Analysis of chitinase gene specific transcript accumulation in tea \textit{[Camellia sinensis (L.) O. Kuntze]} during induced systemic resistance by methyl jasmonate

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Chitinase gene (Pr-3) specific transcript accumulation was increased by 5.57\% in mature leaf compared to the other \textit{in vitro} grown tissues in tea (\textit{Camellia sinensis}) genotype (T383) during induced systemic resistance by methyl jasmonate. Chitinase (Pr-protein) gene specific two primers were used in RT-PCR reaction to measure the differential transcript accumulation in different tissue systems (young leaf, mature leaf, callus and shoot regenerated from somatic embryo). The time-course expression patterns resulted in differential expression of two transcripts (254 & 366 bp) in response to induction. The 366 bp transcript was sequenced bidirectionally and deposited into the GenBank of NCBI (acc.no. EU373553).

\textbf{Keywords:} \textit{Camellia sinensis}, chitinase gene transcript, ISR, RT-PCR.

\section*{Introduction}

Plant chitinases belong to a family of pathogenesis-related (PR) proteins, which are over-expressed by plants in response to a pathogen attack\textsuperscript{1-3}. Chitinases catalyze the hydrolysis of the linked $\beta$-1,4-$N$-acetyl-$D$-glucosamine (GlcNAc) polymers that form chitin chains, a major component of fungal cell walls, and is involved in the inducible defenses of plants. During the local and systemic responses of plants, a large group of defense enzymes, PR-proteins and signal molecules is synthesized in high amounts to display a broad spectrum of antimicrobial activity\textsuperscript{4-6}. The mRNA accumulation for many plant defense genes is more rapid during interactions involving a plant that expresses resistance to a particular pathogen\textsuperscript{7}. Researchers have tried to isolate different chitinase genes from different plant species to use it in crop improvement programme in order to increase the plant’s own immune system\textsuperscript{8-11}. Wounding can induce formation of jasmonates and ethylene, each of which is capable of independently inducing signal transduction pathways leading to resistance against plant pests. Jasmonic acid and related compound methyl jasmonate are part of the octadecanoid pathway in plants\textsuperscript{12} and in some cases contribute to the resistance to insects\textsuperscript{13,14} and pathogen\textsuperscript{15}. There is no report on chitinase gene specific transcript accumulation in tea plants and no initiative has been taken for the improvement of Indian tea crop by manipulating the chitinase like defense protein. So, the present investigation provides novel insights into the chitinase specific gene expression in tea in response to defence signalling compound.

\section*{Materials and Methods}

\textbf{Defense Induction by Foliar Application of Methyl Jasmonate}

Four categories of plant material were used to induce transcript accumulation after induction with methyl jasmonate. The 12-months-old tea plants (genotype T383) were grown in earthen pots and maintained in greenhouse in ambient light and temperature. Leaf development was separated into two stages: Stage I- young leaves, 2-4 cm long (YL), and Stage II- mature leaves, 5-9 cm long (ML). The test solution, methyl jasmonate (0.2 mM) was hand sprayed in the form of foliar application at a rate of 40 mL/m\textsuperscript{2}, so as to wet the leaves completely on both the ventral and dorsal surfaces. Similarly tissues (tea genotype T383) from different growth and developmental stages of \textit{in vitro} grown calluses (2-month-old) and \textit{in vitro}-regenerated shoots of somatic embryos (6-month-old) were treated with test solution after filter sterilization through a 0.22 µm disposable filter. Controls were mock sprayed with distilled water plus Tween-20. Sprays were applied...
between 10.00 am to 11.00 am, while stomata remained consistently open. The leaves, callus and shoots were harvested from different time courses (0, 0.3, 2, 6, 12, 16, 24, 36, 48, 72 h), then frozen in liquid nitrogen and stored at −80°C before RNA isolation. Experiments were repeated twice with three replicates.

**Total RNA Isolation (Transcripts), Purification and Quantification by Spectrophotometry**

Total RNA isolation was carried out from the above abiotically induced tea plant tissues as described before. The purified total RNAs were quantified with a UV-vis Spectrophotometer (Shimadzu, 160) at a wavelength of 260 nm. The integrity and the quality of total RNA were verified by running samples on 1.2% denaturing agarose gel. RNA was fractionated on 1.2% agarose gel containing 2.2 M formaldehyde (commercial formaldehyde is 12.3 M) to check its quality and integrity.

**One-step RT-PCR for Chitinase Gene Specific Transcript Accumulation**

Chitinase gene specific transcript accumulation (mRNA transcription level) was obtained through RT-PCR method using RNA from treated and untreated (control) samples. 15 µg RNA (total, including the chitinase gene transcript, mRNAs) was taken from each of the treated samples in different time courses as a starting material in RT-PCR reaction. The one step M-MuLV RT-PCR kit of Bangalore Genei was used in the present study and the reaction was carried out according to the manufacturer’s instruction manual. The first strand cDNA synthesis was performed using the above one-step RT-PCR reaction mixture by incubating the reaction for 30 min at 50°C. The cDNA obtained was PCR amplified in 35 cycles consisting of 94°C for 1 min, 50°C for 1 min and 72°C for 7 min for final extension in a thermocycler using the chitinase gene specific primer 5′-TTTTCGTCGAAAATGGAAAG-3′ (forward primer) 5′- ACCAGCTTCTTCGTTGCCAA-3′ (reverse primer). The second DOP-primer pair constructed from the chitinase protein sequences which were also used in the RT-PCR to quantify the chitinase gene specific transcript accumulation in both treated (induced) and untreated samples. Forward DOP-primer was 5′-CTGTGGCCTGGCAAGTGGkgytggwrytg-3′ and reverse primer was 5′-CAGTAGCGACGACGTGGCArywccarcmsma-3′. In case of DOP-primer, the cycling temperature for 35 cycles was as follows: 94°C for 1 min, 60°C for 1 min and 72°C for 2 min and 72°C for 7 min for final extension. After the amplification in RT-PCR, the cDNA product was extracted, purified and quantitated with spectrophotometer.

**RT-PCR Product Quantification on Gel**

The amplified cDNA product obtained from the RT-PCR was analyzed by electrophoresis on 1% agarose gel for visual quantification of the chitinase gene specific transcript accumulation in different samples (treated and control) after staining with ethidium bromide on UV-Transilluminator and photographed.

**Quantification of Purified RT-PCR Product (cDNAs) to Measure Transcript Accumulation**

The cDNA product of RT-PCR was purified according to Sambrook and Russell. The purified RT-PCR products (cDNAs) from each of the treated and untreated samples during induced systemic resistance by methyl jasmonate were measured using spectrophotometer.

**DNA Sequencing**

The RT-PCR product of 366 bp was sequenced bidirectionally through the BigDye terminator technology (Applied-Biosystem, at Bangalore Genei, Bangalore, India). The 254 bp product of RT-PCR has not been sequenced because it was the product of chitinase gene specific primer and not from DOP-primer.

**Results and Discussion**

**Differential Gene Expression**

Differential gene expression was estimated through the quantification of RNA after induction with methyl jasmonate. The yield of total RNA (µg/g fresh wt, FW) was as follows: 657, 660, 645 and 651 for young leaf, mature leaf, callus tissues and regenerated shoots, respectively. For all samples, the A260/280 ratios ranged from 1.88 to 1.93, indicating lack of protein contamination. Further, the A260/230 ratio was higher than 2.0 for all the samples, which shows that the RNA was of high purity and without polyphenol and polysaccharide contamination. The RNA integrity was assessed by the sharpness of RNA bands visualized on a denaturing 1.2% agarose gel (Fig. 1). Total gene expression pattern (RNA transcriptions) was increased in the treated samples, which could be judged by the increasing amount of RNA synthesis (Table 1). RNA transcript accumulation was enhanced...
2.0-5.75% in the treated samples as compared to the control samples. Highest rate of transcription (698 µg RNA/g FW) was found in the treated mature tea leaves, followed by young leaves (684 µg RNA/g FW). Gene’s transcription rate was very slow in both the treated and control samples of callus tissues. The relative transcription rate varied from 645 to 660 (RNA µg/g FW). Transcripts accumulation was moderate in case of shoots regenerated from somatic embryos, varied from 651 to 680. It was noticed that young leaves were not induced by the elicitor, methyl jasmonate at a rate which can be comparable with the induction level of mature leaves (657-684). The mature leaves induced by the application of methyl jasmonate showed quite a high rate of transcript accumulation (660 to 698), which is 4.24% more than that of the control transcription level. This indicates that some of the genes are expressed more in the treated samples than that of the control. The expression may include some of the defense-related genes, i.e., pathogenesis related genes. Induction level was highest at a time course of 16 h from the time of treatment and gradually decreased with the further increase in the time course beyond 16 h. After 72 h of treatment, the level of induction was similar to the level of the control conditions (Table 1).

Table 1—Quantification of total transcripts accumulation (RNA µg/g FW) in young, mature green leaves and in vitro grown tissues after treatment with methyl jasmonate in different time courses

<table>
<thead>
<tr>
<th>Time course (h)</th>
<th>Young leaf</th>
<th>Mature leaf</th>
<th>In vitro grown callus tissues</th>
<th>In vitro regenerated shoots of somatic embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Induced*</td>
<td>Control</td>
<td>Induced*</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>657±0.35</td>
<td>660±0.33</td>
<td>645±0.08</td>
<td>651±0.23</td>
</tr>
<tr>
<td>0.3</td>
<td>660±0.07</td>
<td>665±0.09</td>
<td>645±0.05</td>
<td>654±0.08</td>
</tr>
<tr>
<td>2</td>
<td>664±0.05</td>
<td>670±0.11</td>
<td>645±0.11</td>
<td>659±0.51</td>
</tr>
<tr>
<td>6</td>
<td>673±0.33</td>
<td>677±0.04</td>
<td>645±0.44</td>
<td>663±0.88</td>
</tr>
<tr>
<td>12</td>
<td>679±0.24</td>
<td>684±0.33</td>
<td>645±0.23</td>
<td>672±0.32</td>
</tr>
<tr>
<td>16</td>
<td>684±0.49</td>
<td>698±0.06</td>
<td>645±0.09</td>
<td>680±0.21</td>
</tr>
<tr>
<td>24</td>
<td>674±0.20</td>
<td>678±0.44</td>
<td>645±1.11</td>
<td>676±0.09</td>
</tr>
<tr>
<td>36</td>
<td>662±0.05</td>
<td>669±0.32</td>
<td>645±0.43</td>
<td>669±0.41</td>
</tr>
<tr>
<td>48</td>
<td>663±0.39</td>
<td>669±0.72</td>
<td>645±0.99</td>
<td>664±0.19</td>
</tr>
<tr>
<td>72</td>
<td>658±0.58</td>
<td>667±0.08</td>
<td>645±0.56</td>
<td>653±0.32</td>
</tr>
</tbody>
</table>

*Each value represents the mean±standard error of three replicates.

Quantification of cDNAs in Treated and control samples after RT-PCR

The RT-PCR amplification product was fractionated on 1% agarose gel for visual estimation. Fig. 2 clearly shows the two different band positions in the gel, one band of 254 bp and another of 366 bp. Chitinase gene specific transcript accumulated more in mature leaves compared to young leaf. But callus and shoot showed less amount of transcript accumulation (Tables 1 & 2). Quantity of cDNAs were 203, 253, 154 and 183 µg in YL, ML, callus and shoot, respectively, because the mRNA has been
Table 2—Semi-quantification of RT-PCR products (µg cDNAs/g FW) relating to chitinase gene specific transcript accumulation in young, mature leaf and in vitro grown tissues in different time courses after treatment with methyl jasmonate

<table>
<thead>
<tr>
<th>Time course (h)</th>
<th>Control (µg)</th>
<th>Induced* (µg)</th>
<th>Control (µg)</th>
<th>Induced* (µg)</th>
<th>Control (µg)</th>
<th>Induced* (µg)</th>
<th>Control (µg)</th>
<th>Induced* (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young leaf</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>In vitro grown callus tissues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>134</td>
<td>134±0.09</td>
<td>143</td>
<td>143±0.47</td>
<td>121</td>
<td>121±0.04</td>
<td>124</td>
<td>124±0.27</td>
</tr>
<tr>
<td>0.3</td>
<td>134</td>
<td>138±0.30</td>
<td>143</td>
<td>165±0.39</td>
<td>121</td>
<td>124±0.11</td>
<td>124</td>
<td>130±0.31</td>
</tr>
<tr>
<td>2</td>
<td>134</td>
<td>145±0.89</td>
<td>143</td>
<td>172±0.08</td>
<td>121</td>
<td>131±0.06</td>
<td>124</td>
<td>144±0.05</td>
</tr>
<tr>
<td>6</td>
<td>134</td>
<td>167±0.45</td>
<td>143</td>
<td>187±0.10</td>
<td>121</td>
<td>142±0.23</td>
<td>124</td>
<td>153±0.11</td>
</tr>
<tr>
<td>12</td>
<td>134</td>
<td>183±0.39</td>
<td>143</td>
<td>223±0.21</td>
<td>121</td>
<td>149±0.20</td>
<td>124</td>
<td>172±0.36</td>
</tr>
<tr>
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<td>134</td>
<td>214±0.44</td>
<td>143</td>
<td>253±0.29</td>
<td>121</td>
<td>154±0.38</td>
<td>124</td>
<td>183±0.81</td>
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<tr>
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<td>187±0.18</td>
<td>143</td>
<td>231±0.04</td>
<td>121</td>
<td>140±0.06</td>
<td>124</td>
<td>170±0.55</td>
</tr>
<tr>
<td>36</td>
<td>134</td>
<td>169±0.26</td>
<td>143</td>
<td>198±0.15</td>
<td>121</td>
<td>134±0.01</td>
<td>124</td>
<td>159±0.06</td>
</tr>
<tr>
<td>48</td>
<td>134</td>
<td>154±0.08</td>
<td>143</td>
<td>165±0.05</td>
<td>121</td>
<td>127±0.57</td>
<td>124</td>
<td>137±0.32</td>
</tr>
<tr>
<td>72</td>
<td>134</td>
<td>127±0.22</td>
<td>143</td>
<td>149±0.77</td>
<td>121</td>
<td>123±0.91</td>
<td>124</td>
<td>127±0.99</td>
</tr>
</tbody>
</table>

*Each value is mean±standard error of three replicates

The BLAST (basic local alignment search tool) algorithm BLASTN gave the following significant alignment information about the 366 bp DNA sequence of tea plants. The DNA sequence matches with basic chitinase gene of *Nepenthes thysanum* (1717 bp, acc.no. gb|AY61883.1|) complete cds of 171 bp, Score=65.8 bits (35), Expect=1e-07, Identity=35/35 (100%), Gaps=0/5 (0%), Strand=Plus/Minus, as follows:

```
query 2  CAATGGCGAATGGGTGACAGCAGTGGTTACATCT
        ||||||||||||||||||||||||||||||||||)||||
subject 1709 CAATGGCGAATGGGTGACAGCAGTGGTTACATCT 1675
```

The TBLastX has shown the significant alignments with chitinase protein, acc.no. AY518881.1 N. khasiana basic chitinase 1-1 gene, complete cds, Length=1572, Score=37.7 bits (76), Expect=4.1, Identites=14/15 (93%), Positives=14/15 (93%), Gaps=0/15 (0%), Frame=2/2:

```
Query 46 STREITWATPSGHL.2
      ST EITWTATPSGHL
Shjet 1520 STREITWATPSGHL 1564
```

Fig. 3—Bioinformatics analysis of 366 bp RT-PCR product of DOP primer.

amplified so many times (starting RNA concentration was 15µg in each sample). Further, treated samples accumulated more amount of mRNA transcript (cDNAs) in comparison with the control samples (Table 2). DOP-primer pair produced 366 bp cDNA products, while chitinase gene specific primer pair produced 254 bp cDNA products. Methyl jasmonate treatment rapidly induced the accumulation of chitinase gene specific mRNA transcript. The expression of defence related gene was initiated after 0.3 h of treatment, which reached to peak after 16 h and later declined to near control levels within 72 h of the treatment (Tables 1 & 2).

Sequencing of 366 bp RT-PCR Product and Analysis using BLAST Algorithms

The sequence information of 366 bp DNA from tea plant was deposited into the GenBank of NCBI (acc.no. EU373553) as a hypothetical protein gene of *C. sinensis*. This sequence information was then analyzed through BLASTN program, which indicated that the 366 bp sequences contain the genetic information of basic chitinase gene (Fig. 3A), similar to *Nepenthes khasiana* (1717 bp, acc.no. gb|AY61883.1|). The result of GENSCAN showed that it can encode protein peptide containing 81 amino acids (Fig. 3B) similar to chitinase of *N. khasiana*. 
In the present study, effect of methyl jasmonate on gene expression was studied in tea (clone T383) at four different developmental stages: young leaf, mature leaf, in vitro grown callus tissues and in vitro regenerated shoots of somatic embryos. Defence-related differential gene expression was induced by the treatment of methyl jasmonate in leaves, callus and regenerated shoots in tea where constitutive expression was low. Chitinase may function in plant defence as inducible and/or constitutively expressed genes in many cases\textsuperscript{19}, and have been shown in several cases to be inducible by wounding, ethylene, and methyl jasmonate\textsuperscript{20,21}. The present results hint at the complexity of the interaction between methyl jasmonate and tea leaves and tissues. The response of tea leaves and tissues to methyl jasmonate was strongly influenced by the stage of leaf development at different time courses. It was found that transcript accumulation was induced by methyl jasmonate in mature leaves where the quantities of accumulation were more compared to the callus tissues. In callus tissues, transcript accumulation was very low. As described here, accounting for tissue ontogeny is critical when we study the molecular responses of tea in defence induction to methyl jasmonate. The constitutive and inducible defence strategies used by tea are dependent on the developmental stage of the tissues involved. With this understanding, differences in constitutive and inducible expression of tea defence genes in divergent tea germplasm source can be more accurately characterized. The results indicated the sharp enhancement of defence protein gene expression in tea by methyl jasmonate, including the chitinase gene. The peak accumulation was observed after 16 h of spray of methyl jasmonate. If, it was not induced by the methyl jasmonate induction, total RNA quantity would not have increased at this level. The total RNA consists of mRNAs, rRNAs, tRNAs, and other special RNAs. Within the total mRNAs, chitinase gene specific mRNA molecules are present at their own concentration. Subsequently, this chitinase gene specific mRNAs are amplified through RT-PCR to quantify the mRNA transcript accumulation during induced systemic resistance (ISR). Chitinase gene specific RT-PCR product of 254 bp cDNAs concentration was more in induced material in comparison to control material. RT-PCR produced more number of cDNA copies of an mRNA whose copy number was more in the starting material and amplified accordingly. In earlier report, it was observed that during induced systemic resistance, induction with specific inducer can enhance the differential gene expression of some of the proteins that may be DNA binding protein, cell division regulatory protein, peroxidases, glucanases, chitinases, caffeine or light harvesting proteins\textsuperscript{14}. Similar type of gene expression induction was observed in the present study. The knowledge gained from the present investigation can be applied in future to understand the detailed pathways involving induced systemic resistance mechanisms against plant pathogens in tea plant.

References


