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ORIGINAL ARTICLE

Differential host responses of sugarcane to *Colletotrichum falcatum* reveal activation of probable effector triggered immunity (ETI) in defence responses

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Abstract

Key message **The differential compatibility responses of sugarcane to Colletotrichum falcatum pathotypes depend on the nature of both host primary defence signalling cascades and pathogen virulence.**

Abstract The complex polyploidy of sugarcane genome and genetic variations in different cultivars of sugarcane remain a challenge to identify and characterise specific genes controlling the compatible and incompatible interactions between sugarcane and the red rot pathogen, *Colletotrichum falcatum*. To avoid host background variation in the interaction study, suppression subtractive hybridization (SSH)-based next-generation sequencing (NGS) technology was used in a sugarcane cultivar Co 7805 which is compatible with one *C. falcatum* pathotype but incompatible with another one. In the incompatible interaction (ICI—less virulent) 10,038 contigs were assembled from ~ 54,699,263 raw reads, while 4022 contigs were assembled from ~ 52,509,239 in the compatible interaction (CI—virulent). The transcripts homologous to CEBiP receptor and those involved in the signalling pathways of ROS, Ca^{2+}, BR, and ABA were expressed in both interaction responses. In contrast, MAPK, ET, PI signalling pathways and JA amino conjugation related transcripts were found only in ICI. In temporal gene expression assays, 16 transcripts showed their highest induction in ICI than CI. Further, more than 17 transcripts specific to the pathogen were found only in CI, indicating that the pathogen colonizes the host tissue whereas it failed to do so in ICI. Overall, this study has identified for the first time that a probable PAMP triggered immunity (PTI) in both responses, while a more efficient effector triggered immunity (ETI) was found only in ICI. Moreover, pathogen proliferation could be predicted in CI based on transcript expression, which were homologous to *Glomerella graminicola,* the nearest clade to the perfect stage of *C. falcatum (G. tucumanensis*).

Keywords Sugarcane · *Colletotrichum falcatum* · Differential host response · Illumina high throughput sequencing · Defense signalling

Abbreviations

ABA	Abscisic acid
ABAREBF	Abscisic acid responsive element binding factor
AGT	Glyoxylate aminotransferase
BR	Brassinosteroid
BRSK	Brassinosteroid signalling kinase
CBPCML	Calcium binding protein CML
CDPK	Calcium dependent protein kinase
CEBiP	Chitin elicitor binding protein
CNGC	Cyclic nucleotide gated channel
DAMPs	Damage associated molecular patterns
DRPRPM1	Disease resistant protein RPM1
DRPRPS5	Disease resistant protein RPS5
ER	Ethylene receptor
ET	Ethylene
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
GO	Gene ontology
HR	Hypersensitive
JA	Jasmonic acid
JAAS	Jasmonic acid amino acid synthetase
KAAS	KEGG automatic annotation server

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KEGG	Kyoto encyclopedia for genes and genomes
LVir	Less virulent
MAPK	Mitogen activated protein kinase
MAPKK1	Mitogen activated protein kinase kinase 1
MAPKKK1	Mitogen activated protein kinase kinase kinase1
NADH	Nicotinamide adenine dinucleotide phosphate
PAMP	Pathogen associated molecular pattern
PI	Phosphoinositide
PR	Pathogenesis-related
PRRs	Pattern recognition receptors
PTI	PAMP triggered immunity
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
SA	Salicylic acid
SOD	Superoxide dismutase
SSH	Suppression subtractive hybridization
SSH	Suppression subtractive hybridization
Vir	Virulent
VTPATPase	V type proton transporting ATPase subunit1

Introduction

Plants, being sessile are continuously exposed to various biotic and abiotic stresses during their lifetime. Among them, biotic stresses caused by the invading pathogen often result in complete devastation of the diseased plant. To cope up with the pathogen stress, plants have evolved sophisticated defense strategies to recognize and restrict pathogen invasion by activating their immune responses. At the plant-pathogen interface, plant perceives pathogen's cell wall associated conserved molecules termed as pathogen associated molecular patterns (PAMPs) that is recognized by the cognate pattern recognition receptors (PRRs) located in plasma membrane of host plant. Recognition of PAMP by PRR, results in triggering of basal defense responses termed as PAMP triggered immunity (PTI). Successful pathogens evade this host detection by secreting effector proteins and make the host susceptible to pathogen invasion known as effector triggered susceptibility (ETS). Whereas, the effector proteins of pathogens are recognized by a set of resistance (R) genes present in the plant, which further activate strong host defense termed as effector triggered immunity (ETI). This mode of defense is stronger and often termed as R-gene mediated defense or gene for gene interaction. PTI emerges as a basal defense to prevent further colonization of the pathogen. ETI leads to hypersensitive response mediated programmed cell death and enhanced resistance at the whole plant which is long lasting (Hamdoun et al. 2013). Resistance is determined by a set of R genes localized at the surface of the plant plasma membrane or the cytoplasm. Flor's gene for gene hypothesis states that a specific R gene in the plant recognizes its cognate avirulence (Avr) gene in the pathogen. Specific recognition results in provoking defense gene expression in the plant system (Ali & Reddy, 2000; Beers & McDowell, 2001). During evolution, new resistant genes are generated to cope with the newly evolved virulent strains of pathogens. Some R genes such as Hm1 and RPM1 are present as single copy in resistant plants and are absent in the susceptible plants (Flor, 1971) but most of the R genes are organized in complex loci containing an array of homologous genes. For example, Rp1, Rpp5, Xa21, Pto, Dm3, I2, N, M and Cf genes are localized in a cluster (Gao et al. 2000; Grant et al. 1995). In a crop with complex polyploidy and aneuploidy, the R genes are organized in a complex locus. The polyploid nature and genetic complexity of sugarcane makes it difficult to find a specific R gene for a particular disease/pathogen. So far, *Bru*1 is the only known resistant gene conferring resistance to brown rust and is found to segregate in a Mendelian pattern of 3:1 (Asnaghi et al. 2004).

Sugarcane (*Saccharum* spp. hybrid) is an economically important crop cultivated in tropical and subtropical regions of the world. India ranks second in sugarcane production next to Brazil (www.fao.org/corp/statistics/en/). Red rot caused by the ascomycete fungal pathogen *Colletotrichum falcatum* (Teleomorph: *Glomerella tucumanensis*) is a serious threat for sugarcane cultivation in the tropical regions of the world (Viswanathan 2010, 2021a). Management of the disease depends solely on cultivating red rot resistant cultivars. However, during the past decades, severe disease epidemics have occurred which resulted in removal of many elite cultivars from cultivation. Frequent emergence of new variants of *C. falcatum* leads to the varietal breakdown (Viswanathan and Rao 2011). Hence, detailed studies were conducted on the molecular basis of the interaction between sugarcane and *C. falcatum* to understand the host resistance mechanism by our research group. In our previous studies, we documented the early and prominent induction of pathogenesis-related (PR) proteins as induced defense response against *C. falcatum* (Viswanathan et al. 2003, 2005) and accumulation of phytoalexins at the pathogen infection site was documented as marker for red rot resistance (Malathi et al. 2008; Kumar et al. 2015; Nandakumar et al. 2021a). The chitinase gene from sugarcane has been characterized as a class IV glycosyl hydrolase based on full gene sequence and in silico 3D structure prediction. Further, the differential expression of the chitinase gene in red rot resistant and susceptible sugarcane cultivars was monitored through qRT-PCR (Rahul et al. 2015). Differential display (DD)-RT-PCR was used to identify differential transcripts upregulated during pathogenesis of *C. falcatum* in resistant

and susceptible cultivars of sugarcane (Prathima et al. 2013; Rahul et al. 2016).

The NGS based sequencing technology plays a vital role in exploring genes and genomes of an organism. The whole genome and transcriptome of *C. falcatum* were sequenced using Illumina Hi-Seq 2500 (Viswanathan et al. 2016; Prasanth et al. 2017). Earlier we adopted suppression subtractive hybridization (SSH) strategy to identify the time specific and initial defense responses of sugarcane during *C. falcatum* pathogenesis which hypothesized involvement of jasmonic acid (JA), ethylene (ET), reactive oxygen species (ROS), phosphoinositide (PI) and calcium (Ca^{2+}) signals in disease resistance (Sathyabhama et al. 2015, 2016). In our previous transcriptomic studies, a set of sugarcane cultivars varying in red rot resistance, either a resistant or a susceptible host were used to determine differential transcripts upregulated during *C. falcatum* pathogenesis (Prathima et al. 2013; Sathyabhama et al. 2015, 2016). The variation in transcript accumulation between resistant and susceptible varieties cannot rule out the changes in defense transcriptomes due to their genetic complexity. This may have a profound influence on identifying the genes/proteins involved in pathogen defense in sugarcane. In our experimental trials, certain cultivars of sugarcane, exhibiting differential host responses to different pathotypes of *C. falcatum* was established (Viswanathan et al. 2020). In this study, the sugarcane cultivar Co 7805 exhibiting incompatible (ICI) and compatible (CI) interactions to *C. falcatum* pathotypes *Cf*87012 and *Cf*94012, respectively was used for SSH coupled with subtracted transcriptome sequencing and to identify the initial defense responses exhibited in sugarcane against *C. falcatum*. Illumina HiSeq 2000 sequencing platform was used to sequence subtracted transcripts derived from the two responses. This study generated comparative transcriptomes of ICI and CI in sugarcane against *C. falcatum* and identified probable resistance mechanism in sugarcane to *C. falcatum* for the first time.

Materials and methods

Plant material and pathogen culture

A tropical sugarcane cultivar Co 7805 was planted in sugarcane field at ICAR-Sugarcane Breeding Institute (SBI) (ICAR), Coimbatore, India and the crop was raised following standard field practices for a tropical sugarcane. Two pathotypes of *C. falcatum* viz., *Cf*87012 and *Cf*94012 were isolated from infected stalk of sugarcane cultivar Co 87012 and Co 94012, respectively and maintained as part of *C. falcatum* culture collections at Plant Pathology lab, ICAR-SBI, Coimbatore. Plug method of pathogen inoculation was performed by making boreholes on the third internode from the base of the canes (Mohanraj et al. 2012, Viswanathan et al. 2021). Separate sets of sugarcane stalks of the cultivar Co 7805 were inoculated in triplicates with the two *C. falcatum* pathotypes and mock inoculated samples served as control. Seed canes of sugarcane free from all the designated pathogens were planted to raise a healthy crop for pathogen inoculation and also to prevent interference from other pathogens for the current genomic work. Stalk tissue samples were collected in triplicates with their respective controls at 36 h post inoculation and stored in − 80 °C till RNA isolation.

RNA extraction and suppression subtractive hybridization (SSH)

Total RNA was extracted from all the samples with TRI reagent (Sigma-Aldrich, USA). The quality of RNA was checked in an agarose gel and quantified in a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, USA). 1 µg of purified RNA was used for cDNA synthesis following the manufacturer's instructions of Smarter™ PCR cDNA synthesis kit (Clontech, CA, USA). Forward and reverse subtractions for the cDNAs were done by following the manufacturer's instructions of PCR-Select™ cDNA subtraction kit (Clontech, CA, USA). Forward subtraction represents incompatible interaction library (ICI), in which cultivar Co 7805 challenged with pathotype *Cf*87012 was used as tester and cultivar Co 7805 challenged with *Cf*94012 and mock sample of cultivar Co 7805 were used as the driver. In reverse subtraction representing compatible interaction library (CI), the cultivar Co 7805 challenged with *Cf*94012 was used as tester and cultivar Co 7805 challenged with the pathotype *Cf*87012 and mock sample of cultivar Co 7805 were used as driver.

Illumina library construction and sequencing

The cDNA pools of the subtracted two transcripts were sequenced by Illumina HiSeq 2000 paired end (PE) sequencing platform at Xcelris Genomics Pvt Ltd, Ahmadabad, Gujarat India. The two subtracted ds cDNAs were fragmented using Covaris S2 (Covers Inc., Massachusetts, USA). After fragmentation, Illumina indexing adapters were added to the blunt ends and size selected in the range of 300–600 bp in 2% agarose-Etbr gels. The two subtracted ds cDNAs were prepared for sequencing according to the Illumina TruSeq DNA sample preparation guide v2 (August 2011, rev. A) (Illumina Inc, San Diego, USA) for Illumina Paired-End (PE) Multiplexed sequencing. Cluster generation was carried out for the PE library by hybridization of template DNA molecules onto the oligonucleotide-coated surface of flow cell v3 (Illumina Inc., San Diego, USA). Immobilized DNA template copies were amplified by bridge

amplification to generate clonal DNA clusters. The process of cluster generation was performed on cBOT using TruSeq PE Cluster kit v3-cBot-HS (Illumina Inc., San Diego, USA). TruSeq SBS v3-HS kits (Illumina Inc., San Diego, USA) were used to sequence DNA of each cluster on a flow cell using sequencing by synthesis technology on the Illumina HiSeq 2000 flow cell v3. Samples were sequenced using 100 bp PE runs.

Transcript assembly and annotation

The raw reads were quality trimmed; adaptor sequences were removed and the reads were size selected using Trimmomatic v0.17. After adaptor trimming, high quality reads with mean quality scores more than 25 and sequence length longer than 50 bp were selected for assembly. De novo assembly of the subtracted transcripts assembled contigs were performed in a CLC genomics workbench v6.0. Functional annotation of the assembled transcript contigs were predicted with gene ontology (GO) terms through BLASTx analysis using BLAST2GO program. An e-value threshold of e^{-5} and a high scoring segment pair (hsp) filter of 33 were kept as default parameters for similarity search. Sequences less than 100 were filtered and removed.

Pathway analysis by KEGG-KAAS

The transcript assembled contigs that belong to the metabolic pathways that are expressed in the interaction were identified through mapping the assembled transcripts to Kyoto Encyclopedia for Genes and Genomes (KEGG) eukaryotic database using KEGG Automatic Annotation Server (KAAS). All the transcripts were compared against KEGG-KAAS database using BLASTx with default threshold bit-score value of 60.

Validation of differential gene expression through qRT-PCR

Temporal gene expression of 16 transcripts mapped to KEGG-KAAS database was further analysed in (Step One Plus™ Real-Time PCR Systems, Applied Biosystems Inc., Life Technologies, USA). Representative transcripts involved in recognition, signalling and defense were further evaluated. The transcripts include chitin elicitor binding protein (CEBiP), mitogen activated protein kinase kinase kinase 1 (MAPKKK1), mitogen activated protein kinase kinase 1 (MAPKK1), Brassinosteroid signalling kinase (BRSK), disease resistant protein RPM1 (DRPRPM1), disease resistant protein RPS5 (DRPRPS5), cyclic nucleotide gated channel (CNGC), calcium dependent protein kinase (CDPK), calcium binding protein CML (CBPCML), superoxide dismutase Cu Zn (SOD Cu Zn), V type proton transporting ATPase subunit1 (VTPATPase), jasmonic acid aminoacid synthetase (JAAS), Abscisic acid responsive element binding factor (ABAREBF), ethylene receptor (ER), 14-3-3 protein epsilon (14-3-3 PE) and chitinase. The sugarcane cultivar Co 7805 stalks inoculated with *C. falcatum* pathotypes *Cf*87012 and *Cf*94012 were collected at 12, 36 and 72 h post inoculation. Total RNA was extracted by TRI reagent (Sigma-Aldrich, USA). 1 µg of total RNA was used for cDNA synthesis and first strand cDNA was diluted further to 200 ng and used as templates for qRT-PCR. SYBR green PCR master mix (Applied Biosystems Inc., Life Technologies, USA) was used for the analysis. Cane samples inoculated with sterile distilled water served as control. Melt curve analysis was performed at 60 °C to check amplification specificity in the assays. Sugarcane 25S rRNA gene was used as the internal control. Each of the reaction was repeated three times with two biological replicates. To determine relative expression of up regulated ESTs, 2^{-CT} method was used (Livak and Schmittgen 2001) and plotted against time series of gene expression. The list of primers is given in Table 1.

Results

Differential interaction of *C. falcatum* pathotypes

The differential responses of sugarcane cultivar Co 7805 to the two *C. falcatum* pathotypes *Cf*87012 and *Cf*94012 were assessed over a period of three years. The cultivar consistently showed incompatible response upon inoculation against the pathotype *Cf*87012 and compatible response to the pathotype *Cf*94012. Phenotypic symptoms caused by *C. falcatum* were clearly visible on the 5^{th} and 10^{th} days after inoculation as progressive reddish lesions on the inoculated internode and transgressed to the upper internodes in the CI. Whereas, in ICI, the pathogen could not make any progress beyond inoculated spot (Fig. 1).

Transcriptome sequencing and de novo assembly

The number of raw reads generated by Illumina HiSeq 2000 were 54,699,263 and 52,509,239 and the number of filtered reads were 41,025,151 and 42,001,812 for ICI and CI, respectively. Quality trimming, adaptor sequence removal and size selection of transcript reads resulted in a total of 10,038 and 4,022 high quality reads for ICI and CI, respectively. After BLASTx analyses the respective figures were reduced to 7849 and 2899. About 2189 and 1123 reads did not have homology for ICI and CI, respectively and were described as novel genes or hypothetical proteins. The transcripts were submitted in NCBI sequence read archive

Table 1 Primer sequences of selected transcripts used in gene expression assays

Transcript name	Primer sequences 5′-3′	
Chitin elicitor binding protein	F	TGCGCGTCCAGGATGTGTG
	R	ATGACGGACGTCCTCCACATGG
Mitogen activated protein kinase kinase kinase1	F	GGTGCTGCTGATATACAGACAGGC
	R	GGACCAGACTTGGGGCGTG
Mitogen activated protein kinase kinase1	F	GGTGCTGCTGATATACAGACAGG
	R	ACCAGACTTGGGGCGTGTAAT
Brassinosteroid signaling kinase	F	CAGCACACGGCCCGTTA
	R	CTGTGACGCATTTTTTGGGCC
Disease resistance protein RPM1	F	GTGCAGGGTGGTTCCCTAAGCT
	R	GGTGGAGTGTCCTAAGGTACTGGATG
Disease resistance protein RPS5	F	GGCAGGGTTGGAAGGACCAAG
	R	CCATATTCTGGGAAAAGGGTGCAG
Cyclic nucleotide gated channel	F	CGCGATTGCCAACGACAG
	R	TGCAGCAGTCTTGCAGGCA
Calcium dependent protein kinase	F	GCACACCGGCTTGACAGACC
	R	GTGGCTGTGCCAAGTTCATTTGCC
Calcium binding protein CML	F	ACACCAGTCAATAAGCACACGCC
	R	AGGATTGCCAAAGAGCTGGGTG
Superoxide dismutase Cu–Zn family	F	GCTGAGGGCGTAGCTGAGG
	R	GCCAACAACACCACATGCCAGT
V type proton transporting ATPase subunit1	F	GAGCTTGGCGCATTCAGAGCTG
	R	GCAGTGCATGCAGGAAGGCA
Jasmonic acid amino acid synthetase	F	AGCCATTGGCCCACTTGAG
	R	GGTGGTGTTCCTACTTAGTATCTGC
Abscisic acid responsive element binding factor	F	GGCACTGGTACACTCGACTCTG
	R	CGATGCCTGCGGAGAACATTGA
Ethylene receptor	F	TCGGTTTCCTCCGCTTTGAG
	R	TGTGAAGGCTGACTCTTTGAGAGA
14-3-3 protein epsilon	F	CGCATGCTCCCTCGCCA
	R	CGTCAGGCTTGCTTGCATCCC
Chitinase	F	GCTGCAGATCTCGTGGAACTACAAC
	R	GTGCACGTTGTTCGTCCAGAAC
25SrRNA	F	GGCAGCCAAGCGTTCATAGC
	R	GGGTAAAACTAACCTGTCTCACGAC

(SRA) database, with accession numbers SRR2992210 and SRR 2,992,249 for ICI and CI, respectively.

BLAST homology with other species and annotation

In ICI, the BLAST hits constituted to maximum homology of 34% with *Sorghum bicolor* followed by 32% with *Zea mays* and 4% with *Oryza sativa*. In case of CI, the respective homologies were 42%, 27% and 5% for *Sorghum bicolor, Zea mays* and *Oryza sativa*. In ICI, 1% homology in *Triticum urartu*, *Vitis vinifera*, *Glycine max*, *Hordeum vulgare* and *Medicago truncatula* species were found. In CI, no homology was found with those species. *Saccharum officinarum* and *Populus trichocarpa* had 1% homology in CI, and no homology was found in ICI. Both ICI and CI had 1% homology with *Saccharum* hybrid cultivar (Suppl. Figure 1a, 1b). In both ICI and CI, many of the transcripts from BLAST annotation were found to be hypothetical proteins or novel genes. Therefore, to know the functional ontology of the differential transcripts, GO distribution and KEGG-KAAS functional categorization were done.

Gene ontology (GO) distribution

The high-quality assembled transcripts were annotated with gene ontology (GO) terms. The GO terms were distributed as biological processes, molecular functions and cellular components (Fig. 2). In biological process, transcripts pertaining to aromatic amino acid family biosynthetic process, protein N-linked glycosylation, small GTPase mediated

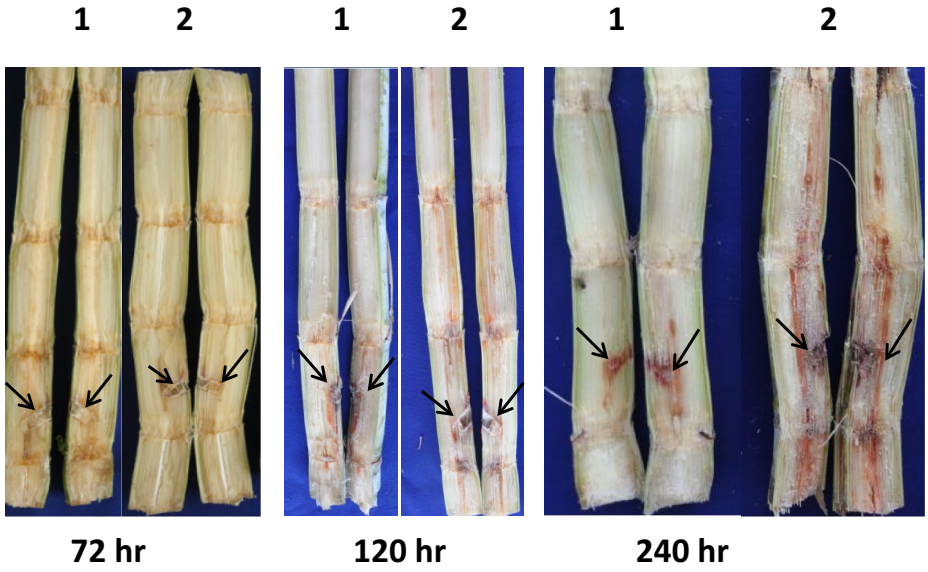


Fig. 1 Phenotypic symptoms of sugarcane cultivar Co 7805 exhibiting differential response to inoculation with two different *C. falcatum* pathotypes *Cf*87012 (1) incompatible and *Cf*94012 (2) compatible; arrows indicate point of pathogen inoculation

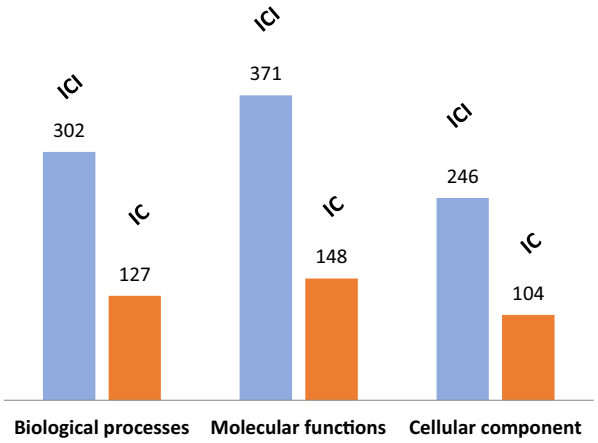


Fig. 2 Gene ontology distribution in resistant (ICI) and susceptible (CI) responses in SSH libraries of sugarcane challenged with virulent and less virulent pathotypes of *C. falcatum*

signal transduction, GPI anchor biosynthetic process and IMP biosynthetic process were expressed differentially in ICI. In CI, transcripts pertaining to sucrose biosynthetic process, negative regulation of peptidase activity and protein deubiquitination were present. In cellular component, transcripts pertaining to RNA polymerase complex, cis-golgi network, anaphase-promoting complex, transcription factor TFIID complex and photosystem I & II were present in ICI. In CI, a single differential transcript pertaining to cullin-RING ubiquitin ligase complex was present. In molecular function, transcripts pertaining to shikimate kinase activity, cellulose synthase (UDP-forming) activity, P-P-bond-hydrolysis-driven protein transmembrane transporter activity, aspartic-type endopeptidase activity, serine-type endopeptidase activity and mannose-1-phosphate guanylyl transferase (GDP) activity were present in ICI. In CI, serine-type endopeptidase inhibitor activity and quinone binding—oxidoreductase activity, acting on NADH or NADPH were present. The gene ontology of the 3 GO terms is presented in Table 2.

KEGG-KAAS functional annotation of subtracted transcriptome

High quality reads corresponding to 10,038 for ICI and 4022 for CI were mapped in KEGG-KAAS database. The transcripts were mapped to 12 categories pertaining to carbohydrate metabolism, energy metabolism, lipid metabolism, nucleotide metabolism, amino acid metabolism, glycan metabolism and biosynthesis, metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, biosynthesis of other secondary metabolites, genetic information processing, environment information processing and plant pathogen interaction. Of the total transcripts mapped, 42% were found to be present in both the interactions or unchanged during *C. falcatum* pathogenesis, 47% of the transcripts were upregulated in ICI and 11% of the transcripts were found to be upregulated in CI. In all the categories several transcripts were mapped in common i.e., those transcripts were present in both the responses or unchanged during the interaction (Fig. 3). The representative transcripts are listed in Table 3.

Differential transcripts from *Glomerella graminicola*

From the BLAST homology search, a total number of 17 transcripts homologous to *Glomerella graminicola* were

Table 2 Representative GO of ICI and CI through BLAST homology search

	ICI		CI	
GO biological process				
1	transcript_266	Aromatic amino acid biosynthesis process	transcript_177	Sucrose biosynthesis process
2	transcript_681	Protein N-linked glycosylation	transcript_254	Negative regulation of peptidase activity
3	transcript_789	Small GTPase mediated signal transduction	transcript_373	Spermine biosynthetic process; spermidine biosynthetic process
4	transcript_2602	D-amino acid catabolic process	transcript_449	Protein deubiquitination; post-translational protein modification
5	transcript_2826	One-carbon metabolic process		
6	transcript_2942	GPI anchor biosynthetic process		
7	transcript_3315	Two-component signal transduction (phosphorelay); peptidyl-histidine phosphorylation		
8	transcript_4821	IMP biosynthetic process		
9	transcript_4947	Small GTPase mediated signal transduction		
10	transcript_5013	Meiotic chromosome segregation		
11	transcript_5441	Oxidation-reduction process; terpenoid biosynthetic process		
12	transcript_6927	Inositol triphosphate metabolic process		
GO cellular component				
13	transcript_1452	RNA polymerase complex	transcript_2078	Cullin-RING ubiquitin ligase complex
14	transcript_2324	Cis-Golgi network		
15	transcript_3744	Low density lipoprotein particle; high density lipoprotein particle;		
16	transcript_4014	Anaphase promoting complex		
17	transcript_4349	Cell junction; cell wall		
18	transcript_7732	Transcription factor TFIID complex		
19	transcript_7780	Photosystem-I; chloroplast thylakoid membrane		
20	transcript_7849	Photosystem-II; integral to membrane; chloroplast thylakoid membrane		
GO molecular function				
21	transcript_226	Shikimate kinase pathway; ATP binding; magnesium ion binding	transcript_254	Serine type endopeptidase inhibitor activity; peptidase activity
22	transcript_855	P-P bond hydrolysis-driven-protein transmembrane transporter activity	transcript_2789	Quinone binding; oxidoreductase activity; acting on NADH or NADP
23	transcript_1184	Mannosyl-oligosaccharide 1,2 alpha mannosidase activity; calcium ion binding		
24	transcript_1517	Cellulose synthase (UDP) forming activity		
25	transcript_1884	SNAP receptor activity		
26	transcript_3369	P-P bond hydrolysis-driven-protein transmembrane transporter activity		
27	transcript_4062	N-acetyltransferase activity		
28	transcript_4097	Aspartic-type endopeptidase activity		
29	transcript_6112	Serine-type endopeptidase activity		
30	transcript_6441	Mannose-1-phosphate guanylyl transferase (GDP) activity		
31	transcript_6927	Inositol-1,3,4 triphosphate 5/6-kinase activity; inositol tetrakisphosphate 1-kinase activity; ATP-binding; magnesium ion binding		
32	transcript_1987	NADH dehydrogenase (ubiquinone) activity		

present only in CI. The transcripts represented pathogenic determinants of *G. tucumanensis,* the perfect stage of *C. falcatum*. The transcripts were found to be involved in fungal morphogenesis (alanine glyoxylate aminotransferase), intra cellular signal transduction (Ras), translation (ribosomal proteins), glycolysis (hexokinase), RNA splicing and the

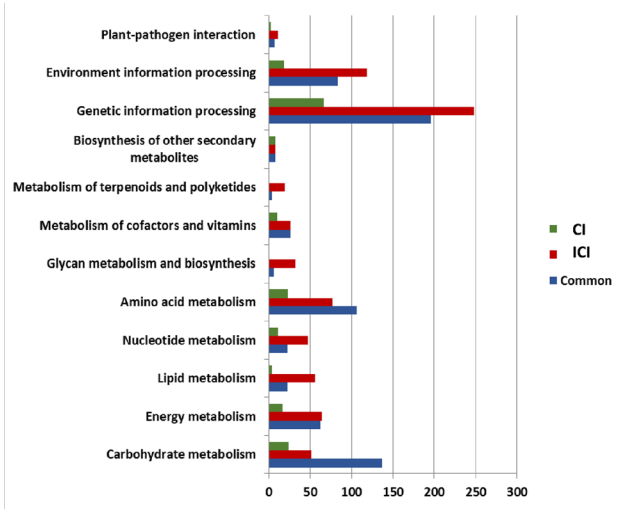


Fig. 3 Functional categorization of transcripts involved in resistant (ICI) and susceptible (CI) responses in SSH libraries of sugarcane challenged with virulent and less virulent pathotypes of *C. falcatum*

E3 Ub ligase of the Ub-26S proteasome pathway (Table 4). The expression of transcripts corresponding to the pathogen even after subtraction of cDNA suggested that the *C. falcatum* could colonize the host tissues in compatible interaction whereas transcripts related to *C. falcatum* colonization was not found in the incompatible interaction.

Validation of gene expression through qRT-PCR

The gene expression levels of the following transcripts viz., *CEBiP, MAPKKK1, MAPKK1, DRPRPM1, DRPRPS5, CBPCML, JAAS* and *ABAREBF* decreased gradually at 12 h and 36 h and reached to low level at 72 h post inoculation in ICI. Whereas, in CI, the transcripts levels of *CEBiP, MAPKKK1* and *CBPCML* exhibited an inconsistency in their expression. Expression of *MAPKK1* and *JAAS* gradually decreased from 12 to 72 h. Expression of *DRPRPM1* showed a similar response as observed in ICI but the transcript level was less than twofold. Expression of *ABAREBF* gradually increased from 12 to 72 h and reached to 2.5-fold higher levels at 72 h in CI compared to that in ICI. The defense gene chitinase gradually increased in both ICI and CI from 12 to 72 h. However, the transcript accumulation was found to be higher in ICI and reached a maximum of fourfold at 72 h post *C. falcatum* inoculation. The transcripts of *CNGC* and *CDPK* gradually increased in ICI and gradually decreased in CI from 12 to 72 h post inoculation. The transcripts of *14-3-3 PE* and *SOD Cu Zn* increased from 12 to 36 h and declined at 72 h in the CI. In ICI, there was an unstable expression. fivefold expression was noticed in ICI for *SOD Cu–Zn* at 12 h post *C. falcatum* inoculation. For, VTP ATPase, the expression in ICI was unstable and CI showed a constant sixfold expression at 36 and 72 h post *C. falcatum* inoculation. *BRSK* and *ER* showed an inconsistent expression in both the responses. At 12 h, *BRSK* showed more than fivefold expression in ICI. At 36 h, *ER* showed more than tenfold expression in CI. Overall, incompatible interaction revealed higher expression of different transcripts associated with host resistance to defense upon *C. falcatum* inoculation whereas in the compatible interaction, except for a few transcripts where the gene expression was not prominent.

Discussion

Red rot, caused by the fungal pathogen *Colletotrichum falcatum* is a devastating disease of sugarcane crop. The survival of a sugarcane cultivar in India is highly linked to red rot resistance in almost all parts of sugarcane cultivating regions of the country. Once, the popular cultivars are affected by red rot, they cannot be propagated in the field and has to be removed from cultivation. The pathogen infection causes complete devastation of the crop under field conditions. Hence, concerted efforts were given to identify red rot resistant cultivars in sugarcane varietal development programmes (Viswanathan, 2021b). During the last two decades, considerable efforts were made to understand defense strategies adopted by sugarcane in response to *C. falcatum*. Evidence of induction of PR- proteins and 3-deoxyanthocyanidin phytoalexins as biochemical defense responses during *C. falcatum* pathogenesis were found (Viswanathan et al. 2005; Malathi et al. 2008; Kumar et al. 2015).

Recently, a large number of transcripts at the interface of plant – pathogen interaction has been sequenced by the use of parallel NGS, and many candidate genes responsible for resistance or susceptibility were identified. In our preliminary SSH studies, we assessed initial responses of sugarcane tissues at 12 h and 36 h after post-inoculation in two cultivars Co 93009 and CoC 671 along with gene expression assays (Sathyabhama et al. 2015). Based on that, in this study, forward and reverse subtracted transcriptomes captured in sugarcane during 36 h post *C. falcatum* challenge were sequenced through an NGS-Illumina Hi-Seq 2000 sequencing platform. Further, 36 h sampling is expected to capture transcript profiling just before visible symptoms appearance. The pathotype *Cf*87012 (LVir) exhibited incompatible interaction (ICI) and the pathotype *Cf*94012 (Vir) exhibited compatible interaction (CI) when inoculated on the sugarcane cultivar Co 7805, which exhibited a differential host interactions. A total of 10,038 and 4022 transcripts were derived for ICI and CI, respectively. In that, only 7849 and 2899 transcripts had BLAST homology for ICI and CI, respectively. The transcripts were mapped in KEGG-KAAS for functional categorization and biochemical

Table 3 Representative differential transcripts from ICI and CI and their putative functions as mapped in KEGG-KASS database

S.No	Transcripts	Function	ICI	CI
1	Chitin elicitor binding protein (CEBiP)	Recognition	√	√
2	Serine threonine protein kinase (STPK)	Signalling	√	√
3	Mitogen activated protein kinase kinase kinase1 (MAPKKK1)	Signalling	√	x
4	Mitogen activated protein kinase kinase1 (MAPKK1)	Signalling	√	x
5	Disease resistance protein RPM1 (DRP RPM1)	Defense	√	x
6	Disease resistance protein RPS2 (DRP RPS2)	Defense	√	x
7	Disease resistance protein RPS5 (DRP RPS5)	Defense	√	x
8	Protein kinase (PK)	Signalling	√	x
9	Brassinosteroid signalling kinase (BRSK)	Signalling	√	√
10	14-3-3 protein (14-3-3 P)	Multifaceted roles	√	√
11	Phospholipase C (PLC)	Signalling	√	√
12	Phospholipase D (PLD)	Signalling	√	√
13	Phosphoinositide 3 kinase (PI3K)	Signalling	√	x
14	Phosphoinositide 4 kinase (PI4K)	Signalling	√	x
15	Linoleate 9S lipoxygenase (LOX)	Signalling	√	x
16	Jasmonic acid amino synthetase (JAAS)	Signalling	√	x
17	Calcium binding protein CML (CBP CML)	Signalling	√	√
18	Calcium binding protein 39 (CBP 39)	Signalling	√	x
19	Calmodulin (CaM)	Signalling	√	√
20	Calcium dependent protein kinase (CDPK)	Signalling	√	√
21	Cyclic nucleotide gated channel (CNGC)	Redox homeostasis	√	x
22	Respiratory burst oxidase (RBO)	Oxidative stress	√	√
23	Catalase (CAT)	Oxidative stress	√	√
24	Superoxide dismutase (SOD)	Oxidative stress	√	√
25	Glutathione peroxidase (GPOX)	Oxidative stress	√	√
26	L-Ascorbate peroxidase (L-APX)	Oxidative stress	√	√
27	Peroxidase (POX)	Oxidative stress	√	√
28	Glutathione S transferase (GST)	Redox homeostasis	√	√
29	Glutathione S reductase (GSR)	Redox homeostasis	√	√
30	Abscisic acid receptor PYR/PYL (ABAR PYR/PYL)	Signalling	√	√
31	Protein phosphatise 2C (PP2C)	Signalling	√	√
32	Ethylene receptor (ETR)	Recognition & Signalling	√	x
33	Ethylene insensitive 2 (EIN2)	Signalling	√	x
34	Ethylene insensitive 3 (EIN3)	Signalling	√	x
35	S-adenosyl methionine synthetase (SAMS)	Secondary metabolism	√	√
36	S-adenosyl methionine decarboxylase (SAMDC)	Secondary metabolism	√	√
37	Hexokinase (HK)	Glycolysis	√	x
38	Phosphoglycerate mutase (PGM)	Glycolysis	√	x
39	Pyruvate dehydrogenase (PDH)	Glycolysis	√	x
40	Succinate dehydratase (SDH)	Tricarboxylic acid cycle	√	x
41	Phenylalanine ammonia lyase (PAL)	Phenyl propanoid biosynthesis	√	x
42	4-Coumarate CoA ligase (4-CouCoAL)	Phenyl propanoid biosynthesis	√	x
43	Coniferyl aldehyde dehydrogenase (ConADH)	Phenyl propanoid biosynthesis	√	x
44	Cinnamyl alcohol dehydrogenase (CinAlcDH)	Phenyl propanoid biosynthesis	√	x
45	Trans-cinnamate 4 monooxygenase (TC4MO)	Phenyl propanoid biosynthesis	x	√
46	Tyrosine aminotransferase (TyrAT)	Alkaloid biosynthesis	√	√
47	Tyrosine decarboxylase (TyrDC)	Alkaloid biosynthesis	√	√
48	Phosphomevalonate kinase (PMK)	Terpenoid biosynthesis	√	x
49	Diphosphomevalonate decarboxylase (DPMDC)	Terpenoid biosynthesis	√	x
50	Farnesyl diphosphate synthase (FDPS)	Terpenoid biosynthesis	√	x

Table 3 (continued)

S.No	Transcripts	Function	ICI	CI
51	Phytoene synthase (PhyS)	Carotenoid biosynthesis	√	x
52	Zeaxanthin epoxidase (ZXEOX)	Carotenoid biosynthesis	√	x
53	Violaxanthin de-epoxidase (VXDOX)	Carotenoid biosynthesis	√	x
54	Chalcone synthase (CS)	Flavonoid biosynthesis	x	√
55	Photosystem I P 700	Light harvesting complex	√	√
56	Photosystem II	Light harvesting complex	√	x
57	Cytochrome b6	Light harvesting complex	√	x
58	Apocytochrome f	Light harvesting complex	x	√
59	F-type H + -transporting ATPase subunit	Ion transport	√	√
60	Clathrin heavy chain	Secretory pathway	√	x
61	Charged multivesicular body protein	Secretory pathway	√	√
62	Vacuolar protein sorting associated protein	Secretory pathway	√	√
63	Ras-related protein	Secretory pathway	√	x
64	Programmed cell death 6 interacting protein (PCD6IP)	Secretory pathway	√	x
65	E3 ubiquitin protein ligase (E3UbL)	Secretory pathway	√	x
66	Vesicle transport protein	Secretory pathway	√	x
67	Signal recognition particle receptor subunit	Protein processing in ER	√	√
68	Signal peptidase	Protein processing in ER	√	√
69	Chitinase	Defense	√	√
70	Endoglucanase	Defense	√	x
71	Pathogenesis related 1 (PR-1)	Defense	√	x
72	Pectin esterase	Cell wall metabolism	x	√

ICI incompatible interaction, *CI* compatible interaction, √ presence of the transcripts, x absence of the transcripts

Table 4 Transcript homologous to *Glomerella graminicola* from SRL identified through BLASTx homology search

S.No	Transcripts	Function
1	Endoribonuclease L-PSP	Translation
2	Hexokinase	Glycolysis
3	Ribosomal L2 domain containing protein	Translation
4	G-patch domain containing protein	RNA splicing
5	25 s ribosomal protein	Translation
6	Ribosomal protein s28e	Translation
7	Ribonucleotide reductase	Translation
8	Hypothetical protein GLRG	Unknown
9	Ribosomal protein L18ae protein family	Translation
10	Ras family protein	Intracellular signal transduction
11	AMP binding enzyme	Intracellular signal transduction
12	Sulfate permease	Transmembrane protein
13	Zinc finger containing protein	Proteasome system
14	Translation elongation factor EF-1	Translation
15	Ribosomal L29e family protein	Translation
16	Ribosomal protein s12	Translation
17	Alanine-glyoxylate amino transferase	Fungal morphogenesis

pathway analysis (Fig. 3). Finally, a hypothetical model representing the probable occurrence of PTI in both ICI and CI, ETI in ICI and ETS in CI has been proposed in sugarcane for the first time.

We found a transcript homologous to chitin elicitor binding protein (CEBiP), and a Serine/Threonine protein kinase induction in both ICI and CI. In rice, the chitin molecule/elicitor of the fungal pathogen, *Magnaporthe grisea*

is perceived by an extracellular LysM receptor containing CEBiP, a pattern recognition receptor (PRR) (Kaku et al. 2006). PRRs recognize both pathogen-derived nonself PAMPs/MAMPs and plant-derived damage associated molecular patterns (DAMPs), which trigger PTI. However, the transcripts involved in downstream signalling namely MAPK and PI were present only in ICI. Even though, there is probable occurrence of PTI in both ICI and CI, the magnitude of signals activated by MAPK, JA and PI may be higher in ICI, which is probably responsible for prolonged defense than the CI (Fig. 4). The pathogen responsive MAPK activation is likely to promote the generation of

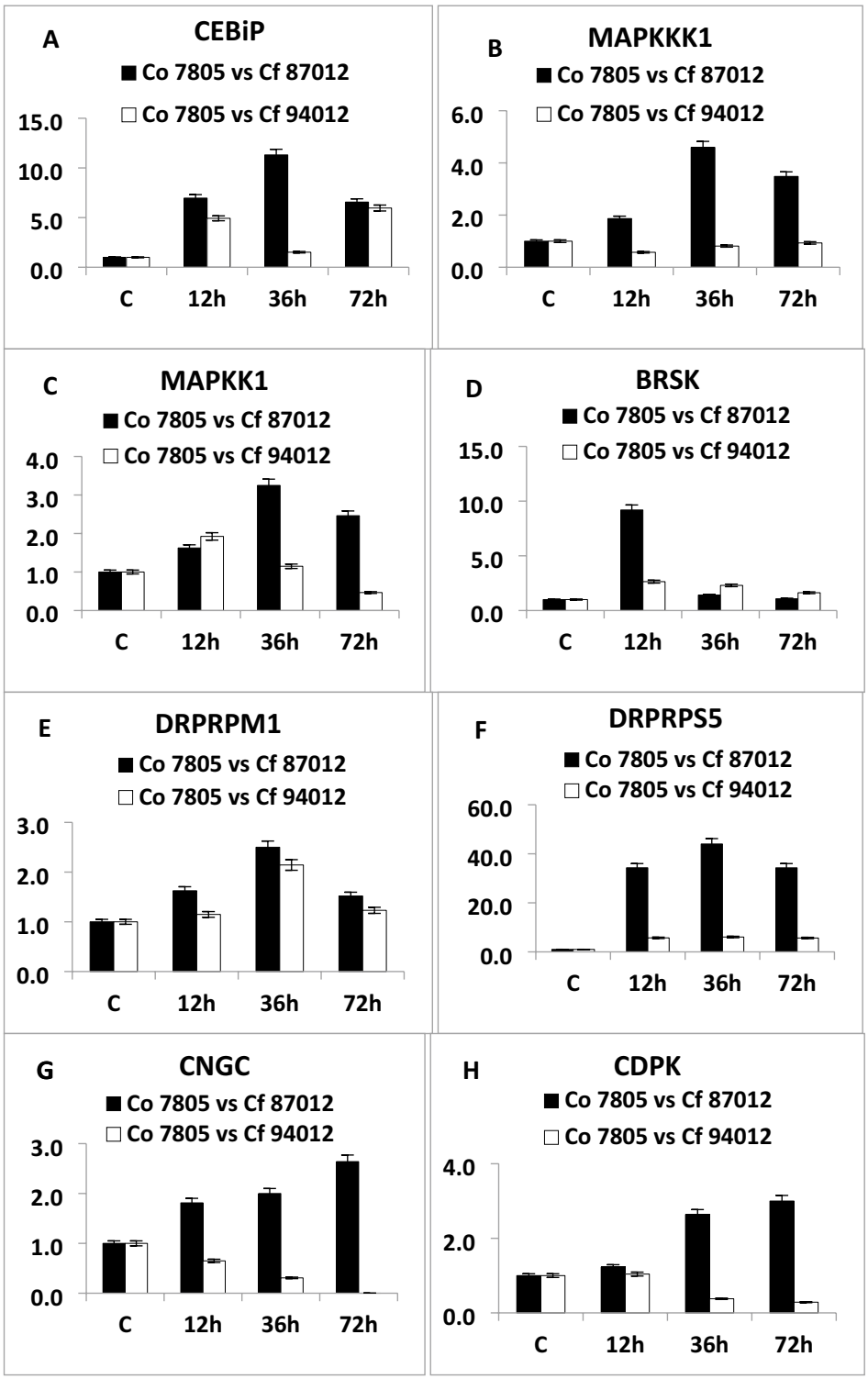
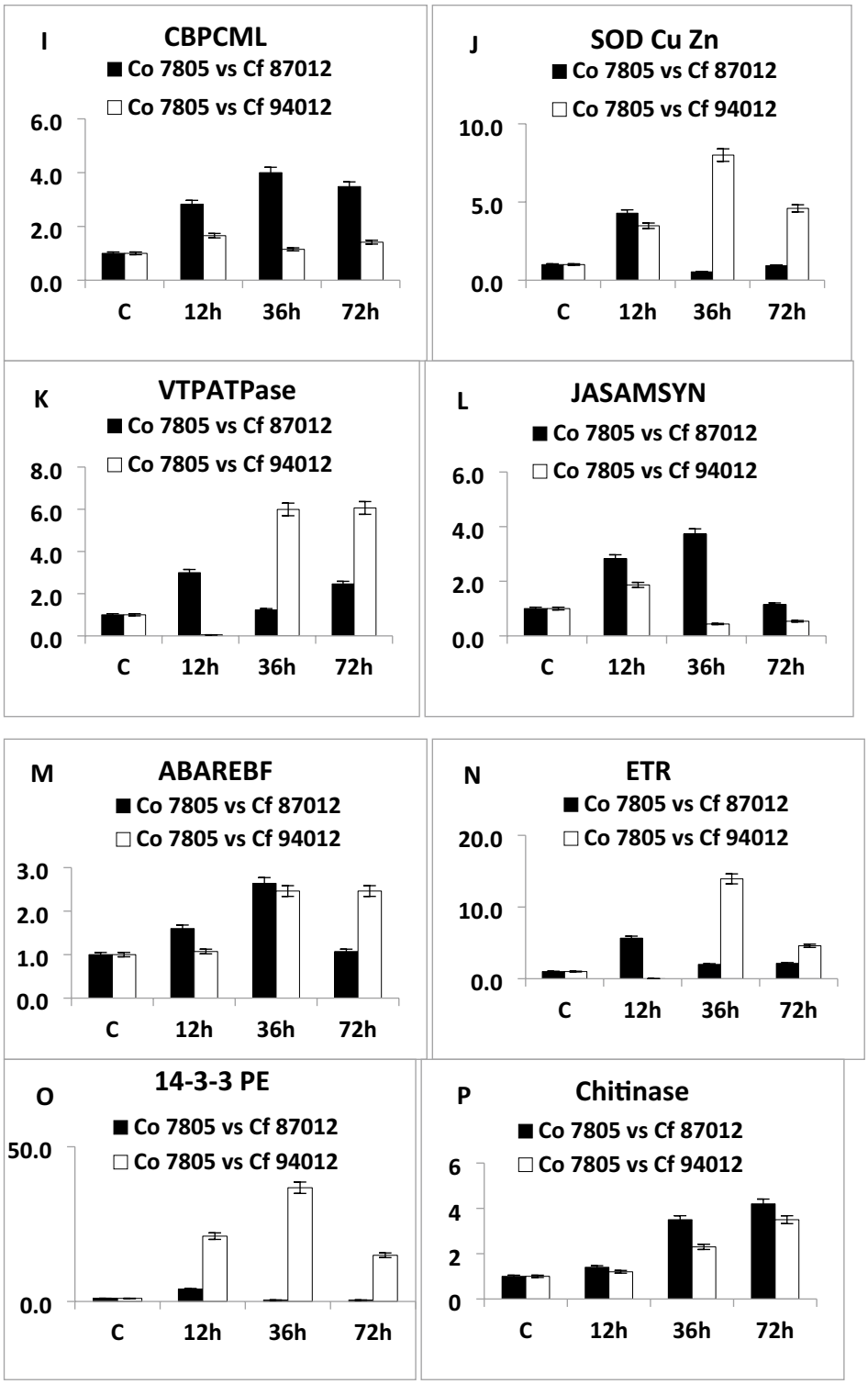


Fig. 4 Gene expression profiling of transcripts in incompatible (Co 7805 vs *Cf* 87,012) and compatible interactions (Co 7805 vs *Cf* 94,012). *C* mock control, *h* hours post *C. falcatum* inoculation, *X- axis* hours post *C. falcatum* inoculation, *Y-axis* RQ values

Fig. 4 (continued)



ROS in chloroplasts and JA signalling, which plays an important role in execution of hypersensitive (HR) cell death in plants. Usually, the virulent pathogen secretes effectors to make the plant susceptible or to evade PTI termed ETS (Jones et al. 2006).

The pathogenic transcripts homologous to *G. graminicola* involved in primary metabolites production and the transcript; alanine-glyoxylate amino transferase involved in fungal morphogenesis were upregulated only in CI (Table 4). The transcripts captured in CI homologous to *G. graminicola* indicate successful pathogenesis and the pathogen's

proliferating stage inside the susceptible host. In addition, studies by Bhadauria et al (2012a, b) have demonstrated the essential role of the enzyme alanine: glyoxylate aminotransferase (AGT1) in the rice blast pathogen *Magnaporthe oryzae*. AGT may provide a means to maintain redox homeostasis in appressoria and contribute to the triglyceride mobilization from conidia to appressoria. Similarly, in the interaction between sugarcane and *C. falcatum*, the role of AGT must be essential to transfer nutrients and enhance lipid mobilization, which is essentially required for melanisation of appressorium utilizing the glycerol during pathogenesis.

In the gene expression assays, the transcripts homologous to disease resistance proteins RPM1, RPS2 and RPS5 were upregulated only in ICI. qRT-PCR experiments carried out in ICI and CI, revealed the transcriptional gene expression of RPM1 and RPS5 in both the responses (ICI and CI). However, the magnitude of expression was higher in ICI (Fig. 4). In addition, RPS5 was found to be more than 40-fold in ICI, but a constant expression of fivefold was noticed in CI throughout the period of study (Fig. 4). Probably, here, proteins involved in the decoy model of defense may exist and the pathogenic effectors secreted by *C. falcatum* may be recognized by guarded/decoy proteins and ETI gets activated. Whereas, in CI, the effector may have the ability to inactivate the R gene products and induce pathogenicity, which can be termed as effector triggered susceptibility (ETS) (Jones and Dangl 2006). The *C. falcatum* pathotype *Cf*94012 has probably induced ETS in host cultivar Co 7805 by secreting effector proteins. Recently, two probable molecular signatures from *C. falcatum* viz., CfEPL1 (eliciting plant response-like protein 1, a ceratoplatanin protein) and CfPDIP1 (plant defense inducing protein 1, a novel protein) were found and their functional characterization of the respective genes revealed that they induce HR in tobacco and systemic resistance against *C. falcatum* in sugarcane. These studies have indicated that these PAMPs/Effectors of *C. falcatum* may govern PAMP-triggered immunity (PTI)/effector-triggered immunity (ETI) in sugarcane (Ashwin et al. 2017, 2018). The gene expression of three transcripts differentially regulated from ICI and CI pertaining to Ca^{2+} signals, the CDPK, CNGC and calcium binding protein CML (CBP CML) were quantified in qRT-PCR in cultivar Co 7805 inoculated with two different *C. falcatum* pathotypes. For all the transcripts, the ICI showed upto 3.8-fold expression whereas in CI, less than 1.5-fold expression was noticed (Fig. 4). This proves the probable involvement of all the transcripts of Ca^{2+} signalling in host resistance to *C. falcatum*

Several transcripts involved in provoking defense responses like clathrin heavy chain, programmed cell death (PCD)-6 interacting protein and transcripts involved in PI signalling were found only in ICI. In addition, this study has documented the crucial role of secretory pathway and vesicle trafficking in HR-PCD. The presence of clathrin heavy chain, PCD 6 interacting protein and signal peptidase transcripts in ICI and its absence in CI is a convincing factor to determine that PCD takes place at a rapid phase only in ICI. In CI, PCD may not be a response, which gives place for successful pathogenicity and disease spread.

In secretory pathway, only a few transcripts pertaining were found to be commonly expressed in both ICI and CI. However, most of the important transcripts namely PCD6 interacting protein and signal peptidase were upregulated only in the ICI. This proves the involvement and possible role of secretory pathway in HR-PCD. In a typical R-Avr gene interaction, few rapidly elicited proteins like Avr9/Cf9 are reported and in sugarcane, the transcript was found upregulated in the resistant cultivar (Sathyabhama et al 2015). Avr9/Cf-9-induced F-Box1 (ACIF1; ACRE189) is an F-box protein with a leucine-rich-repeat domain found in a screen to identify proteins involved in Cf9-mediated ETI in *N. benthamiana* (Rowland et al. 2005). ACIF1 is widely conserved and is closely related to F-box proteins that regulate plant hormone signaling in Arabidopsis. Silencing of ACIF1 Arabidopsis homologs (VFBs) induced a subset of methyl jasmonate- and ABA-responsive genes, supporting a regulatory role of ACIF1/VFBs in hormone-mediated plant defense responses (van den Burg et al. 2008). Janjusevic et al. (2006) gave direct evidence on how the bacterial-secreted proteins act as an E3 Ub ligase in plant cells and affect the host defense response. When the *Pseudomonas syringae*-type III effector AvrPtoB is delivered into tomato plants containing the *Pto* resistance gene, it elicits PCD that inhibits pathogen invasion. In contrast, AvrPtoB suppresses PCD in the Pto-absent plants, leading to rapid pathogen spread in the infected tissues.

Structural analysis indicated that the C-terminal region of AvrPtoB is highly homologous to the U-box and RING-finger domain of eukaryotic E3 ligases. In vitro E3 ligase activity assay indicated that it has ubiquitin ligase activity. Mutation of the conserved residues in the U-box/RING-finger motif of AvrPtoB significantly compromised AvrPtoB's anti-PCD activity in tomato leaves and dramatically reduced disease symptom on infected plants. These results clearly demonstrated that *P. syringae* use an E3 ligase effector protein to suppress plant PCD and probably other defense-related processes in the infected cells. Similarly, in this study, a few Ub-ligases are upregulated only in the ICI and a specific F-box and DNA damage binding proteins were found only in the CI (Table 3). This could be because of the host modification strategies followed by the pathogen. Still this study provides a new insight on the involvement of ubiquitin/26S proteasome system (UPS) in sugarcane and *C. falcatum* interaction. It is novel information in this particular host pathogen interaction.

Our recent studies suggest that micro(mi)RNAs regulate many target genes that are involved in inciting early responses to *C. falcatum* infection during the incompatible and compatible interactions in sugarcane against *C. falcatum*. We identified miRNA miR5568b involved in chloroplast and mitochondrial function, HR response, enhancing JA and SA accumulation, a fungal responsive miRNA miR169b.3p regulating phenylpropanoid biosynthesis, post-transcriptional gene regulation, inner membrane transporter by HR response and defense-related miRNA, miR166b.5p involved in increasing resistance by activating ETI, PTI and ER stress in the host–pathogen interaction (Nandakumar et al. 2021b). The network of miRNAs identified in sugarcane—*C. falcatum* interaction has validated the present findings in the role of signalling molecules and regulatory genes.

Conclusion

This study has provided new insights into the molecular mechanisms of resistant and susceptible responses of sugarcane in response to *C. falcatum* through a detailed transcriptomic approach. This is the first report which indicates

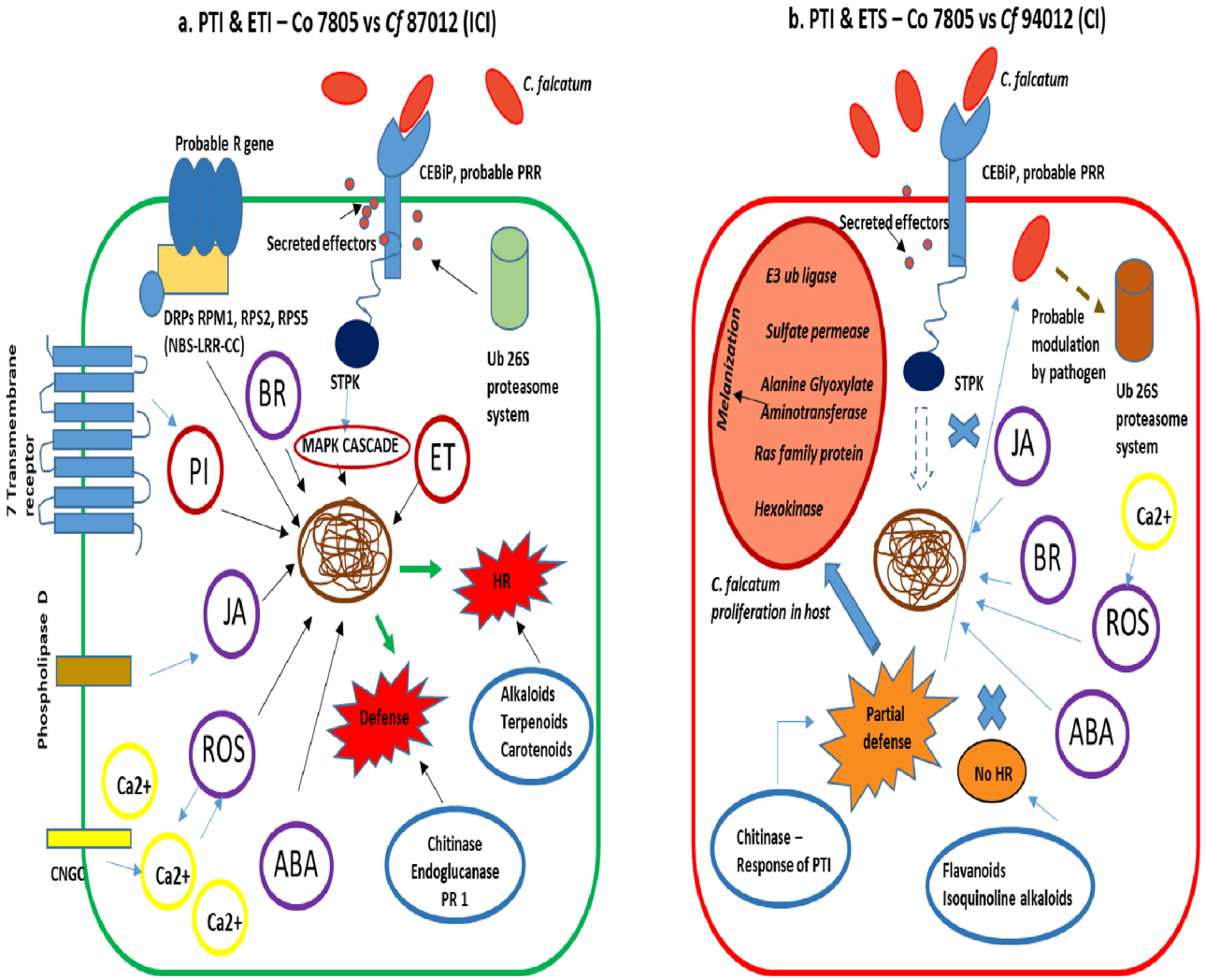


Fig. 5 Schematic representation of probable hypothetical events occurring in sugarcane cultivar Co 7805 to two different *C. falcatum* pathotypes during **a** incompatible (*Cf*87012) and **b** compatible (*Cf*94012) interactions. *MAPK* mitogen activated protein kinase, *PI* phosphoinositide & *ET* ethylene, *JA* jasmonic acid, *BR* brassinosteroid, *ABA* abscisic acid, *ROS* reactive oxygen species, *CEBiP* chitin elicitor binding protein, *LRR-NBS-CC*- leucine rich repeat- nucleotide binding site, coiled coil, *DRP* disease resistant protein, *RPM1- RPS2-RPS5- STPK*- serine threonine protein kinase, *CNGC* cyclic nucleotide gated channel, *HR* hypersensitive response, pathogenesis related protein 1. In the incompatible interaction (**a**), defense strategies are evidenced by the exhibition PTI + ETI by the host; In the compatible interaction (**b**), the pathogen proliferation is favoured by melanization, a protective strategy for the pathogen (PTI + ETS). CEBiP elicits PTI, DRP elicits ETI and Pathogenic determinants favours ETS

the association of signalling molecules such as MAPK, Ca^{2+}, JA, PI, ET, ROS, ABA and BR in an incompatible interaction, whereas in compatible interaction, the absence of MAPK, PI and ET signalling molecules indicated that the resistance mechanism is confined to MAPK, PI and ET signalling molecules. In addition, in CI, the pathogen clearly evaded host detection. The upregulation of AGT in CI is a convincing factor to determine the pathogenesis of *C. falcatum* at the transcript level. Also, the involvement of chloroplastic photosystem proteins, the ubiquitin proteasome system and differential expression of a CNGC protein in providing defense responses against the pathogen in sugarcane are novel findings in this study. Further, this study has provided evidence on the essential role of pathogenic determinants of *C. falcatum* to establish inside the host tissue. The probable adaptive mechanisms exhibited by the pathogen and its ability to modify host defense mechanisms are reported for the first time. Further, the hypothesis developed by this study goes in parallel with the zig zag model of plant immunity, based on the transcripts upregulated, the immune reaction is mediated through PTI + ETI in ICI whereas in CI, PTI + ETS operates (Fig. 5). This study has revealed a new dimension in the enigmatic sugarcane – *C. falcatum* interaction and provided cues for further research on manipulating susceptibility-associated genes through gene editing to manage the disease.

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Author contribution statement RV conceived, designed and received funds for the research work. MS conducted the experiments. PM and ARS analyzed the data. MS and CNP analyzed NGS data. MS and RV wrote the manuscript. All authors read and approved the manuscript.

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Declarations

Competing interests The authors have not disclosed any competing interests.

Ethical approval The present research did not involve human participants and/or animals.

Informed consent Informed consent was obtained from all individual participants included in the study.

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