Transcriptomic Analyses of Differential Host Responses to Red Rot Pathogen Colletotrichum Falcatum in Sugarcane Through Subtractive Library and NGS Approach

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Abstract

The fungal pathogen *Colletotrichum falcatum* causes the stalks, the economically important for sugar extraction. Although, disease management is achieved by cultivating resistant cultivars, the complex polyploidy of sugarcane genome complicates understanding the inheritance of disease resistance. Earlier attempts of using resistant and susceptible varieties to understand host-pathogen interaction resulted in cultivar specific expression of genes due to different genomic background of the varieties. To avoid host background variation in the interaction, suppression subtractive hybridization (SSH) based next generation sequencing technology was utilized in the same cv Co 7805 which behaves differently as incompatible and compatible to two different *C. falcatum* pathotypes. In the incompatible interaction (ICI) with *C. falcatum* pathotype *Cf*87012 (Less virulent, LVir) 10,038 contigs were assembled from ~54,699,263 raw reads. In the compatible interaction (CI) to the *C. falcatum* pathotype *Cf*94012 (Virulent, Vir) 4022 contigs were assembled from ~52,509,239 raw reads. The transcripts homologous to CEBiP receptor and transcripts involved in the signals ROS, Ca^{2+}, BR, JA and ABA were exhibited in both the responses. Additionally, MAPK, ET, PI signals and JA amino conjugation related transcripts were found only in ICI. Finally, the temporal gene expression of a total number of 16 transcripts was monitored in qRT-PCR. Most of the transcripts exhibited highest induction in ICI in comparison with 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 to do so in ICI. Overall, this study has identified for the first time, the differential responses of a single sugarcane host to two different *C. falcatum* pathotypes and PAMP triggered immunity (PTI) is exhibited in both the responses, but the more efficient effector triggered immunity (ETI) was found only in ICI at the molecular level.

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 results 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 pathogenicity determinants termed pathogen associated molecular patterns (PAMPs) such as chitin and glucans present in the pathogen cell wall by the cognate pattern recognition receptors (PRRs) located at the surface of plasma membrane and triggers defense responses termed 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 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 (HR-PCD) and enhanced resistance at the whole plant level 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 specificities 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 must be 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* Went (Teleomorph: *Glomerella tucumanensis* [Speg.] Arx and Muller) 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 that resulted in removal of many elite cultivars from cultivation. Frequent emergence of new variants of the pathogen *C. falcatum* contributes 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. Firstly, early and prominent induction of pathogenesis-related (PR) proteins was documented as a defense and induced defense response against *C. falcatum* (Viswanathan et al. 2003, 2005). In addition to induction of the PR proteins, 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 (Pratima 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). The 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 (Pratima et al. 2013, Sathyabhama et al. 2015, 2016). The variation in transcript accumulation between resistant and susceptible varieties we cannot rule out the changes in transcriptomes due to their genetic complexity and 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 cv Co 7805 exhibiting incompatible (ICI) and compatible (CI) interactions to *Cf*87012 and *Cf*94012, respectively was used for SSH and subtracted transcriptome sequencing and to identify the initial defense responses exhibited in sugarcane against *C. falcatum* in fool-proof manner. 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 detailed information on resistance mechanism in sugarcane to *C. falcatum* for the first time.

Materials And Methods

Plant material and pathogen culture

A tropical sugarcane cv 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* isolated from infected stalk tissue samples of sugarcane cv Co 87012 and Co 94012 named as pathotypes *Cf*87012 and *Cf*94012 respectively, maintained as part of *C. falcatum* culture collections, Plant Pathology lab, ICAR-SBI, Coimbatore were used for this study. The differential response of sugarcane cv Co 7805 to the two pathotypes *Cf*87012 and *Cf*94012 was assessed over a period of three years. The cultivar consistently showed incompatible response upon inoculation with the pathotype *Cf*87012 and compatible response to the pathotype *Cf*94012 (Fig. 1). Pathogen inoculation was performed by plug method in the third internode from the base of the cane following standard protocol for sugarcane cultivar as reported earlier (Mohanraj et al. 2012, Viswanathan et al. 2021). Separate sets of sugarcane stalks of the cv Co 7805 were inoculated in triplicates with the two *C. falcatum* pathotypes and mock inoculated samples served as controls for the experimental samples. Stalk tissue samples were collected in triplicates with their respective controls 36h post *C. falcatum* inoculation and stored in -80^{o}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^{TM} 1000 spectrophotometer (Thermo Scientific, USA). 1µg of purified RNA was used for cDNA synthesis following the manufacturer's

instructions of Smarter^{TM} PCR cDNA synthesis kit (Clontech, CA, USA). Forward and reverse subtractions for the cDNAs were done following the manufacturer's instructions of PCR-Select^{TM} cDNA subtraction kit (Clontech, CA, USA). Forward subtraction represents resistance response library (RRL), in which cv Co 7805 challenged with pathotype *Cf*87012 was used as tester, cv Co 7805 challenged with *Cf*94012 and mock sample of cv Co 7805 were used as the driver. In reverse subtraction representing susceptible response library (SRL), the cv Co 7805 challenged with *Cf*94012 was used as tester and cv Co 7805 challenged with the pathotype *Cf*87012 and mock sample of cv 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 100bp PE runs.

Transcript assembly and annotation

The raw reads were quality trimmed; adaptor sequences 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 50bp were selected for assembly. De novo assembly of the subtracted transcript assembled contings 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 gene expression through qRT-PCR

Temporal gene expression of 16 transcripts mapped to KEGG-KAAS database was studied in (Step One Plus™ Real-Time PCR Systems, Applied Biosystems Inc., Life Technologies, USA). Representative transcripts involved in recognition, signalling and defense were studied. 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 cv Co 7805 stalks inoculated with *C. falcatum* pathotypes *Cf*87012 and *Cf*94012 were collected at 12, 36 and 72h post *C. falcatum* inoculation. Total RNA was extracted by TRI reagent (Sigma-Aldrich, USA). 500 µg of total RNA was used for cDNA synthesis; first strand cDNA was diluted further to 200ng and used as templates for qRT-PCR. SYBR green PCR mastermix (Applied Biosystems Inc., Life Technologies, USA) was used for the analysis. 25SrRNA was used as the internal control. The list of primers is represented in Table 1.

Results

Transcriptome sequencing and *denovo* assembly

The number of raw reads generated by Illumina HiSeq 2000 was 54,699,263 and 52,509,239 for RRL and SRL with respective the number of filtered reads was 41,025,151 and 42,001,812. 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 RRL and SRL, respectively. Analyses of two sets of transcripts assigned the transcripts to the 7,849 and 2,899 transcripts for RRL and SRL through BLASTx. There was no homology for 2,189 and 1,123 for RRL and SRL, respectively and they are described as novel genes or hypothetical proteins. The transcripts were submitted in NCBI sequence read archive (SRA) database, with accession numbers SRR2992210 and SRR 2992249 for RRL and SRL respectively.

BLAST homology with other species and annotation

In RRL, 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 SRL, the respective homologies were 42%, 27% and 5% for *Sorghum bicolor, Zea mays* and *Oryza sativa*. In RRL, 1% homology in *Triticum urartu*, *Vitis vinifera*, *Glycine Max*, *Hordeum vulgare* and *Medicago truncatula* species were found. In SRL, no homology was found with those species. *Saccharum officinarum* and *Populus trichocarpa* had 1% homology in SRL, and no homology was found in RRL. Both RRL and SRL had 1% homology with *Saccharum* hybrid cultivar (Suppl. Fig. 1a, 1b). In both RRL and SRL, many of the transcripts from BLAST

annotation were found to be hypothetical proteins or novel genes. So, 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 signal transduction, GPI anchor biosynthetic process, IMP biosynthetic process and so on were expressed differentially in RRL. In SRL, 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, photosystem I & II were present in RRL. In SRL, 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 RRL. In SRL, 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 RRL and 4022 for SRL 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 RRL and 11% of the transcripts were found to be upregulated in SRL. 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 BLAST homology search

From the BLAST homology search, a total number of 17 transcripts homologous to *Glomerella graminicola* were present only in SRL. 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 E3 Ub liagase 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 of the following transcripts CEBiP, MAPKKK1, MAPKK1, DRPRPM1, DRPRPS5, CBPCML, JAAS and ABAREBF showed a gradual increase in their expression at 12h and 36h and a decline at 72h post *C. falcatum* inoculation in RR. Whereas, in SR, CEBiP, MAPKKK1 and CBPCML exhibited an inconsistency in their expression at all the time intervals. MAPKK1 and JAAS showed gradual decrease from 12h to 72h. DRPRPM1 showed a similar response as RR but the transcript level was less than 2-fold. ABAREBF showed gradual raise from 12 to 72h and reached 2.5-fold at 72h in SR which is higher than the RR. The defense gene chitinase showed a gradual raise in both RR and SR from 12 to 72h. However, the transcript accumulation was found to be higher in RR and reached a maximum of 4-fold at 72h post *C. falcatum* inoculation. The transcripts CNGC and CDPK showed a gradual increase in RR and a gradual decrease in SR from 12 to 72h post *C. falcatum* inoculation. The transcripts 14-3-3 PE and SOD Cu Zn showed an increase in expression from 12 to 36h and a decline at 72h in the SR. In RR, there was an unstable expression. 5-fold expression was noticed in RRL for SOD Cu-Zn at 12h post *C. falcatum* inoculation. For, VTP ATPase, the expression in RR was unstable and SR showed a constant 6-fold expression at 36 and 72h post *C. falcatum* inoculation. BRSK and ER showed an inconsistent expression in both the responses. At 12h, BRSK showed more than 5-fold expression in RR. At 36h ER showed more than 10-fold expression in SR. 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*. Evidences 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). In recent years, through massively parallel next generation sequencing (NGS), a large number of transcripts at the interface of plant – pathogen interaction has been sequenced and identified many candidate genes responsible for resistance or susceptibility. In this study, forward and reverse subtracted transcriptomes captured during sugarcane – *C. falcatum* interaction were sequenced through an NGS-Illumina Hi-Seq 2000 sequencing

platform. The pathotype *Cf*87012 (LVir) exhibited incompatible interaction (ICI) and the pathotype *Cf*94012 (Vir) exhibited compatible interaction (CI) when inoculated on the sugarcane cv Co 7805 which exhibited a differential host interactions. A total of 10,038 and 4,022 transcripts were derived for ICI and CI respectively. In that, only 7,849 and 2,899 transcripts had BLAST homology for ICI and CI, respectively. The transcripts were mapped in KEGG-KAAS for functional categorization and biochemical 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.

In this study, a transcript homologous to chitin elicitor binding protein (CEBiP), and a S/T protein kinase were induced 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 chitin elicitor binding protein (CEBiP), a PRR (Kaku et al. 2006). PRRs recognize both pathogen-derived nonself PAMPs/MAMPs and plant-derived DAMPs, which triggers PTI. But 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 resistance than the CI (Fig. 4). The pathogen responsive MAPK activation is likely to promote the generation of ROS in chloroplasts and JA signalling, which plays an important role in execution of 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). In this study, from BLAST homology search, it is evident that, unlike the pathotype *Cf*87012, *Cf*94012 penetrated the host surface. 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* must be indicating successful pathogenesis and the pathogen's proliferating stage inside the susceptible host. Also, recent studies by Bhadauria et al (2012 a, 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 very much needed for melanisation of appressorium utilizing the glycerol during pathogenesis.

In the present study, only in ICI, the transcripts homologous to disease resistance proteins RPM1, RPS2 and RPS5 were upregulated. qRT-PCR experiments carried out in ICI and CI, at three-time intervals post pathogen inoculation revealed the transcriptional gene expression of RPM1 and RPS5 in both the responses (ICI and CI). But the magnitude of expression was higher in ICI (Fig. 4). Also, RPS5 was found to be more than 40-fold in ICI, but a constant expression of 5-fold 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 cv 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 RRL and SRL pertaining to Ca^{2+} signals, the CDPK, CNGC and calcium binding protein CML (CBP CML) were quantified in qRT-PCR in cv 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*.

In this study, several transcripts involved in provoking defense responses like clathrin heavy chain, 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 this study, only few transcripts pertaining to secretory pathway were commonly expressed in both the interactions ICI and CI. But 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 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 programmed cell death (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 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 me has validated the present findings in the role of signalling molecules and regulatory genes.

Conclusion

This study has provided new insights in to 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 the association of signals likes 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 signals indicated that the resistance mechanism is confined to MAPK, PI and ET signals. Also, in CI the pathogen has developed its dynamic nature to evade 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 the differential expression of a CNGC protein in providing defense responses to this pathogen in sugarcane system are novel finding in this study. Further, this study has provided evidence on the essential role of pathogenic determinants of *C. falcatum* establishment 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, where in ICI based on the transcripts upregulated, the immune reaction is PTI-ETS+ETI exists. In CI, PTI-ETS+ETI exist (Fig. 5). This study has framed a new dimension to look into the enigmatic sugarcane – *C. falcatum* interaction and has proved its occurrence in a logical way.

Declarations

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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.

Compliance with Ethical Standards

The authors declare that they have no conflict of interest.

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

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

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Tables

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

Table 2: Representative GO of RR and SR through BLAST homology search

		RRL		SRL
	GO biological process			
1	transcript_266	aromatic amino acid family biosynthetic process;	transcript_177	sucrose biosynthetic 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 system (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 trisphosphate 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_266	shikimate kinase activity; 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 acti	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;		
28	transcript_4062	N-acetyltransferase activity;		
29	transcript_4097	aspartic-type endopeptidase activity;		
30	transcript_6112	serine-type endopeptidase activity;		
31	transcript_6441	mannose-1-phosphate guanylyltransferase (GDP) activity;		
32	transcript_6927	inositol-1,3,4-trisphosphate 5/6-kinase activity; inositol tetrakisphosphate 1-kinase activity; ATP binding; magnesium ion binding;		
33	transcript_1987	NADH dehydrogenase (ubiquinone) activity;		

Table 3. Representative differential transcripts from RRL and SRL and their putative function as mapped in KEGG-KASS database

S.No	Transcripts	Function	RR	SR
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
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	√

RR- Resistance response, SR – Susceptible response, √ - 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	25s 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

Figures

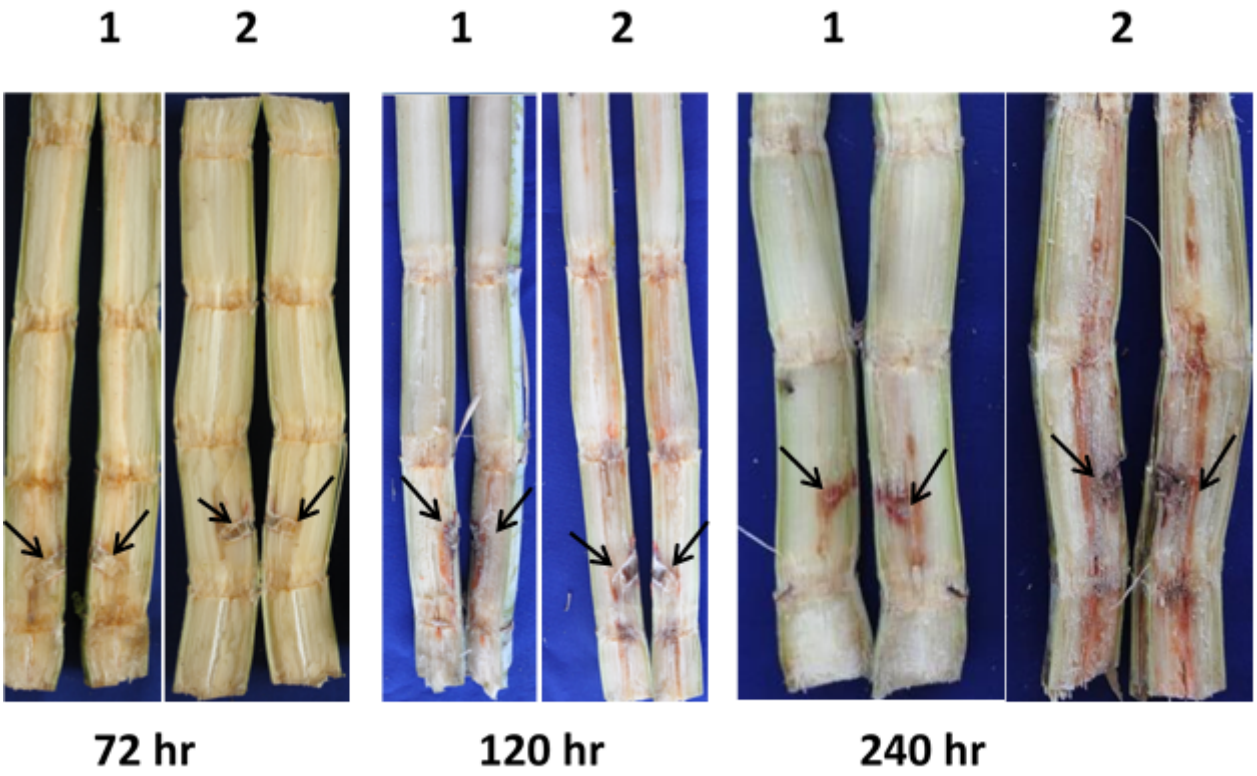

Figure 1

Phenotypic symptoms of sugarcane cv Co 7805 exhibiting differential response to inoculation with two different C. falcatum pathotypes Cf87012 (1) and Cf94012 (2); arrows indicate point of pathogen inoculation.

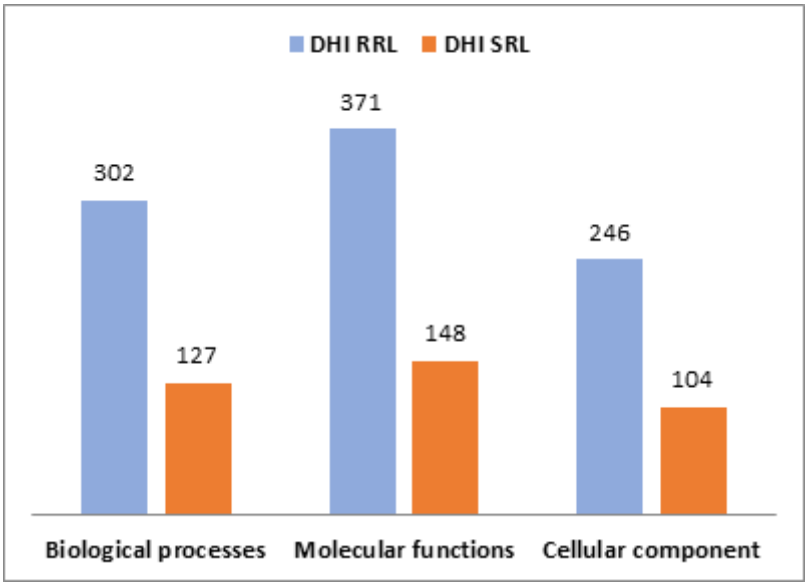

Figure 2

Gene ontology distribution in resistant (RRL) and susceptible (SRL) responses in SSH libraries of sugarcane challenged with virulent and less virulent pathotypes of C. falcatum.

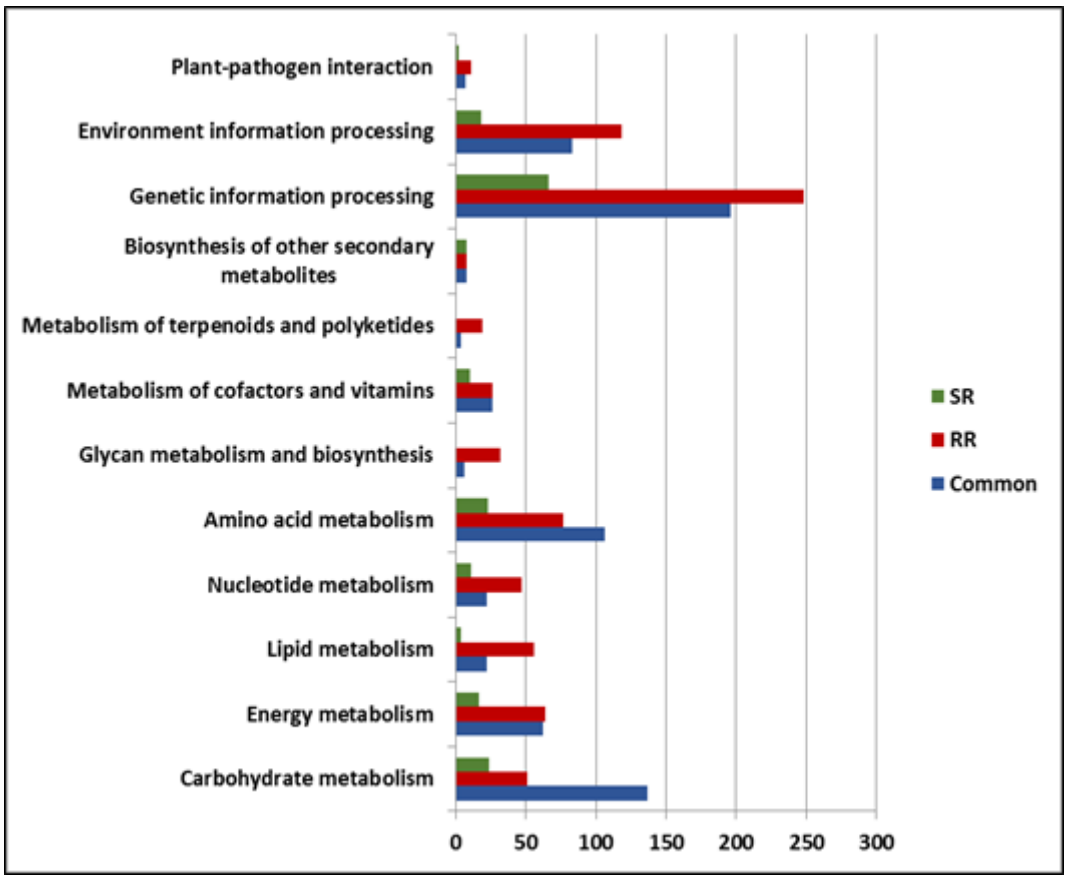

Figure 3

Functional categorization of transcripts involved in resistant (RR) and susceptible (SR) responses in SSH libraries of sugarcane challenged with virulent and less virulent pathotypes of C. falcatum.

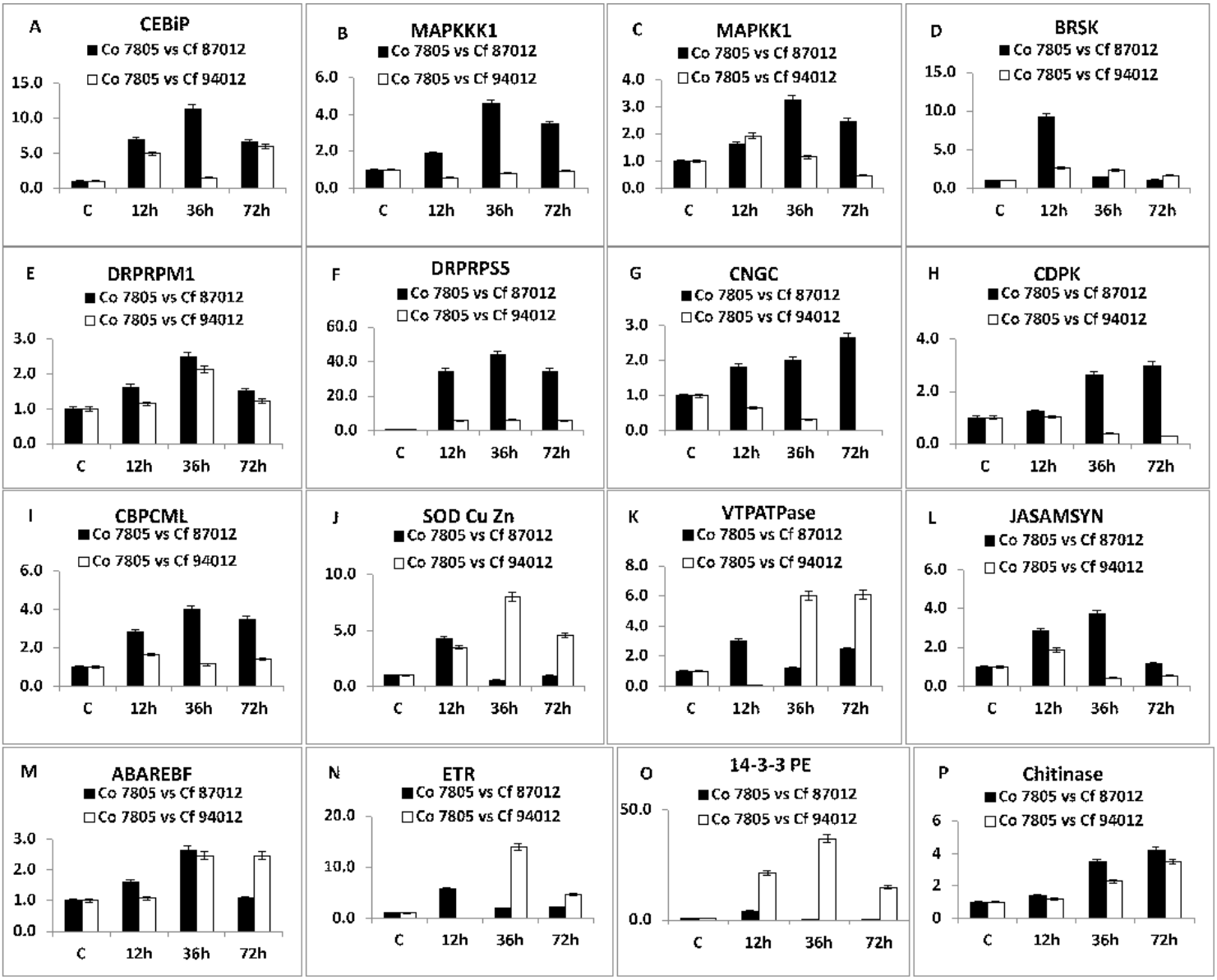

Figure 4

Gene expression profiling of transcripts in incompatible (Co 7805 vs Cf 87012) and compatible interactions (Co 7805 vs Cf 94012). C – Mock control, h – hours post C. falcatum inoculation, X- axis – hours post C. falcatum inoculation; Y – axis – RQ values

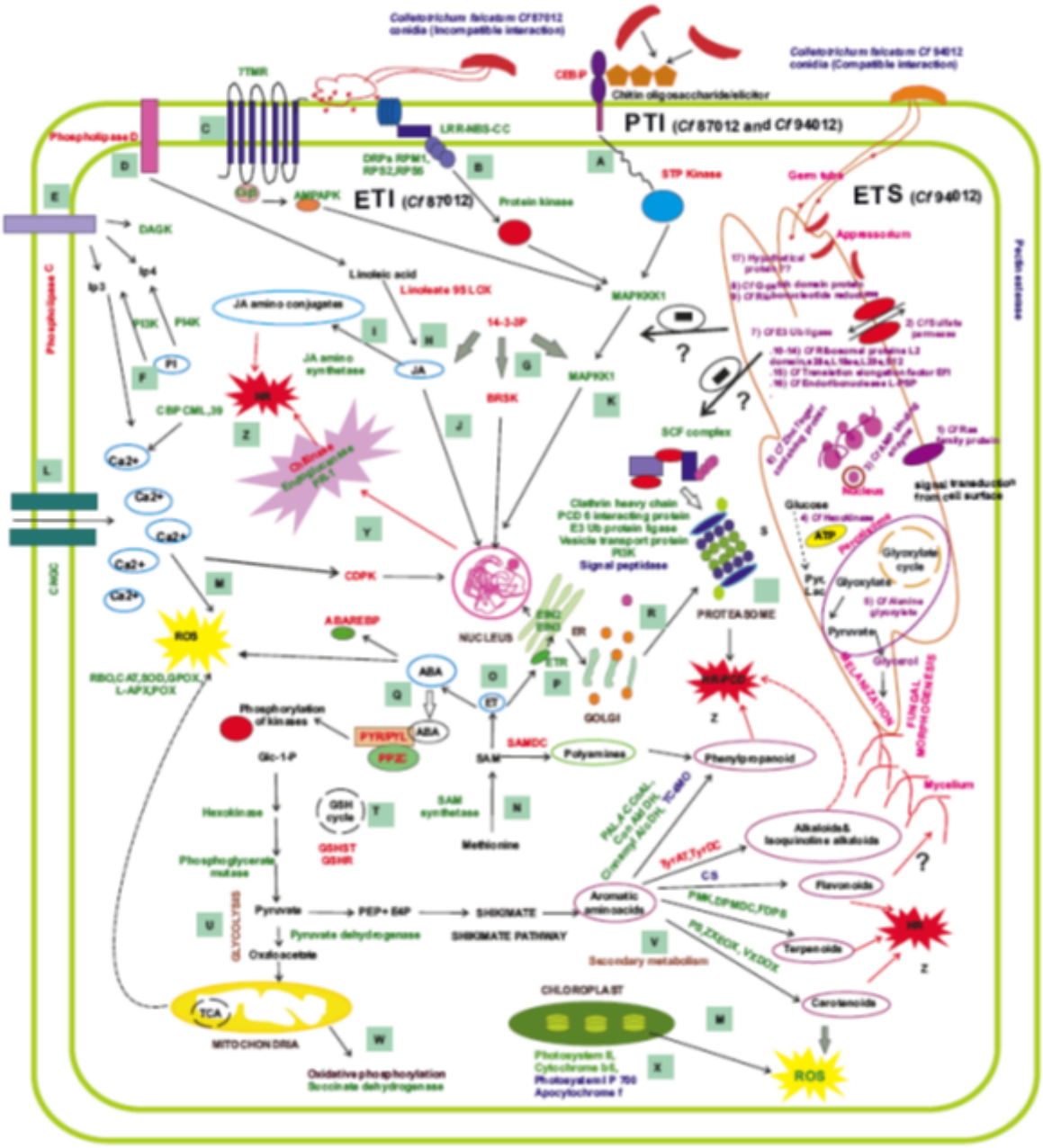

Figure 5

Schematic representation of probable hypothetical events occurring in sugarcane variety Co 7805 to two different C. falcatum pathotypes during incompatible (Cf87012) and compatible (Cf94012) interactions. Transcripts in green represents those involved in incompatible interaction. Red- transcripts involved in both compatible and incompatible interactions. Blue- transcripts involved compatible interaction. Lavender-represents transcripts involved in the reproductive stage of C. falcatum pathotype Cf94012 inside the host cells. 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, MAPKKK1 – Mitogen activated protein kinase kinase kinase 1, MAPKK1 – Mitogen activated protein kinase kinase 1, BRSK – Brassinosteroid signalling kinase, 14-3-3P – 14-3-3 Protein, JA-

Jasmonic acid, LOX – Lipoxygenase, DAG – Diacylglyceol, PI, PI3K, PI4K, IP3, IP4,CBP- Calcium binding protein, Ca2+- Calcium, CDPK – Calcium dependent protein kinase, CNGC – Cyclic nucleotide gated channel, ROS- Reactive oxygen species, RBO – Respiratory burst oxidase, CAT – Catalase, SOD – Superoxide dismutase, GPOX- Glutathione peroxidase, L-APX – L-Ascorbate peroxidase, POX – Peroxidase, ABA – Absicisic acid, ABAREBP – ABA response element binding protein, PYR/PYL – ABA receptor, PP2C -, SAM – S-adenosyl methionine, SAMDC – SAM decarboxylase, ET- Ethylene, ETR – ET receptor, EIN2 – Ethylene insensitive 2, EIN3 - Ethylene insensitive 3, Glc-1-P – Glucose 1 phosphate, PDH – Pyruvate dehydrogenase, TCA – Tricarboxylic acid cycle, SDH – Succinate dehydrogenase, PEP – phosphoenol pyruvate, E4P – Erythrose 4 phosphate, PAL – Phenylalanine ammonia lyase, 4-CCoAL-, CoADH – Coniferyl aldehyde dehydrogenase, CiADH – Cinnamyl alcohol dehydrogenase, TyrAT-, TyrDC-, PMK-, DPMDC-, FDPS-, PS-, ZXEOX-, VXDOX-, PCD – Programmed cell death-, Ub- Ubiquitin, SCF – Skp1-Cullin- F-box, HR – Hypersensitive response, TC4MO-, CS – Chalcone synthase,

Supplementary Files

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