



Carbohydrate active enzymes (CAZy) regulate cellulolytic and pectinolytic enzymes in *Colletotrichum falcatum* causing red rot in sugarcane

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Abstract

Colletotrichum falcatum, an ascomycete pathogen causes red rot of sugarcane which is specialized to infect cane stalks. Cellulolytic and pectinolytic enzymes are necessary for degradation of plant cell wall which stands as barrier for successful fungal pathogenesis. In the study, we have confined to the CAZy genes that regulate cellulolytic and pectinolytic enzymes in two distinctive pathotypes of *C. falcatum*. Comparative transcriptome analysis revealed that a number of CAZy genes producing cellulolytic and pectinolytic enzyme were present in the virulent (Cf671) and least virulent (RoC) pathotypes. Two consecutive transcriptome analyses (in vitro) were performed using Illumina Hi Seq 2500, further analysis was done with various bioinformatic tools. In vitro expression analysis of cutinase, glycoside hydrolyase and pectin-related genes revealed number of genes that attributes virulence. Numerous pectin-related genes involved in degradation of plant cell wall, pectinase and pectin lyase are considered to be key precursor in degradation of pectin in sugarcane. These results suggest that cellulolytic enzymes, cutinase and pectin-related genes are essential for degradation of sugarcane cell wall and considered to be an important pathogenic factor in *C. falcatum*. This is the first detailed report on sugarcane cell wall-degrading enzymes during its interaction with *C. falcatum* and also this comparative transcriptome analysis provided more insights into pathogen mechanism on *C. falcatum*.

Keywords *Colletotrichum* · Cellulose · Hemicellulose · Pectin related genes · Red rot of sugarcane · Transcript profiling

Abbreviations

CAZy	Carbohydrate active enzymes
CSEPs	Candidate secretory effector proteins
CMB	Complete media broth
SBS	Sequencing by synthesis
RTA	Run time analysis
CDS	Coding sequences
TGICL	Transcript gene indices clustering tool
KAAS	KEGG automatic annotation server
KEGG	Kyoto encyclopedia of genes and genomes
GO	Gene ontology
GH	Glycoside hydrolyase

CYP	Cytochrome P450
NGS	Next generation sequencing

Introduction

The aerial parts of plants have outer layer which is considered to be first line of defence against intervention of plant pathogens; cuticle is the first challenging factor for the pathogen during its invasion (Chen et al. 2013). Fungal pathogens have an ability to produce arsenal of proteins and enzymes that can overcome and interfere pathogen-associated molecular pattern (PAMP) triggered immunity (PTI) and effector triggered immunity (ETI) (Jones and Dangl 2006). The serine esterase made of α/β hydrolase superfamily serves as cutinase will facilitate the pathogen invasion into the host system by hydrolysing the cuticle layer with the help of small monomers of cutin (Lu et al. 2018; Nikolaivits et al. 2018). There are several evidential reports suggesting that cutinases are involved in spore attachment, appressorium formation, initial attachment/

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degradation of plant cutin layer in various ascomycetes, and still there exists catalogue of confusion in determining the role of cutinase (Lu et al. 2018; Deising et al. 1992; Stahl and Schafer 1992; Koschorreck et al. 2010; Zhang et al. 2014). Though there are plenty of reports suggesting that cutinase has various roles in host pathogen interaction, the role of cutinase in sugarcane during interaction with *C. falcatum* has not been exploited. The damage associated molecular pattern (DAMP) which is considered to be rapid in triggering innate response than PAMP triggered response, helps plant cell wall reinforcement, biosynthesis of antimicrobial compounds and phytoalexins. Pectin is considered to be one of the major components in plant cell wall, especially in between the cells/xylem vessels and in woody tissues, which acts as secondary cell wall. The role of pectin lyase, polygalacturonase in sugarcane serves as inducer in producing high biomass yield and this pectin is composed of acidic and neutral sugar-containing side chains which is utilised highly as renewable biomass co-product (Xiao and Anderson 2013). Pectin layer in sugarcane is considered to be most important primary cell wall component which comprises 35% approximately in dicot and several monocots (Lara-Marquez et al. 2011).

Colletotrichum falcatum Went (Perfect state; *Glomerella tucumanensis* (Speg.) Arx & Muller) infects the cane stalks and causes symptoms of rotting of internodal tissues, inversion of sucrose by producing of pathogen induced invertases and death of cane stalks. Numerous references were recorded on economic importance in many sugarcane growing countries over early years of the past century (Viswanathan 2010, 2021a). The disease remains a major constraint to sugarcane production in India, Pakistan, Bangladesh, Indonesia, Myanmar, Thailand, Vietnam, USA, Sudan, etc. (Singh et al. 2000; Viswanathan 2021a). Epidemics of the disease have been very common ever since its occurrence in India in 1901 which spread from *Saccharum officinarum*/*S. barberi* clones to the newly released varieties developed through nobilization (Viswanathan 2021b). Further, the cane cultivation and sugar industries in the past century have witnessed lots of high-yielding sugarcane varieties being uprooted from the field due to red rot. Although the disease is effectively managed by releasing disease-resistant varieties (Viswanathan and Samiyappan 1999; Viswanathan and Rao 2011), the resistance in the varieties is not static due to emergence of new pathogenic strains that cause breakdown of resistance in the popular sugarcane varieties (Viswanathan 2021b). *C. falcatum* exhibits enormous variation for pathogenicity and such extensive pathogenic variation has been documented by comparing virulence pattern in 117 *C. falcatum* pathotypes/ isolates under tropical and subtropical conditions (Viswanathan et al. 2017). The same study reported pathogenicity/ virulence of the designated pathotypes CF01,

-02, -03, -04, -05, -06, -07, -08, -09, -10, -11 and -12 that destroyed the cvs Co 1148, Co 7717, CoJ 64, CoS 767, Co 419, Co 997, CoC 671, Co 94012 and 89V74 and also of other isolates and sugarcane varieties.

Further, the mechanism governing red rot resistance has not been fully understood due to genomic complexity. The differential induction of chitinases and thaumatin-like proteins (TLP's) in sugarcane in response to infection by *C. falcatum* has been demonstrated using biochemical and Western blot studies (Viswanathan et al. 2005). Differential expression of genes during interaction of sugarcane x *C. falcatum* was established through DD RT-PCR assays (Pratima et al. 2013; Rahul et al. 2016). 2D based core proteome analysis classification was performed during sugarcane challenged with *C. falcatum* (Ganesh Kumar et al. 2020). Suppression subtractive hybridization (SSH) based time specific and initial defense responses of sugarcane during *C. falcatum* pathogenesis were recorded (Sathyabhama et al. 2015, 2016). However, the molecular tools have yet to be fully explored to characterize the genes activated during pathogen invasion. In this new-generation sequencing era, it is easy to exploit the genes responsible for an action using Next-generation sequencing (NGS) technology. Especially RNA-Seq technology will help us to explore the genes and their expressions to a great extent with their relative expression level. We have sequenced the whole genome and transcriptome of *C. falcatum* using Illumina Hi-Seq 2500 (Viswanathan et al. 2016; Prasanth et al. 2017). *C. falcatum* was found to have large number of plant cell wall-degrading enzymes (PCWDE) such as CAZy, secondary metabolites, cutinase and secretory effector proteins. Earlier we reported candidate secretory effector proteins from genome and transcriptome of *C. falcatum* and virulent strain-specific SSR marker from the genome of *C. falcatum* (Prasanth et al. 2019, 2021). In that, CAZy profusely found to play a vital role in cell wall degradation, host target identification, biotrophy–necrotrophy switch, etc. In the present study, a comparative transcriptome analysis of cellulolytic and pectinolytic enzymes in two pathotypes Cf671 (virulent) and CfRoC (least virulent) of *C. falcatum* has brought a revolutionary identification a large number of CAZy genes contributing to plant cell wall degradation, regulating cellulolytic enzymes and pathogenic virulence.

Materials and methods

Fungal culture used

The *C. falcatum* pathotype CfRoC (isolated from the sugarcane variety RoC) maintained at the red rot culture collection of the institute (ICAR-Sugarcane Breeding Institute,

Coimbatore India) was used for the current study. The fungus was multiplied on oatmeal agar (oatmeal 30 g, agar 20 g, water 1L) for 7 days. Monoconidial culture of the fungus was inoculated on complete media broth (CMB) and incubated under room temperature (28 ± 2 °C) for 12 days (without shaking) for RNA isolation. Previously sequenced *C. falcatum* pathotype Cf 671 (isolated from the sugarcane variety CoC 671) was taken for comparative transcriptome analysis (Prasanth et al. 2017).

RNA extraction and library preparation for transcriptome sequencing

Total RNA was extracted from *C. falcatum* CfRoC using TRI Reagent (Sigma-Aldrich, USA) and treated with Rnase free DNase I (Promega, USA). Subsequently, the quality of RNA was checked in 1% denatured agarose gel electrophoresis for the presence of intact 28 and 18S bands and RNA was quantified using Nanodrop-8000. The paired-end cDNA sequencing library was prepared using Illumina TruSeq SBS Kit v3 as per the manufacturer's protocol. Library preparation was started with mRNA fragmentation followed by reverse transcription, second-strand synthesis, paired-end adapter ligation and finally ended with index PCR amplification of adaptor-ligated library. Library quantification and quality check were performed on Caliper Lab Chip GX using HT DNA High Sensitivity Assay Kit. The libraries of all samples were in the size range of 200 bp to 600 bp. The resulting libraries were validated using the Agilent Bio Analyzer 2100 onto the Agilent High Sensitivity Chip performed at Nucleome Informatics Pvt. Ltd, Hyderabad, India.

Cluster generation and sequencing run

Paired-End sequencing allows the template fragments to be sequenced in both forward and reverse directions. Cluster generation was carried out by hybridization of template molecules onto the oligonucleotide-coated surface of the flow cell. Immobilized template copies were amplified by bridge amplification to generate clonal clusters. This process of cluster generation was performed on cBOT using TruSeq PE Cluster kit v3-cBot-HS. The kit reagents were used for binding of samples to complementary adapter oligos on paired-end flow cells. The adapters were designed to allow selective cleavage of the forward cDNA strand after resynthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment. TruSeq SBS v3-HS kits were used to sequence cDNA of each cluster on a flow cell using sequencing by synthesis technology on the HiSeq 2500.

Transcriptome sequencing, assembly and annotation

The cDNA libraries prepared and sequenced on the Illumina HiSeq 2500 and both ends of the sequenced cDNA were analysed using FastQC v0.11.3. Clean reads were obtained by removing the empty reads, the adaptor sequences and the low-quality sequences (reads with unknown base pairs 'N') using TRIMMOMATIC V 0.33. The clean reads were then assembled into contigs and transcripts based on pair-end information using CLC genomics workbench V 6.0 on default parameters. The CDS regions were predicted using Trans Decoder V 2.0.1. The *C. falcatum* transcriptome was annotated using BLASTX and BLAST2GO. The KEGG pathway annotation was performed using KAAS automated server.

Gene enrichment analysis

GO sequence distribution, helped in specifying all the annotated nodes comprising of GO functional groups. CDS of transcripts associated with similar functions were assigned to the same GO functional group. The GO sequence distributions were analysed for all the three GO domains, i.e. biological processes, molecular functions and cellular components.

KEGG pathway identification

Orthologous assignment and mapping of the transcripts to the biological pathways were performed using KEGG automatic annotation server (KAAS) (Moriya et al. 2007). All the transcript contigs were compared against the KEGG database (Kanehisa and Goto 2000; Kanehisa et al. 2012) using BLASTx with threshold bit-score value of 60. The mapped transcript contigs represented metabolic pathways of major biomolecules such as carbohydrates, lipids, nucleotides, amino acids, glycans, cofactors, vitamins, terpenoids, polyketides, etc. The mapped contigs also represented the genes involved in genetic and environmental information processing and cellular processes.

Screening protein families involved in fungal pathogenesis

Protein families were classified by searching the assembled transcripts against Pfam (Punta et al. 2012) and InterProScan (Quevillon et al. 2005). Protease families were identified using BLASTp against MEROPS peptidase database release 9.6 (Rawlings et al. 2012). Cytochromes (CYPs) were named according to classification details collected from BLASTp against Fungal Cytochrome P450 database version 1.2 (Park et al. 2008). Carbohydrate-degrading enzymes selected from

InterProScan and Pfam analyses were classified according to GH (Glycoside hydrolase) family as classified in CAZy database (Cantarel et al. 2009). Proteases, carbohydrate-degrading enzymes and membrane transporters were predicted from *C. falcatum* transcriptome and compared with *C. graminicola*, *C. sublineola*, *C. higginsianum*, *C. gloeosporioides* and *C. orbiculare*.

RT-PCR validation

To validate the candidate genes selected from each family of CAZy based on the transcriptome data, cell wall glycosyl hydrolase, glycoside hydrolase family 5, glycoside hydrolase family 3, glycoside hydrolase family 10, glycoside hydrolase family 35, glycoside hydrolase family 43, glycoside hydrolase family 28, glycosyl transferase, polysaccharide deacetylase, polysaccharide deacetylase 3 and carbohydrate esterase by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The assay was performed with a Step-One plus Real-Time PCR system (Applied Bio systems, USA) to profile the expression of cellulolytic, hemi cellulolytic and pectinolytic genes which are involved in plant cell wall degradation in virulent (*Cf671*) and avirulent (*CfRoC*) *C. falcatum* pathotypes. The CAZys retrieved from comparative transcriptomics gave a clear view about up regulation and down regulation between the pathotypes of *C. falcatum*. Each reaction was carried out in triplicate; the primers used for qRT-PCR were designed based on the transcriptome data except for actin and are listed in Table ESM 4. The actin primers described by Chakravarthi et al. (2015) were used for normalization. For qRT-PCR experiments, cDNA concentration was standardized for each sample and dissociation

curve analysis was performed to check primer specificity. The cocktail for qRT-PCR reaction contained 50 ng cDNA, 2.5 pmol primers, 10 µl SYBRGREEN Master Mix, and nuclease free water to make up the reaction mixture to 20 µl. The reaction was performed for 40 cycles (denaturation for 10 min at 95 °C followed by annealing and extension at 1 min for 58 °C).

Results

Culture morphology and transcriptome assembly

Cf671 and *CfRoC* pathotypes were morphologically distinct and they have their uniqueness in their survival and development. The histopathological study on *C. falcatum* pathotypes brought a clear outline that virulent pathotype had ability to produce orange/salmon coloured acervuli whereas *CfRoC* was non-sporulating did not produce any visible conidia (Figs. 1 and 2). The pathotype *Cf671* generated 56, 637,987 reads and the pathotype *CfRoC* generated 55,664,294 reads (Table ESM 1). The trimmed high-quality reads were assembled using TRINITY v2.0.6; the short reads passed QC were assembled into transcripts. The contig length of *Cf671* pathotype was 31,194,966, whereas in case of *CfRoC* pathotype it was 33,092,606 (Table ESM 1).

Annotations

The transcriptome of *Cf671* pathotype was assembled into 24,732 transcripts and 30,510 transcripts in case of *CfRoC* pathotype (Table ESM 2). The assembled transcripts were

Fig. 1 Cultures of *C. falcatum* sporulating and non sporulating pathotypes on oat meal agar; (A) sporulating highly virulent pathotype *Cf671*. (B) Non sporulating and less virulent pathotype *CfRoC*

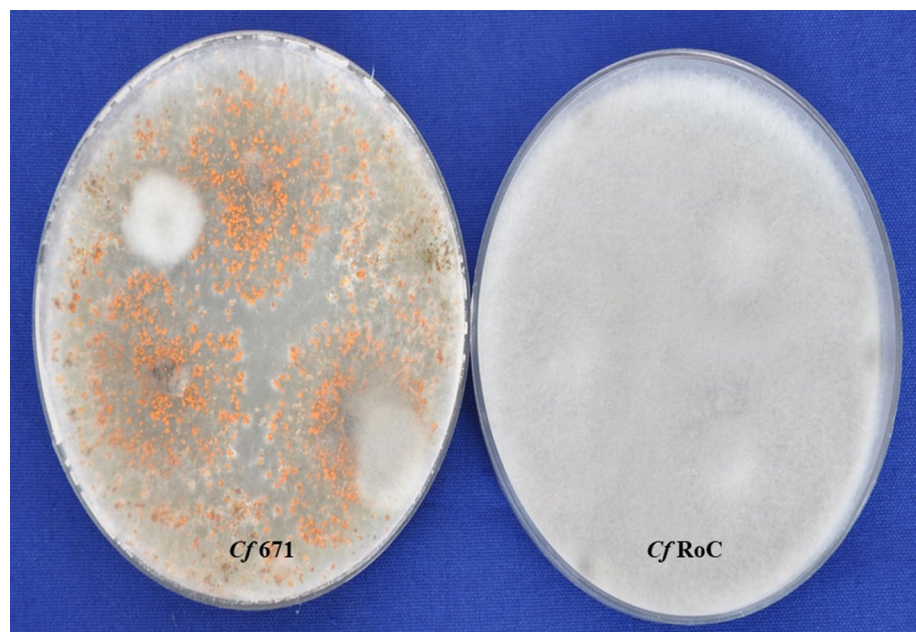
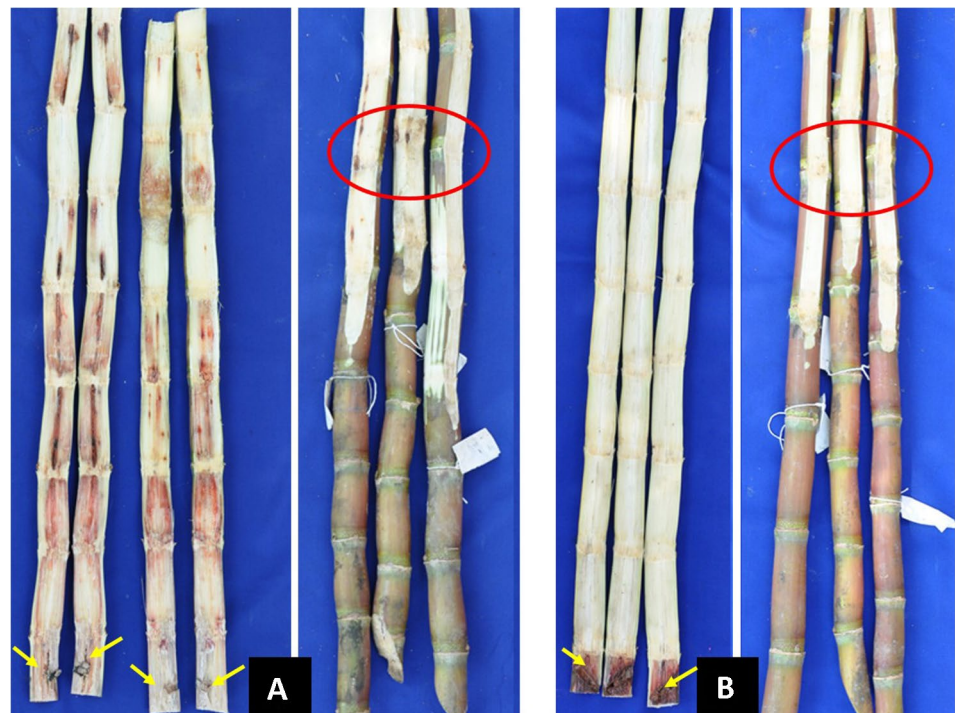


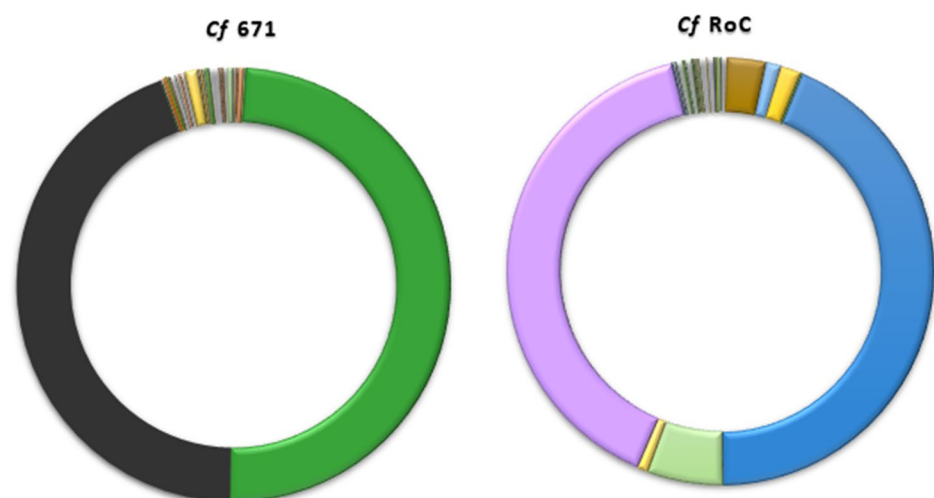
Fig. 2 Phenotypic disease symptoms of *C. falcatum* after plug and nodal swab inoculation in sugarcane stalks of susceptible cv CoC 671. **A** *Cf671* pathotype inoculated; **B** *CfRoC* pathotype inoculated. Arrows indicate site of pathogen inoculation in the plug method and circles indicate the pathogen inoculated node in nodal swabbing method. Only in the pathotype *Cf671* inoculated canes disease progress is noticed in both the methods



further annotated using CD-HIT-EST v4.6.1 (12), TGICL v2.1 (13), TransDecoder v2.0.1, BlastX and Blast2GO. This TransDecoder revealed that there were 23,136 CDS regions in the pathotype *Cf671* and 17,830 CDS regions in *CfRoC*. The transcriptome of the pathotype *Cf671* was further annotated, based on BlastX search in that 19,183 were found to be annotated while 5549 had no significant blast hits among the 24,732 transcripts. About 21,221 were found to be annotated and 9289 unigenes had no significant blast hits in case of *CfRoC*. The species distribution for the *Cf671* using BlastX revealed that 9046 genes have best matches (first hit) with *C. graminicola*, followed by *C. sublineola* (8125), *C. higginsianum* (1158), *C. gloeosporioides*

(465), *C. orbiculare* (295), *Nectria haematococca* (161), *Verticillium albo-atrum* (77) *Magnaporthe oryzae* (41), *Chaetomium globosum* (41), *Metarhizium acridum* (28), *Neurospora crassa* (16), other fungal organisms (296), other organisms (845) (Fig. 3). The annotations from *CfRoC* revealed that the *C. graminicola* has 9243 common genes between them, followed by *C. sublineola* (8448), *C. higginsianum* (1206), *C. gloeosporioides* (568), *C. orbiculare* (182), *C. fiorinae* (629), other fungal organisms (694)*, and other organisms (199) (Fig. 3). Even though the genes sharing between *Colletotrichum* species are closer, they do have their own novel genes which make differences among survival in their hosts system. The total

Fig. 3 Hierarchical gene cluster analysis and common fungal transcript annotation statistics based on organisms; the majority of gene sharing is between *C. graminicola* and *C. sublineola* in both the pathotypes



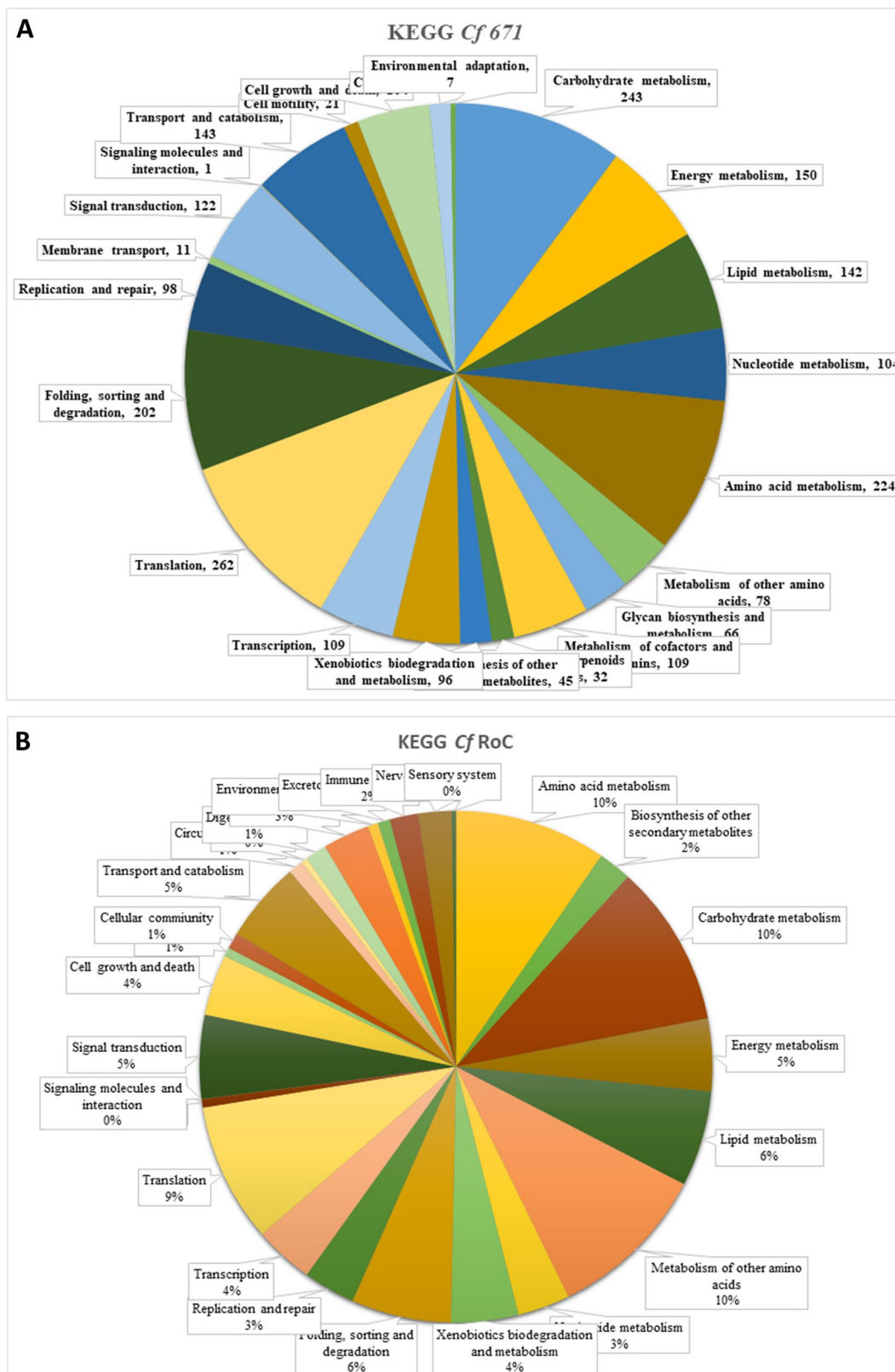


Fig. 4 KEGG distribution between *C. falcatum* pathotypes of Cf671 and CfRoC

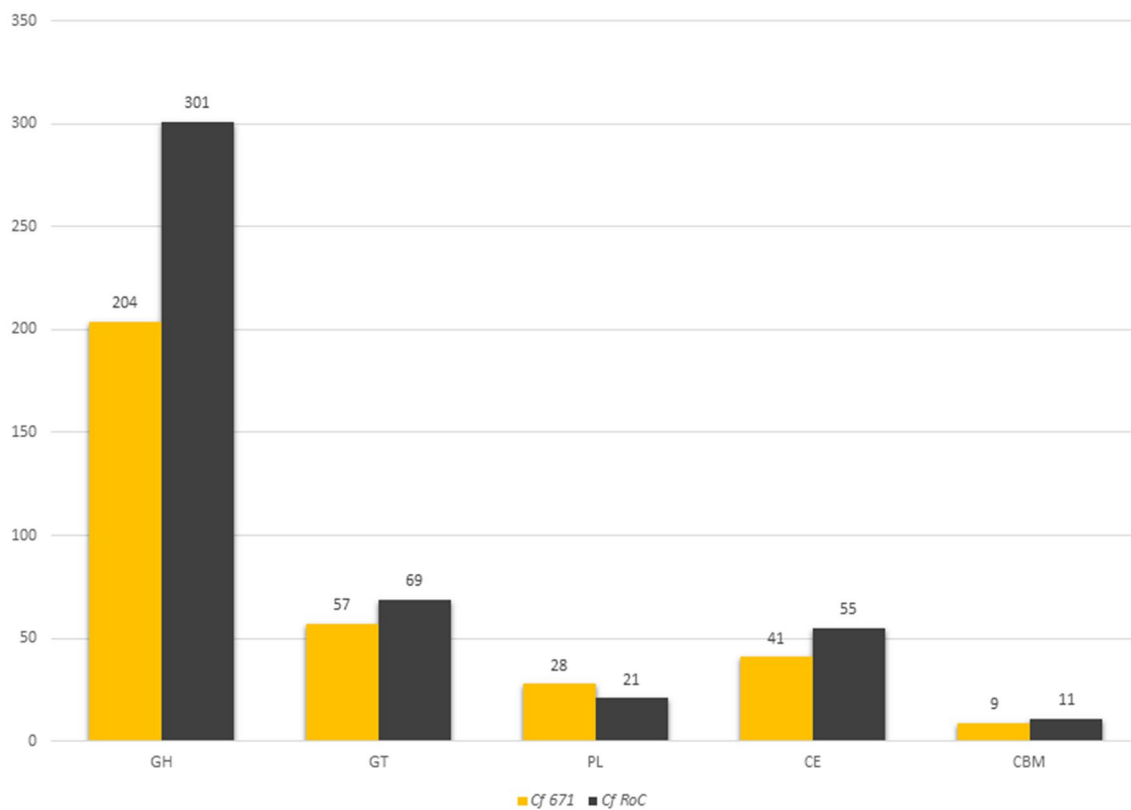


Fig. 5 Different classes of CAZy families and its distribution in *C. falcatum* pathotypes *Cf671* and *CfRoC* transcriptome analyses

number of unique annotated proteins from the pathotype *Cf671* was 4356 and 12,142 proteins from the pathotype *CfRoC*. The common annotated transcripts between the pathotypes *Cf671* and *CfRoC* were found to be 5251 and 8169, respectively.

Gene family's expansion and contraction

The gene family's expansion and contraction in the fungal pathogen are associated with the particular host range. In case of *C. falcatum*, it is more specific and the pathotype *Cf671* which is considered to be virulent has contracted several classes of genes for colonization and establishing virulence in the host system, whereas the least virulent pathotype *CfRoC* had a greater number of genes than the virulent pathotype. The major group of genes which contributes virulence in a hemibiotrophic pathogens CSEPs, Secondary Metabolites, Transporters and CAZy, are found to be contracted in the pathotype *Cf671* and expanded in the pathotype *CfRoC* (Table ESM 3).

GO and KEGG distribution

The GO sequence distributions for *Cf671* and *CfRoC* pathotype were analysed for all the three GO domains,

i.e., biological processes, molecular functions and cellular components. The *Cf671* had 8098 genes with 23 functional groups, whereas the pathotype *CfRoC* had 23,910 genes distributed from transcripts (Fig. 4). A total of 2399 unigenes were predicted as pathway regulatory genes and were annotated in the KAAS database, assigned to several KEGG pathways in *Cf671*; however, 5547 unigenes were found to be present and predicted several regulatory pathway genes in *CfRoC*.

CAZy distribution

Carbohydrate active enzymes (CAZy) play a vital role in degradation of plant cuticle layer by triggering the fungal plant cell wall-degrading enzymes (PCWDEs). *C. falcatum* has plenty of PCWDEs which belong majorly to glycoside hydrolyase (GH) and also CAZy contributes to biotrophy–necrotrophy switch. The major classes of CAZy glycoside hydrolyase (GH), glycosyl transferase (GT), polysaccharide lyase (PL), carbohydrate esterase (CE) and carbohydrate-binding modules (CBM) are found to be present in *C. falcatum* (Fig. 5). The heat map and distribution of CAZy family among the pathotypes of *C. falcatum* brought a clear view that they are mainly involved in cellulose degradation and pathogenicity development in *Cf671* and *CfRoC*.

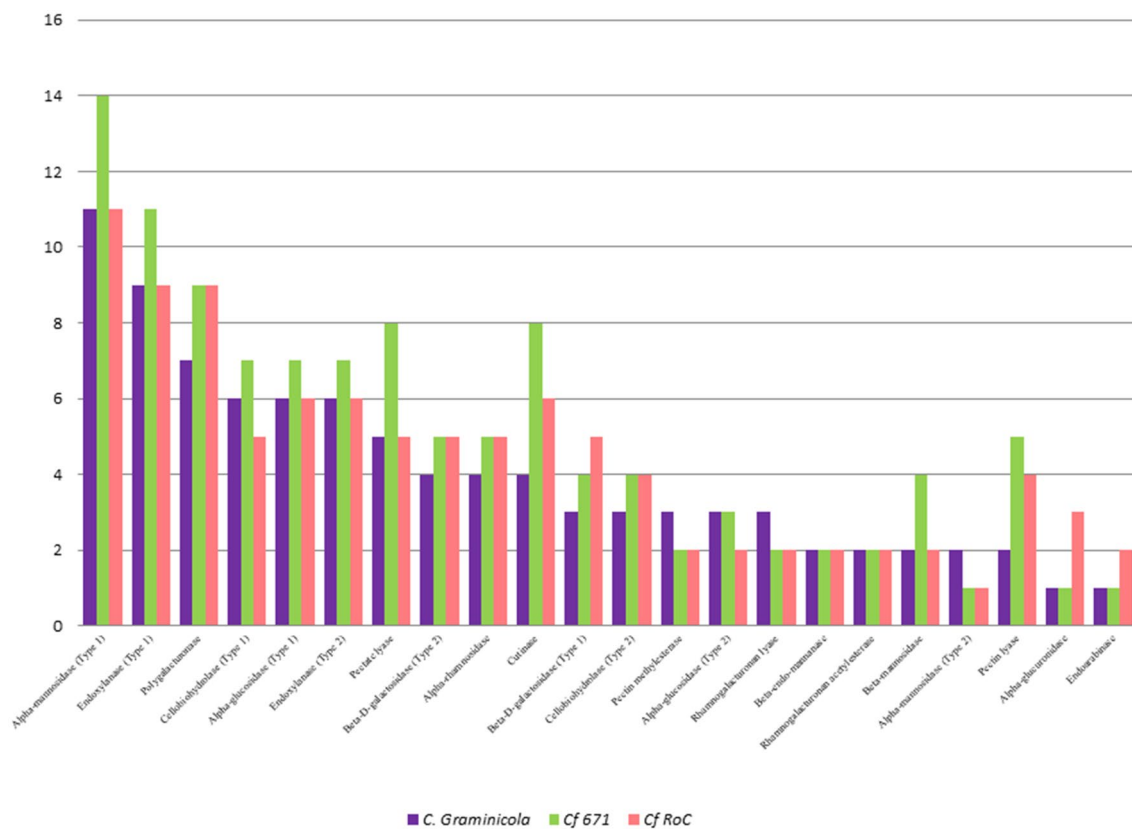


Fig. 6 Distribution of cellulolytic, hemicellulolytic and pectin-related genes compared between *C. graminicola* and *C. falcatum* pathotypes *Cf671* and *CfRoC*. The genes in *Cf671* are up regulated compared to *C. graminicola* and *CfRoC*

RoC and also for survival in the host system, respectively (Fig. 7).

Enzymes involved in plant cell wall degradation

Most of hemibiotrophic fungal organism secrete cell wall-degrading enzymes in large numbers, among them cellulose, hemicellulose and pectin-degrading enzymes play a vital role. Transcriptome analysis revealed presence of several enzymes likes xylanases, cutinases, cellobiohydrolase, alpha-glucosidase, alpha rhamnosidase, pectate lyase, galactotomannan, alpha-mannosidase and ligninolytic enzymes like laccases and peroxidases in *C. falcatum*.

Functional analysis of CAZy in *C. falcatum*

qRT-PCR assay was performed for the candidate genes selected from each family of CAZy to profile the expression of cellulolytic, hemicellulolytic and pectinolytic genes in *C. falcatum*. The CAZys retrieved from comparative transcriptomics gave a clear view about up regulation and down regulation between the pathotypes of *C. falcatum*. The qRT-PCR profiles showed that the candidate genes are considered

to be putative pathogenicity genes which are involved in virulence losses/gain. Candidate genes for cellulose such as cell wall glycosyl hydrolase, glycosyl hydrolase 3 and 5 were up regulated in both the pathotypes *Cf671* and *CfRoC* at in vitro stage. During *in planta* interaction, glycosyl hydrolase 10 (xylanases) and glycosyl hydrolase 35 (β -galactosidases) from hemicellulose and polysaccharide deacetylase (pectin degrading) were upregulated in the pathotypes *Cf671* but downregulated in *CfRoC* (Fig. 8). In contrast, the up regulation and down regulation between the *Cf671* and *CfRoC* enzymes such as GH, GT and PL may probably regulate the pathogenicity in *C. falcatum*.

Discussion

Plant cell wall is the major barrier to the establishment of fungal infection on a host system. Cellulose, the major polysaccharide is present in the plant cell wall; therefore, fungal organism produces specific enzymes to degrade rigid wall and to surpass the layer of protection by hydrolysing cellulose and its derivatives (Laine et al. 2000; Wanjiru et al. 2002). The pathogen invasion and biotrophic development

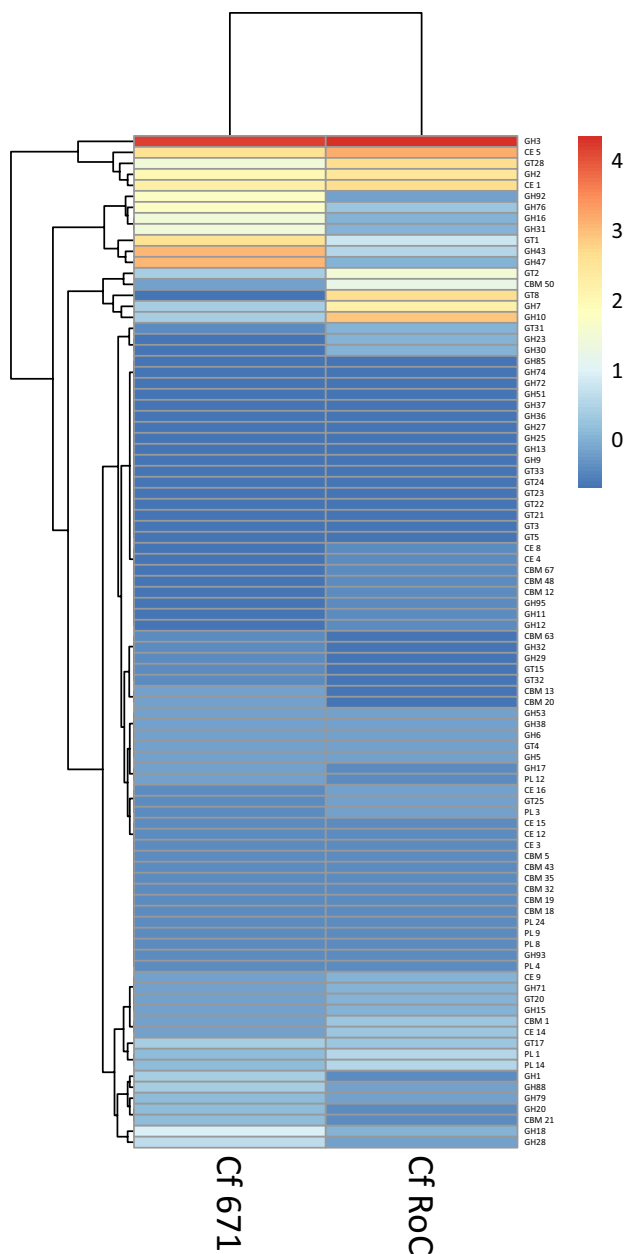


Fig. 7 Heat map for the expressed cellulolytic, hemicellulolytic and pectin degrading enzymes from two pathogenic pathotypes *Cf671* and *CfRoC*. Glycoside hydrolase is found to be expressed higher in virulent pathotype, whereas glycosyl transferase expressed higher in the least virulent pathotype *CfRoC*

mask its surface by converting the hyphal surface-exposed chitin by deacetylation. This chitin degrades plant cuticle layer and elicits defense responses (Gueddari et al. 2002). Most of the *Colletotrichum* spp. produces a number of cell wall-degrading enzymes both in *in planta* and in vitro conditions (Gan et al. 2013; Prasanth et al. 2017). There are several reports in *Colletotrichum* that PCWDEs have been implicated in facilitating successful invasion and

colonization in the host system (Choi et al. 2013; Rao and Nandineni 2017; O'Connell et al. 2012; Gan et al. 2013). The arsenal of *C. falcatum* genes involved in hemibiotrophic lifestyle is found to be similar to *C. graminicola* and *C. sublineola* infecting related hosts maize and sorghum, respectively (Viswanathan et al. 2016). The genome of *C. falcatum* revealed presence of PCWDEs, candidate secretory effectors (CSEPs), transposable elements, primary and secondary metabolites, membrane transporters, signalling molecules, CAZy and mating proteins involved in development and sclerotic management proteins. Though PCWDE are essential for successful pathogenesis and regulation of virulence in *C. falcatum*, their role during the process of pathogenesis is poorly understood. To exploit the ability to produce PCWDE in *C. falcatum* a comparative transcriptome approach has been performed with NGS platform using Illumina Hi Seq 2500. Two different pathotypes of *C. falcatum* varying in virulence were sequenced and their transcriptome analyses of gene expression profiles between *Cf671* and *CfRoC* revealed that virulent pathotype produces a greater number of PCWDE genes when compared with other hemibiotrophs (Fig. 5). Most of the enzymes screened were produced in in vitro conditions; PCWDE enzymes in *C. falcatum* are capable enough to degrade pectin cellulose, and polysaccharides though cell wall possess a greater number of polysaccharides for their defence (Prasanth et al. 2017, 2019). This study aimed to advance the understanding of the PCWDEs such as cellulolytic, hemi cellulolytic and pectin-related genes present in *C. falcatum* causing red rot in sugarcane. As described above, this plant pathogenic fungus has large number of CAZy genes which are putatively involved in pathogenicity and they were characterized using virulent pathotype (*Cf671*) and a least virulent pathotype (*CfRoC*). In this study, we conducted a transcriptome sequencing of least virulent pathotype (*CfRoC*) and compared it with virulent pathotype (*Cf671*) which was sequenced using NGS platform. Comparative transcriptomics brought an understanding that distribution of CAZy genes in each pathotype regulates their pathogenic mechanism which helps in their survival in the host system and also to be the successor in causing red rot in sugarcane.

Sugarcane stalk contains more of cellulose, pectin, cutin and wax that favour hard rigid cell wall as first barrier to fungal invasion (Viswanathan 2010). For pathogenic virulence in *C. falcatum*, it requires biochemical weapons to breach cell wall of sugarcane. Most of CAZy genes involved during appressorial phase in *Colletotrichum* spp, for example 18 different families, were reported in *C. graminicola* and 35 different CAZyme families in *C. higginsianum* (O'Connell et al. 2012). The role of CAZy is to potentially degrade polymers of plant cell wall such as cutin, cellulose, hemicelluloses and pectin. CAZyme regulates genes which are involved in initial penetration of host epidermal cell wall; large number of

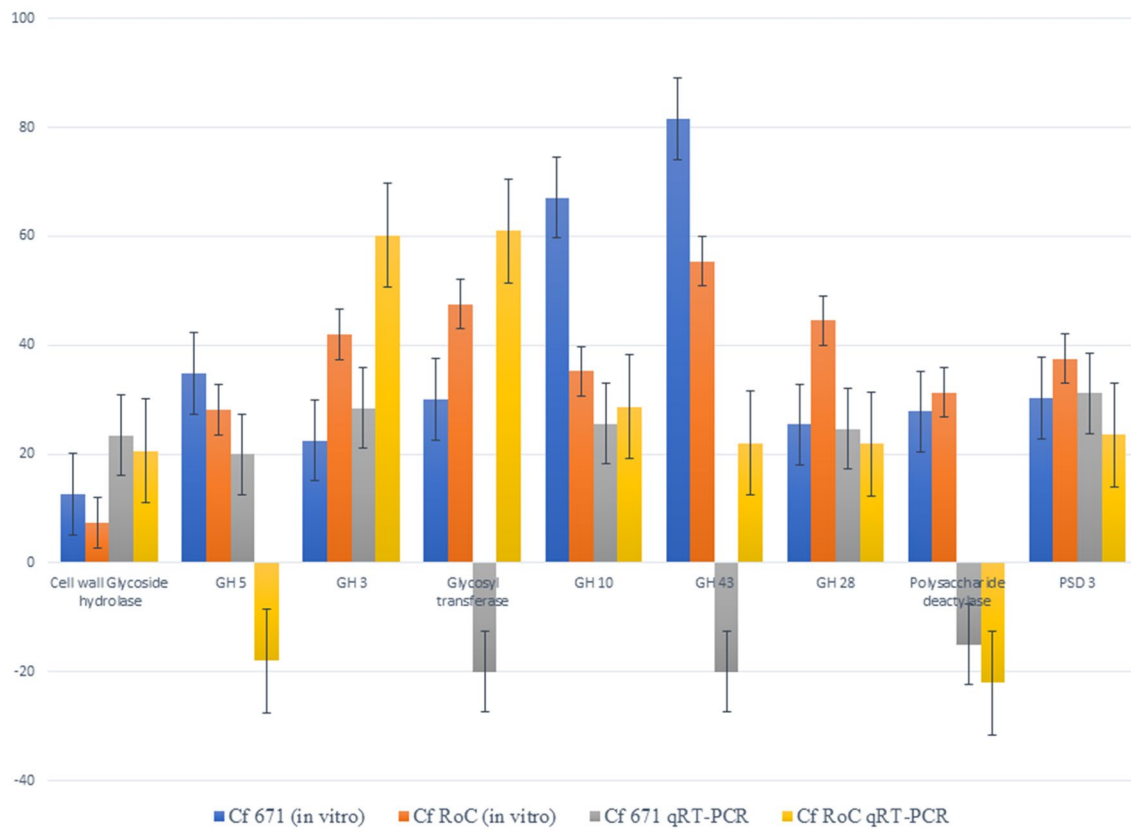


Fig. 8 Transcriptome profiling of CAZy regulatory candidate genes based on their RPKM values, A-*Cf* cell wall glycosyl hydrolase; B-*Cf* glycosyl hydrolase 5; C-*Cf* glycosyl hydrolase 3; D-*Cf* glycosyl hydrolase 10; E-*Cf* glycosyl hydrolase 35; F-*Cf* glycosyl hydrolase 43; G-*Cf* glycosyl hydrolase 28; H-*Cf* polysaccharide deacetylase; I-*Cf* polysaccharide deacetylase 3

Table 1 List of cellulolytic enzymes involved in degradation of plant cell wall by hemibiotrophs

Organism	Cellulases					β-Glycosidases		Accessory enzymes	
	GH 6	GH 7	GH 5	GH 12	GH 45	GH 1	GH 3	GH 61	CBM
<i>Cachliobolus sativus</i>	3	6	15	3	3	3	15	29	11
<i>Dothistroma septosporum</i>	0	1	11	3	1	2	10	4	1
<i>Fusarium graminearum</i>	1	2	14	4	1	3	22	14	12
<i>Glomerella graminicola</i>	3	6	15	6	2	1	18	32	21
<i>Magnaporthe oryzae</i>	3	5	13	4	1	2	18	24	25
<i>Moniliophthora perniciosa</i>	2	3	11	4	2	2	11	16	5
<i>Mycosphaerella graminicola</i>	0	1	9	0	1	1	16	4	0
<i>Colletotrichum falcatum</i>	4	11	4	1	2	0	42	16	4

genes induced both in biotrophic and necrotrophic phases of *Colletotrichum* (O’Connell et al. 2012; Prasanth et al. 2017; Gan et al. 2012). Regulation of cellulolytic, hemi cellulolytic and pectin degradation by CAZy makes the pathogen to become great potential and induces virulence. The CAZy genes such as GH, GT, PL, CBM and CE gene families between *Cf671* and *CfRoC* have been extensively studied using comparative transcriptome approach which revealed that the fungal pathogen *C. falcatum* establishes in the host

tissues via fungal host-induced virulence effectors. Enzymes that encode modifications in fungal cell wall are glycoprotein synthesis/processing (GH47, GH76, and GH92), chitin deacetylases (CE4), chitinases and β-1,3-glucanases (GH16, GH17, GH55, GH64) which were found to be present in *C. falcatum*. β-1,3-glucanases (GH16) induced in *C. graminicola* appressoria and involved in mechanical penetration were found to be present in both the pathotypes of *C. falcatum* (Fig. 6). Hemicellulase and pectinase activities and

Table 2 List of Hemi cellulolytic enzymes involved in degradation of plant cell wall by hemibiotrophs

Organism	β-Glucanase		Xylanases		Xyloglucanase		α-Galactosidases		β-Mannase		α-Arabinosidase			β-Galactosidases	
	GH 67	GH 115	GH 10	GH 11	GH 30	GH 74	GH 27	GH 36	GH 26	GH 43	GH 51	GH 54	GH 62	GH 35	
<i>Cachitobolus sativus</i>	1	3	5	5	2	4	3	1	1	16	2	1	2	4	
<i>Dothistroma septosporum</i>	1	0	0	2	2	3	2	1	0	11	1	1	1	1	
<i>Fusarium graminearum</i>	1	3	5	2	0	5	2	2	0	19	2	1	1	3	
<i>Glomerella graminicola</i>	1	3	9	6	2	7	3	1	1	17	2	1	2	5	
<i>Magnaporthe oryzae</i>	1	4	6	5	1	5	3	0	0	19	3	1	4	0	
<i>Moniliophthora perniciosa</i>	0	1	4	2	1	3	1	0	0	3	1	0	0	2	
<i>Mycosphaerella graminicola</i>	0	1	2	1	1	4	1	1	0	10	2	1	1	2	
<i>Colletotrichum falcatum</i>	1	0	13	0	0	0	0	0	0	17	0	0	0	1	

expanded class of CWDEs were reported in *C. truncatum*. The genes containing CBM42 binding to arabinofuranose were found in all fungi except in *C. truncatum* and *C. incanum* (Rao and Nandineni 2017). Analysis of major polysaccharide-degrading enzymes with wide range of substrates revealed linkage-specific expansion in glycosyl hydrolyase family. Our previous report on screening the effectors from the *C. falcatum* genome and transcriptome revealed that cell wall-degrading glycosyl hydrolyase was found to be highly induced at biotrophic stage (Prasanth et al. 2019). The fruit rot causing pectin-degrading enzymes such as GH 28, GH 78, PL 1 and PL 3 are found to be expanded in *C. truncatum* and *C. fruticola*, but in case of *C. falcatum* the number of genes was found to be on par but their roles vary as stage-specific genes. The contraction of auxiliary activity (AA) enzymes reported in *C. graminicola* and *C. sublineola* was found to be same in *C. falcatum*. The Heat map of cellulolytic, hemicellulolytic and pectin-degrading enzymes showed variation in their expression patterns between two pathotypes of *C. falcatum* (Fig. 7) which clearly shows that CAZy regulates cell wall-degrading enzymes and may play a vital role in plant penetration and successful pathogenesis (Fig. 8).

Cutinase plays a vital role not only in breakdown of leaf cuticle layers during penetration but also for degradation of plant cell wall during colonization and hyphal invasion. But in case of *C. falcatum*, a stalk-infecting pathogen, there are numerous cutinase genes such as glycosyl hydrolase and their roles vary depending on the developmental stages of the host. It is considered to be stage-specific expressive gene; their enzymes are involved in colonization and hyphal development in the host system. Pectate lyase in *C. gloeosporioides* promotes pathogenicity; to test their contribution in penetration and colonization in avocado fruit, a heterologous expression study was carried out and it was found that complexity of system requires more than one factor for pathogenicity (Yakoby et al. 2000). *C. falcatum* has a greater number of pectate lyase genes which may be putatively involved in pathogenesis and virulence factors. Based on stalk infecting nature, the pectin lyase sequences are different compared to other hemibiotrophs. The evolutionary effect of enzyme diversity is due to the substrates that are encountered during interactions with an extended variety of hosts. The *Colletotrichum* spp. have a wide range of hosts and close relationship. During the developmental stages they bypass plant defense and penetrate cell walls with the use of lytic enzymes. Host plants also have strategic approaches to detect and to defend against attack of pathogens by producing phytoalexins and inhibitor enzymes (Viswanathan and Rao 2011). Hemibiotrophic lifestyle in *Colletotrichum* spp. makes greater variability of unique enzymes based on the host system and most of them are regulated by CAZy genes. The relationships of lytic enzymes

Table 3 List of pectin-degrading enzymes in hemibiotrophs

Organism	Polygalacturonase		Polygalacturonate lyase				
	GH 28	GH 78	PL 1	PL 3	PL 4	PL 9	PL 11
<i>Cachliobolus sativus</i>	4	6	0	5	4	0	5
<i>Dothistroma septosporum</i>	4	1	0	0	2	0	1
<i>Fusarium graminearum</i>	6	9	0	7	3	1	7
<i>Glomerella graminicola</i>	7	7	0	4	3	1	4
<i>Magnaporthe oryzae</i>	4	2	0	1	1	0	4
<i>Moniliophthora perniciosa</i>	3	9	0	2	0	0	0
<i>Mycosphaerella graminicola</i>	2	2	0	1	0	0	3
<i>Colletotrichum falcatum</i>	2	1	0	4	2	1	4

and the lifestyle of fungi are very well exploited after the advent of next generation sequencing technology, this high throughput sequencing method made several evidential reports in *Colletotrichum* spp that reveals about plant cell wall-degrading enzymes and role during pathogenesis. It is very well demonstrated that pathogenic fungi of monocotyledonous plants are better adapted to degrade the cell wall of plants reflecting host preference (Choi et al. 2013). Comparative transcriptome analysis of *C. falcatum* pathotypes reveals that there are three major PCWDEs such as cellulolytic, hemi cellulolytic and pectin-degrading enzymes which are regulated by CAZymes. Those enzymes were compared with other hemibiotrophs based on the following classification: The cellulolytic enzymes were classified as cellulases (GH6, GH7, GH5, GH12, and GH45), β -glycosidases (GH1 and GH3) and accessory enzymes such as GH 61 and CBM (Table 1) and the hemi cellulolytic enzymes as β -glucuronidases (GH67, GH115), xylanases (GH10, GH11, GH30), xyloglucanases (GH74), α -galactosidases (GH27, GH36), β -mannase (GH26), α -arabinosidases (GH43, GH51, GH54) and β -galactosidases (GH62, GH35) (Table 2). The pectinolytic enzymes such as polygalacturonases (GH28, GH78) and polygalacturonate lyase (PL1, PL3, PL4, PL9 and PL11) (Table 3) are found to be present. The obtained results suggested that *C. falcatum* pathotypes produce cellulolytic and pectinolytic enzymes in large numbers that helps to degrade sugarcane cell wall and involvement of these enzymes in development of red rot in sugarcane. The CAZy from Cf671 pathotype were found to be abundant than CfRoC pathotype and this is the first investigation on pathotype variation, genetic mechanism and simulation of pathogenicity between the *C. falcatum* pathotypes.

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Author contributions RV conceptualised, designed, initiated, and supervised the project. CNP prepared materials and performed whole transcriptome data analysis. CNP performed lab experiments and contributed to data analysis and data interpretations. RV, PM and ARS analysed the interpreted whole information retrieved from the transcriptome analysis. CNP and RV wrote the manuscript. All authors read and approved the final manuscript.

Data access All data contributing to this transcriptome initiative have been deposited at the NCBI under BioProject PRJNA272832 and PRJNA380334. The accession number of Sequence Read Achieves (SRA) is SRR1765657. The Bio sample accession number of CfRoC is SAMN06640823 and SRA: SRS2076621.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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