Research Article

Study of Expression Pattern of a Set of Defense Genes in Response to *Alternaria brassicae* Infection and Salicylic Acid and Jasmonic Acid Treatments in *Brassica juncea*

Ravi P. Saini[1*], Deepika K. Umesh[2], Shivani Nagar[2] and Anita Grover[1]

[1]Division of Molecular Biology and Biotechnology
[2]Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi-110012

Abstract

Alternaria blight is one among the serious diseases of *Brassica juncea* causing up to 45-58% loss in the yield with no proven source of transferable resistance in any of the hosts. The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are major players in the regulation of signaling networks that are involved in induced defense responses against pathogens and insects. In order to develop resistance thoughtful information of defense mechanism in terms of defense signaling molecules involved and defense genes induced during the plant-pathogen interaction need to be understood at the molecular level. In the present study, *Brassica juncea* (Varuna) was taken for studying expression analysis of important defense genes with SA, JA and *in vitro* fungal infection as three separate treatments. We found that chitinase and thionin transcript levels were elevated in mustard leaves upon treatment with JA and during infection with necrotrophic fungal pathogen *Alternaria brassicae*. Conversely expression levels of *PR1* and *NPR1* were induced exclusively upon SA treatment. They were not induced either by JA or after fungal infection. These results clearly indicate existence of two separate hormone dependent pathways i.e. SA and JA in *Brassica juncea* similar to *Arabidopsis thaliana* reported earlier [1]. *Glucanase* expression was also seen during SA treatment although some expression was also seen by JA treatment.

Keywords: Salicylic acid, Jasmonic acid, Fungal infection, *PR1*, *Glucanase* (*PR2*), *Chitinase* (*PR3*), *Thionin* (*PR13*), *NPR1*

***Correspondence**
Ravi Prakash Saini
raviprakashsaini2@gmail.com

Introduction

Brassica juncea is economically very important crop because of its high oil content and superior oil quality. Despite the fact that India is one of the leading oilseed producing countries in the world, it is not able to meet the edible oil requirement for its vast population[2]. The average yield per hectare of the crop is very low (1190 kg/ha) when compared to the world average of 1622 kg/ha. The major factors for poor yield in *Brassica juncea* are insects and diseases. The crop is susceptible to a number of pathogenic diseases among which the most important and devastating fungal disease is *Alternaria* blight, caused by *Alternaria brassicae*. It alone accounts for about 57% loss in yield in *Brassica*[3].

A. brassicae is a necrotrophic pathogen[4], which produces lesions surrounded by chlorotic areas on the leaves, stem and siliquae causing reduction in photosynthetic areas, defoliation and early induction of senescence. The

absence of resistance genes within crossable germplasms of *Brassica* necessitates use of genetic engineering strategies to develop genetic resistance against this pathogen. Therefore, efforts are being done in order to have sufficient knowledge about the genes induced during infection and their regulation measures. This is particularly important for *B. juncea* as there is very little information available on defense mechanisms in this crop.

All over the world biotechnology based fungal disease resistance programmes are being carried out for developing resistance against *Alternaria brassicae* which includes over-expression of genes coding for antifungal compounds, use of specific R genes and manipulation of regulatory genes which encodes signal proteins required for expression of downstream antifungal genes. In order to proceed forward in this area it is necessary to understand the defense response of *Brassica juncea* in molecular terms, when it is infected by *A. Brassicae*.

Plant hormones are essential for the regulation of plant growth, development, reproduction, and survival. The importance of salicylic acid (SA), jasmonate (JA), and ethylene (ET) as primary signals in the regulation of plant defense is well established [5&6]. Pathogens that require a living host (biotrophs) are commonly more sensitive to SA mediated defense responses, whereas pathogens that kill the host and feed on the contents (necrotrophs) and herbivorous insects are generally affected by JA/ET mediated defences[7&8]. Interestingly, SA- or JA-dependent signaling pathways are not always activated exclusively in response to biotrophs or necrotrophs [7] for example, basal resistance against the fungus *Botrytis cinerea* is regulated by SA in tomato but by JA and ET in tobacco[9]. Thus, there is not always a clear cut demarcation of defense signaling pathways against same pathogen between different crop species.

Therefore it is necessary to understand the effects of various harmones and pathogen on the expression analysis of defense genes and to understand signalling pathways mediated by pathogen and hormones and their cross talk. Genes namely *NPR1, PR1, Thionin(PR13), Glucanase(PR2), Chitinase(PR3)* were analysed for this study

Experimental

Materials and Methods

Biological material and growth conditions

Brassica juncea var. Varuna was used in this study. Plants were grown in pots, kept at 25^0C, 16 hrs light/8 hours dark photoperiod and 80% humidity in glass house at National Phytotron Facility, IARI, New Delhi. (**Figure 1**) Collection of leaf samples (medium size) for various treatments was done from one month old seedlings. 20mM SA and 100 µM JA solutions were used for hormonal treatments. Water was used as control in the case of salicylic acid while 1ml of ethanol dissolved in 1 litre of water was used as control in the case of jasmonic acid. *Alternaria* culture was grown on Potato Dextrose Agar media at 22^0C under 70-80% relative humidity for 14 hrs photoperiod.

Figure 1 Plants of Brassica juncea (Varuna) at Phytotron

Treatments

Harmonal treatments: SA and JA treatments were done *in vitro* by using the above mentioned concentration of both the hormones. SA and JA solutions were taken in two separate beakers (volume should be sufficient so that petioles could be dipped) and freshly plucked leaves of one month old seedlings were put, with petioles dipped into it nicely. Collection of leaf samples were done after definite time intervals of 4,8,12 and 24 hrs and kept safely at -70^0C.

***In vitro* fungal infection by spore suspension:** Petriplates having moist filter paper (two layers) were used for *in vitro* studies. Healthy *Brassica juncea* leaves were plucked out and kept in the petriplates, infected with the spore suspension (1.5×10^3spores/ml). For infection 2µl of spore suspension was placed onto two to three randomly selected places on the leaf surface. The leaves were incubated in a BOD incubator for 10 hrs dark and 14 hrs light, moisture content was maintained by periodically watering the filter paper. Incubation was done for definite time intervals of 24, 48, and 72 hrs and necrotic lesions were appeared after prescribed time. (**Figure 2**), leaf samples for RNA isolation were collected from the area surrounding the spot of spore suspension.

Figure 2 Leaf showing necrotic spots after infection

Plant RNA isolation and RT-PCR

Total RNA was isolated by using Trizol method as mentioned in the section from treated and control samples after definite time intervals of 4,8,12 and 24 hrs in the case of hormonal treatments. Total RNA was also isolated from *in vitro* infected leaves after 24, 48 and 72 hrs post inoculation.

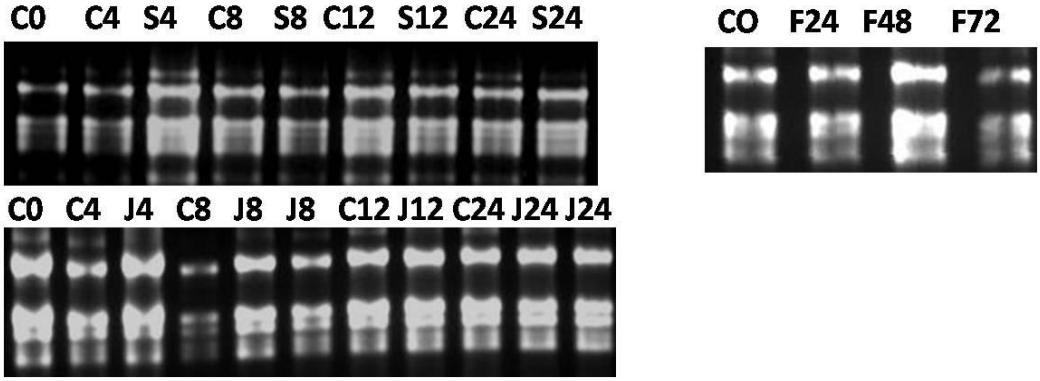

Figure 3 Total RNA isolated after fungal inoculation of leaves; C= Control, S= Salicylic acid, J= Jasmonic acid, F= fungal infection; Numerical digit shows duration in hours after which sample was collected

Table 1 Gene specific primers used for RT-PCR

Sl. No.	Gene	Primer	Sequence	Tm (^oC)
1	*NPR1*	Forward	**5'** TACTGACCTCCTGAAACGTGAG**3'**	66
		Reverse	**5'** TGTCCCGGGTAACTCTGTAACAC'	66
2	*PR1*	Forward	**5'**AGCTCTTGTTCATCCCTCGAAAGC **3'**	72
		Reverse	**5'**TGCAAGAAATGATGGTTCCACC **3'**	64
3	*Glucanase* (*PR2*)	Forward	**5'**TGATGGCTTCCTTCTTCGACACC **3'**	58
		Reverse	**5'**TGCATAGCCTGGATGAGAACC **3'**	46
4	*Thionin* (*PR13*)	Forward	**5'**TGTCATGGCGCAGAATCAGG **3'**	62
		Reverse	**5'**TGCTTTAGCTGAGCCCTTGTTGC **3'**	70
5	*Chitinase* (*PR3*)	Forward	**5'**ATGGGGCTACTGTTTCAAGGACG **3'**	70
		Reverse	**5'**ACCACCACACTCCAATCCACCG **3'**	70

Quality and purity of RNA was checked on 1% agarose gel prepared in 1X MOPS solution. (**Figure 3**). Quantification of same RNA samples was also done through nanodrop instrument. Then RNA samples were reverse transcribed into cDNA by using Fermentas life sciences first strand synthesis kit. Finally RT-PCR was carried out to check the expression analysis of above mentioned defense genes using gene specific primers.(**Table 1)**. Actin gene was used as a loading control in all the three treatments. PCR products were then visualized under a UV light and picture was taken in the Alpha Image gel document system (**Figure 4A,5A,6A**.).

Expression analysis of *PR1* gene

SA treatment

It was seen that *PR1* gene was induced under SA treatment and the increase was detected as early as 4hrs after treatment. The induced expression reached a maximum of about 4-6 folds after 24hrs over control plants (water treated). (**Figure 4B**)

JA treatment

JA treatment did not induce *PR1* gene expression at least till 24hrs. (**Figure 5B**)

Fungal infection treatment

PR1 was not induced by fungal infection till 72hrs of fungal infection. (**Figure 6B**)

Expression analysis of *glucanase* (*PR2*)gene

SA treatment

Expression of *glucanase* gene was induced as early as 4hrs and remains continued till 24hrs. The plateau of expression was observed at 4hrs of treatment and decreased sharply in later time intervals. Induction at 4hrs was about 4-6 folds over control plants. (**Figure 4C**)

JA treatment

Induction of *glucanase* gene was absent after jasmonic acid treatment. (**Figure 5C**) A faint band can be seen at 4hrs of jasmonic acid treatment.

Fungal infection treatment

Expression of *glucanase* gene was absent by fungal infection. (**Figure 6C**)

Expression analysis of *chitinase* (*PR3*)gene

SA treatment

SA did not induce expression of *chitinase* gene but there seems to be very little basal expression in control plant also along with S4 and S8. (**Figure 4D**)

JA treatment

It was seen that expression of *chitinase* gene began at about 4hrs after JA treatment and continued till 24hrs. Induction was about 6-10 folds over control plants (water treated).Gel picture clearly showed a marked induction of chitinase gene by JA. (**Figure 5D**)

Fungal infection treatment

Expression of *chitinase* gene was seen at 24 hrs post inoculation and diminished completely at later time intervals. (**Figure 6D**)

Expression analysis of *NPR1* gene

SA treatment

It was seen that expression of *NPR1* gene began at about 4hrs upon SA treatment and continued till 24hrs. Induction was about 2-3 folds over control plants (water treated). (**Figure 4E**)

JA treatment

Induction of NPR1 gene was not seen clearly, very little expression was observed after 4hrs of treatment.(**Figure 5E**)

Fungal infection treatment

NPR1 gene was not expressed during fungal infection. (**Figure 6E**)

Expression analysis of *thionin* (*PR13*)gene

SA treatment

As seen in the figure induction of *thionin* gene was absent during salicylic acid treatment. (**Figure 4F**)

JA treatment

Expression of *thionin* gene was induced as early as 4hrs and increased constantly till 12 hrs, with plateau of expression at 12hrs and then decrease subsequently at 24 hrs. Induction was about 6-10 folds over control plants (water treated).Gel picture (**Figure 5F**) clearly showed a marked induction of *thionin* gene by JA.

Fungal infection treatment

Induction of *thionin* gene was very prominent at 24hrs after infection and it totally subsides by 48hrs.(**Figure 6F**)

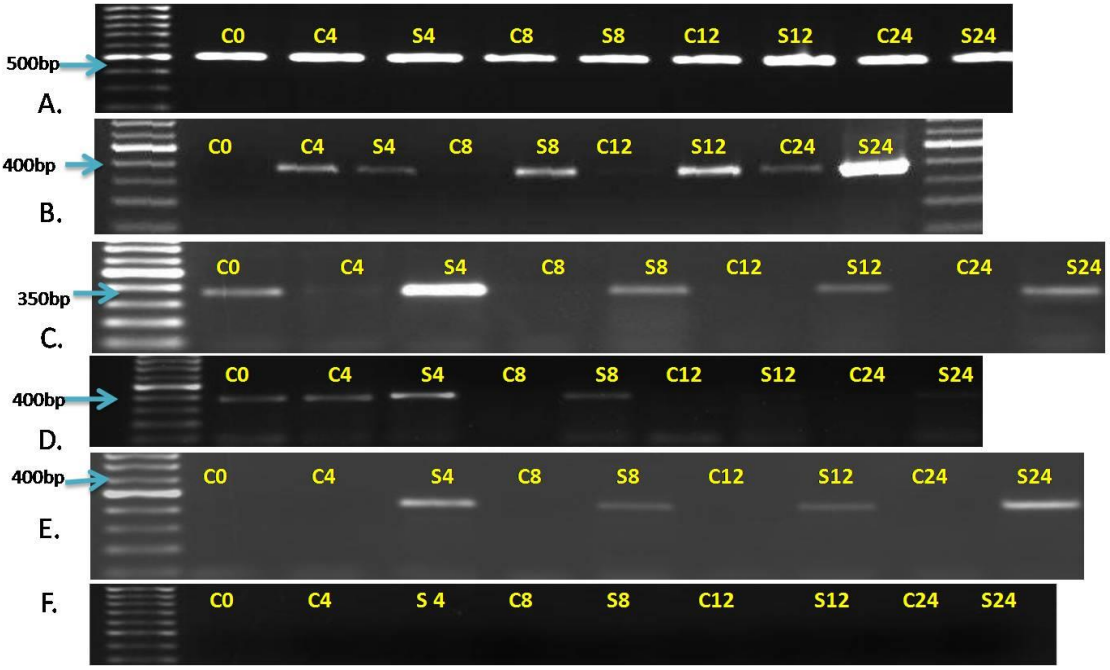

Figure 4 RT-PCR showing expression of A = Actin gene,B = PR1 gene , C.= PR2 gene, D= PR3 gene; E. = NPR1 gene; F.= PR13gene; C= Control; S= Salicylic acid treatment; M= DNA ladder
Numerical digit shows duration in hours after which sample was collected

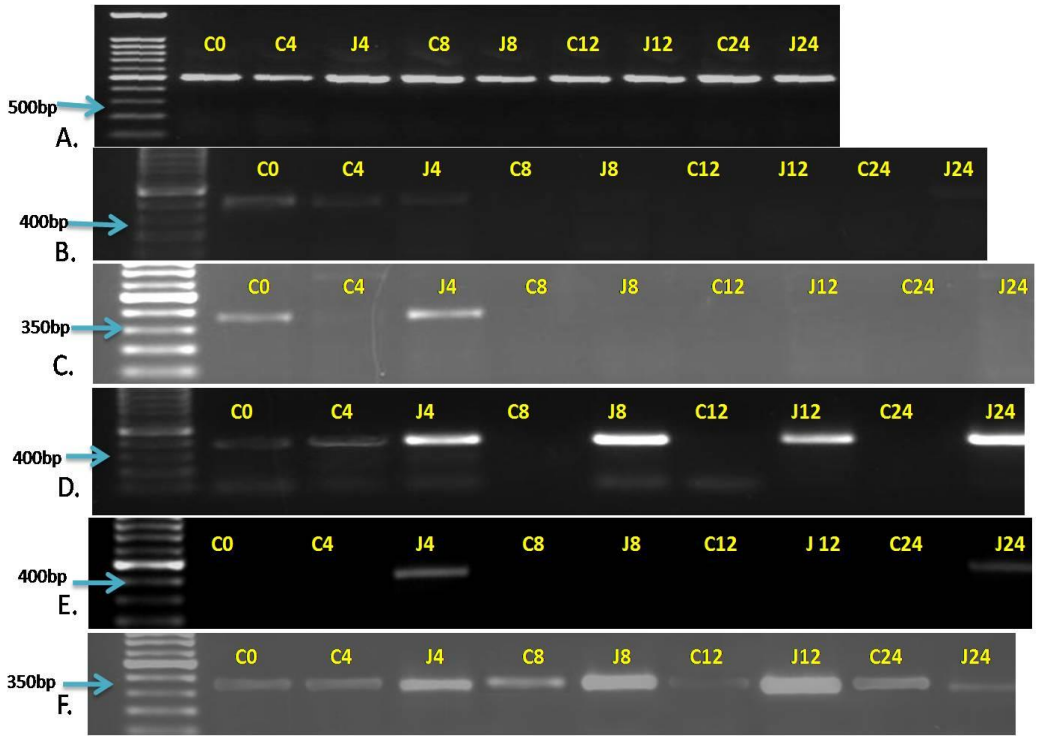

Figure 5 RT-PCR showing expression of A = Actin gene,B = PR1 gene , C.= PR2 gene, D= PR3 gene, E.= NPR1 gene, F.= PR13gene; C= Control, J= Jasmonic acid treatment; M= DNA ladder
Numerical digit shows duration in hours after which sample was collected

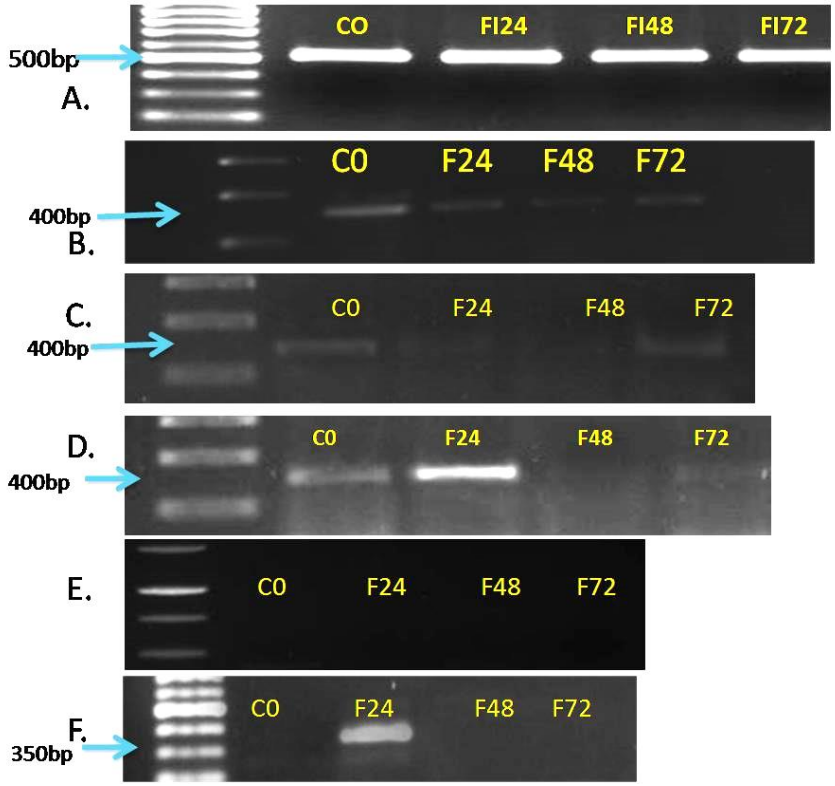

Figure 6 RT-PCR showing expression of A = Actin gene, B = PR1 gene , C.= PR2 gene, D= PR3 gene, E.= NPR1 gene, F.= PR13gene; C= Control, F= Fungal infection, M= DNA ladder
Numerical digit shows duration in hours after which sample was collected

Discussion

When a pathogen infects a plant there is a immediate local response expressed in the form of necrotic lesions (hypersensitive response) and there is a systemic signal which spreads to other uninfected parts of the plant, this involves the expression of several gene families called SAR genes, five of these gene families encode PR proteins, some of them have been demonstrated to have antifungal activity. These observations suggest that the SAR proteins may play a causal role in the immunized state. Role of SA in SAR is already well established[10] classically thought to be important against biotrophic pathogens. By contrast, recent studies implicate lipid signals such as JA-derived molecules are required for SAR [11&12], and a putative lipid transfer protein is required in challenged tissue to initiate a mobile signal [13]

In the present study we examined the effect of salicylic acid(SA), jasmonic acid(JA) (defense signaling hormones) and *Alternaria brassicae* infection on the expression analysis of various defense genes as compared to control (water treated) conditions in mustard leaves. RT-PCR (with gene specific primers) was done to analyze the differential expression of defense genes. It was found that the expressions of *PR1* and *NPR1* genes were exclusively induced by salicylic acid but not induced at all during JA and *Alternaria* infection. SA responsive transcriptional induction of *PR1* and *NPR1* has been reported in *Arabidopsis* earlier[14].

We further observed that highest level of expression of *PR1* and *NPR1* reached at 24 hours upon SA treatment. Similar report of *PR1* gene expression was reported by[15] through northern blot analysis. Above mentioned results clearly indicate that SA act as an inducer molecule for the expression of *PR1* and *NPR1*[16]. Expression of *chitinase* and *thionin* genes was seen upon JA and *Alternaria* infection but not through SA. Analogous result of induction of

thionin gene upon JA treatment was reported by[17]. By seeing the expression of both chitinase and thionin genes during fungal infection and JA treatment we might suggest role of JA as a defense signaling molecule during *Alternaria* infection.Expression of *glucanase* gene was found only in salicylic acid treatment, with highest level of expression during 4 hours which proposes role of SA in its induction. These results indicate existence of two separate hormone dependent pathways i.e. SA and JA in *Brassica juncea* similar to *Arabidopsis thaliana* reported earlier by [1].

Conclusion

There are two separate, SA and JA signaling pathways in *Brassica juncea* which results in the induction of different defence responsive genes.In *Brassica juncea*, *Alternaria brassicae* induces JA signaling pathways there by inducing chitinase and thionin genes which are marker genes for JA pathway.

Sl. No.	Gene name	Treatments		
		Salicylic Acid	Jasmonic Acid	Fungal Infection
1	*PR1*	+++	-	-
2	*PR2*	++	-	-
3	*PR3*	-	+++	++
4	*PR13*	-	+++	++
5	*NPR1*	++	-	-

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