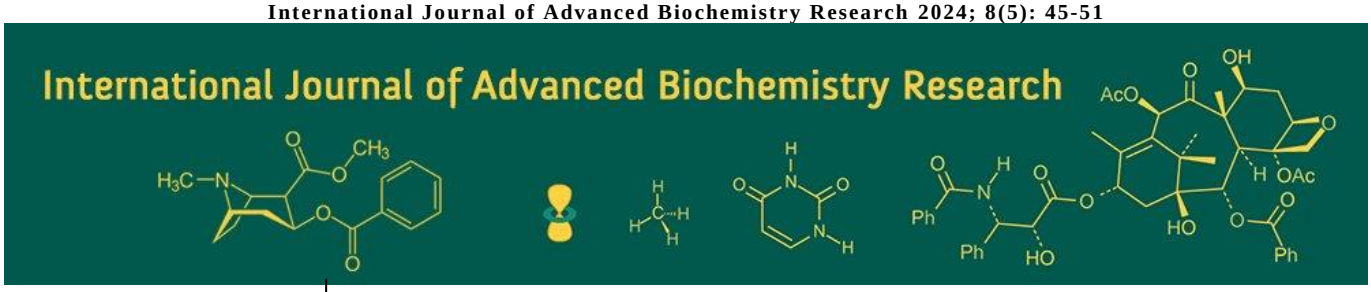

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Monika Bisht
Department of Molecular Biology & Genetic Engineering, College of Basic Sciences & Humanities, GBPUA&T, Pantnagar, Uttarakhand, India

Shivanshu Garg
Department of Biochemistry, College of Basic Sciences & Humanities, GBPUA&T, Pantnagar, Uttarakhand, India

Pooja Nain
Department of Soil Science, College of Agriculture, GBPUA&T, Pantnagar, Uttarakhand, India

Himanshu Punetha
Department of Biochemistry, College of Basic Sciences & Humanities, GBPUA&T, Pantnagar, Uttarakhand, India

Dinesh Pandey
Department of Molecular Biology & Genetic Engineering, College of Basic Sciences & Humanities, GBPUA&T, Pantnagar, Uttarakhand, India

Corresponding Author:
Dinesh Pandey
Department of Molecular Biology & Genetic Engineering, College of Basic Sciences & Humanities, GBPUA&T, Pantnagar, Uttarakhand, India

Salicylic acid modulated expression of MAPK3 and NPR1 gene during pathogenesis of Alternaria blight in *Arabidopsis thaliana*

Monika Bisht, Shivanshu Garg, Pooja Nain, Himanshu Punetha and Dinesh Pandey

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Abstract
In order to explore the role of salicylic acid mediated defense at different stages of Alternaria blight, the relative transcript expression analysis of MAPK3 and NPR1 was performed on the plants of two ecotypes (WS and Columbia) of *Arabidopsis thaliana* inoculated distinctly with pathogen, salicylic acid (SA) and salicylic acid pre-conditioning followed by pathogen inoculation. Upon application of SA, the disease index significantly reduced in both ecotypes in response to pathogen challenge. Higher expression of MAPK3 and NPR1 was observed at critical stages of disease progression in plants–preconditioned with SA before pathogen inoculation as compared to only pathogen challenged plants of both the ecotypes. This reflects triggering of SA mediated expression of MAPK3, NPR1 dependent defense response against the pathogen. High and rapid induction of this defense pathway in Columbia ecotype contributes to its tolerance to Alternaria blight as compared to WS. Salicylic acid triggered lower expression of MAPK3 and NPR1 in response to pathogen encounter at later stages seems to increase disease severity while compared to early and middle stages. The role of SA in defense through modulation of MAPK3 and NPR1 is visualized as soon as 4 hours after pathogen inoculation. MAPK3 and NPR1 expression levels were upregulated and downregulated in a cyclic fashion in both ecotypes in all treatments. SA thus acts by cyclically upregulating MAP Kinase3 and NPR1 at critical initial and late stages of disease progression. The analysis of real time expression of MAPK3 & NPR 1 gene helps in gaining insights for Alternaria blight pathogenesis in model plant *Arabidopsis thaliana.*

Keywords: Mitogen-activated protein kinase 3 (MAPK3), Non-expressor of pathogenesis-related genes 1 (NPR1), Wassilewskija ecotype (WS), alternaria blight, pathogenesis

Introduction
In plants the genus *Brassica* belonging to the cruciferous family is the economically most important genus consisting of various important oilseed crops. *Alternaria brassicae* is major pathogen of the disease, which is responsible for causing 15-71% yield loss in India. It is proposed that genetic engineering methods can be utilized to develop durable resistance against the disease provided that molecular mechanism of pathogenesis is delineated. Despite the destructive nature of this pathogen, very little is known about the molecular mechanisms through which it causes the disease. Phytohormones play central roles in abiotic and biotic stress signaling and activation of various defense genes. The role of Salicylic acid, Jasmonic acid and Ethylene have already been implicated in biotic stress signaling while they are involved during systemic and induced defense responses. Salicylic acid mediated plant defense responses are triggered against biotrophic pathogens whereas necrotrophic pathogens involve Jasmonic acid or ethylene dependent induced defense responses. Therefore, a semi/hemibiotrophic pathogen like *Alternaria brassicae* could invoke both Salicylic acid and Jasmonic acid dependent defense responses on its biotrophic or necrotrophic "lifestyle" at different stages of pathogenesis. Recently MAP kinases have been demonstrated to be important regulators of salicylic acid (SA) and Jasmonic acid (JA) mediated signaling pathways. Mitogen activated protein kinases (MAPK) are known to be important mediators in signal transmission, connecting the perception of external stimuli to cellular responses. Mitogen-activated protein kinase (MAPK) cascades are three tiered signaling kinase modules that can be found in all eukaryotes (Jagodzik *et al.*, 2018) [5].

In *Arabidopsis* MAPK3, MAPK4 and MAPK6 are all activated by bacterial and fungal pathogens (pathogen-associated molecular patterns) (Xie *et al.*, 2023) [9]. It was shown that MAP kinase 4 and MAP kinase 6 are reciprocally regulated during pathogenesis of Alternaria blight using *Arabidopsis thaliana* as host (Kannan *et al.*, 2012) [6]. The expression of some MAP kinases has been associated with increase in SA level during basal defense and inducible defense mechanisms, SAR. For example, expression of MAPK3 is linked with induction of SA dependent genes/proteins (Li *et al.*, 2017) [7]. A report in Arabidopsis shows that priming (a physiological process by which a plant prepares to more quickly or aggressively respond to future biotic or abiotic stress)by the salicylic acid analog benzo-(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester results from accumulation of inactive MAPK3 (Frąckowiak *et al.*, 2019) [4]. MAPK3 activity is then induced in response to biotic stress (pathogen infection), thereby enhancing defensive gene expression and the induction of antifungal metabolites. Whether MAPK3 is a general pre-stress marker or works in concert with other markers remains to be seen. Another gene that has been found to play a crucial role in SA mediated defense is NPR1.The *Arabidopsis* NPR1 protein is a key regulator of salicylic acid (SA) mediated gene expression in systemic acquired resistance. NPR1 has been demonstrated to be an important transducer of the SA signal in the SA-mediated activation of PR gene expression and broad-spectrum resistance (Chen *et al.*, 2019) [2]. Both MAPK 3 and NPR 1 gene has been shown to be induced by the generation of ROS (reactive oxygen species). There are a number of intermediatory proteins that act an important link between MAPK 3 and NPR 1 gene induction after generation of reactive oxygen species. One such important protein is OXI1. The expression and activation of the *Arabidopsis* serine/threonine kinase *OXIDATIVE SIGNAL-INDUCIBLE1* (OXI1) are induced in response to hydrogen peroxide. OXI1 is required for activation of MAPKs (MPK3 and MPK6, orthologs of WIPK and SIPK, respectively, in Arabidopsis) (Asai and Yoshioka, 2008) [1].

Keeping the above information in mind in this study, we attempted to address the influence of SA pre-conditioning on the pathogenicity (in terms of disease index) of Alternaria blight on susceptible and tolerant ecotypes of *Arabidopsis thaliana*, the effect of SA pre-conditioning on the transcript profiling of MAP Kinase 3 and NPR 1 at different stages of disease progression of Alternaria blight in *Arabidopsis thaliana* spp. by using semi–quantitative and Real Time PCR and to correlate the relationship between pathogen colonization on Arabidopsis thaliana spp. and expression of MAPK 3 and NPR 1 gene involved in SA signaling.

Materials and Methods

Seed sowing and maintenance of *Arabidopsis* plants

Arabidopsis plants were grown and maintained in aseptic condition so as to avoid contamination from other pathogens.

Isolation of *Alternaria brassicae* spores

Alternaria brassicae spores were isolated from naturally infected *Brassica juncea cv. Varuna* plants in the field condition growing in Crop research center, G.B.P.U.A & T. Pantnagar. By using soft brush all the spores in the diseased leaf are rubbed, so that at last all the spores are mixed well in water.

Pre-treatment of leaves of *Arabidopsis* with Salicylic acid

Arabidopsis leaves were treated with Salicylic acid concentration 200 µM for 48 hrs before inoculation with *Alternaria brassicae* spores. Salicylic acid was dissolved in ethanol to make up the final concentration.

Infection of *Arabidopsis* plants

Artificial inoculation was done on one and half month-old plants grown in glass house. Based on the concentration of spores which was adjusted to 10^4 spore's ml^{-1}, 10 µl suspension was sprayed on the leaves of *Arabidopsis* plants with the help of atomizer.

Calculation of Disease index of leaf

Observation on disease severity was recorded using 0-6 disease rating scale (Conn *et al*., 1990) [3]. Average number of spots was recorded based on the observation taken on fully developed middle leaves and Diameter of randomly selected spot of leaf was measured in cm including yellow halo, chlorotic area with necrotic brown area from the center, with the help of a thin plastic scale. Average size of spot was then calculated. The average disease index was calculated by taking observation on three leaves (Conn *et al.*, 1990) [3].

Collection of infected *Arabidopsis* leaves and Storage of plant Material

For the expression profiling at the transcriptional level, samples were collected at different time intervals following the inoculation of leaves with *Alternaria* spores at 4 HAI (hours after inoculation), 12 HAI, 1 DAI (days after inoculation), 3DAI, 7DAI, 14DAI, 21DAI. The samples were transferred to ultra-cool refrigerator at - 80˚C for storage after properly labeling.

MAP Kinase 3 and NPR 1 Expression profiling

RNA was isolated using RNA express reagent prepared in our lab, quantified and then checked for quality in 1.2% agarose gel. Once quality and quantity were confirmed reverse transcriptase PCR was performed to obtain cDNA from isolated RNA. For expression study real time PCR was performed of this cDNA using primers designed for MAPK3, NPR1 and actin. For designing primers nucleotide search for the query namely MAP Kinase 3 and NPR 1 from *Arabidopsis thaliana* was performed at NCBI. The cds sequences for MAP Kinase 3 and NPR 1 were retrieved in FASTA format. The retrieved sequence was analyzed with BLAST in Arabidopsis gateway and then the sequence showing maximum identity with *Arabidopsis thaliana* MAPK3 and NPR1 was retrieved. The retrieved sequences were fed into Primer 3 (the online primer designing tool) where all the parameters were adjusted according to the need of experiment to design both forward & reverse primers. The primer sequences were then fed into primer Blast to make sure it is a single hit for *Arabidopsis thaliana* desired genes. Primer parameters were also calculated using oligo calc (an online tool).The details of sequence of primers are provided in the following Table 1.

Table 1: The details of primers along with their properties

Primer Name	Sequence	Product size	T_m (°C)
ACT.F	5′GAATCCACGAGACGACTTACAAC3′	200 bp	55.4
ACT.R	5′CGATCCAGACACTGTACTTCCTC3′		56.6
MPK3.F	5′TCGTTTGCTCTGTGTTGGATAC 3′	181 bp	55.2
MPK3.R	5′TGTCTTCTTAGTGGTGGTGGAA 3′		55.7
NPR 1.F	5′AGAAGACGACACTGCTGAGAAA3′	187 bp	56
NPR 1.R	5′ACGACGATGAGAGAGTTTACGG 3′		56.2

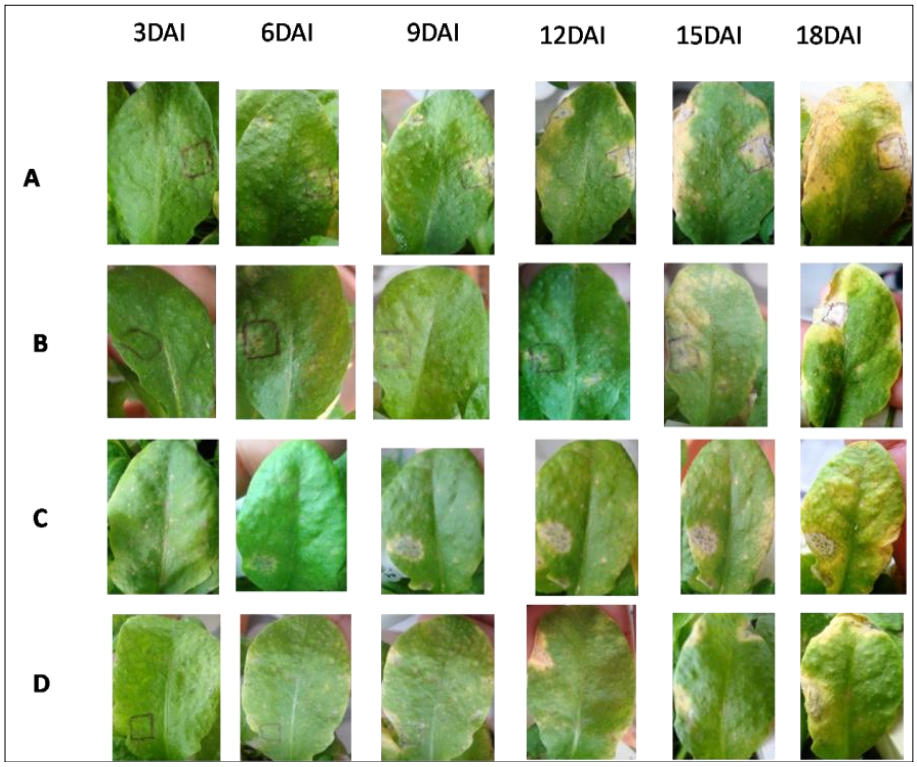
Results and Discussion

Calculation of percent disease index

Upon inoculation with spores of pathogen disease symptoms appeared at 3 days after inoculation in both ecotypes of Arabidopsis thaliana. In order to study the differential response in both the ecotypes to SA pretreatment, percent disease index was calculated by taking observation on pathogen inoculated leaves and salicylic acid pre-conditioned but pathogen inoculated leaves of each ecotype. Percent disease index on leaf was recorded at 3, 6, 9, 12, 15 and 18 days after inoculation (DAI) in both Columbia and WS maintained in glass house Fig. 1. Disease index for Alternaria blight was calculated by measuring the size of spots and their number on the infected leaves as per the formula (Conn *et al*., 1990) [3]. The results on number of spots observed under different treatments are given in Table 2. No disease spots were observed in the presence of salicylic acid. In the presence of pathogen alone, the number of spots is more than the number of spots in presence of salicylic acid and pathogen. This provided substantial evidence that salicylic acid is inhibiting pathogenesis process. When compared between two ecotypes, Col had a smaller number of spots than the WS ecotype. As the disease progresses, the number of spots increases but throughout disease progression the number of spots in Columbia are always less than that of WS. This is true in the case of both the treatments of pathogen alone and pathogen along with salicylic acid. This supports the view that Columbia is a tolerant ecotype as compared to WS.

Table 2: Number of disease lesions appeared at different stages of disease progression under the influence of salicylic acid, salicylic acid preconditioning with pathogen and pathogen alone

Average no. of spots						
Stages of infection	**Salicylic acid**		**Salicylic acid + pathogen**		**Pathogen**	
Ecotypes	WS	Col	WS	Col	WS	Col
Control	0	0	0	0	0	0
Early (7 DAI)	0	0	15	13	32	28
Middle (14DAI)	0	0	20	17	35	30



A: Ecotype WS inoculated with pathogen.
B: Ecotype WS with SA (200 µM) preconditioning and pathogen inoculation.
C: Ecotype Columbia inoculated with pathogen.
D: Ecotype Columbia with SA (200 µM) preconditioning and pathogen inoculation.

Fig 1: Determination of disease index under the influence of SA preconditioning in leaves of *Arabidopsis thaliana* at 3DAI, 6DAI, 9DAI, 12DAI, 15DAI and 18DAI (DAI- Days after inoculation with pathogen).

Percent disease index is taken as a measure of susceptibility or resistance of a particular ecotype to a particular disease. Higher disease index means an ecotype is susceptible to the disease whereas a lower index suggests resistance to the disease.WS ecotype reported a higher disease index (60%) than Columbia ecotype (56.6%) in case of inoculation of only pathogen. In case of the treatment of salicylic acid along with pathogen, also WS reported a higher disease index (53.3%) as compared to Columbia (46.6%). This again supports the fact that WS is susceptible to the disease as compared to Columbia ecotype as it has reported a higher disease index in both the cases. Salicylic acid has played a significant role in disease reduction as is evident from the significantly reduced disease index in salicylic acid pretreated plants as compared to only pathogen treatment in both the ecotypes Fig. 2. and Table 3.

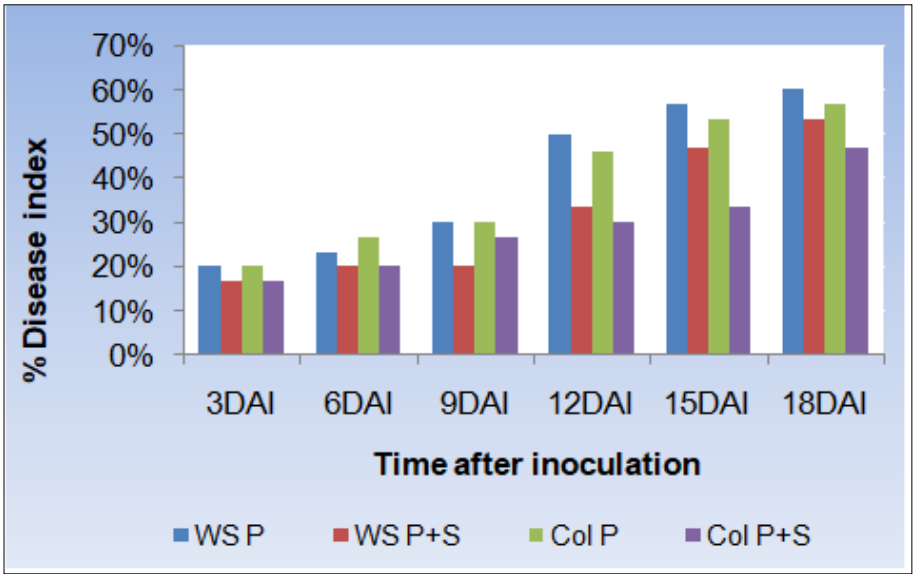


Fig 2: Influence of salicylic acid preconditioning (200 µM) on disease index of Columbia and WS ecotype of *Arabidopsis thaliana* (P: pathogen inoculated; P+S: SA preconditioned with pathogen inoculation.

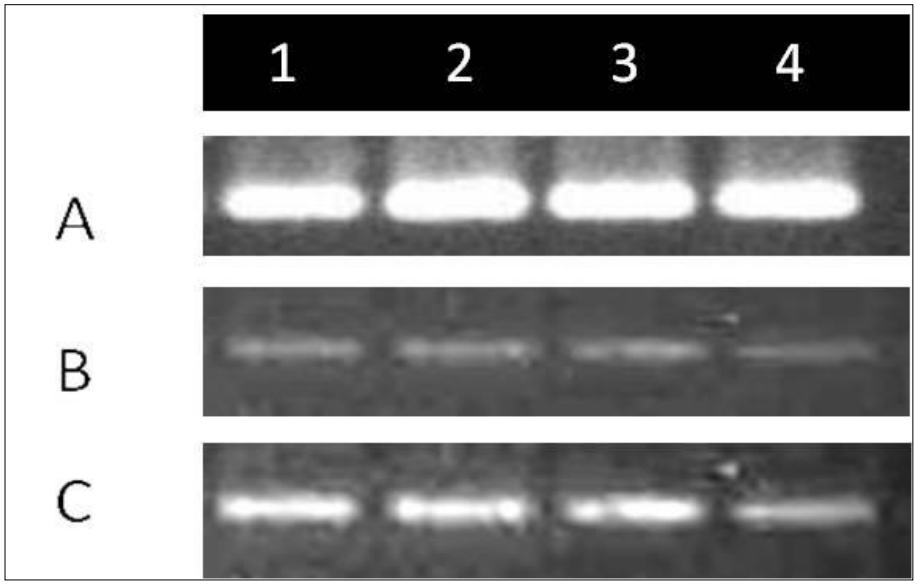
It is seen that percent reduction in disease index (from pathogen to salicylic acid preconditioning before inoculating with pathogen) is higher in almost all time periods in Columbia except for 9DAI where WS has higher decrease/reduction in the disease index. Highest reduction in disease index is seen in Columbia at 15DAI (20%).

Table 3: Influence of salicylic acid, salicylic acid preconditioning with pathogen and pathogen alone on disease index of susceptible and tolerant ecotypes of *Arabidopsis thaliana*

	Disease index (%)											
	3 DAY		6 DAY		9 DAY		12 DAY		15 DAY		18 DAY	
	P	P+S	P	P+S	P	P+S	P	P+S	P	P+S	P	P+S
Number of leaves observed	5	5	5	5	5	5	5	5	5	5	5	5
WS	20%	16.6%	23.3%	20%	30%	20%	50%	33.3%	56.6%	46.6%	60%	53.3%
COl	20%	16.6%	26.6%	20%	30%	26.6%	46%	30%	53.3%	33.3%	56.6%	46.6%

Expression Profiling of MAP Kinase 3 and NPR1 using Semi Quantitative and real time PCR

For expression analysis of MAPK 3 and NPR1, total RNA was isolated from leaves of different stages of disease progression and subjected to different treatments. Semi-quantitative RT –PCR and real time PCR was performed taking actin gene as the internal standards. It has been reported that MAPK 3 & 6 regulate ROS amplification and scavenging pathway along with regulating NPR1 activation followed by activation of salicylic acid pathway (Torres *et al*., 2006) [8]. In order to probe the involvement of MAPK3 dependent SA pathway transcript profiling of MAP Kinase 3 was performed during different stages of disease progression and in various treatments by quantitative real time PCR. Expression pattern of MAPK3 was compared in two different ecotypes of *Arabidopsis* during different stages of disease progression in presence or absence of salicylic acid pre-conditioning. Clear high intensity bands were visible in case of actin supporting the fact that actin was found to be constitutively expressed in all samples and Expression of both the genes (MAPK3 and NPR1) was seen as early as 4 hours after inoculation (HAI) in case of both ecotypes and all treatments.



L1: MAPK 3 Columbia
L2: MAPK 3 WS
L3: NPR 1 WS
L4: NPR 1 Columbia

Fig 3: Gel strips showing amplified bands at 4HAI (hours after inoculation) for different treatments of *Arabidopsis thaliana* ecotypes depicting expression of MAPK3 and NPR1 as early as 4HAI (A-Actin; B -Pathogen, C- Salicylic acid preconditioned (200 µM)before Pathogen inoculation). Time interval for all lanes is 4HAI (hours after inoculation)

The expression data obtained from quantitative Real time analysis of MAPK3 and NPR1 was subjected to analysis which provided expression values of various treatments in the two ecotypes throughout all the intervals along with the relationship between these values. In both the ecotypes, WS and Columbia of *A. thaliana* application of 200 µM salicylic acid triggered an upregulation in expression of MAPK3 and NPR1 gene at as early as 4 hours after treatment. Similarly in only pathogen inoculated and salicylic acid pre-conditioned with pathogen inoculation samples, the expression of MAPK3 is not constantly maintained at all-time intervals and is rather cyclically upregulated and downregulated, probably due to interaction with other intercellular or developmental cues or due to feedback regulation of MAPK3 and NPR1.

In both the ecotypes, higher expression of MAPK3 and NPR1 was observed at important stages (as per histological study in our lab) in salicylic acid pre-conditioned and pathogen inoculated plants than in pathogen inoculated plants at initial stages of disease progression. The expression of both MAPK3 and NPR1 shoots up at 3DAI (stage where pathogen germ tube interaction with stomatal and cellular components). This suggests the triggering of salicylic acid induced defense against the pathogen. Upon correlating the data with that of disease index and cell death it can be concluded that induction of salicylic acid mediated MAPK3 dependent defense and NPR1 gene expression curtails the hemibiotrophic growth of pathogen and reduced disease severity in both the ecotypes. Further on the basis of expression analysis of MAPK3 and NPR 1, it was observed that high magnitude of salicylic acid mediated defense is triggered as early as at 4 hours after pathogen encounter in Columbia for both only pathogen and pathogen after salicylic acid treatment than WS when compared to healthy plant. Irrespective of treatments, Columbia ecotype showed high expression levels of MAPK3and NPR1 as compared to WS ecotype. Thus, the high and rapid induction of salicylic acid mediated MAPK3 and NPR1 dependent defense in Columbia contributes to its tolerance to Alternaria blight disease. However, the fluctuations in the level of MAPK3 and NPR1 suggest an arms race between the host plant and the pathogen.

In Columbia and WS, MAPK3 and NPR1 gene expression levels were seen to be both upregulated and downregulated at various time intervals in both pathogen challenged plants and pathogen challenged salicylic acid pre-conditioned plants. This implies that salicylic acid mediated defense and MAPK3, NPR1 gene expression is either paralyzed by pathogen in order to support its necrotrophic growth or its biosynthesis is regulated by feedback inhibition or depends on other proteins that are being regulated. In Columbia ecotype, this defense pathway involving MAPK3 and NPR1 is upregulated first at 4 hours after pathogen inoculation and then continuously down regulated till first day after inoculation. It is again upregulated at 3rd day after inoculation and then downregulated up to middle stage i.e., 14th day and then again upregulated at 21 DAI (late stage). This implies inherent ability of this ecotype to mount salicylic acid mediated defense and restrict the probable biotrophic growth of the pathogen. However, eventually, this defense response appears to be paralyzed by the pathogen by the time late stage of disease is reached although salicylic acid mediated and MAPK3 and NPR1 dependent defense response is still functioning.

Salicylic acid application on both ecotypes increases the expression levels of MAPK3 with its maximum value 4 hours after treatment with pathogen and at 3 DAI in WS and Columbia ecotype respectively and for NPR1 at 3 days after inoculation with pathogen in Columbia and WS ecotype of *A. thaliana*. However, this upregulation is not constantly maintained. In only salicylic acid preconditioned plants a high level of expression of MAPK 3 and NPR 1 is seen after 48 hours of pre-conditioning suggesting that SA triggers the expression of these genes even in absence of pathogen also (Figs.4a, 4b, 4c, 4d and Figs.5a, 5b, 5c, 5d).

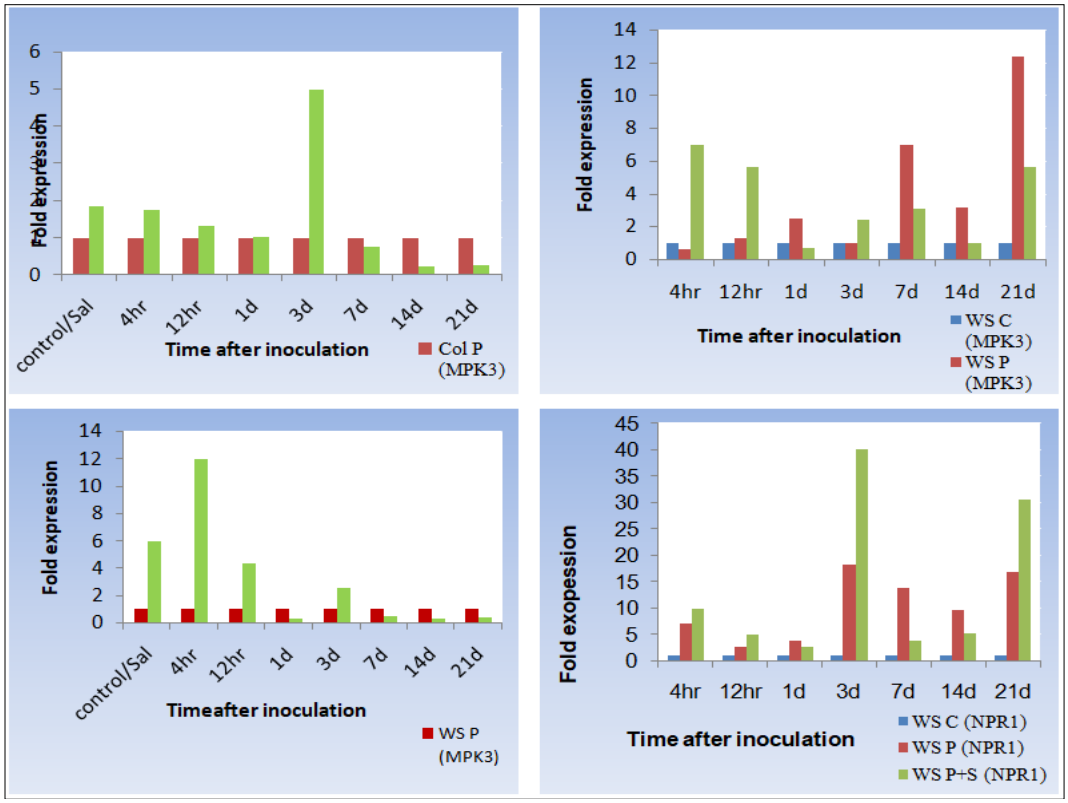


Fig 4a, 4b, 4c, 4d: Graphical representation for expression patterns of MPK3 in various treatments of *A. thaliana* ecotype Columbia (C: control (Healthy leaf); P: pathogen inoculated; P+S: SA preconditioned (200 µM) before pathogen inoculation. Here healthy leaf is reference for expression fold calculation).

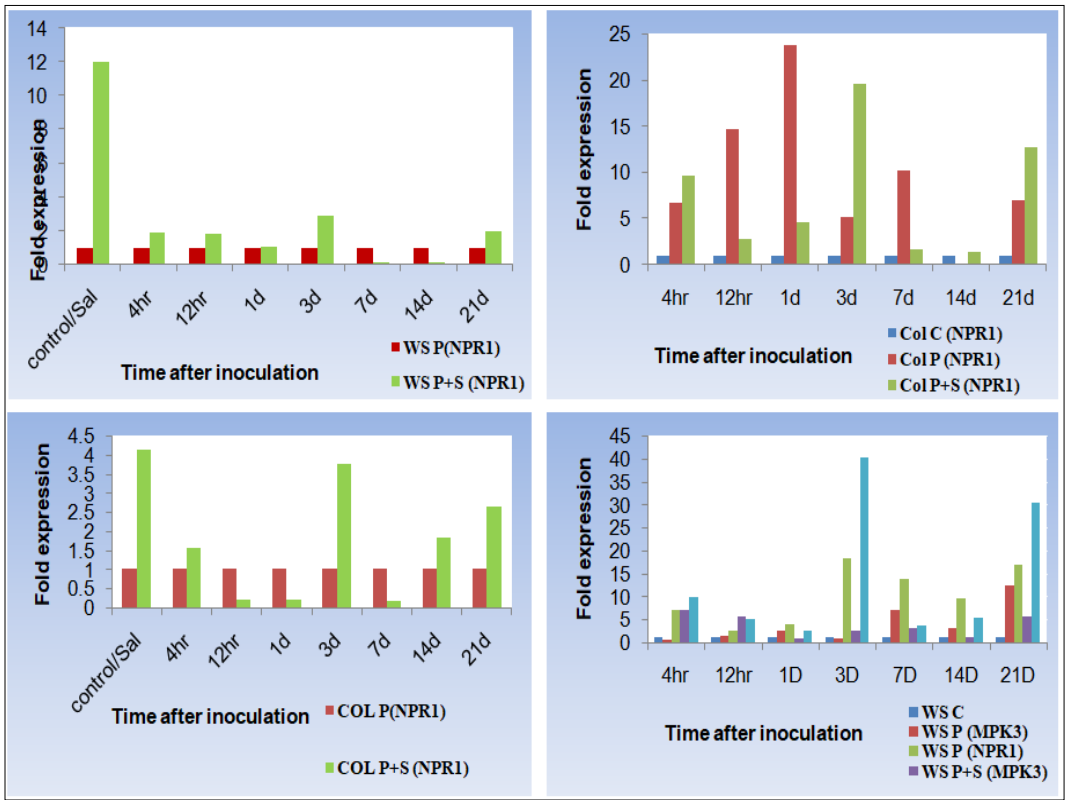


Fig 5a, 5b, 5c, 5d: Graphical representation for expression patterns of NPR1 in various treatments of *A. thaliana* ecotype WS (C: control (Healthy leaf); P: pathogen inoculated; P+S:SA preconditioned(200 µM) before pathogen inoculation. Here healthy leaf is reference for expression fold calculation)

SA thus acts by upregulating MAP Kinase 3 and NPR1 soon after pathogen inoculation (4HAI) and again upregulating these genes at 3DAI (a critical stage of pathogen establishment) of disease progression. Most of the times both the genes are upregulated and downregulated (in SA preconditioned plants) at the same time implying these genes function together in SA mediated defense pathway (Fig. 6).

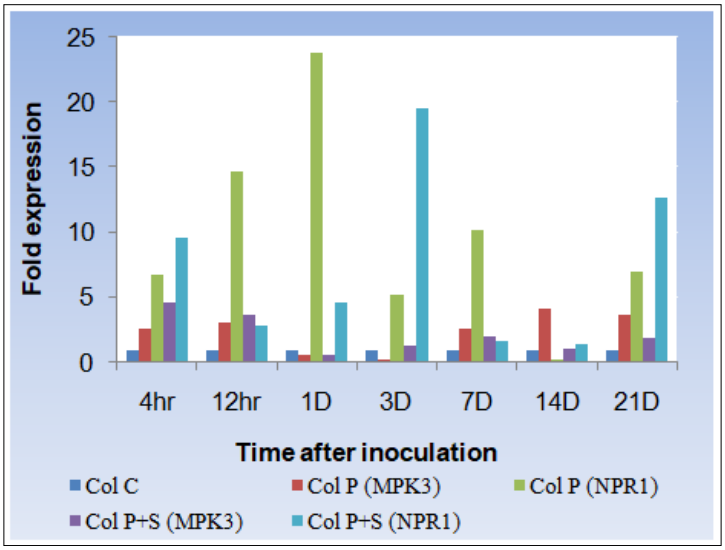


Fig 6: Graphical representation for expression patterns of MPK3 and NPR1 in various treatments of *A. thaliana* ecotype Columbia (C: control (Healthy leaf); P: pathogen inoculated; P+S: SA preconditioned (200 µM) before pathogen inoculation. Here healthy leaf is reference for expression fold calculation).

Conclusion

The present study describes the involvement of MAPK3 and NPR1 during salicylic acid mediated defense response against the pathogen of Alternaria blight in Arabidopsis thaliana. Salicylic acid induces the defense response against the pathogen by modulating the expression of MAPK3 and NPR1 at various stages of disease progression. This defense pathway appears to reduce disease index and curtail the hemibiotrophic growth of the pathogen in both ecotypes. Hence, in near future, development of salicylic acid mimicking agro-chemicals can be developed against agronomically important pathogens which trigger salicylic acid dependent defense pathway in crop plants. However, this defense pathway is eventually overcome by the pathogen during arms race and disease is incited. This study thus illustrates that induction of just salicylic acid dependent defense signaling pathway is not sufficient to completely restrict the pathogen of Alternaria blight as it just delays the disease incidence and its severity. It would be worthwhile to explore the role of other defense pathways and components therein to curtail the spread of the pathogen. Most notably there is a need to identify the common MAPK cascades and other proteins associated in its pathway which id influenced during multiple defense responses. This would help in biotechnological based engineering of defense against Alternaria blight in near future.

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