Gene expression profiling of *Arabidopsis thaliana* chitinase genes in response to *Alternaria brassicae* challenge

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ABSTRACT: The experiment was conducted to understand the gene expression profile of ten Arabidopsis chitinase genes upon *Alternaria brassicae*- *Arabidopsis thaliana* pathogenesis, both locally as well as systemically. At3g12500 (basic endochitinase), class I chitinase gene induced significantly in distal regions of infected plants. At3g16920 (Chitinase-like protein), member of class II chitinase induced 6 h post-inoculation both locally as well as distally. Among the four class IV, chitinase genes, At2g43590 (Chitinase family protein) displayed significant induction locally after infection. However in distal regions, At2g43570 (Putative chitinase) and At3g47540 (Chitinase-like protein) showed visible differential expression profile. Among class V genes, At4g19810 (Glycosyl hydrolase superfamily protein) was the only gene to give significant expression profile locally and also systemically. The genes expressed locally are presumed to have catalytic function to cleave β-1, 4-glycoside bond present in biopolymers of N-acetylglucosamine of chitin layer in fungal cell wall and can be candidate genes for imparting PAMP triggered immunity (PTI) and effector triggered immunity (ETI). The genes having significant expression profile for different time intervals upon *A. brassicae* challenge systemically, viz. At3g12500, At2g43590, At2g47540 and At4g19810, which are considered to be as excellent candidates of induced systemic resistance (ISR). The information obtained would be utilized for characterization of pathogen inducible chitinase promoters and development of transgenic plants for fungal resistance and can also be used to dissect transcription factors interacting with these genes in the signaling cascades.

Key words: *Alternaria brassicae*, chitinase, local and systemic response, pathogenesis, induced systemic resistance

Fungi are important pathogens causing major yield losses in cereals, pulses, oilseeds, vegetables and fruits, leading to severe crop losses both in quantity and quality. About 125 million tonnes of crops are vanished every year. This is sufficient to feed at least 600 million people across the world (Mathew et al., 2012). Brassicaceae family consists of about 3500 species in 350 genera. The *Alternaria brassicae* and *A. brassicicola* are two important necrotrophic pathogens for yield loss of *Brassica* species all over the world (Westman et al., 1999). One of the major constraints on productivity of Indian mustard is damage due to Alternaria leaf spot, which is caused by *Alternaria brassicae*. Depending upon severity, the yield losses have been estimated as 35-46% (Mishra et al., 2010) in India and up to 70% all over the world with no proven source of transferable resistance in any of the hosts (Vishwanath et al., 1997). Disease management strategies utilizing fungicidal chemicals are practically insufficient in addition to being environmentally hazardous. Grover and Gowthaman (2003) opined that utilizing desirable genes for the development of transgenics to improve crop yields and helps to understand complex molecular phenomemns that occur during pathogenesis. Therefore, transgenic *Brassica* plants over-expressing antifungal genes like chitinases are required.

Pathogenesis related proteins are defined as proteins encoded by host plant induced under pathological or related conditions. They are set of novel low molecular weight compounds associated with host defense mainly in incompatible interactions which impedes the pathogen progress (Mehrotra and Aggarwal, 2003). Chitinases (EC 3.2.1.14) are PR proteins under family PR-3, PR-4, PR-8 and PR-11 constituting five classes. They catalyse the hydrolytic cleavage of β-1, 4-glycoside bond present in biopolymers of N-acetylglucosamine. The fungal cell wall is constituted of chitin as its major component and chitinases expressed upon fungal infection have an ability to degrade their cell walls, restricting fungal growth and development. They are induced in hypersensitive response (HR) and induced systemic resistance (ISR) defense mechanisms mediated by jasmonic acid (JA) and/or ethylene (ET).

Complete genome sequencing of *Arabidopsis thaliana* in 2000, has paved the way for identification of 25 putative Arabidopsis chitinase genes distributed among five chromosomes. The microarray data from geneinvestigator on gene expression studies of chitinase genes reveal that, it responds to various biotic and abiotic stresses. Natural variations in susceptibility and resistance to Alternaria have been shown to exist in model plant Arabidopsis ecotypes and several mutants have been identified that confer increased susceptibility to Alternaria. Further, enormous genomic resources that are available for Arabidopsis make it an ideal system to work (Kagan and Hammerschmidt, 2002).

Several transgenic plants harbouring chitinase genes have been developed (Punja, 2001) and they showed resistance to fungal pathogens. The role of plant chitinases against fungi has been characterized, the first document of transgenics for fungal resistance was...
developed by constitutively expressing bean chitinase gene in tobacco and *Brassica napus* (Broglie et al., 1991). Overexpression of *Momordica charantia* chitinase gene (*McCHI1*) significantly enhanced resistance to *Phytophthora nicotianae* in transgenic *N. benthamiana* and Verticillium wilt in transgenic cotton by increasing the endochitinase activity. Mondal et al. (2003) have transformed Indian mustard with chitinase gene under 3SS promoter and showed that these transgenics had reduced infestation with Alternaria leaf spot by 12-56% as compared with non-transgenic plants. Sheath blight fungal-resistant finger millet has been obtained by inserting rice chitinase gene (*chi11*) (Ignacimuthu and Ceasar, 2012).

Arabidopsis chitinase genes have not been utilized for the development of transgenics to impart fungal resistance through over expression either driven by 3SS promoter or by pathogen inducible promoter. Since *Arabidopsis thaliana* and *A. brassicae* pathosystem exhibit incompatible interaction, the identification of chitinase genes induced under this situation would be better candidates for heterologous transgenics development and can also be utilized for characterization of fungal pathogen responsive chitinase promoters. With this background the gene expression profiling of chitinase genes in response to Alternaria was performed.

**MATERIALS AND METHODS**

**Isolation of pathogen**

The infected leaves of mustard (*Brassica juncea*) cv. Varuna having clear symptoms were brought from the field of IARI, New Delhi and used to isolate *Alternaria brassicae*. Initially, it was isolated by using PDA and then sub-cultured for pure culture maintenance on Radish Dextrose Agar (RDA) medium (Thakur and Kolte, 1985) in a growth chamber set at 22°C with 16h/8h light/dark periods. Conidial suspensions were prepared by scraping mycelium from 25-day-old cultures and suspending in sterilized distilled water. The conidial suspensions were centrifuged at 4000 rpm for 4 min, the residue re-suspended in 0.03% tween-20 surfactant solution, and then re-centrifuged to a working concentration of 100ng/µl.

**Sample collection**

Leaf samples were harvested from Alternaria challenged plants for RNA isolation at an interval of 6, 12, 24, 48, 72 and 96 h post inoculation (hpi). The leaf portion surrounding the infected area was collected from infected leaves to study local response and samples were obtained from distal leaves from infected plants in order to study systemic response. Samples were immediately frozen in liquid nitrogen and stored at -80°C for isolating RNA.

**RNA isolation for RT-PCR**

Arabidopsis total RNA was extracted from harvested leaf samples using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Formaldehyde agarose gel was used to run isolated RNA in order to check its integrity and it was quantified by NanoDrop 1,000 (NanoDrop Technologies, Inc., DE, USA). First-strand cDNA was synthesized from 1µg of total RNA using Oligo (dT) primer (Thermo Scientific, USA) according to the manufacturer's instructions and quantified as above mentioned. The cDNAs were diluted to a working concentration of 100ng/µl.

**Designing and synthesis of chitinase/house-keeping genes primers for RT PCR**

*In-silico* analysis was performed prior to the design of primers for RT-PCR. Ten chitinase genes were shown to be up-regulated in Arabidopsis by biotic stress based on the available genevestigator microarray data. The PCR primers were designed using Clone manager and Primer-3 Software’s based on the sequence information available in Arabammon database and primers of *α-tubulin* were designed from cDNA sequences of Arabidopsis. The corresponding primers and gene accession numbers are listed in Table 1. All primers used were submitted to NCBI database for BLASTn search and confirmed to specifically anneal only with their corresponding genes. During designing primers, all the parameters for RT primers were considered and followed systematically. All the designed chitinase primers were synthesized by SIGMA Company.
with their experimental results and are normalized with alpha-tubulin as a house-keeping gene. All the genes expression patterns are explained as local expression (gene expression from RNA isolated from Alternaria infected leaves per se) and distal expression (gene expression from leaves other than Alternaria infected but from the same plant to look for the systemic response).

Class I chitinase

At3g12500 was the only member represented the Class I chitinase from Arabidopsis thaliana. This is a basic endochitinase involved in JA and/or ethylene signal transduction pathways.

Local expression:

There was no expression of this gene locally in A. brassicae infected leaves across time course of 6h to 96h and also there was no basal expression in control. In order to eliminate confusion about PCR functioning, the positive control was included.

Distal expression:

Interestingly AT3g12500, basic endochitinase induced distally giving good expression at 48h and 96h post inoculation.

Alternaria being a necrotrophic fungus, the plant resistance is obtained through ISR mediated by JA and

### Table 1. List of chitinase/house-keeping genes primers used for semi quantitative RT-PCR

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Primer sequence</th>
<th>bp</th>
<th>A.T °C</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT3G12500F</td>
<td>5<code> AATGATGCGCTTGTGCTTG 3</code></td>
<td>522</td>
<td>60</td>
<td>Basic chitinase</td>
</tr>
<tr>
<td>AT3G12500R</td>
<td>5<code> TCCATAACCGGTAATCTCCC 3</code></td>
<td>569</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT3G16920F</td>
<td>5<code> GGATGGTGAGTTGTTG 3</code></td>
<td>673</td>
<td>57</td>
<td>Chitinase-like protein</td>
</tr>
<tr>
<td>AT3G16920R</td>
<td>5<code> CGTTATCGAATCCGGTGTTG 3</code></td>
<td>532</td>
<td>55</td>
<td>Glycosyl hydrolase superfamily protein</td>
</tr>
<tr>
<td>AT4G19730F</td>
<td>5<code> GAAACCCTCAGGTGAAGAC 3</code></td>
<td>505</td>
<td>55</td>
<td>Glycosyl hydrolase superfamily protein</td>
</tr>
<tr>
<td>AT4G19730R</td>
<td>5<code> CAGCCAACGTAGGAATC 3</code></td>
<td>515</td>
<td>55</td>
<td>Glycosyl hydrolase superfamily protein</td>
</tr>
<tr>
<td>AT4G19750F</td>
<td>5<code> CAAGCCACTTACAACC 3</code></td>
<td>515</td>
<td>55</td>
<td>Glycosyl hydrolase superfamily protein</td>
</tr>
<tr>
<td>AT4G19750R</td>
<td>5<code> CTCCAGCTTACGAAAC 3</code></td>
<td>554</td>
<td>55</td>
<td>Glycosyl hydrolase superfamily protein</td>
</tr>
<tr>
<td>AT4G19810F</td>
<td>5<code> GTCATCGCGTTGAATAG 3</code></td>
<td>554</td>
<td>55</td>
<td>Putative chitinase</td>
</tr>
<tr>
<td>AT4G19810R</td>
<td>5<code> CAGTGTAGCCGCGTAGTAG 3</code></td>
<td>534</td>
<td>58</td>
<td>Putative chitinase</td>
</tr>
<tr>
<td>AT2G43570F</td>
<td>5<code> TACGACGTAGAGATCTG 3</code></td>
<td>532</td>
<td>57</td>
<td>Chitinase family protein</td>
</tr>
<tr>
<td>AT2G43570R</td>
<td>5<code> CTCCAGCTTACGAAAC 3</code></td>
<td>532</td>
<td>57</td>
<td>Chitinase family protein</td>
</tr>
<tr>
<td>AT2G43580F</td>
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<td>505</td>
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<td>Chitinase-like protein</td>
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<tr>
<td>AT2G43580R</td>
<td>5<code> GTCCAGCTTACGAAAC 3</code></td>
<td>505</td>
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<td>Chitinase-like protein</td>
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<tr>
<td>AT2G43590F</td>
<td>5<code> GGCTGGCGAATCAGGATAG 3</code></td>
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<tr>
<td>AT2G43590R</td>
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<td>431</td>
<td>58</td>
<td>Chitinase-like protein</td>
</tr>
<tr>
<td>AT3G47540F</td>
<td>5<code> CCGGCTGCCGTTAACTTAG 3</code></td>
<td>613</td>
<td>53</td>
<td>Housekeeping gene</td>
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<tr>
<td>AT3G47540R</td>
<td>5<code> CGCTGGCGAATCAGGATAG 3</code></td>
<td>613</td>
<td>53</td>
<td>Housekeeping gene</td>
</tr>
</tbody>
</table>

*Note: bp: RT-PCR product size, A.T: Annealing temperature

### Thermal conditions followed for RT-PCR

The PCR was performed using a thermocycler (Applied Biosystems) in a 25-µL final volume including 2µL of diluted cDNA template, 2.5 µL of 10X amplification buffer (Thermo Scientific, USA), 0.5 µL of 10 mM Deoxyribonucleotide triphosphates (Thermo Scientific, USA), 0.5 µL of 10 pico-molar of each primer, and 0.2 µL (1 U) of dreamtaq DNA polymerase and 18.8 µL of PCR grade water. The PCR steps included an initial denaturation at 94°C for 4 min, followed by 30 cycles of 94 °C for 30 s, 53-60°C for 30 s, 72°C for 1 min with a final extension at 72°C for 10 min. The PCR products were separated using 1.2% agarose gels, stained with ethidium bromide and observed on a UV transilluminator. Experiments with all primers were carried out at least for three times.

### RESULTS AND DISCUSSION

The Arabidopsis chitinases represented all the classes (Class I- V) of chitinases classified to date based on their sequence and structural analysis. Class I, II and IV belonged to the family 19, whereas class III and V come under family 18 of chitinases. The 10 chitinase genes considered under this study are discussed below along with their experimental results and are normalized with alpha-tubulin as a house-keeping gene. All the genes expression patterns are explained as local expression (gene expression from RNA isolated from Alternaria infected leaves per se) and distal expression (gene expression from leaves other than Alternaria infected but from the same plant to look for the systemic response).

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**Distal expression:** Interestingly AT3g12500, basic endochitinase induced distally giving good expression at 48h and 96h post inoculation.

Alternaria being a necrotrophic fungus, the plant resistance is obtained through ISR mediated by JA and
ethylene. Since this pathosystem showed compatible interaction initially as the leaf blight symptoms appeared in our study, indicating IARI strain was virulent. The symptoms also evidenced to show that the leaf margin or outer area of leaf was more susceptible than inner or basal portion of leaf. There was no spreading of symptoms after four days post inoculation (dpi), proving that Arabidopsis displayed incompatible interaction with Alternaria after duration of time course. This clearly demonstrated presence of either ETI or PTI to display HR in local infected regions. The HR in turn activates ISR to provide broadspectrum resistance throughout the plant. This gene induction at site of infection not showed any fold change increase at series of time intervals after pathogen inoculation (Fig. 1). This indicated that at the site of infection, it has no role to play, means there is no endochitinase activity showed at point of infection. At3g12500 might not be the candidate gene for ETI and/or PTI, may be other associated genes and PR genes would involve in these actions. But the expression of this gene showed excellent induction in the distal portion (Fig. 1) of infected plants. It means there was the systemic response and the signal transduction may be mediated through JA and ET as it is known that basic endochitinase is involved in these pathways (Thomma et al., 1999).

The ATCHIB (At3g12500) was shown to inhibit Trichoderma reesei. At the same time, it was not effective against other fungi tested by Verburg and Huynh (1991). They suggested that its action was specific for a particular fungus. Thomma et al. (1999) demonstrated that this gene induced by fungal pathogen infection but in presence of ethylene, this evidenced to show that class I chitinase of Arabidopsis chitinase might be induced in distal portions because of the activation of ISR.

Class I chitinase gene involved in the signal transduction pathways would be a good candidate for further basic research to identify transcription factors interaction and the protein activity in the infected plants. The promoter characterization of such genes enables us to identify the cis-elements involved in signal transduction interacting with the transcription factors.

Class II chitinase

In Arabidopsis, there are four class II chitinase members identified to date and not much study has been done on them. Based on the sequence information, it was revealed that due to lack of essential amino acid residues these might not perform chitinase like functions (Passarinho and De Vries, 2002).

The class II putative chitinase gene, At3g16920 having other functions like regulation of protein trafficking between Golgi and plasma membrane in-trans has been studied. The expression pattern of this gene showed significant induction 6hpi in both the samples of local as well as distal portions (Fig. 1). The gene expression was not observed after 12hpi in local infected leaves. But slightly lower levels of gene expression persisted in distal leaves of infected leaves. It might indicate that gene expression may be higher during early stages of infection; means needs to analyze the expression at each hour time course immediately after pathogen inoculation. The results suggest that it can be used to develop transgenics for fungal defense because of its early induction.

Class IV chitinases

Chitinases survey and sequence analysis of class IV chitinases from Arabidopsis enlightened that, they are not prominently involved in defense mechanisms rather they are more likely to be involved in developmental regulation (Passarinho and De Vries, 2002). But in our studies on this class of chitinases revealed that they too are involved in defense mechanisms. The genes studied under this class are, At2g43570, At2g43580, At2g43590 and At3g47540 and their expression pattern is as under:

**Local expression:** The gene At2g43590 displayed significant early induction after 6hpi and also 24hpi (Fig. 2) but its expression seemed to be seldom decreased after 24hpi. However, genes At2g43570 and At3g47540 showed very little induction upon pathogen infection. The gene, At2g43580, had no induction across the time course of Alternaria challenge.

**Distal expression:** The genes At2g43570, At2g43590 and At3g47540 showed highly significant expression profile across time intervals after Alternaria infection (Fig. 2). However, At2g43580 did not induce in response to Alternaria pathogen and it is as like local expression, it

![Fig. 1. Time course transcript accumulation of class I and Class II chitinase genes in local and distal leaves after Alternaria brassicae inoculation of Arabidopsis plants in vivo.](image-url)

*Total RNA was isolated from leaves of treated plants as per the time course. Semi quantitative RT-PCR was performed from cDNA made from each RNA sample. α-Tubulin transcripts mentioned in Fig:2 are same for class I and II and were used to normalize the samples. 30 PCR cycles were performed. Note: -ve and +ve controls are kept to eliminate doubts of any contamination and PCR functionality.*
might be induced specifically upon challenge of other fungal pathogens other than Alternaria. The analysis by Passarinho and De Vries (2002) revealed that some of the class IV chitinases are pseudogenes. This gene might be the representative of pseudogene and had no induction after infection.

The results evidenced to say that, gene, At2g43590, induced very early upon pathogen attack and may have role in HR to restrict pathogen spread. It is also imperative that this could be member of ISR mediated gene as its expression persisted throughout the experimental post inoculations in distal portions of the plant. The genes At2g43570 induced systemically in distal portions and showed significantly visible expression profile after 6, 48 and 96 hpi and it also holds good for At3g47540 as well. This infers that their expression depends upon production of phytohormones such as JA and ET and their transport to the other parts of the plant. Nayanakantha et al. (2014) demonstrated that, EF586206.1 chitinase gene of *Brassica juncea* is the class IV gene, its expression in *Camelina sativa* upon *A. brassicae* challenge was negligible in local regions where as in the distal parts it showed significant expression. These results support our investigation that, class IV genes displayed significant induction systemically.

They seem to be excellent candidates from class IV chitinases to be considered for further characterization of their functions upon pathogenesis of various fungal pathogens. It would be helpful for characterization of pathogen responsive promoters and development of transgenics for fungal pathogens.

**Class V chitinases**

This class has the diversified chitinase members speculated to play role in defense as well as developmental regulation but very limited aspects have been uncovered about their full functions (Melchers et al., 1994). Among nine available class V Arabidopsis chitinase genes, four genes were selected for gene expression profiling based on microarray data from plant membrane databases. The genes studied under this class were, At4g19730, At4g19750, At4g19760 and At4g19810.

**Local expression:** At4g19810 showed highly significant visible expression immediately after 6hpi and its expression was almost negligible after 24hpi (Fig. 3). Whereas At4g19730 and At4g19750 induced at non significant levels across the time intervals upon inoculation. But At4g19760 gene expression was not found locally.

**Distal expression:** Interestingly only the gene At4g19810 showed enhanced expression profile from systemic response (Fig. 3) however At4g19760 had visible expression after 96hpi.

At4g19810 induced very early in the local regions to have catalytic function and also showed spectrum of expression profile in the distal regions. It encompasses its role in signal transduction pathways to restrict pathogen spread. The functional characterization of this gene in events such as HR (Hypersensitive Response), programmed cell death and ISR will give better insights into its role in plant protection (Fig. 3). From the above
outcome it is evident that At4g19810 would be the potential member from the class V chitinase for the development of fungal resistant transgenic plants.

Thus, expression profile of Arabidopsis chitinase genes in response to Alternaria infection reveals that they are induced both locally as well as systemically of infected plants. It indicates their role, in early defense mechanisms against Alternaria through ETI and/or PTI and in ISR mediated by JA and/or ET to provide broad spectrum resistance. Brassica juncea is a susceptible host for Alternaria, causing severe yield losses and there is no potential source of transferable resistance in Brassica. The induced genes of Arabidopsis can be positively utilized in Brassica transgenic development programmes to give better resistance to increase yield potential. In addition, the promoter regions of these genes can be characterized to identify cis and trans elements responsible for the induction of these genes under pathogen stress. This information would be useful for the development of synthetic pathogen inducible promoters and can be directly utilized for the development of fungal resistant transgenic plants in several crop plants under pathogen inducible promoters instead of constitutive promoters.

REFERENCES


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