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In-silico Interaction Studies of *Alternaria brassicae* Toxin Destruxin B and Potential Partners of MAPK4 Cascade

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Abstract

Alternaria blight is one of the important fungal diseases of *Brassica* plant which leads to major yield losses as well as deterioration in quality. Plant responds toward fungal attack through intricate signal transduction pathways involving MAPKs. In the present study an effort was taken to delineate the MAP kinase pathway involving MAPK4 through *in-silico* interaction studies. Here we have reported that destruxin B has strong interaction with Lys M receptor kinase and Lys M receptor and toxin interaction was differential with different host. Lys M receptor kinase showed strong interaction with MAPKKK19. Out of ten MAPKKs known four MAPKK *viz.* MAPKK3, MAPKK5, MAPKK8 and MAPKK9 showed good interaction with MAPK4. Among these four interacting MAPKKs, MAPKK9 showed best interaction with MAPKKK19. At the downstream of the cascade MAPK4 showed strong interaction with WRKY 25 and WRKY 40.The results of the present study clearly indicate the role of MAPK4 in plant defense against *Alternaria* blight.

Highlights

- Destruxin-B shows strong interaction with Lys M receptor kinase.
- Lys M shows strong interaction with MKKK19.
- MKK9 shows strong interaction with MKKK19.
- MKK3, MKK5, MKK8 & MKK9 show strong interaction with MPK4.
- MPK4 shows strong interaction with WRKY25 & WRKY40.

Keywords: *alternaria* blight, *brassica*, destruxin B, MAPK, superimpose, *In-silico* interaction

Alternaria blight is one of the most devastating fungal diseases which affects the majority of cruciferous crops and is one among the important diseases of rapeseed mustard caused by *Alternaria brassicae* leading to substantial yield losses. No *Brassica* species are known to be resistant to *Alternaria* blight (Pedras *et al*., 2003). Molecular level studies have shown that there are at least three components involved in *Alternaria* blight disease development- two toxin (chlorotic and necrotic) and a phytohormone.

Alternaria brassicae produces four cyclic depsipeptide as phytotoxin, named as destruxin. Destruxin B is the main phytotoxin which is produced by *Alternaria brassicae* (Bains *et al.,* 1987; Tewari *et al*., 1997; Agarwal *et al.,* 1994; Ayer *et al*., 1987; Buchwaldt *et al*., 1992) Homo destruxin B, Destruxin B2 and desmethyl destruxin B are the other phytotoxic compounds which is secreted by this fungus (Tewari *et al*., 1997; Montemurro *et al.,* 1992). Out of these Destruxin-B is considered as concentration dependent host selective toxin. Over the years much effort has been placed to understand the plant-pathogen /host specific toxin interaction. There are evidences in literature which support the view that toxin act through action of cell signal transduction (Taj *et al*., 2004).

MAPK cascade is most conserved signaling cascade across the eukaryotes which act downstream of receptors or sensors to transduce extracellular stimuli, including abiotic and biotic stresses into adaptive intracellular responses (Qiu *et al*., 2008). MAPK cascade minimally comprises the mitogen activated protein kinase kinase kinase (MAPKKK), which phosphorylate the mitogen activated protein kinase kinase (MAPKK), further MAPKK activate mitogen activated protein kinase (MAPK) through phosphorylation. Phosphorylation events comprise a fundamental element of signal transmission in living cells. Mitogen-activated protein kinase (MAPK) signaling pathways have important roles in both basal defense and R gene–mediated resistance (Ishihama *et al*., 2011).

Different MAPK cascades are present in a single cell and often share common components. There is some crosstalk between pathways, but MAPK cassettes appear to be insulated from each other by the intrinsic specificity of the MAPKKs and MAPKKKs and by binding interactions that are thought to organize the cassette into multi-enzyme complexes. Despite of the extensive study of the involvement of the MAPK in plant defense, the cascades through which MAPKs transduce the signals are largely unknown (Ishihama *et al*., 2011).

There are number of reports indicating that both biotrophic and necrotrophic pathogens are generally recognized by plant specific receptor protein (Gao *et al*., 2008; Jones and Dangl, 2006). This recognition of pathogen leads to the activation of multiple signaling cascades (Hammond-kosak and parker, 2003; Llorente *et al.,* 2005) through the receptor mediated activation of MAPKKK. Activation of MAPKKK ultimately activate MAPK cascade (Taj *et al*., 2010). Activated MAPK regulate the expression of defense proteins through phosphorylation of transcription factor. Some members of WRKY transcription family are regulated by MAPKs at the transcriptional and posttranscriptional levels in defense related signaling pathways (Ishihama *et al*., 2011). It is possible that the PAMP of *Alternaria brassicae* and its toxin affects some of the key components of this highly conserved MAP kinase cascade. A current challenge in this research area is to determine which signaling pathways in the plant make use of which MAPK components during elicitation of plant defense response.

Therefore, it would be logical to assume that knowledge of pathogen recognition and transmission of stimuli to the signaling pathway could play a crucial role in disease resistance. Although there are some wet lab methods for addressing protein-protein interaction or probing protein partners, these methods have some limitations like identification of correct solutions, proper dealing with ûexibility and conformational changes. For single protein in a cell various possible binding partners ranging from few to several hundred are reported (Zacharias 2010; Gavin *et al*., 2002; Rual *et al*., 2005). Nowadays computational approaches are often used to predict how two proteins interact and form a complex. Several protein-protein docking servers have been developed for this purpose. In the present study, attempt has been made to decipher the cascade regulated by MAPK 4 with the study of interaction of destruxin B, host selective toxin of *Alternaria brassicae*, with different receptor kinase reported for necrotrophic fungi like Lys-M RLK, CHRK1, LRPK1, WAK and ERECTA and the potential partners of cascade. It was realized that such studies will help in identification of members of MAPK cascade regulated by MAPK4.

Materials & methods

In-silico analysis to find out possible MAP Kinase cascade involving MAPK 4

Molecular modelling

Homology modelling of Dextruxin B, receptor like kinases, MAPKs, MAPKKs, MAPKKKs and WRKY transcription factors were done with the help of MOE (Molecular Operating Environment). For constructing the structures of all, a template for homology modelling 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. Three dimensional Structures of all substrate

were homology modeled using MOE software (Chemical Computing Group's Molecular Operating Environment) taking the different PDB Id as a template. 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 structure 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 online by PROCHECK.

Superimposition of protein structures

Superimposition of protein structures were performed by MOE (Molecular operating environment) software. Superimposition indicates that till what extent the two structures are similar. Lower RMSD value indicates better superimposition of structures and vice versa.

Molecular docking

To find out the potential partners of MAPK cascade involving MAPK4, we performed the *in silico* docking by the patch dock on-line server (Schneidman-Duhovny *et al*., 2005) http://bioinfo3d.cs.tau.ac.il/PatchDock/ which is based on shape complementarity principles and results were refined using FireDock on-line server (Andrusier *et al*., 2007; Mashiach *et al*., 2008) http://bioinfo 3d. cs. tau. ac.il/FireDock/ which rearranges the interface side chains and adjusts the relative orientation of the molecules. Structures of different receptor kinases from different species were molecularly docked with Dextruxin B toxin of *Alternaria brassicae*. MAPK4 was docked with the all known MAPKKs. To find out the upstream members of MAPKKs in the signaling module, again the docking was performed with some of the members of MAPKKKs family whose activity in plant defense has been reported these were MAPKKK1, MAPKKK2, MAPKKK12 and MAPKKK19. Docking of these four MAPKKKs were performed with that receptor like kinases in different species that showed better interaction with dextruxin B. To find out the downstream targets of MAPK4, the docking was performed with some WRKY transcription factors whose activity was already been reported in plant defense to complete the cascade.

Results

Superimposition of structures among MAPKKs, MAPKKKs and WRKYs.

To correlate the tertiary structures with function, we superimposed the MAPKKs structures, MAPKKKs structures and WRKY structures among themselves. On the basis of superimposition result MAPKK could be categorized into three groups- A, B and C. Group A have MAPKK1 and MAPKK4, group B have MAPKK 2, MAPKK 3, MAPKK 6, MAPKK7, MAPKK 8 and MAPKK 10 and group C contains MAPKK 5 and MAPKK 9. MAPKKK1 shows superimposition with MAPKKK2, MAPKKK12 and MAPKKK 19 but MAPKKK12 shows better superimposition with MAPKKK19 than other MAPKKK. WRKY is a large family of transcription factor, out of several member of WRKY we have selected few in our study and these were WRKY 21, 25, 29, 33, 40, 53 and 69. Again on the basis of superimposition WRKY studied in this work could be categorised in to two groups .Group A include WRKY 21, WRKY 53 and WRKY 69 and Group B contains WRKY 25, WRKY 33, WRKY 29 and WRKY 40 (Table 1, 2 & 3).

***In-silico* Protein- Protein interaction**

Since Destruxin B is the chlorotic toxin produced by the fungus *Alternaria brassicae* and is primarily responsible for the disease development, we performed the docking of different receptor like kinases from different species with the destruxin B. We found that lysM receptor like kinase (*Arabidopsis thaliana*, *Oryza sativa*, *Ricinus communis* and *Glycine max*), CHRK1 receptor like kinase in *Senecio squalidus* and WAK receptor like kinase in *Arabidopsis thaliana* showed better interactions on the basis of global energy (it means lowest the energy more is the stability of the interaction) (Table 4). Docking results also showed the differential interaction of Destruxin B with LysM receptor kinase in different host plants .To predict the downstream members of cascade, we performed the docking of those MAPKKKs (MAPKKK1, MAPKKK2, MAPKKK12 and MAPKKK19) which have been reported in the literature that takes part in the defense with those receptor like kinases in different species that showed better interaction with dextruxin B. It was found that MAPKKK19 showed better interaction with LysM receptor like kinase (*Ricinus communis*), WAK receptor like kinase (*Arabidopsis thaliana*) and

Table 1: Table showing the RMSD value of superimposition of different MAPKKs

Superimposition	RMSD value	Superimposition	RMSD value
MAPKK1-MAPKK2	5.09	MAPKK3-MAPKK10	2.92
MAPKK1-MAPKK3	5.23	MAPKK4-MAPKK5	9.45
MAPKK1-MAPKK4	2.11	MAPKK4-MAPKK6	6.16
MAPKK1-MAPKK5	8.79	MAPKK4-MAPKK7	6.45
MAPKK1-MAPKK6	5.11	MAPKK4-MAPKK8	6.13
MAPKK1-MAPKK7	5.28	MAPKK4-MAPKK9	8.56
MAPKK1-MAPKK8	5.23	MAPKK4-MAPKK10	5.86
MAPKK1-MAPKK9	9.10	MAPKK5-MAPKK6	9.76
MAPKK1-MAPKK10	5.16	MAPKK5-MAPKK7	9.63
MAPKK2-MAPKK3	3.02	MAPKK5-MAPKK8	9.89
MAPKK2-MAPKK4	6.12	MAPKK5-MAPKK9	0.73
MAPKK2-MAPKK5	9.89	MAPKK5-MAPKK10	8.61
MAPKK2-MAPKK6	1.94	MAPKK6-MAPKK7	2.21
MAPKK2-MAPKK7	2.38	MAPKK6-MAPKK8	2.18
MAPKK2-MAPKK8	2.36	MAPKK6-MAPKK9	10.2
MAPKK2-MAPKK9	10.5	MAPKK6-MAPKK10	2.05
MAPKK2-MAPKK10	2.02	MAPKK7-MAPKK8	0.98
MAPKK3-MAPKK4	9.72	MAPKK7-MAPKK9	10.4
MAPKK3-MAPKK5	9.98	MAPKK7-MAPKK10	1.95
MAPKK3-MAPKK6	2.79	MAPKK8-MAPKK9	10.7
MAPKK3-MAPKK7	2.87	MAPKK8-MAPKK10	1.92
MAPKK3-MAPKK8	2.79	MAPKK9-MAPKK10	9.98
MAPKK3-MAPKK9	10.8		

Table 2: Table showing the RMSD value of superimposition of different MAPKKKs

Superimposition	RMSD value
MAPKKK1- MAPKKK2	2.56
MAPKKK1- MAPKKK12	2.30
MAPKKK1- MAPKKK19	2.84
MAPKKK2- MAPKKK12	4.09
MAPKKK2- MAPKKK19	4.06
MAPKKK12- MAPKKK19	2.57

MAPKKK2 was found to interact better with CHRK1 receptor like kinase (*Senecio Squalidus)* (Table 5). Docking results of all 10 MAPKKs with MAPK4 showed that MAPKK5, MAPKK8, MAPKK3 and MAPKK9 have better interaction with MAPK4 (Table 6). To complete the cascade and to predict the upstream members of MAPKs in the signaling module, again the docking of all 10 MAPKKs was performed with same members of MAPKKKs family which were docked earlier with receptor like kinases.Docking result suggests that MAPKK9 is activated by MAPKKK2/MAPKKK19 (Table 7).

Table 3: Table showing the RMSD values of superimposition of different WRKY transcription factors

Superimposition	RMSD value	Superimposition	RMSD value
WRKY21-WRKY25	9.01	WRKY29-WRKY33	3.05
WRKY21-WRKY29	9.31	WRKY29-WRKY40	2.03
WRKY21-WRKY33	8.80	WRKY29-WRKY53	9.46
WRKY21-WRKY40	8.73	WRKY29-WRKY69	9.21
WRKY21-WRKY53	1.41	WRKY33-WRKY40	3.05
WRKY21-WRKY69	0.91	WRKY33-WRKY53	8.71
WRKY25-WRKY29	3.00	WRKY33-WRKY69	8.82
WRKY25-WRKY33	1.14	WRKY40-WRKY53	8.55
WRKY25-WRKY40	3.26	WRKY40-WRKY69	8.67
WRKY25-WRKY53	8.69	WRKY53-WRKY69	4.82
WRKY25-WRKY69	8.97		

Table 4: Global energy of interaction between dextruxin B and different receptor like kinases of various species.

Receptor/species	CHRK1	ERECTA	LYS	WAK	LRPK1
Arabidopsis thaliana	-40.34	-36.94	-60.39	-55.94	-36.19
Ipomoea trifida	-46.63				
Nicotiana tobacum	-43.77				
Oryza sativa	-48.56	-51.41	-55.06		-35.24
Populus trichocarpa	-42.73	-39.37	-51.17	-53.65	
Ricinuscommunis	-38.65	-30.33	-56.12	-42.48	-34.03
Senecio squalidus	-58.42				
Zea mays	-45.55	-44.08	-48.24		
Glycine max		-44.28	-54.04		-44.28
Hordeum vulgare		-44.24	-51.10		
Marchantia polymorpha		-43.59			-41.31
Physcomitrella patens		-34.43			-36.13
Gossypium hisutum					-51.45
Malus x domestica					-41.13
Piacea glauca					-40.20
Lotus japonicus			-49.75		
Madicago truncatula			-46.06		

Table 5: Global energy of interaction between MAPKKKs and different receptor like kinases of various species.

Receptor/ MAPKKK1, MAPKKK2,MAPKKK12 &MAPKK19	LYS				CHRK1	WAK
Species	*Arabidopsis thaliana*	*Glycine max*	*Oryza sativa*	*Ricinus communis*	*Senecios qualidus*	*Arabidopsis thaliana*
Global energy	-45.85	-60.17	-41.26	-42.29	-36.81	-42.02
	-53.09	-49.43	-56.63	-58.19	-71.28	-35.46
	-36.28	-30.14	-27.25	-45.29	-57.01	-49.09
	-27.46	-49.03	-43.13	-70.81	-29.46	-70.14

Table 6: Global energy of interaction between MAPKKs and MAPK4

MAPKK /MAPK4	1	2	3	4	5	6	7	8	9	10
Global energy	-32.52	-24.49	**-53.82**	-39.63	**-63.05**	-41.37	-23.72	**-55.23**	**-53.08**	-42.02

Table 7: Global energy of interaction between MAPKKKs and MAPKKs.

MAPKKK/MAPKK3,MAPKK5,MAPKK8 &MAPKK9	1	2	12	19
Global energy	-40.41	-49.00	-44.34	-28.52
	-37.62	-23.74	-23.73	-41.51
	-33.86	-48.10	-44.58	-48.59
	-39.98	-53.06	-28.40	-53.59

Table 8: Global energy of interaction between MAPK4 and different WRKY transcription factors.

WRKY/MAPK4	21	25	29	33	40	53	69
Global energy	-27.61	-61.99	-38.51	-42.34	-49.16	-42.05	-36.52

To predict the downstream targets of MAPK4, the docking was performed with some WRKY transcription factors whose activity was already been reported in plant defense *viz* WRKY 21, 25, 29 , 33, 40, 53 , 69 (Popsecu *et al.,* 2008; Teena *et al*., 2011; Asai *et al*., 2002; Fill *et al*., 2009; Cristna *et al.,* 2010; Andersson *et al*., 2005). Docking studies indicated that WRKY 25 and 40 could be the better interacting partner of MAPK4 (Table 8).

Discussion

In-silico protein-protein interaction Studies

Cell perceives the signal through the cell surface receptors including receptor like kinases (RLK), receptor like proteins (RLP) and extracellular binding proteins .These receptors are known to interact with MAMPs (Microbes Associated Molecular Pattern) or PAMPS (Pathogen Associated Molecular Pattern) and effectors. Receptor like kinases interact with pathogen associated molecular pattern, which are small peptides like chitin- oligosaccharide Hamel and Beaudoin (2010) & flagellin Pitzschke *et al*., (2009). Destruxin B which is a small peptide, produced by the fungus *Alternaria brassicae*, is found to interact with Lys M receptor like kinase and WAK receptor through *in silico* interaction studies. The results of docking interaction of Destruxin B with different receptor like kinases from different species indicated that Lys M receptor like kinase in *Arabidopsis* showed better interaction with it. A mutation in Lys M receptor-like protein (Lys M RLK1) gene blocked the induction of almost all chito-oligosaccharide-responsive genes and led to increased susceptibility to fungal pathogens indicating that Lys M RLK1 is essential for chitin signaling in plants (Wan *et al.,* 2008).

Arabidopsis genome has more than 80 MAPKKKs but very few members of MAPKKKs were studied. To continue the downstream cascade, docking was performed with those MAPKKKs (MAPKKK1, MAPKKK2, MAPKKK12 and MAPKKK19) which have been reported in the literature to take part in the defense along with those receptor like kinases in different species which showed better interaction with dextruxin B. It was found that MAPKKK19 showed better interaction with lysM receptor like kinase (*Ricinus communis*), WAK receptor like kinase (*Arabidopsis thaliana*) and MAPKKK2 was also found to interact better with CHRK1 receptor like kinase (*Senecio Squalidus*).

Activity of MAPK4 has been identified in plant defense against biotic stress (Desikan *et al*., 2001)**.** Out of all 10 MAPKKs known, activity of some MAPKKs in plant defense has already been identified against biotic stress viz; MAPKK3, MAPKK5 (Asai *et al.,* 2002). Docking results of all 10 MAPKKs with MAPK4 showed that MAPKK5, MAPKK8, MAPKK3 and MAPKK9 have better interaction with MAPK4. There are several evidences in the literature that show MAPKK3, MAPKK5, MAPKK1, MAPKK2 are the upstream kinase of MAPK4 (Popescu *et al*., 2008; Anderson & Ellis 2010) and MAPKK9 is the upstream kinase of MAPK3/MAPK6 (Teena *et al*., 2011). According to the superimposition results MAPKK9 and MAPKK5 also belong to the same group C. So it could be hypothesized that MAPKK 9 might be the upstream kinase not only for MAPK3/MAPK6 but also for MAPK4. Recently one group also tried to find out the interaction of all MAPKs with all upstream MAPKKs through functional protein microarrays, they found that MAPK4 interacts with MAPKK1, MAPKK 2, MAPKK3, MAPKK 5 & MAPKK 7 (Popescu *et al.,* 2008). Teena *et al*., (2011) showed that activation of MAPK4 cascade is required for sustainable activation of defense pathway. Since our docking result does not show the better interaction of MAPK4 with MAPKK1/MAPKK2, it implies that this interaction occurs through scaffold protein or after post translational modification of the protein. At the early stage of infection of Alternaria blight all three MAPKs (MAPK3, MAPK6 and MAPK4) were expressed. It should be further investigated by the wet lab experiment if all these three kinases (MAPK3, MAPK4 and MAPK6) activated by specific MAPKKs or by a common MAPKK. To find out the upstream members of MAPKKs in the signaling module, again the docking was performed with same members of MAPKKKs family which already been docked with receptor like kinases. Docking result suggested that MAPKK9 is interacting with MAPKKK2 /MAPKKK19. To find out the downstream targets of MAPK4, the docking was performed with some WRKY transcription factors whose activity was already been reported in plant defense *viz* WRKY 21, 25, 29, 33, 40, 53 , 69 (Popsecu *et al*., 2008; Teena *et al*., 2011; Asai *et al*., 2002; Fill *et al*., 2009; Cristna *et al*., 2010 Andersson *et al.,* 2005). Docking studies indicated that WRKY 25 and 40 could be the better interacting partner of MAPK4. As other groups have shown by different molecular and biochemical approaches that MAPK4 interacts with all above listed WRKY proteins except WRKY 29 which is downstream partner of MAPK3/MAPK6, we assume that it might be due to the specificity provided by the scaffold proteins. The best studied example of scaffold protein is interaction of MAPK4 with WRKY 33 interaction via MKS1 protein (Fill *et al.,* 2009). By protein microarray studies and Y2H studies WRKY 40 and WRKY 25 was reported as the downstream interacting protein of MAPK4 (Popsecue *et*

al., 2008; Anderson *et al.,* 2005) . Our superimposition result also grouped both WRKY 25 and WRKY 40 in a same group and this is also supported by the docking results.

In conclusion present study describes the role of MAPK4 in plant defense against *Alternaria* blight and predicts some potential candidate for MAPK4 cascade. Our observations lead us to propose the model of MAPK cascade involving MAPK4 in pathogenesis of *Alternaria* blight disease which has left us with some open question which should be further answered through wet lab experiments.

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