:** Ten motifs were obtained by MEME software. Different motifs are indicated by different filled boxes with numbers 1 to 10. Names of all the members from different protein and E values are shown on the left side of the figure, and a scale is located at the bottom of the figure to indicate relative sizes of the motifs.

S.No.	E value	Len	Sequence Pmk1
1.	4.2e-1204	50	MHRVIRTQELSDDHCQYFIYQTLRALKAMHSANVLHRDLKPSNLLLNANC
2.	1.3e-1075	50	KISFNVSEQYDIQDVVGEGAYGVVCSAIHKPSGQKVAIKKITPFDHSMFC
3.	4.2e-941	39	DNSGFMTEYVATRWYRAPEIMLTFKEYTKAIDVWSVGCI
4.	5.4e-993	50	AFNPVKRITVEEALKHPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLSK
5.	1.5e-972	50	GKDYHHQLTLILDVLGTPTMEDYYGIKSRRAREYIRSLPFKKKVPWRTMF
6.	2.5e-746	36	RYFNHENIISILDIQKPRNYETFNEVYLIQELMETD
7.	1.3e-279	14	DLKVCDFGLARSAA
8.	1.4e-174	14	PKTSDLALDLLEKL
9.	4.9e-161	10	EMLSGKPLFP
10.	6.1e-147	10	LRTLREMKLL

Table 1: Multilevel consensus sequences for the MEME defined motifs of Pmk1.

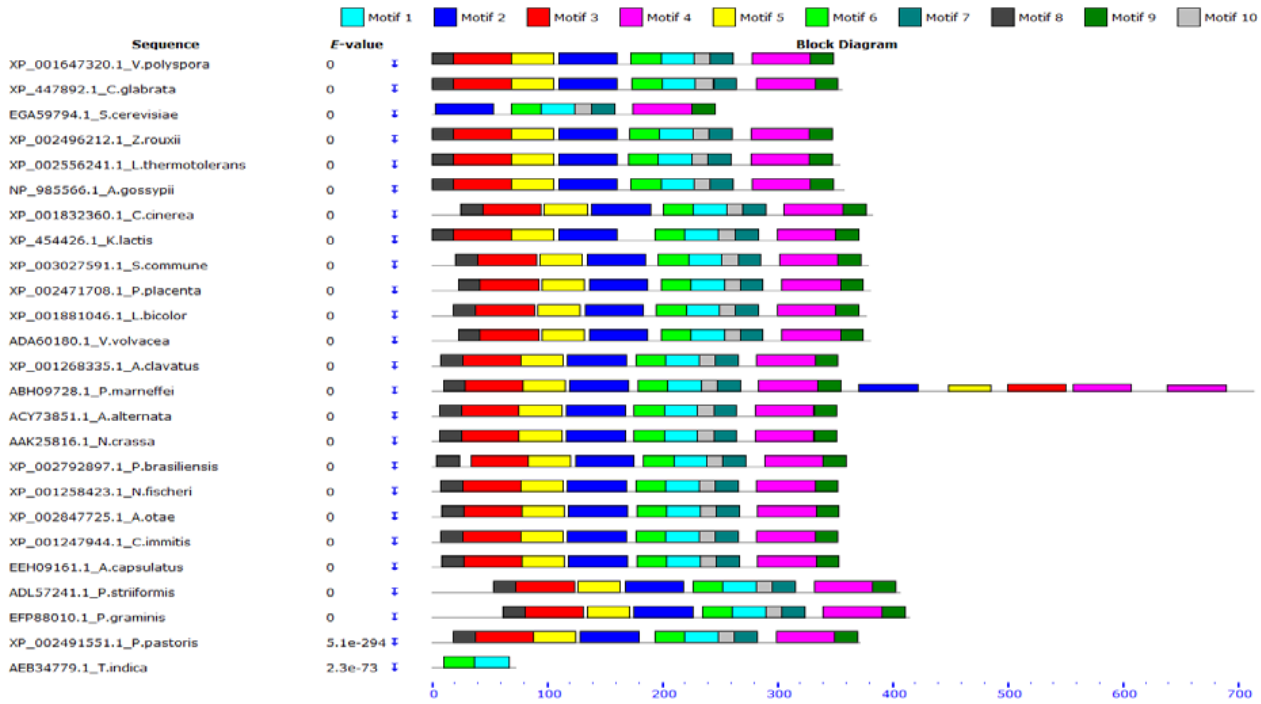

Figure 5: **Block Diagram of Multilevel consensus sequences for the MEME defined motifs of Fus3 proteins:** Ten motifs were obtained by MEME software. Different motifs are indicated by different filled boxes with numbers 1 to 10. Names of all the members from different protein and E values are shown on the left side of the figure, and a scale is located at the bottom of the figure to indicate relative sizes of the motifs.

S.No.	E value	Len	Sequence
1.	7.8e-607	29	AIDVWSCGCILAEMLSGKPLFPGKDYHHQ
2.	4.5e-1029	50	DDHCQYFIYQTLRALKAMHSANVIHRDLKPSNLLLNANCDLKVCDFGLAR
3.	5.2e-879	50	GEGAYGVVCSAVHKPSGQKVAIKKITPFDHSMFCLRTLREMKLLKYFNHE
4.	2.6e-763	50	VDLLEKMLTFNPKKRITVEEALRHPYLEPYHDPDDEPTAPPIPPEFFDFD
5.	8.4e-620	36	NIISILDIQKPRSYESFNEVYLIQELMETDMHRVIR
6.	7.8e-553	26	GFMTEYVATRWYRAPEIMLTFKQYTK
7.	2.6e-231	19	DYYCIKSRRAREYIRSLPF
8.	2.3e-195	19	MGRKISFNVSSQYQIQDVV
9.	1.8e-187	19	HKDDLSKEQLKQLIWNEIM
10.	1.9e-160	14	LTLIFDVLGTPTME

Table 2: Multilevel consensus sequences for the MEME defined motifs of Fus3.

of Fus3 gene for the MEME defined motifs is shown in Figure 5 and Table 2.

Understanding of molecular mechanism of KB disease and pathogenesis is not explored so far. However, it is possible that conserved pathogenicity factors common to all phytopathogenic fungi are also present in *T indica*. Over the past decade, a family of serine/threonine protein kinase known as MAPKs is involved in the transduction of a variety of extracellular signals which regulate key

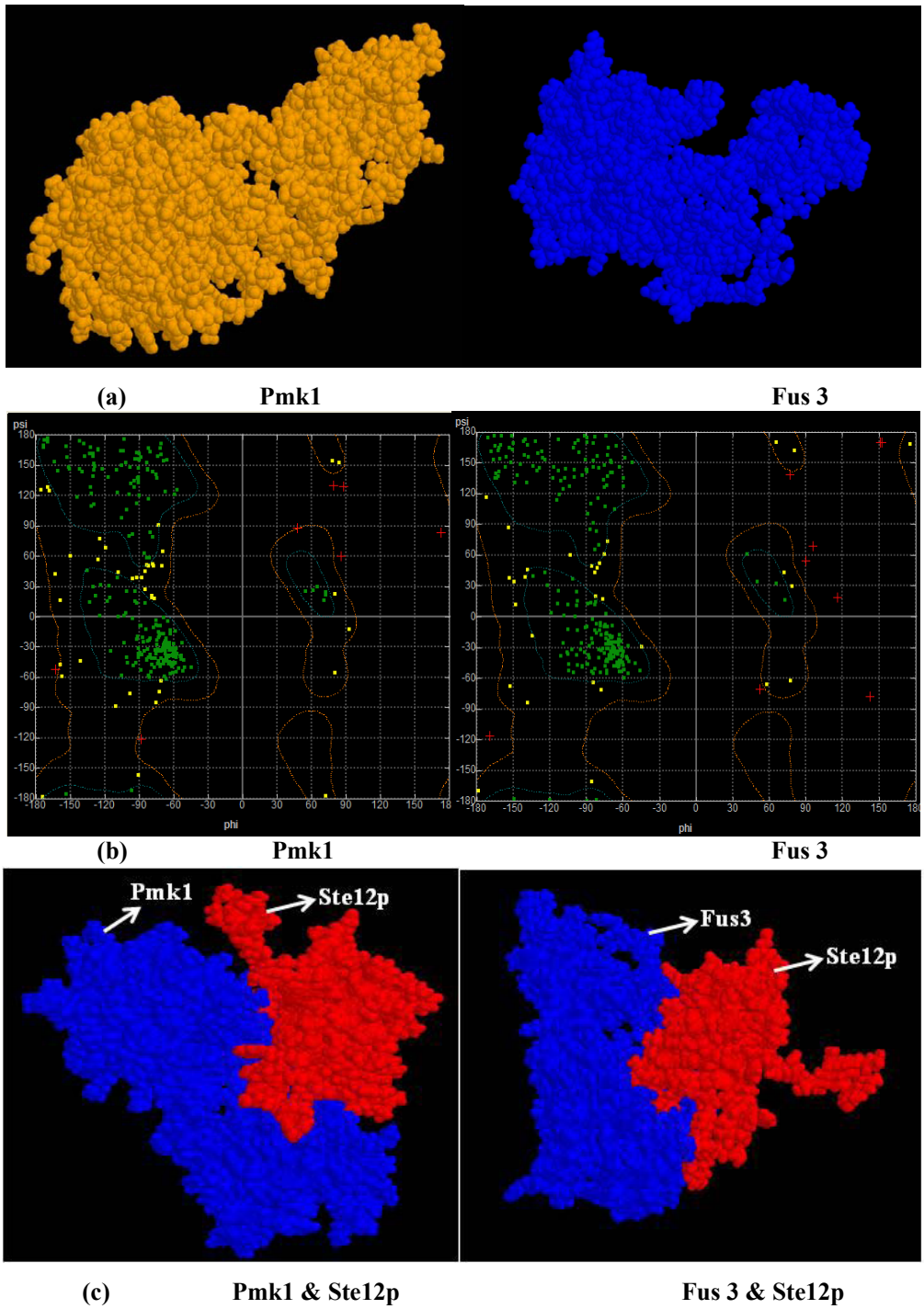

Figure 6: **(a)** Space fill model representation of Pmk1 & Fus3: 3 D structures were constructed by MOE & visualization done by Rasmol.
(b) Ramachandran's plot of Pmk1 & Fus3 respectively
(c) Protein–Protein Interaction between Pmk1 & Ste12p and Fus3 & Ste12p

virulence functions are highly conserved across a wide range of plant pathogenic fungi.

Structure analysis

Some species such as *M grisea* develop specialized infection structures in order to directly penetrate the plant surface, others meanwhile seem to rely more heavily on the digestion of the host surface by lytic enzymes or, as is the case for *T indica*, gain entry through the stomata and probably also triggers MAPK dependent signaling pathways mediated by Pmk1 and Fus3. In order to elucidate the involvement of MAPK components, protein – protein interaction has been done using bioinformatics tool for getting clues about pathogenesis of diseases.

Structures of *T indica* Fus3 and Pmk1 were homology modeled using MOE software taking the NMR structure of PDB accession no. 2B9H.A and PDB Id 2B9.A respectively as a template (Figure 6a). For each molecule 11 structures were generated, out of which the minimized average model with maximum score was selected. The initial energy of the protein was calculated (in kcal/mol) by GizMOE using MMFF94 X force field. The energies of the designed structures were minimized using the energy minimization tool of MOE. For energy minimization we used two parameter MMFF94x Force field and 0.05 gradient.

For interaction homology modeling of fungal Ste12p was also done through MOE version 2010.03 taking PDB ID 3HEJ.A as a template. Energies of these structures were also minimized through MOE. The 3-D structures were verified at PROCHECK and Protein Geometry tool of MOE. Correspondingly, Ramachandran's plot was also constructed to validate the structure (Figure 6b). The results showed that these models are authentic with 88.9% residues are found in most favored regions in Fus3 while 83.1% residues in Pmk1. 10.4% and 15.3% residues are also observed in additional allowed regions in Fus3 & Pmk1 respectively. The stable structures are further used for interaction with Ste12p protein.

Key functional regulations of numerous biological processes involved in the pathogenesis of diseases can also be achieved through targeting protein- protein interaction. Therefore interaction between two different pairs of protein i.e. Pmk1 with Ste12p and Fus3 with Ste12p was done using HEX 5.1 software (Figure 6c). Pmk1 interact with Ste12p with -551.19 kcal total energy while Fus3 interact with Ste12p -157.76 k cal total energy. The residues involved in interaction are shown in the Table 3.

Residues involved in interaction b/w Pmk1 & Ste12p		Residues involved in interaction b/w Fus3 & Ste12p	
Pmk1	**Ste12p**	**Fus3**	**Ste12p**
Val26	Leu14	Asn70	Met1
Glu29	Ala18	Arg79	Val3
Gly30	Leu43	Phe83	Gln4
Tyr32	Phe46	Met179	Asn7
Gly33	Pro51	Phe236	Ser8
Val34	Trp54	Ile238	Arg9
Val35	Gln55	Ile239	Thr10
Met59	Gln58	Thr241	Glu11
Phe60	Ile59	His243	Phe45
Thr64	Ile60	Arg257	Ala50
Gln140	Arg61	Glu258	Trp54
Glu174	Arg62	Tyr259	Arg55
Glu176	Tyr63		Asn57
Arg262	Tyr64		Ile58
Pro295	Leu65		Ile60
Arg296	Asn66		Phe99
Lys297	Lys98		
Glu302	Phe99		
Pro316	Leu128		
Glu317	Pro131		
Glu319	Lys132		
Ala322	Leu139		
Glu323	Lys146		
Pro324	Lys152		
Leu325	Phe155		
Asp326	Trp156		
Glu351	Glu317		

Table 3: Residues involved in interaction.

During evolution phytopathogenic fungi have developed diverse mechanisms by which to infect their hosts. In response to pheromone, regulation of Ste12p activity in *S cerevisiae*, the Fus3 MAPK pathway (Ste11-Ste7-Fus3) relays a phosphorylation signal to reach the Dig1p/2p complex, leading to dissociation and Ste12p phosphorylation [29]. In true filamentous fungi, Ste12 like proteins play essential roles in sexual development and pathogenicity. Ste12p was also identified as a major regulator of yeast invasive growth and pseudohyphal development, as a target of the Kss1p MAPK cascade which is homologous of Pmk1 [29]. The sequential activation of the MAPK cascade eventually results in the activation of specific class of fungal transcription factors, grouped under the term Ste12- like factors and expression of specific sets of genes in response to environmental stimuli. The corresponding Ste12 protein was originally found to be a target of the Fus3 MAPK cascade regulating mating. TiPmk1 and TiFus3, MAP kinase genes isolated from the *T indica*, are probably involved in the signaling infection structure formation. Based on the analysis of the Pmk1, Fus3 & Ste12 p and the available data in the literature it is also hypothisized that in *T indica* interaction between TiPmk1 & Ste12p may be responsible for invasive growth and pseudohyphal development and interaction between TiFus3 & Ste12 p may be responsible for disease development in host plant. The specific role of TiPmk1 and TiFus3 in infection related morphogenesis and interaction between downstream partner suggest that conserved fungal signal transduction pathways might provide lead to identify useful target for the future designing of the antifungal molecules.

Conclusion

With the advent of genomics approaches that have been applied to monitor downstream components of specific MAPK pathways viz. Pmk1 and Fus3 interact with their downstream partner Ste12p like transcription factor and conserve as good model to elucidate their role in the disease in response to different environmental stimuli. This will enable to provide opportunities in better understanding of mechanism(s) of pathogenesis which will ultimately lead to the development of anti-fungal molecule(s) for the management of fungal diseases. This wills also opens the possibilities to identify and distinguish a diversity of responses controlled by Ste12 and Ste12-like proteins activated by either TiPmk1 or TiFus3 and in their upstream activated by different stimuli, allowing the adaptation of various *Tilletia sp* to their ecological niche, differential sexual development, morphological transition and exerting virulence mechanisms.

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References

1. Mitra M (1931) New bunt on wheat in India. Ann Appl Biol 18: 178-179.
2. Mundukur BB (1943) Karnal bunt air borne disease. Curr Sci 12: 230-249.
3. Lev S, Sharon A, Hadar R, Ma H, Horwitz BA (1999) A mitogen – activated protein kinase of the corn leaf pathogen Cochliobolus heterostrophus is involved in conidiation, appressorium formation and pathogenicity: Diverse

roles for mitogen-activated protein kinase homologous in foliar pathogens. PNAS 96: 13542-13547.

4. Chang L, Karin M (2001) Mammalian MAP kinase signaling cascades. Nature 410: 37-40.
5. Gustin MC, Albertyn J, Alexander AM, Davenpont K (1998) MAP kinase pathways in the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev 62: 1264-1300.
6. Xu JR (2000) Map kinases in fungal pathogens. Fungal Genet Biol 31: 137-152.
7. Zheng L, Campbell M, Murphy J, Lam S, Xu JR (2000) The BMP1 gene is essential for pathogenicity in the gray mold fungus Botrytis cinerea. J Molecular Plant-microbe interactions.13: 724-732.
8. Collopy PD, Amey RC, Sergeant MJ, Challen MP, Mills PR, et al. (2010) The pmk1-like mitogen-activated protein kinase from Lecanicillium (Verticillium) fungicola is not required for virulence on Agaricus bisporus. Microbiology 156: 1439-1447.
9. Pandey A, Roca MG, Read ND, Glass NL (2004) Role of a Mitogen-Activated Protein Kinase Pathway during conidial germination and hyphal fusion in Neurospora crassa. Eukaryotic Cell 3: 348-358.
10. Zhao X, Mehrabi R, Xu JR (2007) Mitogen-activated protein kinase pathways and fungal pathogenesis. Eukar Cell 6: 1701-1714.
11. Chen RE, Thorner J (2007) Function and regulation in MAPK signaling pathways: lessons learned from the yeast Saccharomyces cerevisiae. Biochim Biophys Acta 1773: 1311-1340.
12. Kramer B, Thines E, Foster AJ (2009) MAP kinase signalling pathway components and targets conserved between the distantly related plant pathogenic fungi Mycosphaerella graminicola and Magnaporthe grisea. Fungal Genet Biol 46: 667-681.
13. Zhao X, Kim Y, Park G, Xu JR (2005) A mitogen-activated protein kinase cascade regulating infection-related morphogenesis in Magnaporthe grisea. Plant Cell 17: 1317-1329.
14. Park G, Xue C, Zhao X, Kim Y, Orbach M, et al. (2006) Multiple upstream signals converge on the adaptor protein Mst50 in Magnaporthe grisea. Plant Cell 18: 2822-2835.
15. Brückner S, Köhler T, Braus GH, Heise B, Bolte M, et al. (2004) Differential regulation of Tec1 by Fus3 and Kss1 confers signaling specificityin yeast development. Curr Genet 46: 331-342.
16. Chou S, Huang L, Liu H (2004) Fus3-regulated Tec1 degradation through SCFCdc4 determines MAPK signaling specificity during mating in yeast. Cell 119: 981-990.
17. Bardwell L, Cook JG, Voora D, Baggott DM, Martinez AR, et al. (1998) Repression of yeast Ste12 transcription factor by direct binding of unphosphorylated Kss1 MAPK and its regulation by the Ste7 MEK. Gen Dev 12: 2887-2898.
18. Seneviratne JM, Gupta AK, Dinesh P, Indu S, Anil K (2009) Determination of genetic Divergence based on DNA markers amongst monosporidial strains derived from fungal isolates of Karnal bunt of Wheat. J Plant Pathol 25: 303-316.
19. Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res 8: 4321-4325.
20. Sambrook J, Fritch EF, Maniatis T (1989) Molecular cloning a laboratory manual 2nd ed. N. Y. cold spring Harbor Laboratory, cold spring Harbor Laboratory Press, 1959.
21. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W, improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680.
22. DNASTAR, Madison, Wisconsin.
23. Tamura K, Dudley J, Nei M, Kumar S, et al. (2007) MEGA4, Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol Biol Evol 24: 1596-1599.
24. Bailey TL, Williams N, Misleh C, Li WW (2006) MEME: discovering and analyzing DNA and protein sequence motifs, Nucleic Acids Res 34: W369-W373.
25. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, et al. (2000) The protein data bank. Nucleic Acids Res 28: 235-242.
26. Molecular Operating Environment (MOE), (2010) Chemical Computing Group Inc 1010 Canada.
27. Laskowski RA, MacArthur MW, Moss DS, Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Crystallogr 26: 283-291.
28. Khokhlatchev AV, Canagarajah B, Wilsbacher J, Robinson M, Atkinson M, et al. (1998) Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell 93: 605-615.
29. Wong Sak Hoi J, Dumas B (2010) Ste12 and Ste12-Like Proteins, Fungal Transcription Factors Regulating Development and Pathogenicity Eukaryot Cell 480-485.