Transcripts of pectin-degrading enzymes and isolation of complete cDNA sequence of a pectate lyase gene induced by coffee white stem borer (*Xylotrechus quadripes*) in the bark tissue of *Coffea canephora* (robusta coffee)

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Abstract Of the two commercially cultivated coffee (*Coffea*) species, *C. arabica* (arabica) is highly susceptible and *C. canephora* (robusta) is highly resistant to the insect pest *Xylotrechus quadripes* (Coleoptera: Cerambycidae), commonly known as coffee white stem borer (CWSB). We constructed a forward-subtracted cDNA library by Suppression Subtractive Hybridization (SSH) from robusta bark tissue for profiling genes induced by CWSB infestation. Among the 265 unigenes of the SSH EST library, 7 unigenes (5 contigs and 2 singletons) matching different pectin-degrading enzymes were discovered. These ESTs matched one pectate lyase, three polygalacturonases, and one pectin acetylsterase gene. Quantitative real-time PCR (qRT-PCR) revealed that CWSB infestation strongly induces the pectate lyase gene at 72 h. Complete cDNA sequence of the pectate lyase gene was obtained through 3' and 5' RACE reactions. It was a 1595 bp long sequence that included full CDS and both UTRs. Against *C. canephora* genome sequences in Coffee Genome Hub database (<http://coffee-genome.org/>), it had 22 matches to different pectate lyase genes mapped on 9 of the 11 pseudochromosomes, the top match being Cc07_g00190 Pectate lyase. In NCBI database, it matched pectate lyase sequences of several plants. Apart from *C. canephora*, the closest pectate lyase matches were from *Sesamum indicum* and *Nicotiana tabacum*. The pectinolytic enzymes discovered here are thought to play a role in the production of oligogalacturonides (OGs) which act as Damage-Associated Molecular

Pattern (DAMP) signals eliciting innate immunity in plants. The pectate lyase gene, induced by CWSB infestation, along with other endogenous pectinolytic enzymes and CWSB-specific elicitors, may be involved in triggering basal defense responses to protect the CWSB-damaged tissue against pathogens, as well as to contain CWSB in robusta.

Keywords *Coffea canephora* · *Xylotrechus quadripes* · Suppression subtractive hybridization (SSH) · Expressed sequence tag (EST) · Pectate lyase · Polygalacturonase · Pectin acetylsterase · Pectin-degrading enzymes

Introduction

Coffee liked for its characteristic aroma, flavor and stimulating effects of caffeine is prepared from the roasted and ground beans of coffee plants (*Coffea* spp). Although the *Coffea* genus includes more than 124 species (Davis 2011), *C. arabica* L., popularly known as arabica coffee and *C. canephora* Pierre called as robusta coffee are the two species under commercial cultivation, contributing around 60 and 40% to the total global coffee production, respectively (International Coffee Organization, ICO: <http://www.ico.org>). Arabica produces beverage of superior quality, but it is susceptible to several pests and pathogens. *Xylotrechus quadripes* Chevrolat (Coleoptera: Cerambycidae), commonly known as coffee white stem borer (CWSB), is the most destructive insect pest of arabica coffee plants in India (Anonymous 2014; Venkatesha and Dinesh 2012). It is also distributed in other Asian countries such as Bangladesh, Myanmar, Nepal, China, Thailand, Vietnam, Indonesia, and Sri Lanka (Rhains et al. 2002; Lan and Wintgens 2004). The CWSB beetles lay eggs in

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cracks and crevices on the bark of woody stems (Seetharama et al. 2005; Venkatesha and Dinesh 2012). After initial tunneling and feeding in the bark, the larvae enter the wood and make extensive galleries in the main stem and thick primary branches. Apart from affecting the quality and quantity of the produce, severe infestation can heavily damage the main stem up to roots and kill the plants. Conventional breeding has not been successful in developing arabica cultivars resistant to CWSB. Currently available control measures have not been able to control the pest effectively. The pest causes substantial capital loss and heavy financial distress to arabica coffee farmers as millions of severely infested arabica plants are killed or have to be uprooted every year to prevent further spread of the pest. As a consequence, arabica is getting replaced by robusta. Robusta and other species like *C. liberica* are resistant to CWSB. It is essential to understand the genetic mechanisms of defense mounted by the resistant species against CWSB to come out with strategies to breed arabica varieties resistant to CWSB.

Feeding by chewing insects such as CWSB can cause severe wounding and damage large portions of the plant tissues. Plants have evolved sophisticated mechanisms to promptly respond to wounding, rapidly heal the tissue, and prevent microbe infections (Savatin et al. 2014; Duran-Flores and Heil 2016). Endogenous molecules released from wounded tissue may act as Damage-Associated Molecular Patterns (DAMPs) that activate the plant innate immunity. Typical examples of DAMPs involved in response to wounding are the peptide systemin, and the oligogalacturonides (OGs), which are oligosaccharides released from the pectic component of the cell wall (Savatin et al. 2014). OGs are released from the plant cell walls upon partial degradation of homogalacturonan, the main component of pectin, by wound-induced hydrolytic enzymes or, during infections by microbial hydrolytic enzymes (Boller and Felix 2009; De Lorenzo et al. 2011). OGs induce in several plant species a wide range of defense responses including production of ROS, nitric oxide, phytoalexins, glucanase, chitinase, and callose (Galletti et al. 2008). Genes involved in biosynthesis of jasmonic acid (JA) and ethylene (ET), as well as, genes for general stress responses are rapidly induced by wounding (Reymond et al. 2000; Delessert et al. 2004).

Robusta is highly resistant to CWSB. Robusta plants occasionally exhibit symptomatic ridges of CWSB incidence on the stem, but there is no further damage to the plants (Santosh et al. 2011). Robusta seems to mount strong induced defense response against CWSB larvae in the bark tissue. Profiling of transcriptional changes in the bark tissue of robusta due to CWSB infestation may be expected to provide an insight into the molecular mechanisms of its resistance against the pest. Towards this

direction, we constructed a forward-subtracted cDNA library by Suppression Subtractive Hybridization (SSH) (Diatchenko et al. 1996) from robusta. Around 265 uni-genes obtained from the sequences of more than 700 randomly selected clones of the library became a valuable EST database for defense gene mining. In this study, we attempted to identify the transcripts of pectinolytic enzymes in the EST database. These pectinolytic enzymes are thought to have role in the production of OGs which are DAMP signals in eliciting innate immunity in plants. Further we proceeded to obtain complete cDNA sequence of a pectate lyase gene based on a partial EST obtained in the SSH library. We also assessed the changes in the expression levels of the pectate lyase gene in response to CWSB infestation.

Materials and methods

Biological materials, artificial infestation and tissue sampling

About twelve-year-old robusta (*C. canephora* Cv CXR), plants grown in the farm of Ramakrishna Ashram in Mysore were used in the study. Neonatal larvae of CWSB used for artificial infestation of the robusta plants were collected as follows: Arabica plants showing typical symptoms of CWSB infestation were collected from coffee farms and stored in the laboratory and covered with nylon mosquito nets to trap emerging beetles. Emerged beetles were collected manually and released in wide-mouthed, plastic bottles. The mouths of bottles were covered with thin, cotton cloth, and secured in place by rubber bands. The bottles were kept inverted over blotting paper circles so that the cloth remained in close contact with blotting paper. After mating, the female beetles inserted their ovipositors through the holes in the cloth and laid eggs on the surface of the blotting paper. The eggs got tightly fixed on the blotting paper by a gummy coating on the eggs. Blotting papers laden with CWSB eggs were placed in Petri plates and incubated at 22 °C. The eggs hatched in 6–7 days releasing the neonatal larvae.

For artificial infestation, about one inch thick, healthy, undamaged stems were chosen. On these stems, a small thin flap (about 1 cm long and wide) was cut open from the papery, brown outer most layer of the bark using a sharp scalpel blade to expose the inner bark. Two neonatal larvae were inoculated in the pocket between the membranous flap and soft fleshy inner bark, using a soft brush. The flap was secured in place by wrapping a cellophane tape loosely around the stem at the inoculation site. To get enough tissues for RNA isolation, several such inoculations were made on each stem with a gap of 2–3 inches between them.

The larvae were allowed to infest the stem for different periods. At each time point, the inoculation sites were observed by removing the cellophane tapes and the flap. Up to 1.5-cm-long characteristic narrow galleries made in the inner bark tissue by CWSB larval feeding could be seen at inoculation sites. Bark tissue along the edges of galleries had turned brownish and tiny frass particles were also seen. The larvae were removed from the sites of inoculation and 1.5–2 cm wide and 1-mm-thick inner bark tissues up to underlying wood were collected with a sharp scalpel blade in 2 ml microcentrifuge tubes and frozen in liquid nitrogen for RNA isolation. For SSH library, tissues were collected 36 h post inoculation (hpi). For real-time PCR, tissues were collected 4, 8, 16, 24, 36, and 72 hpi. In the same way, mock inoculation sites were made to collect non-infested control tissues. For this, flaps of outer bark were opened up and closed loosely with cellophane tapes without inoculating CWSB larvae. The bark tissues were collected from these mock inoculation spots at respective time points to serve as non-infested controls for preparing driver cDNA for SSH library and for quantitative real-time PCR. Mock inoculation sites were made to neutralize the possible effect of mechanical wounding made by opening a flap for inoculation of CWSB larvae. As only small quantity of sample tissue could be obtained from each infestation site, tissue samples from the same time points were pooled to get enough RNA.

mRNA isolation and double-stranded cDNA preparation

Total RNA for all the experiments was isolated using RNeasy Plant Mini Kit (Qiagen) essentially following manufacturer's protocol. Double-stranded cDNA for SSH library and 3' and 5' RACE reactions was prepared using SMARTer™ PCR cDNA Synthesis Kit (Clontech). Procedure followed for RNA isolation and preparation of double-stranded cDNA is described earlier (Santosh and Sreenath 2012).

Construction of forward-subtracted cDNA library by suppression subtractive hybridization (SSH)

Clontech PCR-Select™ cDNA Subtraction Kit (Clontech) was used for preparing the forward-subtracted cDNA by Suppression Subtractive Hybridization (SSH) (Diatchenko et al. 1996) according to the manufacturer's protocol. Double-stranded cDNA prepared using SMARTer™ PCR cDNA Synthesis Kit (Clontech) was digested with Rsa I restriction enzyme to form shorter, blunt-ended fragments. cDNA from the bark tissue subjected to CWSB larval infestation for 36 h was used for 'tester' and that from non-infested control bark tissue was used for the 'driver'

cDNAs. The tester cDNA was divided into two populations; each of the populations was ligated with adaptors 1R and 2R accordingly. Subtracted cDNA was prepared after two rounds of hybridization and PCR amplifications which result in reduced background and enrich differentially expressed sequences. The subtracted cDNA was ligated to pGEM-T Easy Vector (Promega) and transformed into JM109 *E. coli* bacterial cells to construct forward-subtracted SSH (fSSH) cDNA library. Transformed cells were selected on LB agar plates containing Xgal/IPTG/Ampicillin.

Identification of ESTs of pectin-degrading enzymes in SSH library

Positive colonies were grown in LB broth culture and plasmid DNA was isolated from the overnight grown bacterial cultures using Qiaprep Spin Mini Prep Kit (Qiagen). More than 700 fSSH clones were selected randomly and sequenced at Eurofin Genomics, Bangalore. The sequences were screened, analyzed, and assembled into 265 unigenes (non-redundant sequences derived from contigs and unigenes) using DNASTAR software (Lasergene). The sequences matched several genes, including plant defense genes (results not shown here). The transcripts of pectin-degrading enzymes in the 265 unigenes of the SSH library were identified by Blast searches of the sequences against *C. canephora* genome sequences available in the websites of Coffee Genome Hub (<http://coffee-genome.org/>, Dereeper et al. 2015) and Sol Genomics Network (<https://solgenomics.net/tools/blast/>), as well as the general databases of NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Default expected values of Blast algorithms of respective databases were used.

Analysis of expression levels of pectate lyase gene by quantitative real-time PCR (qRT-PCR)

To check the effect of CWSB larval infestation on expression of pectate lyase gene, quantitative real-time PCR (qRT-PCR) was performed in a LightCycler 480 real-time PCR instrument (Roche Diagnostics) using the LightCycler 480 SYBR Green I Master kit. Total RNA for qRT-PCR analysis was obtained from bark tissues infested with CWSB larvae for 4, 8, 16, 24, 36, and 72 h, as well as non-infested control bark tissues using RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from 1 µg total RNA using M-MuLV Reverse Transcriptase and oligo dT primer (Genei). Primer pair suitable for qRT-PCR and RACE reactions was designed using PrimerSelect module of DNASTAR (Lasergene) software based on the partial EST sequence of pectate lyase gene obtained in the SSH library (Table 1). Both primers were 29 bases long. The forward

Table 1 Sequences of PCR primer pairs used for qRT-PCR and RACE reactions

Forward primer, 5'–3'	Reverse primer, 5'–3'
GGAGCGCAGCCCCTACCATCAACAGC	AAGTAGGCCCCATTGAGCATCAAGTCTCC
Also used the sequence as GSP1 for 3' RACE	Also used the sequence as GSP2 for 5' RACE

primer had T_m of 69.6 °C and the T_m of reverse primer was 65.9 °C. The expected size of PCR product was 160 bp. cDNA was diluted 100 times with sterile DEPC-treated water for PCR. The reaction mixture (25 μ l) contained 2 μ l diluted cDNA, 10 μ l of LightCycler 480 SYBR Green I Master Mix (Roche), and 5 μ l of each primer (10 μ M). qRT-PCR assay was performed according to the instructions supplied with the Users' manuals. The PCR conditions were as follows: 95 °C for 5 min for polymerase activation, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min (combined annealing and extension step). Three replicated amplifications were performed for each time point and the results were averaged. *C. canephora* GAPDH was used as reference gene for normalization of the values. Specificity of the amplified PCR products was assessed by performing melting curve analysis on the instrument. Amplification of a single product of expected size was verified by gel electrophoresis on a 2% agarose gel and ethidium bromide staining. LightCycler 480 software (version 1.5; Roche Diagnostics) was used to collect the fluorescence data. Relative quantification of pectate lyase at different time points of CWSB larval infestation was carried out through $2^{-\Delta\Delta C_p}$ method (Livak and Schmittgen 2001).

Obtaining complete cDNA sequence of pectate lyase gene by RACE reactions and its analysis

Rapid amplification of cDNA ends (RACE) (Frohman et al. 1988) technique was used to obtain complete cDNA sequence of the pectate lyase gene based on EST fragment of the pectate lyase gene obtained in the SSH library. RACE reactions were done using SMARTer RACE cDNA Amplification Kit (Clontech) following the protocol of the manufacturer. Two rounds of PCR were done using Advantage 2 PCR Kit (Clontech) for amplification. Some double-stranded cDNA prepared using SMARTerTM PCR cDNA Synthesis Kit (Clontech) was remaining after construction of the SSH library. Same DNA was used as substrate for first round of PCR in both 3' and 5' RACE reactions instead of preparing separate 5' and 3' RACE-ready cDNAs. The same PCR primers (Table 1) that were used for qRT-PCR were used for RACE reactions also. In the first round PCR, the forward primer was used as gene-specific primer 1 (GSP1) for 3' RACE and reverse primer

was used as gene-specific primer 2 (GSP2) for 5' RACE along with the Universal Primer A Mix (UPM) provided in the kit for both reactions. Touchdown protocol was followed for first round of PCR. For second round (nested) of PCR, diluted primary PCR product was used as substrate. The nested PCR also used the same gene-specific primers (GSP1 and 2) instead of designing gene-specific nested primers (NGSPs) along with Nested Universal Primer A (NUP) provided in the kit. Thermal cycling conditions used for second round of PCR were 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min for 20 cycles.

The RACE products were ligated to pGEM-T Easy Vector (Promega) and transformed into JM109 *E. coli* bacterial cells. Plasmid DNA was isolated from positive clones using Qiaprep Spin mini prep kit (Qiagen) and sequenced at Eurofin Genomics (Bangalore) by Sanger's method using M13 primers. Sequences of 5' and 3' race products were analyzed and aligned to get a complete cDNA sequence of pectate lyase gene using DNASTAR software (Lasergene). This cDNA sequence was analyzed by Blast searches against *C. canephora* genome sequences in the websites of Coffee Genome Hub (<http://coffee-genome.org/>, Dereeper et al. 2015) and Sol Genomics Network (<https://solgenomics.net/tools/blast/>), as well as the general databases of NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Several top matching protein sequences in NCBI and the top match in Coffee Genome Hub were downloaded and aligned with our translated protein sequence to construct a phylogenetic tree using Mega 6 software (Kumar et al. 1994). Sequences of corresponding regions of the SSH clone and complete cDNA sequence of pectate lyase gene obtained by RACE were aligned using MegAlign module of DNASTAR software to identify polymorphism between them.

Results and discussion

Transcripts of a pectin-degrading enzymes in the SSH library

ESTs matching one pectate lyase, three polygalacturonases, and one pectin acetyltransferase were discovered in the SSH library by blast searches. Totally, 7 unigenes (5

contigs and 2 singletons) from 20 sequences of the SSH library matched different pectin-degrading enzymes (Table 2). While pectate lyase and the polygalacturonase genes had the conserved domains of Polysaccharide Lyase Family 6 (PL-6 superfamily), pectin acetylsterase had the

conserved domains of pectin acetylsterase (PAE) family. While there were several matching sequences of *C. canephora* pectate lyase and polygalacturonase genes, there were only two matching sequences of pectin acetylsterase in Coffee Genome Hub database. For each unigene, the top

Table 2 Transcripts of pectin-degrading enzymes discovered in the fSSH library constructed from *C. canephora* Cv CXR bark tissue artificially infested with CWSB neonatal larvae

Details of unigenes matching pectin-degrading enzymes	Top most matches in Coffee Genome Hub (BlastN against CDS with UTRs)	Conserved domains and top NCBI (BlastX) matches
One unigene Contig 8 of 5 sequences 427 bp	Cc07_g00190 Pectate lyase Matches in CDS and 3' UTR	PL-6 superfamily Unnamed protein product [Coffea canephora] Sequence ID: CDP16631.1 Other NCBI matches are probable pectate lyase 18 or pectate lyase like.
One unigene Contig 15a of 5 sequences, (Slight variation in member sequences due to indel & SNPs, the variants may represent alleles) 298, 312 & 313 bp	Cc03_g15740 Putative Glycoside hydrolase, family 28 Matches in CDS & 3' UTR	PL-6 superfamily Unnamed protein product [Coffea canephora] Sequence ID: CDP08132.1 Other NCBI matches are polygalacturonase C or polygalacturonase like
Three unigenes match 3 parts of the gene from middleof CDS to full 3' UTR till polyA tail First unigene Contig 118a of 2 sequences 926 bp, Matches in CDS Second unigene Contig 125a of 3 seqs 236 bp Matches in CDS & 3' UTR Third unigene Singleton 138, 121 bp before PolyA tail Matches end of 3' UTR	Cc03_g15700 Polygalacturonase First unigene matches in CDS Second unigene matches in CDS & 3' UTR Third unigene matches to end of 3' UTR	PL-6 superfamily First and second unigenes match unnamed protein product [Coffea canephora] Sequence ID: CDP08128.1 third unigene in only short 3' UTR before polyA tail. No NCBI matches Other NCBI matches are polygalacturonase like or polygalacturonase C
One unigene Contig118b1 of 3 sequences 926 bp	Cc03_g15840 Polygalacturonase Matches in CDS	PL-6 superfamily Two very close matches, the second one may correspond to Coffee Genome hub match, Unnamed protein product [Coffea canephora] Sequence ID: CDP08128.1 Unnamed protein product [Coffea canephora] Sequence ID: CDP08142.1 Other NCBI matches are mostly annotated as polygalacturonase like
One unigene Singleton 66 with polyA tail 727 bp preAs	Cc08_g04630 Pectinacetylsterase family protein, matches in CDS and full 3' UTR	PAE (Pectinacetylsterase) family proteins Unnamed protein product [Coffea canephora] Sequence ID: CDP15690.1 Most other NCBI matches are annotated as PREDICTED: pectin acetylsterase 8-like

match with the highest score in Coffee Genome Hub was considered as a correct matching gene. Accordingly, the genes of 5 pectin-degrading enzymes of *C. canephora* Cv CxR identified in our SSH library are Cc07_g00190 Pectate lyase, Cc03_g15740 Putative Glycoside hydrolase family 28 (based on NCBI matches, it is a polygalacturonase), Cc03_g15700 Polygalacturonase, Cc03_g15840 Polygalacturonase, and Cc08_g04630 Pectinacetylsterase family protein. For Cc03_g15700 Polygalacturonase, three unigenes matched three contiguous parts of the gene. Together they represented latter part of the gene from middle of CDS till the end of 3' UTR with polyA tail. In case of other 4 genes, single unigenes matched a part of the corresponding gene (Table 2). In the NCBI database, all top matches were to *C. canephora*, annotated as unnamed protein product, which possibly correspond to respective top matches in Coffee Genome Hub mentioned above.

Unigene (CXr fSSH unigene 8) of Contig 8 was 427 bp long and matched Cc07_g00190 Pectate lyase 9612 gene in Coffee Genome Hub partially in CDS and 3' UTR. It was used to get complete cDNA sequence by RACE.

Expression of pectate lyase gene in response CWSB larval feeding

To check the effect of CWSB larval infestation on expression of pectate lyase gene, quantitative real-time PCR (qRT-PCR) was performed with LightCycler 480 real-time PCR instrument (Roche Diagnostics). The relative expression of the gene increased to 18.69 by 4 h, which further increased to 44.32 by 8 h before slumping to 4.24 by 16 h. The expression level again increased to 43.99 by 24 h and got reduced to 6.36 by 36 h. The gene was again very strongly expressed at 72 h with 786.5 times higher expression levels (Table 3). Thus, the results clearly show strong induction of the pectate lyase gene by CWSB larvae with some fluctuation in the first 36 h. Such a fluctuation was seen for some other genes also (results not shown here). The feeding by CWSB larvae may not be a continuous activity and there may be occasional breaks. The fluctuation in expression levels of pectate lyase gene may be due to variation in the chewing activity of the CWSB larvae and consequent variation in wounding of the host plant tissue. Alternatively, the fluctuations may be due to complex interplay of several genes.

Isolation and analysis of complete cDNA sequence of pectate lyase gene

Rapid amplification of cDNA ends (RACE) (Frohman et al. 1988) technique was used to obtain complete cDNA sequence of a pectate lyase gene. By 3' RACE, the remaining sequence of 3' UTR as well as polyA tail was obtained. By 5' RACE, the upstream sequence comprising CDS and 5' UTR was obtained. The two RACE products produced overlapping sequences which were aligned and merged into a non-redundant, complete cDNA sequence of the gene using DNASTAR software (Lasergene).

The complete cDNA sequence obtained by RACE was 1595 bp before polyA tail. By blast search against *C. canephora* genome sequences in Coffee Genome Hub (<http://coffee-genome.org/>, Dereeper et al. 2015) and Sol Genomics Network (<https://solgenomics.net/tools/blast/>) databases, it was found to have 109 bp 5' UTR, 1242 bp coding sequence (CDS) including TGA stop codon and 244 bp 3' UTR before polyA tail. Translated protein sequence was 413 amino acids long. In BlastN search against CDS with UTR sequences, there were 14 matches to *C. canephora* pectate lyases in Coffee Genome Hub database, the top match being Cc07_g00190 Pectate lyase. In BlastX search, there were 22 matches to different putative pectate lyases in Coffee Genome Hub (Table 4). Nineteen of these matches are mapped on 9 of the 11 pseudochromosomes (Fig. 1) and 3 matches are unmapped (chr0). There were 5 base differences in CDS between our pectate lyase sequence and *canephora* top match Cc07_g00190 pectate lyases (1237/1242 bases; 99.6% match). In the translated protein sequence, there was difference of 4 amino acids (identities of 409/413; positives 411/412). The complete cDNA sequence was searched in NCBI using BlastX for protein matches. In the NCBI, top match was to unnamed protein product (*C. canephora*) with Sequence ID: CDP16631.1. It possibly corresponds to *C. canephora* Cc07_g00190 Pectate lyase. Other NCBI matches are annotated as predicted pectate lyase or hypothetical protein. Top 13 matching protein sequences from NCBI and the top match Cc07_g00190 Pectate lyase from Coffee Genome Hub were aligned with the translated protein sequence of the complete cDNA sequence obtained in this study and a phylogenetic tree was constructed to assess the genetic relationship (Fig. 2). Apart from *C. canephora* pectate lyases, the closest pectate lyase

Table 3 Relative expression levels of pectate lyase gene in robusta bark tissue at different time points (hours after infestation, hai) of CWSB larval infestation

Time points (hai)	4	8	16	24	36	72
Relative expression (normalized)	18.69	44.32	4.24	43.99	6.36	786.5

Table 4 Matches in the Coffee Genome Hub database (BlastX search) for complete cDNA sequence of *C. canephora* Cv. CXR pectate lyase obtained by RACE reactions

Sl. no	Gene name	Locus ID	E-value
1	Pectate lyase ~ 9612 ~ complete	Cc07_g00190	0
2	Probable pectate lyase 15 ~ At4g13710 ~ complete	Cc03_g07230	0
3	Probable pectate lyase 5 ~ At1g67750 ~ complete	Cc11_g16200	0
4	Probable pectate lyase 22 ~ At5g63180 ~ complete	Cc04_g10740	0
5	Probable pectate lyase 8 ~ At3g07010 ~ complete	Cc01_g17310	0
6	Putative pectate lyase 2 ~ At1g11920 ~ complete	Cc09_g01400	5E–88
7	Pectate lyase ~ LAT59 ~ complete	Cc11_g13410	5E–146
8	Putative pectate lyase 2 ~ At1g11920 ~ complete	Cc09_g01390	2E–134
9	Pectate lyase ~ LAT59 ~ complete	Cc07_g03040	3E–149
10	Putative pectate lyase 21 ~ At1g30350 ~ fragment	Cc06_g03170	5E–39
11	Pectin lyase-like superfamily protein ~ At4g13710 ~ fragment	Cc00_g32650	5E–21
12	Putative pectate lyase 2 ~ At1g11920 ~ complete	Cc02_g38080	2E–106
13	Probable pectate lyase 22 ~ At5g63180 ~ complete	Cc05_g14920	1E–171
14	Probable pectate lyase 12 ~ At3g53190 ~ complete	Cc00_g27280	5E–166
15	Putative pectate lyase 2 ~ At1g11920 ~ modules	Cc09_g01370	2E–104
16	Probable pectate lyase 9 ~ At3g24230 ~ fragment	Cc04_g02160	3E–97
17	Probable pectate lyase 16 ~ At4g22080 ~ complete	Cc09_g01380	2E–144
18	Probable pectate lyase 12 ~ At3g53190 ~ complete	Cc06_g04480	1E–160
19	Probable pectate lyase P59 ~ LAT59 ~ complete	Cc07_g03030	4E–156
20	Probable pectate lyase 4 ~ At1g30350 ~ fragment	Cc09_g01410	3E–100
21	Putative pectate lyase 21 ~ At1g30350 ~ fragment	Cc00_g14000	4E–43
22	Probable pectate lyase P59 ~ LAT59 ~ complete	Cc04_g15470	3E–151

matches to our clone are from *Sesamum indicum* and *Nicotiana tabacum*.

Polymorphism between SSH clone and RACE product of pectate lyase

There were a few sequence differences near the 3' end in the corresponding regions of SSH clone and complete cDNA sequence obtained through RACE. There were 6 CT repeats in SSH sequence, while there were 10 CT repeats in RACE sequence. Also, a few bases downstream of CT repeats, there were 5 CTA repeats in SSH clone, while there were 6 CTA repeats in RACE product. After a few more bases, there are 2 SNPs (M and S) between SSH clone and RACE sequence. Thus, the SSH clone and RACE sequences were polymorphic and possibly represent two alleles of the gene. The polymorphic region could be useful to develop genetic markers for tracking the alleles in segregating populations. The SSH clone had closer match in the polymorphic region to Coffee Genome Hub match Cc07_g00190 Pectate lyase than the RACE sequence.

Other interesting findings

Usually, separate RACE-ready cDNAs are prepared for 3' and 5' RACE reactions. However, in this study, we have shown that the double-stranded cDNA prepared using SMARTer cDNA synthesis kit can be used for the two RACE reactions. We used the left over double-stranded cDNA after constructing the SSH library successfully for both RACE reactions. Also, we used the same set of PCR primers successfully for qRT-PCR, as well as, RACE reactions in this study.

As per the multiple matches against Coffee Genome Hub database, pectate lyase belongs to a multigene family comprising of at least 22 genes mapped on 9 of the 11 chromosomes. Interestingly, in our SSH library prepared from CWSB infested robusta bark tissue comprising of 265 unigenes, only one unigene of a pectate lyase gene matching Cc07_g00190 pectate lyase was encountered. Also, the gene was strongly induced by CWSB larval feeding. These findings suggest that the pectate lyase isolated in the current study may be specific to the bark tissue and may be involved in inducing defense responses against CWSB.

Fig. 1 Distribution of matching sequences (BlastX) of complete cDNA sequence of *C. canephora* CxR pectate lyase on pseudochromosomes of *C. canephora* in Coffee Genome Hub database

Fig. 2 Phylogenetic tree prepared by aligning the matching protein sequences of *C. canephora* Cv CXR pectate lyase complete cDNA sequence obtained by RACE. Apart from *C. canephora*, the closest pectate lyase matches are from *Sesamum indicum* and *Nicotiana tabacum*

Role of pectin-degrading enzymes in plant defense response

The ESTs of 7 unigenes matching transcripts of pectin-degrading enzymes were relatively abundant in the SSH library. Strong induction of pectate lyase by CWSB herbivory was confirmed by qRT-PCR analysis. The pectin-degrading enzymes expressed in the bark tissue at the site of CWSB larval feeding may have a defense role. These enzymes may act together on the pectin in cell walls to release OGs. The OGs might act alone or interact with CWSB derived elicitors to induce specific defense genes targeted against the pest. Upregulation of genes encoding insecticidal proteins in tomato is considered to be due to the production of OGs. These OGs are thought to be the causative signal in up-regulating proteinase inhibitor synthesis in wounded tobacco (Bishop et al. 1984). The hydrolysis of pectin to release OGs is thought to be catalyzed by polygalacturonase and pectic lyase (John et al. 1997). Direct evidence that OGs can be produced in plants and function as DAMP signal in the activation of plant immunity is provided (Benedetti et al. 2015). OGs, along with other signaling molecules such as jasmonic acid and hydrogen peroxide which are common to abiotic stresses and pathogen attack, are thought to have a role in induced insect resistance response (Gatehouse 2002).

Plants perceive injury and herbivore attack via the recognition of DAMPs and herbivore-associated molecular patterns (HAMPs) (Duran-Flores and Heil 2016). Application of pure DAMPs or HAMPs frequently activates general downstream responses: membrane depolarization, Ca^{2+} influxes, oxidative stress, MAPKinase activation, and octadecanoid signaling at the molecular level, and the expression of digestion inhibitors, cell wall modifications, and other general defences at the phenotypic level (Duran-Flores and Heil 2016). The general elicitors of the basal defense response in plants OGs (DAMP signal) and Flg22 (the bacterial flagellin peptide, a MAMP signal) both triggered activation of genes CYP83B1, CYP79B2, CYP79B3, SUR1, PAD3, and PAL1 that encode enzymes for the biosynthesis of the tryptophan secondary metabolites indole glucosinolates and camalexin and of phenylpropanoids (Denoux et al. 2008). The protective effects of glucosinolates have been most widely demonstrated in anti-herbivore defense. These facts give support that OGs may also induce defense genes against herbivory apart from microbial pathogens. It would be interesting to check if externally applied OGs on the bark tissue can induce defense responses in coffee plants against CWSB. If so, it could provide a powerful strategy to induce resistance to CWSB in the susceptible arabica plants. External application of OGs in *A. thaliana* increased the expression of a CYP79 and related genes involved in the biosynthesis of

indole glucosinolates which are well-known secondary metabolites with pesticidal activity ((Denoux et al. 2008). External application of OGs in tomato induced proteinase inhibitors (Bishop et al. 1984; Moloshok et al. 1992) involved in plant defense against herbivores. Induction of proteinase inhibitors by externally applied OGs was through jasmonic acid produced in the octadecanoid pathway (Doares et al. 1995). Jasmonic acid and its derivatives activate genes involved in pathogen and insect resistance.

Conclusion

We have identified 7 unigenes matching 5 pectin-degrading enzymes comprising one pectate lyase, three polygalacturonases, and one pectin acetyltransferase in the forward-subtracted cDNA library made by Suppression Subtractive Hybridization (SSH) from bark tissue of robusta (*C. canephora* Cv CXR) artificially infested with CWSB larvae. Complete cDNA sequence matching Cc07_g00190 Pectate lyase in Coffee Genome Hub was obtained through RACE. qRT-PCR analysis showed strong induction of the pectate lyase gene by CWSB larval infestation. The pectin-degrading enzymes discovered here are believed to play role in the production of oligogalacturonides (OGs) which are known to act as damage-associated molecular pattern (DAMP) signals involved in eliciting innate immunity in plants.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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