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In silico-prediction of downstream WRKY interacting partners of MAPK3 in *Brassica*

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Abstract:
Protein-Protein interactions (PPIs) are vital to most biological processes thus the identification of PPIs is of primary importance. Here, we endeavor to identify the downstream interacting partners of (BjMPK3P) in *Brassica juncea* using the docking approach. Out of 63 and 37 members of BrWRKY and BnWRKY transcription factors, 50 and 29 members are showing interactions with BjMPK3P respectively while the rest are showing non-interaction. Twenty two WRKY members are common to both the species. Using minimal sequence motif search as well as through docking approach several novel WRKY interacting proteins were also reported in the present study which need to be confirmed by in vitro kinase assay. Together, the results obtained essentially enhance our knowledge of the MAPK interacting protein network and provide a valuable research resource for developing a nearly important link between pathogen-activated MAPK signaling pathways and downstream transcriptional programming.

Key words: Protein -Protein interactions (PPIs), Transcription factor (TF), docking, MAPK

Background:
Mitogen-activated protein kinases (MAPKs) play central roles in the signaling network as terminal components of MAPK cascades, which are composed of at least three sequentially activated MAPK family modules MAP3Ks (for MAPK kinase kinase), MAP2Ks (for MAPK kinase), and MAPKs **[1].** The MAPKs are known to regulate a myriad of physiological and developmental responses such as cell growth, cell differentiation, hormone signaling, pathogen infection, wounding, drought, low temperature, high salinity etc. **[2].** Although very little is known about MAPK's downstream targets but despite this gap in our knowledge it is clear that MAPKs interact with the transcription factors **[3].** Five major families of plant transcription factors, including bZIP, WRKY, MYB, EREBF and homeodomain protein, have been shown to play roles in the regulation of the plant defense response **[4].** Generally, it is believed that the WRKY family of transcription factors plays major roles in plant responses to biotic and abiotic stresses and during development **[5-6].** WRKY proteins constitute an important link between pathogen-activated MAPK signaling pathways and downstream transcriptional programming. WRKY proteins are characterized by a stretch of the amino acids tryptophan (W), arginine (R), lysine (K), and tyrosine (Y), followed by a typical zinc-finger domain, and constitutes a large class of DNA-binding proteins in plants **[7].** WRKY proteins bind W-box sequences (TTGACC/T) in the promoter region of target genes **[8].** It is difficult to identify the downstream interacting partners or the substrates of MAPKs, primarily because MAPK-substrate interactions are very transient and unstable. Many experimental methods have been developed to study the protein-protein interactions including yeast two hybrid systems, affinity purification followed by mass spectrometry and the phage display libraries, but these methods have its own limitations and suffer from high false positive rate **[9].** In order to overcome these limitations *in silico* studies has been carried out. For studying protein –protein interactions *in silico* ''Docking'' strategy is extensively used in mitogen-activated protein kinase (MAPK) signaling **[10-11].** Regulation of protein activity is required for functional signaling pathways and metabolism. Protein interactions can be regulated by post-translational modifications. Protein phosphorylation is one of the most common posttranslational

modifications in eukaryotic organisms and is involved in almost all cell biological processes. The phosphorylation of serine, threonine and tyrosine residues can affect protein structure, enzymatic activity and subcellular localization, interaction with other proteins as well as it is crucial in signal transduction **[12].** In eukaryotes MAPKs are catalytically inactive in their base state and require phosphorylation. The dual-specificity MAP2Ks phosphorylate MAPKs on both serine/threonine and tyrosine residues in the activation loop **[13].** Once activated, MAPKs phosphorylate many evolutionarily diverged substrates on serine or threonine residues within a minimal S/T-P motif **[14-15].** To mimic this regulation activity we phosphorylated the BjMPK3 protein at threonine (196) and tyrosine (198) residue in TEY motif located in the activation loop (T-loop). In an effort to better understand the protein-protein interactions, we have generated a protein – protein network based on docking approach to predict the downstream interacting WRKY proteins in *Brassica* with BjMPK3P.

Methodology:

The sequences for WRKY transcription factors were downloaded from BRAD (*Brassica* database **[16]** for *Brassica rapa* WRKY TF (BrWRKY), from *Brassica* genome gataeway **[17]** for *Brassica napus* WRKY TF (BnWRKY) and the sequence of *Brassica juncea* (BjMPK3-KF420418) is downloaded from NCBI **[18]** in FASTA format. Homology modeling of the BjMPK3 and WRKY transcription factors was done with the help of MOE (Molecular Operating Environment). For constructing the structures of all, a template for homology modeling was searched with PDB search Programme of MOE. For each molecule 10 structures were generated in the database, out of which the minimized average models with maximum score, lowest E-value and with a cut off sequence identity of < 40% were selected. Structure of BjMPK3 was phosphorylated (BjMPK3P) with MOE as phosphorylation is essential for its enzymatic activity. The final structures were done after constructing and evaluating 3D models. Structural refinement through energy minimization model was performed using energy minimization tool keeping parameter value constant for all structures i.e Gradient: 0.5, MMFF94x Forcefield Cutoff: On=8, Off=10 Solvation: Dielectric=1, Exterior=80. The minimized structures were finally saved as *.pdb files which were validated by Ramachandran plot. After structure formation the refined structure of phosphorylated BjMPK3P was taken as receptor and the structures of WRKY transcription factor family (BrWRKY & BnWRKY) were taken as ligand for the docking studies on the online patch dock server **[19]** which is based on shape complementarity principles and results were refined using FireDock on-line server **[20]** which rearranges the interface side chains and adjusts the relative orientation of the molecules. Taking the global energy of the interacting WRKY transcription factors with MPK3 reported in the literature (WRKY 8, 20, 22, 28, 29, 33, 40, 47, 53 and 69) **[3 & 21-24]** as a criteria, we identified the interacting and non interacting partners of BjMPK3P. After docking, the results were analyzed.

Results & Discussion:

Networks of protein–protein interactions provide a framework for understanding the biological processes and can give insights into the mechanism of diseases. Thus the understanding of biological mechanisms requires the knowledge of protein-protein interactions. MAPK is a conserved link between cell receptor and cell response and is mediated through gene expression which is regulated by transcription factors. To best of our knowledge no work in the literature has been done regarding the prediction of BjMPK3P with WRKY transcription factor family (BrWRKY & BnWRKY) in *Brassica.* As WRKY transcription factor reported to play prominent roles in the regulation of the plant defense response [5-6]. Therefore, the paper focuses on identifying the interacting WRKY transcription factors with BjMPK3P of *Brassica juncea* which is involved in disease signaling process through docking approach. Docking studies use geometric and steric considerations to fit the two proteins (BjMPK3P and WRKY) into a bound complex, the more stable the complex structure (less global energy) higher the probability of their interaction. The docking studies performed here, suggested that out of 63 and 37 members of BrWRKY and BnWRKY transcription factors, 50 and 29 members are showing interaction with BjMPK3P respectively while the rest are showing non-interaction **Table 1 (see supplementary material).** Twenty two WRKY members are common to both the species, whereas (BrWRKY 35 & BrWRKY 72) is showing interaction in *B. napus* and (BnWRKY 25 & BnWRKY 39) is showing interaction in *B. rapa.* These results indicate that protein-protein interactions are might be species specific. A similar study was carried out to predict the downstream interacting partners of MPK3 in *Arabidopsis thaliana* through Support vector machine (SVM) **[25].** 31 WRKY transcription factors (1, 4, 6, 9, 15, 17, 21, 22, 23, 33, 40, 43, 44, 45, 46, 47, 52, 54, 57, 58, 59, 60, 61, 63, 64, 66, 67, 68, 69, 70 and 71) are found to be the common interacting partners of MPK3 through both docking and SVM approach. Recently Sorensson et al. (2012) determined the primary sequence specificity of *Arabidopsis* MPK3 and MPK6 substrates **[26].** They indicated a minimal motif sequence L/P-P/X-S/T-P-R/K by random positional peptide library search to be the substrate for both the kinases. In an another study conducted by Hoehenwarter et al. (2013) they have reported other sequences surrounding the minimal motif S/T-P (Table-2) through the use of tandem metal oxide affinity chromatography to be the MPK3/6 substrate **[27].** Using the minimal sequence motif identified in the above studies, we have derived a list of potential novel substrates (WRKY transcription factors) from *Brassica* which also showed interaction with BjMPK3 through docking studies **Table 2 (see supplementary material).** It could be hypothesized that the amino acids surrounding the minimal S/T-P motif contribute to MAPK specificity. Those motif sequences are also considered in *Brassica* in which the given hydrophobic residue is being replaced by the other hydrophobic residue and so and so for. The results of our study clearly revealed the complexity of BjMPK3P interaction with several WRKY transcription factors triggered in response to diverse upstream stimuli. Number of novel candidate BjMPK3 substrates was predicted and need to be confirmed by in vitro kinase assay.

Conclusion:

The PPI networks can give insights into the mechanisms of diseases and provide a spectrum for the understanding of biological processes. Interaction networks can aid in designing signal transduction pathway and help to find the disease suppressive agents as well as uncover the key genes those are

responsible for senescence and defense responses against pathogens.

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Supplementary material:

Table 1: WRKY transcription factor family (BrWRKY & BnWRKY) genes showing interaction with BjMPK3P.

BrWRKY Showing Interaction with BjMPK3P	50	WRKY1, WRKY7, WRKY8, WRKY9, WRKY11, WRKY15, WRKY16, WRKY17, WRKY18, WRKY19, WRKY20, WRKY21, WRKY22, WRKY23, WRKY25, WRKY26, WRKY27, WRKY28, WRKY29, WRKY30, WRKY32, WRKY34, WRKY37, WRKY39, WRKY40, WRKY43, WRKY44, WRKY45, WRKY46, WRKY47, WRKY49, WRKY50, WRKY51, WRKY52, WRKY53, WRKY54, WRKY57, WRKY58, WRKY59, WRKY60, WRKY61, WRKY62, WRKY63, WRKY64, WRKY66, WRKY67, WRKY68, WRKY69, WRKY70, WRKY71
BnWRKY Showing Interaction with BjMPK3P	29	WRKY4, WRKY6, WRKY7, WRKY8, WRKY11, WRKY15, WRKY17, WRKY18, WRKY20, WRKY21, WRKY22, WRKY26, WRKY27, WRKY28, WRKY29, WRKY32, WRKY33, WRKY35, WRKY40, WRKY44, WRKY45, WRKY46, WRKY50, WRKY53, WRKY69, WRKY70, WRKY72, WRKY74, WRKY75

Table 2: List of potential interacting WRKY transcription factors identified through both minimal sequence motif search and docking approach.

Peptide sequence	***Brassica rapa***	***Brassica napus***
NIMGVESNVQPLTS(ph)PLSK	VQSPL (WRKY 23)	FMSPL (WRKY 74)
	LHSPL (WRKY 60)	LHSPL (WRKY 72)
	FMSPL (WRKY 64)	
FSSLSLLPSQTS(ph)PKESR	TTSPK (WRKY 34)	TTSPK (WRKY 46)
IHHPPS(ph)PR	AASPR (WRKY 9)	-
IHHPSS(ph)PR	VSSPR (WRKY 28)	ISSPR (WRKY 35)
LLPLFPVTS(ph)PR	VSSPR (WRKY 28)	ISSPR (WRKY 35)
TYVADVSEYLGSNS(ph)PRDPYLER	TSSPR (WRKY 16)	TNSPR (WRKY 35)
	TNSPR (WRKY 27)	SSSPR (WRKY 32)
	SSSPR (WRKY 27)	
	QSSPR (WRKY 67	
VAPIPPS(ph)PVKVPQPVPEPVVLEPPQMFVDQR	PASPV (WRKY 57)	-
ATDILQGS(ph)PVESGPTTPLPDKK	MASPV (WRKY 20)	SVSPV (WRKY 32)
	SVSPV (WRKY 53)	
	SLSPV (WRKY 67)	
SHLRPPGNISGSQS(ph)PVESSGLYHSK	WTSPV (WRKY 51)	HTSPV (WRKY 22)
		STSPV (WRKY 22)
YSVDMS(ph)PVKIFK	ENSPV (WRKY 53)	-
YREATNLIPS(ph)PR	AASPR (WRKY 9)	-
TLPVAVVEVVKPES(ph)PVLVIVEKPK	LESPV (WRKY 51)	LESPV (WRKY 20)
	LESPV (WRKY 52)	
	LESPV (WRKY 70)	
SHLRPPGNISGSQSPPVES(ph)PGSYHSK	GESPG (WRKY 20)	-
HATIQPFDVLPS(ph)PTFSAAR	LISPT (WRKY 25)	GLSPT (WRKY 20)
	LASPT (WRKY 29)	LPSPT (WRKY 26)
	LLSPT (WRKY 51)	LASPT (WRKY 29)
	LPSPT (WRKY 58)	
LLHSAYDPQNRPAIEVHLVQVQPAGISADLDSTSNDAGHSS(ph)PTRK	CQSPT (WRKY 53)	
EGYSQSQSRPVYGLS(ph)PTLNHR	LISPT (WRKY 25)	GLSPT (WRKY 20)
	LASPT (WRKY 29)	LISPT (WRKY 25)
	LLSPT (WRKY 51)	LPSPT (WRKY 26)
	LPSPT (WRKY 58)	
SSWTSESYQLKPQSSFSGSHPSGS(ph)PNAR	NPSPN (WRKY 71)	-
IITDYVGS(ph)PATDPMR	GLSPA (WRKY 70)	GLSPA (WRKY 2)
	GLSPA (WRKY 20)	GLSPA (WRKY 4)
MKLPLDIDSPTQSENSSSSQQT(ph)PKSASSR	NSTPK (WRKY 16)	
LLEHFLVQEQTEGSS(ph)PSR	GSSPS (WRKY 11)	ASSPS (WRKY 53)
	LSSPS (WRKY 32)	PTSPS (WRKY 50)
	ASSPS (WRKY 49)	GSSPS (WRKY 6)
MVLFPKS(ph)PSPVNK	VRSPS (WRKY 19)	VRSPS (WRKY 40)
STPVRKPHTSTADLLTWSEVPPPDSP(ph)SSASR	EHPSS (WRKY 20)	-
	DSPSS (WRKY 40)	
	DQPSS (WRKY 60)	