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Co-expression and *in-silico* interaction studies for inter-linking the activation of MAPK3 and LOX genes during pathogenesis of *Alternaria brassicae* in *Brassica juncea*

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Abstract

Alternaria blight disease caused by *Alternaria brassicae* (Berk) Sacc. 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 and jasmonic acid (JAs) which mediate signaling cascades. Genes in these cascades are co-expressed across diverse conditions, suggesting the potential underlying co-regulation mechanism. To understand the co-expressed behavior of genes we have analyzed the expression of LOXs and MAPK3 and it was found that the expression of BjLOX gene is activated at 3 and 6 h of pathogen infection, where as the expression of MAPK3 were found to be increased at 3 h, start decreasing at 6 h and completely depressed after 6 h of infection. As the induction of JA biosynthesis was suggested to require MAPK activation but the defined roles of MAPKs in JA mediated defense signaling is remain unclear, *in-silico* protein- protein interaction were applied to understand the MAPK3 interaction with Lipoxygenase (LOX). Our observations led us to propose that MAPK3 interact with LOX and it might be playing a role in biosynthesis of JA/JA induced expression of defense genes with some open questions.

***Keywords**: LOX, Alternaria blight, MAPKs, Protein- Protein interaction*

Introduction

Lipoxygenase (LOX), an enzyme of JAs pathway have been associated with a number of developmental stages, and in mobilization of storage lipids during germination (Siedow 1991; Kolomiets *et al*., 2001; Feussner *et al*., 2001) and its expression is regulated by different effectors such as the JA, abscisic acid, source/sink status and also by different forms of stresses such as wounding, water deficiency or pathogen attack (Creelman and Mullet, 1997; Melan *et al*., 1993; Fischer *et al*., 1999; Porta *et al*., 1999; Griffiths *et al*., 1999). Induction of LOX genes during plant-pathogen interactions has been reported in several species such as in *Zea mays* 9-LOX, ZmLOX3 may be involved in fungal pathogenesis and its loss-of-function mutants are susceptible to other fungal infection i.e *Aspergillus flavus* and *A. nidulans* (Gao *et al*., 2009). CaLOX1, a pepper gene analysis in pepper and Arabidopsis also explain the role of LOX in defense response and the hypersensitive cell death of plants following pathogen invasion (Hwang and Hwang, 2010). The function of LOX in the defense against pests seems to be related to the synthesis of a number of different compounds with signaling functions, antimicrobial activity or with the development of the HR (Ruste *et al.*, 1999; Creelman and Mullet, 1997; Parchmann *et al.*, 1997; Croft *et al.*, 1993; Weber *et al.*, 1999). It might also be involved in growth and developmental control processes, the biosynthesis of regulatory molecules and volatile compounds involved in insect attraction, as well as its role in defense responses to pathogen, wounding and stress (Feussner and Wasternack, 2002; La Camera, 2004; Baysal and Demirdoven, 2007).

Alternaria blight caused by *Alternaria brassicae* is an important disease in India that leads to major yield losses (Kolte *et al*., 1987). The fungus produces a toxin which causes necrosis of the tissues in and around infected/penetrated area followed by the development of a chlorotic halo in most of plants, including *Brassica sp. A. brassicae* is a semi-biotrophic pathogen and its toxin promotes programmed cell death at the site of infection due to induction of p53 like proteins (Khandelwal *et al*., 2002). Due to lack of *de novo* resistance in Brassica genotypes, biotechnology is one of straight forward option to engineer the resistance against these recalcitrant diseases. However, prior to engineer the disease resistance in Brassica against Alternaria blight, molecular basis of complex signaling pathways leading to induction of defense should be clearly elucidated. Most of the defense or immune responses are generally triggered either salicylic acid (SA) or jasmonic acid (JA) mediated signaling cascades depending upon the types of fungal pathogen.

The plant innate immune response leads the accumulation of salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and nitric oxide (NO) and subsequently, the activation of various defense genes (Kunkel and Brooks, 2002). JAs signaling pathways involves several signal transduction events: the perception of the primary wound or stress stimulus and transduction of the signal locally and systemically, a major component of this response appears to be jasmonate ZIM-domain (JAZ) protein which is a key regulator of jasmonate hormonal response (Chung and Howe, 2009 and Staswick, 2008). The enzyme lipoxygenase along with other enzymes plays key role in the early jasmonate signaling responses (Turner *et al*., 2002).

In recent years, several MAPK-recognition modules that are involved in the specific binding to MAPKs have been reported. MAPKs are proline-directed serine/threonine kinases phosphorylating the serine or threonine in the dipeptide motif S/T-P (Bardwell, 2006). They contain a D domain can be situated on either side of the Ser/Thr Pro, generally D domain include a cluster of basic residues upstream of an LXL motif *i.e*. $(K/R)_{2"3}$-$X_{1"6}$-(L/I)-X-(L/I). The docking interactions with MAPKs must be somehow sequentially organized and it is now becoming clear that these interaction motifs are crucial for efficient and specific signal transduction by MAPKs (Avruch, 2007; Biondi and Nebreda, 2003).

What/where could be the role for MAPK in the jasmonic acid biosynthesis? It is still unclear. As it was known that JA synthesis is initiated in the chloroplasts, while most MAPKs are cytosolic proteins thus it can be explained that, plant MAPKs are likely to participate in the initial extracellular elicitor signal activation processes leading to JA biosynthesis in chloroplasts (Kandoth *et al*., 2007). There are several reports which show that JA plays an important role in the expression MAPK but no direct evidence about the interaction of MAPK with LOX has been reported yet. In order to understand the role of inter-linking of the activation of such genes among host (*B. juncea*)-pathogen (*A. brassicae*) interaction, expression profiling of MAPK-3 and LOX genes were carried out in the present study under the influence of pathogen infections. Furthermore to establish lipoxygenase as an interacting partner of MAPK, protein-protein interaction using *in-silico* approach was also done.

Our approach was to understand the role of lipoxygenase in the host–pathogen interaction and to understand the effects of compatible pathogen on the expression of lipoxygenase. Furthermore, we tried to answer whether lipoxygenase is an interacting partner of MAPK or not by *in-silico* approach.

Materials and methods

Plant material and treatments

Mustard plants were grown under favorable conditions for incidence of the disease *i.e.* for maintaining the humidity of about 80% and a mean air temperature of 18-23ºC. Spore suspension was prepared for giving the infection to mustard plants. Concentration of spore suspension was 75 spores in 100µl of autoclaved distilled water. Leaves challenged with *Alternaria* spores were collected at different time intervals *i.e.* 6h, 12h, 18h, 24h for RNA isolation.

Reverse transciptase-PCR analysis of *Brassica* LOX and MAPK3

For RT-PCR analysis leaves samples that were collected at different time intervals were subjected for RNA isolation using Invitrogen kit (Invitrogen, http://www.invitrogen.com) as per the manufacturer instructions. RT-PCR analysis was performed with the help of One-Step RT-PCR kit with Platinum Taq using the following specific primers LOX-forward, 5'-AGAAATGCTTGCTGGGCTAA-3'and LOX reverse,5'-GCTCAGTGTCGT-TCTGGACA-3'; MAPK3- forward 5'-CGTACA-CTTCGCGAGATCAA-3' and MAPK3- reverse, 5'-ATTCATTCGGTTGCTGCTCT-3'.

Sequence analysis

The similarity searches for sequences were conducted by using the online tool BLASTn. The location of signature sequences was searched by using software ClustalX2. Multiple sequence alignments of related proteins belonging to lipoxygenase group in different plant species were performed using Multalin (http://bioinfo.genotoul.fr/multalin/multalin). Programs INTERPROSCAN (Nucleic Acids Res. 37, D224-228) and ScanSite were employed to detect conserved domains. Phosphorylation sites were located by using the online tool NETPHOS 2.0.

Homology modeling and docking

Homology modeling of BjLOX, *Brassica* MAPK-3 was done by using Molecular Operating Environment (MOE) software. For LOX-P 2BNE.A.pdb, for LOX-N 1JNQ.A.pdb and for MAPK3 2JD5.B.pdb was selected. 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. Docking was performed in between MAPK and LOX-P. The parameters used for the Docking were - Total Runs = 30 Cycle/Runs X= 10 Iteration Limit = 10,000; Potential Energy Grid: ON; Annealing Algorithm: Simulated Annealing.

The active binding sites of the MAPK (fig 4) were detected by using MOE, alpha site finder tool keeping the parameters as Probe Radius 1 = 1.4, Probe Radius 2 = 1.8, Isolated Donor/Acceptor=3, Connection Distance = 2.5, Minimum Site Size = 3, Radius = 2 and docking was performed by selecting these active site.

Results and discussion

Expression profiling of LOX and MAPK3 at different time interval after *Alternaria brassicae* spore suspension challenge

To evaluate the functional role of LOX in relation with MAPK3 during pathogenesis of Alternaria blight, the expression of MAPK3 and LOX gene were examined after the *A. brassicae* spore suspension challenge. If the interaction between MAPK-3 and LOX have any influence on pathogenesis/defense pathway *in vivo*, it is quite pertinent to study for the expression patterns of the MAPK3 and LOX gene to understand the overlap during various stages of infection. Using RT-PCR analysis, MAPK3 gene expression was observed during different time intervals of infection. The expressions of both were found in 3 and 6 h infection of Alternaria spore suspension shown in fig1a and 1b and their densitometry analysis in shown in fig 2. No expression of LOX was seen in control however MAPK-3 expression was seen in control. The expression of genes were analyzed with simultaneous analysis of expression of the constitutive gene expression i.e. Actin expression shown in fig 1c.

In-silico analysis

BjLOX lipoxygenase genes showed 98% identity with *Brassica napus* lipoxygenase 1 mRNA, complete cds and 89% identity with Lox 1 gene present in the *Arabidopsis thaliana*. Translation of the open reading frame present in BjLOX indicated that it codes for 290 amino acids protein. Sequence alignment of the predicted amino acid sequence of BjLOX showed more than 60% similarity with other plant like *B. napus*, *A. thaliana*, Tobacoo and with some other plants LOX. Analysis of the deduced amino acids showed the presence

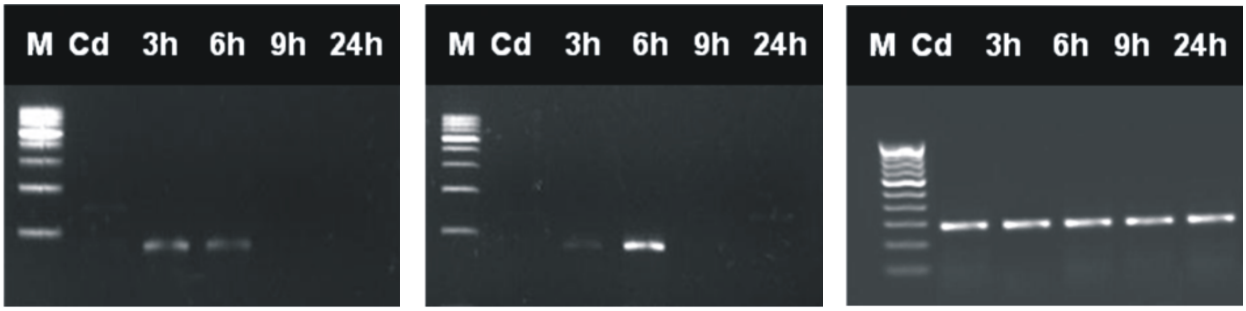

Fig 1: Expression patterns of MAPK-3, LOX and ACTIN genes in different samples of leaves at different time Lane 1: 500bp ladder; Lane 2: Control Lane 3: 3h sample; Lane 4: 6h sample; Lane 5: 9h ..... *Alternaria* spore

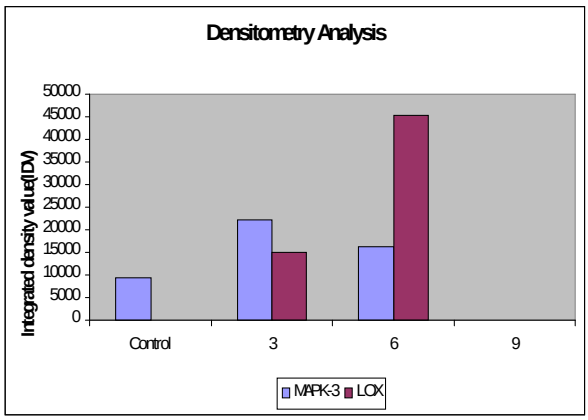

Fig 2: Densitometry analysis of MAPK-3 and LOX at different time interval

of one conserved lox domain which is a characteristic domain of LOX shown in fig 3**.** The deduced amino acid sequence of BjLOX showed the presence of three conserved His residues (118,198 and 207). Analysis of deduced amino-acid sequence of BjLOX showed the presence of phosphorylation site (S)*P-site i.e. RTAQSPKAI for MAPK and the presence of ERK D domain LKPLVIELSLPHPDG respectively. Arabidopsis LOX sequence also showed the presence of ERK D-domain and (S/T)*P site. Multiple sequence alignment and phylogentic analysis revealed the similarity among lipoxygenase gene from different plants fig 4.

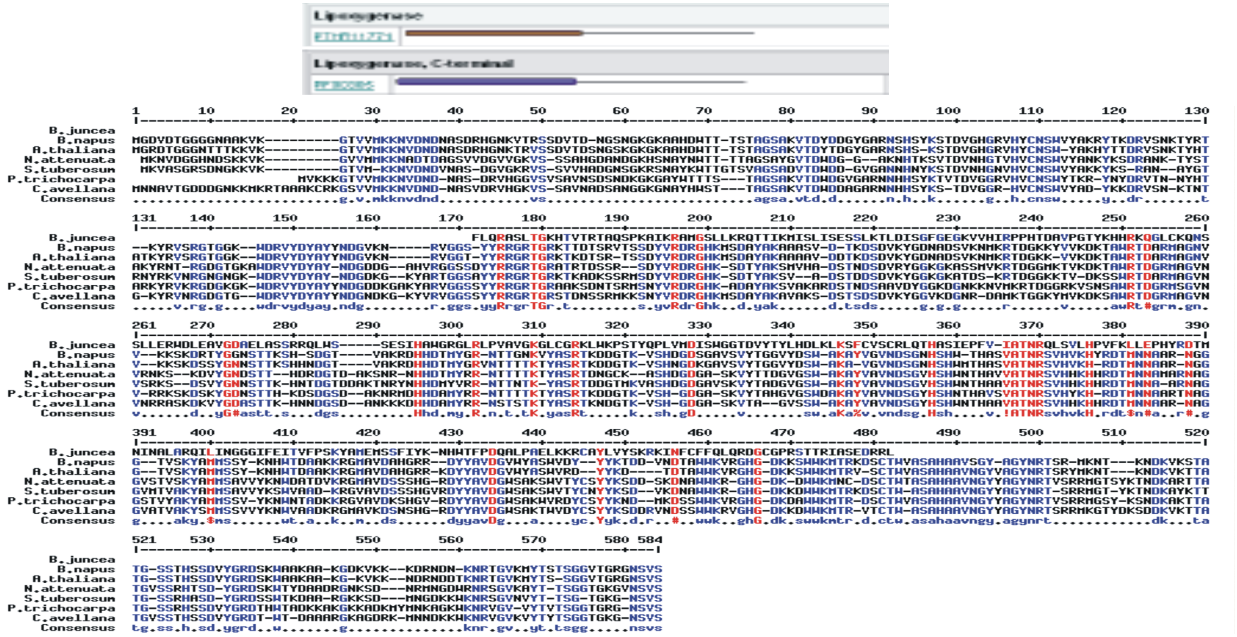

Fig 3: Interproscan result of LOX protein in *Brassica*

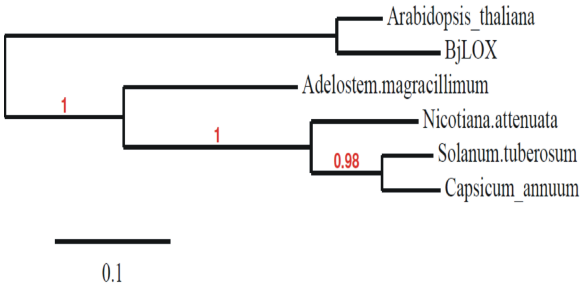

Fig 4: Multiple sequence alignment and phylogentic tree

Protein- Protein interaction

In order to understand the involvement of MAPK signaling that mediates the responses to biotic stresses and triggers the biosynthesis of JA. Identification of LOX as MAPK3 substrates could be an important step towards defining the molecular mechanism involved in the phosphoryaltion/ dephosphorylation dependent regulation of JA biosynthesis or JA induced defense response under present investigation.

In-silico protein-protein interaction of BjLOX and MAPK3 has been done, using MOE Docking software. The binding of the BjLOX with the MAPK3 was analyzed using MOE docking program to find the correct confirmation (with the rotation of bonds, structure of molecule is not rigid) and configuration (with the rotation of whole molecule, structure of the molecule remains rigid) of the ligand, so as to obtain minimum energy structure. GLU147, PRO148, ILE163, ILE165, ASN166, GLY167, GLY168 residues of LOX-P interact with ASP64, LYS66, LEU85, GLU97, TYR98, VAL99, VAL100, THR101, ARG102, ARG105, LEU109, LEU110, ASN112, HIS144, ASN168, LYS172 residues of *Brassica* MAPK3 having threshold value 5.419 shown in fig 5.

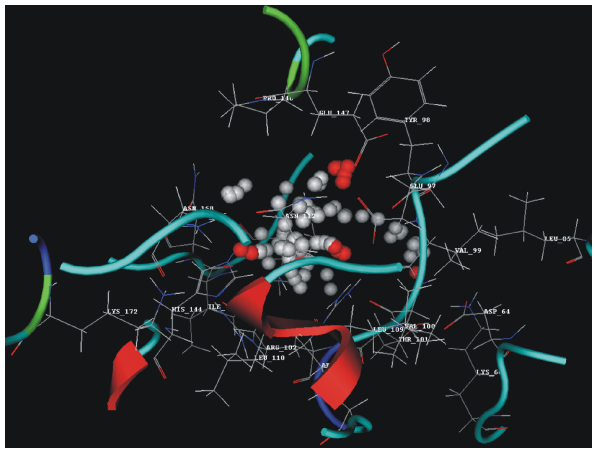

Fig 5: Model describing the protein-protein interaction of MAPK3 represented as red and white sphere *i.e.* Hydrophobic and hydrophilic residues and BjLOX- residues

The speed of plant responses against the fungal attack determines the efficiency of the plant defense and is crucial for the establishment of resistance. Thus the basic knowledge of the molecular mechanism involved in plant's defense response to pathogen attack is obviously of great importance. Genes in the same cascade could be co-expressed. To understand the co-expressed behavior of genes we have analyzed the expression of LOXs and MAPK3 as both the genes responded to stress. The expression of BjLOX gene was found to be activated during 3 and 6 h of pathogen infection, no expression was seen in the control condition, in contrast to this MAPK3 expression were seen in control and found to be increased at 3 h and start decreasing at 6 h and completely depressed after 6 h of infection.

Sequence analysis revealed the presence of MAPK S/T-P sites and ERK D-Domain i.e. LKPLVIELSLPHPDG in BjLOX and the *in- silico* protein-protein docking showed the positive interaction between MAPK3 and LOX, all these findings were giving an indication that LOX might be act as a MAPKs substrate or it might be playing a role in the signal transduction pathway. The overlapping expression behavior of MAPK3 and LOX further helps us in drawing a conclusion that MAPK3 might be phosphorylating LOX proteins.

We have proposed that expression of MAPK3 and LOX played a pivotal role in the pathogenesis/ defense mechanism, based on the lack of

expression of LOX and less expression of MAPK3 in healthy leaves. Hence, from these studies it could be suggested that signaling cascade initiated by pathogen is being mediated through MAPK and it induced the expression of LOX which further activate or regulate the expression of MAPK. Recently clues supporting this hypothesis were reported (Schweighofer and Meskiene, 2008). Further the differential phosphorylation should be done to test this model *in vivo* and *in vitro* interaction.

Our observations lead us to propose a model (fig 6) that MAPK3 interacts with LOX and it might be playing a role in the biosynthesis of JA /JA induced expression of defense genes with certain open questions. Further studies are in progress to address the questions which might shed light on understanding the intimate relationship of MAPK pathways based activation of jasmonate mediated induction of defense response during the pathogenesis of Alternaria blight in *B. juncea*.

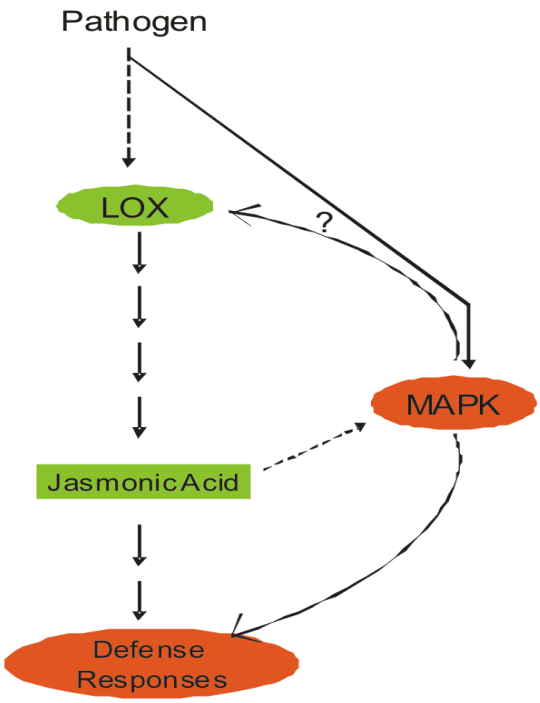

Fig 6: The proposed model depicting interlink between MAPKs and JA biosynthesis

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