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ORIGINAL ARTICLE

Identification of candidate pathogenicity determinants of *Rhizoctonia solani* AG1-IA, which causes sheath blight disease in rice

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Abstract

Sheath blight disease is one of the predominant diseases of rice and it is caused by the necrotrophic fungal pathogen *Rhizoctonia solani*. The mechanistic insight about its widespread success as a broad host range pathogen is limited. In this study, we endeavor to identify pathogenicity determinants of *R. solani* during infection process in rice. Through RNAseq analysis, we identified a total of 65 and 232 *R. solani* (strain BRS1) genes to be commonly upregulated in three different rice genotypes (PB1, Tetep, and TP309) at establishment and necrotrophic phase, respectively. The induction of genes encoding extracellular protease, ABC transporter, and transcription factors were notable during establishment phase. While during necrotrophic phase, several CAZymes, sugar transporters, cellular metabolism, and protein degradation-related genes were prominently induced. We have also identified few putative secreted effector encoding genes that were upregulated during pathogenesis. The qPCR analysis further validated the phase-specific expression dynamics of some selected putative effectors and pathogenicity-associated genes. Overall, the present study reports identification of key genes and processes that might be crucial for *R. solani* pathogenesis. The ability to effectively damage host cell wall and survive in hostile plant environment by managing oxidative stress, cytotoxic compounds, etc. is being proposed to be important for pathogenesis of *R. solani* in rice. The functional characterization of these genes would provide key insights about this important pathosystem and facilitate development of strategies to control this devastating disease.

Keywords RNAseq · Susceptibility · Necrotrophy · CAZymes · Sugar transporters, Effectors

Introduction

The sheath blight disease is one of the most economically important fungal diseases of rice. It is caused by the basidiomycete necrotrophic pathogen *Rhizoctonia solani* Kuhn (telemorph; *Thanatephorus cucumeris*) AG1-IA. In general, *R. solani* strains demonstrate broad host range and cause disease on diverse plants including cereals, potato, bean, cotton, sugar beet, lettuce, melon, forest trees, and ornamental plants, etc (Anderson 1982; Sneh et al. 1991). They also demonstrate considerable variability in terms of their morphological and pathological attributes (Wang et al. 2013). *R. solani* has been classified into 14 different anastomosis groups, i.e., AG-1 to AG-13 and AG-BI (Kuninaga et al. 1997; Guillemaut et al. 2003; Ahvenniemi et al. 2009). Beside hyphal fusion characteristics and sequence polymorphisms in ITS region, karyotype banding patterns have also been used to classify strains into different anastomosis groups (Keijer et al. 1996). The strains belonging to AG1-IA anastomosis group cause disease in rice as well as other agriculturally important crops like corn, barley, sorghum, potato, millet, soybean, and peanut etc (Sneh and Ichielevich-Auster 1998; Fenille et al. 2002). Moreover, different AG1-IA strains demonstrate hypervariable pathological attributes such as number of disease lesions, size of the lesion, RVSC (relative vertical sheath

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colonization), disease score, relative lesion length, etc, on a particular host (Taheri et al. 2007; Das et al. 2013).

The sclerotia formed by *R. solani* serve as a major source of primary inoculum. They can survive for long durations in the soil and infect rice plants during its growing season. Under favorable conditions of high temperature (~28 °C) and humidity (~95%), infection is known to spread rapidly on rice tillers (Lee and Rush 1983). It is worth noting that inspite of extensive global efforts, no source of complete disease resistance has been identified yet. However, some major and minor QTLs providing quantitative resistance against *R. solani* are known (Prasad and Eizenga 2008; Channamallikarjuna et al. 2009; Wang et al. 2011).

With advent of next-generation sequencing platform, it has become easier to perform functional genomic studies involving phytopathogens (Jeon et al. 2007; Rhind et al. 2011; Laabei and Massey 2016; Lee et al. 2016). Also an arduous task of research on filamentous fungi like *R. solani* has gained an impetus in recent years. The draft genome sequences of AG1-IA (36.94 Mb encoding 10,489 ORFs) along with some other *R. solani* strains of different anastomosis groups (AG1-IB, AG2-2IIIB, AG3, and AG8) are now publicly available (Wibberg et al. 2013, 2016; Zheng et al. 2013; Cubeta et al. 2014; Hane et al. 2014). These studies have generated a plethora of genomic information which can be a propitious resource to understand the pathogenicity mechanism of *R. solani*. However due to multinucleate nature of the pathogen (Sinclair 1970), it is difficult to functionally characterize the *R. solani* genes, making it a challenging pathosystem to study.

In our previous study, we observed PB1 and TP309 rice cultivars to be susceptible whereas Tetep to be partially resistant against *R. solani* infections (Ghosh et al. 2014). During the early stage of its pathogenesis (1 dpi; days post inoculation), mycelia growing parallel to rice veins without any anatomical alteration or physiological alteration of host tissues were observed (Ghosh et al. 2017). However, during 3 dpi of pathogenesis, we observed severe anatomical alterations, disintegration of chloroplast integrity along with cell death of infected tissues. Thus, we have considered 1 dpi of *R. solani* pathogenesis as its establishment phase while 3 dpi as its necrotrophic phase. Recently, we have summarized the molecular and physiological alterations in the host, during pathogenesis of *R. solani* (Ghosh et al. 2017). In this study, we are focusing on characterizing the transcriptional dynamics of *R. solani* genes that were commonly expressed during its establishment and necrotrophic phases of pathogenesis on different rice genotypes (PB1, TP309, and Tetep). Several candidate pathogenicity determinants including putative effectors were predicted and their roles during *R. solani* pathogenesis are being postulated in this study.

Materials and methods

Biological materials and pathological assays

Oryza sativa ssp. *indica* (cv. PB1), *O. sativa* ssp. *japonica* (cv. TP309), *O. sativa* ssp. *indica* (cv. Tetep), and *R. solani* AG1-IA strain BRS1 were used in this study. BRS1 sclerotia were cultured on PDA (39 g/L; Potato Dextrose Agar; Himedia, Mumbai, India) plates at 28 °C. The freshly prepared equal-sized sclerotia were used for inoculation. Single sclerotium was used to infect each rice tillers by inserting them into the second sheath. Rice was grown in a PGV36 conviron plant growth chamber at 28 °C temperature, 80% relative humidity, and 12/12 h of day/night cycle. 60-day-old rice sheaths were infected with *R. solani* sclerotia, following the procedures described in (Ghosh et al. 2014). After 1, 2, and 3 day post-inoculation, the infected tissues (including 1 cm up and down from the site of infection) were harvested for transcriptome and expression analysis.

RNAseq library preparation and sequencing

RNAseq were performed for three different *R. solani* infected rice genotypes (PB1, TP309, and Tetep). RNA from *R. solani* infected rice sheath (five infected sheath pooled up for each sample) at 1 and 3 day post-infections (dpi) were isolated using RNeasy Plant Mini Kit (Qiagen). In addition, RNA from sclerotia grown in PDB broth (laboratory media) for 1 and 3 days at 28 °C were also harvested. The transcriptome sequencing was performed using paired end (PE) 2 × 100 bp library on Illumina HiSeq 2000 for each of these samples. The library was prepared using TrueSeq stranded mRNA HT sample preparation kit. TrueSeq PE cluster kit v3-cBot-HS was used for cluster generation, while the TrueSeq SBS kit v3-HS (200 cycles) was used for RNA sequencing. All these procedures were performed using standard protocols recommended by the manufacturer (Illumina Inc.).

Transcriptome assembly, annotation, and differential expression analysis

Trimmomatic version-0.32 was used for pre-processing of reads to remove TruSeq3 pair-end adapter sequences (Bolger et al. 2014). The reads obtained from 1 and 3 dpi laboratory grown *R. solani* strain BRS1 samples were used as bonafide fungal reads. These reads were assembled using Trinity version r20140717 (Haas et al. 2013) to obtain fungal transcripts. These transcripts were utilized to filter out the fungal transcripts from *R. solani* infected rice samples (Online Resource 1). Transdecoder version r20140704 (Haas

et al. 2013) was used to detect coding regions. Annotation was performed using blastx, blastp, hmmscan, and rnammer tools. An e-value cutoff of 1e−6 was applied for all blast searches. RSEM v1.2.19 was used for differential gene expression analysis (Zhao et al. 2011). This program aligns input reads against a reference transcriptome using Bowtie2 and calculates expression values using the alignments. EBSeq program was used to perform differential expression analysis. All genes/transcripts and their associated statistics are reported as differentially expressed only if the FDR is less than 0.05.

Selection of phase-specific in-planta upregulated genes

The log fold change of genes expressed during in-planta infection at 1 and 3 dpi was calculated with respect to their expression in laboratory media and transcripts having fold change ≥ 2 were selected. To determine the in-planta upregulated phase-specific genes, only transcripts showing upregulation at a particular phase in each of the three rice genotypes (PB1, TP309, and Tetep) were selected. This led us to select core genes that were expressed uniquely during establishment, necrotrophic phase, and those that were commonly expressed at both these phases. A flowchart representing steps involved in selection of pathogenesis genes of *R. solani* is depicted in Online Resource 1. Morpheus software was used to plot the heat map of differentially expressed genes of *R. solani* during pathogenesis in three rice genotypes (Online Resource 2).

Functional annotation and metabolic pathway analysis

Physiological relevance and functional categorization of differentially expressed genes were carried out by consolidating genes that share a certain function or participate in a common process. The Gene Ontology terms (GO) were assigned to these genes using the Blast2GO software. GO terms showing high abundance (> 4%) were classified as predominant categories during respective phases of infection. The CAZymes encoding genes were predicted using the CAT software (CAZy Analysis Toolkit). The sugar transporters were predicted from the KEGG and KOG database. Furthermore, *R. solani* transcripts were searched in PHI-base (Pathogen Host Interactions Database) database with *E* value $< 10^{-5}$ to identify putative pathogenicity determinants. The genes obtained were categorized according to their orthologs mutant phenotype (reduced virulence, loss of pathogenicity, lethal, and effector). The term reduced virulence refers to the mutant that shows lesser disease symptoms in comparison to the wild-type strain, loss of pathogenicity refers to the mutant unable to cause disease, and lethal refers to the mutant that is unable to survive. Effector prediction of *R. solani* was performed using SignalP, Phobius, and EffectorP software, and the common genes obtained from these softwares were designated as putative secreted effector genes of *R. solani*. The sequence similarities (obtained through BLASTX) in terms of percent identify along with respective *E* value for each of the PHI-base orthologs and effectors encoding genes across different sequenced strains of *R. solani* were recorded.

qPCR-based verification

To validate the expression pattern of selected pathogenicity-related genes of *R. solani*, qPCR-based expression analysis was carried out at three different time points (1, 2 and 3 dpi) during pathogenesis on susceptible rice cultivar (PB1). qPCR primers were designed in such a manner that they selectively amplify fungal genes but not any rice sequences (Online Resource 3). 2 µg of RNA was used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per specified protocol. 18s rDNA sequence of *R. solani* was used as a reference gene to normalize gene expression and relative expression was calculated using $2^{-\Delta Ct}$ method, wherein ΔCt is the difference between Ct values of target and reference gene (Kiirika et al. 2012). However, to correlate with the RNAseq data, we also estimated fold change of expression of these genes at 1 and 3 dpi by the ΔΔCt method (Livak and Schmittgen 2001).

For some of the pathogenicity-related genes that showed higher fold induction during susceptible interactions (PB1) than in partially resistant interactions (Tetep) in RNAseq analysis, the qPCR analysis was carried out. The fold change in gene expression was calculated at 2 and 3 dpi of pathogenesis with respect to 1 dpi using ΔΔCt method. Each experiment was repeated in at least three biological replicates with three technical replicates.

Results

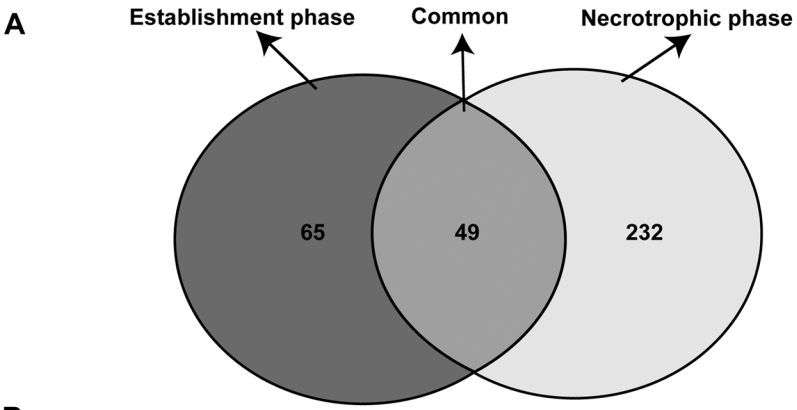
Transcriptome analysis during *R. solani*–rice interactions

Previously, we have reported that *R. solani* strain BRS1 is able to colonize and cause disease on different rice genotypes. Based upon disease severity index, the rice genotypes PB1 and TP309 were susceptible, while Tetep was partially resistant (Ghosh et al. 2014, 2017). During 1 dpi of pathogenesis, the pathogen remains in establishment phase, while at 3 dpi, the pathogen is in its necrotrophic phase. To obtain a robust overview of transcriptional dynamics of *R. solani* during host infection, the RNAseq analysis was performed at 1 dpi (establishment phase) and 3 dpi (necrotrophic phase)

of *R. solani* pathogenesis on three different rice genotypes (PB1, TP309, and Tetep). Furthermore, the transcripts expressed during 1 and 3 days of *R. solani* growth in PDB broth (laboratory media) were identified through RNAseq. The differential expression of transcripts was estimated, by comparing their expression in laboratory media and during pathogenesis on each of the rice genotypes at 1 and 3 dpi (Online Resource 1). In this process, we identified a total of 1196 *R. solani* genes to be differentially regulated during pathogenesis and Online Resource 2 summarizes their expression dynamics (in terms of FPKM values) as heat map. The hierarchical clustering of expression data revealed three distinct clusters of genes being induced during growth in laboratory media, establishment phase, and necrotrophic phase (Online Resource 2). We selected transcripts that showed upregulation in each of the rice genotypes (PB1, TP309, and Tetep), and in this process, we identified 65 and 232 transcripts that were induced exclusively during establishment and necrotrophic phases of *R. solani* pathogenesis, respectively (Fig. 1a; Online Resource 4). Interestingly, 49 transcripts were found upregulated during both establishment and necrotrophic phases (Fig. 1a). It is important to note that although these genes are found expressed in laboratory media, however, they get significantly induced during pathogenesis in rice.

Functional annotation of establishment and necrotrophic phase-associated genes of *R. solani*

The gene ontology annotation of the phase associated genes revealed induction of diverse functions during pathogenesis of *R. solani* (Online Resource 5). The enrichment of GO molecular functions, i.e., catalytic activity and binding as predominant molecular functions were observed during both establishment phase and necrotrophic phase (Fig. 2). The protein kinase activity was prominent catalytic activity during establishment phase, while the hydrolase activity involved in breakdown of glycosyl bonds was abundant during necrotrophic phase. Amongst binding GO term, heterocyclic compound along with organic cyclic compound binding was found induced during both establishment and necrotrophic phases. However, protein binding term was uniquely associated with establishment phase, while carbohydrate binding and pattern (polysaccharide/cellulose) binding GO terms were abundant during necrotrophic phase. In addition, transcripts associated with nutrient reservoir and transmembrane transporter activities were overrepresented during establishment phase.



B

Gene Function	Establishment Phase	Common in Establishment and Necrotrophic Phase	Necrotrophic Phase
CAZymes	9	6	116
Sugar Transporters	3	1	14
Secreted Effectors	4	2	6
PHI-base Category	9	10	44
• Reduced Virulence	7	10	33
• Loss of pathogenicity	2	0	2
• Lethal	0	0	5
• Effectors	0	0	4

Fig. 1 Representation of phase-specific genes of *R. solani* upregulated during pathogenesis. **a** Venn diagram showing the number of in-planta-induced phase-specific genes. **b** Representation of number of upregulated transcripts encoding CAZymes, sugar transporters, effectors, and PHI-base homologs (categories are defined based upon available mutant phenotypes in other pathosystems) during different phases of *R. solani* pathogenesis

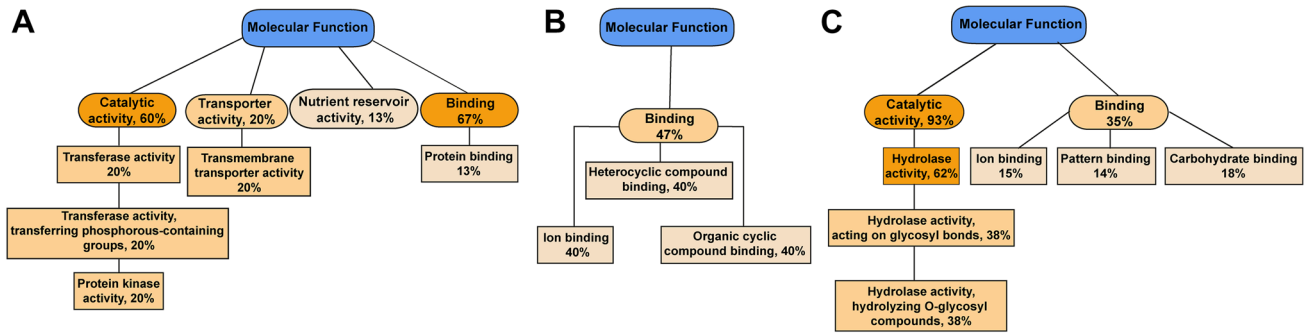


Fig. 2 Gene Ontology classification of *R. solani* transcripts being expressed during different phases of pathogenesis. Box represents percentage of GO molecular function terms associated with upregulated genes during establishment phase (**a**), in both establishment as well as necrotrophic phase (**b**), and during necrotrophic phase (**c**) of *R. solani* pathogenesis

Expression dynamics of putative pathogenesis-associated genes of *R. solani*

Necrotrophic fungi like *R. solani* secrete an array of cell wall degrading enzymes (CAZymes) to break down complex plant macromolecules like cellulose and pectin into simple sugars to facilitate host colonization (Talbot 2010; de Wit et al. 2012; Bennati-Granier et al. 2015). Successful pathogenesis involves availability and transportation of nutrients, such as sugars from infected host tissues to the pathogen (Solomon et al. 2003; Cornell et al. 2007; Talbot 2010). Fungal pathogens are known to utilize secreted effector proteins to promote pathogenesis by either suppressing host defense response or by promoting cell wall damage (De Jonge et al. 2011; Wang et al. 2014). In addition, PHI-base provides information about experimentally curated genes that are associated with reduced virulence, unaffected pathogenicity, loss of pathogenicity, lethal and effectors of different fungi, bacteria, and oomycetes pathogens (Winnenburg et al. 2006). Considering their importance, in this study, we focused on understanding the expression dynamics of in-planta upregulated *R. solani* genes that encode CAZymes, sugar transporters, effectors, and PHI-base orthologs (Fig. 1b).

Cell wall degrading enzymes

We observed 9 CAZymes to be induced during establishment phase, while 116 CAZymes upregulated during necrotrophic phase. The GT32 (glycosyl transferase) and CBM57 (carbohydrate binding-module) family were exclusively induced during establishment phase, while GH18 (glycosyl hydrolase) encoding chitinase, CE10 (carbohydrate esterase) encoding monooxygenase, and CBM12 encoding aminopeptidase were induced during both necrotrophic and establishment phases. The transcripts belonging to AA3|AA8 (auxillary activity family), CE5, GH1, GH10, GH105, GH43, GH5|CBM1, GH51, GH76, GH92, GT1, GT34, PL1 (polysaccharide lyase), and PL4 CAZyme family were found to be particularly induced during necrotrophic phase (Fig. 3; Online Resource 6). These transcripts were encoding important cell wall degrading enzymes like cellobiose dehydrogenase, β-xylanase, β-glucanase, β-glucosidase, arabinosidase, arabinofuranosidase, cutinase, and glucosyl transferase, etc, highlighting their importance during necrotrophy. Upregulation of a chitin deacetylase encoding gene (CL8196Contig1) which converts chitin (a primary constituent of fungal cell wall) into chitosan was also observed during necrotrophic phase. Chitosan is believed to be more elastic and resistant against action of host enzymes (such as chitinases) (Zhao et al. 2010). Thus, upregulation of chitin deacetylase might help the pathogen to protect itself from action of host cell wall degrading enzymes.

Interestingly, we observed ~ 24% the CAZymes induced during necrotrophic phase to show higher induction (≥ twofold) in susceptible cultivars (PB1 and TP309) than in Tetep, partially resistant cultivar (Online Resource 7). The glycoside hydrolase family, chitin deacetylase, cellobiohydrolyase, cellobiose dehydrogenase, aldose epimerase, etc. were prominent amongst them.

Sugar transporters

A total of 18 major facilitator superfamily proteins, comprising of membrane transporters putatively involved in transport of small solutes across the membrane (Cui et al. 2015; Quistgaard et al. 2016), were found induced during pathogenesis in rice (Fig. 1b; Online Resource 8). We detected 3 such transcripts being particularly induced during establishment phase while 14 of them to be induced during necrotrophic phase. Notably, the carboxylic acid transporters (CL7257Contig1 and CL8578Contig1) were upregulated at establishment phase, while genes encoding lactose permease (CL3435Contig1, CL12132Contig1

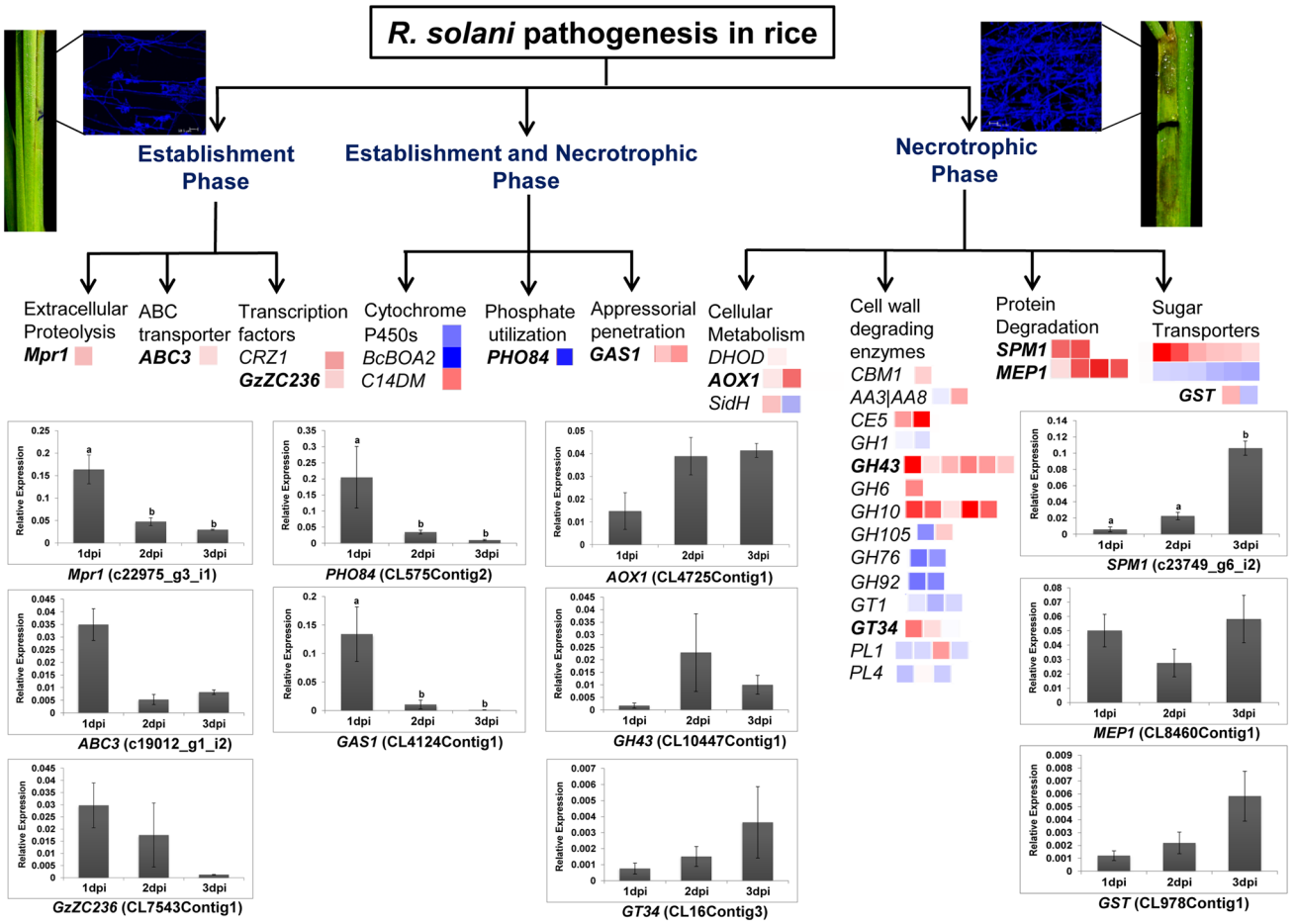


Fig. 3 Schematic representation of genes associated with different phases of *R. solani* pathogenesis. The differentially expressed *R. solani* genes during pathogenesis on susceptible rice genotype (PB1) were classified under three categories, namely establishment phase, necrotrophic phase, and common in both these phases. The prominent functions along with name of particular upregulated genes under each category are summarized. The colored boxes represent number of upregulated paralogous genes in RNAseq analysis and color scale (red to blue in descending manner) represents extent of upregulation. The relative expression with respect to reference gene (18s rRNA) was estimated for few selected genes (highlighted in bold) through qPCR and data is represented as bar graphs. Standard error bars are represented as vertical lines and different letters represent significant difference at p value < 0.05 (estimated by one-way ANOVA). *GST*; refers to general substrate transporter

and CL12687Contig1), sugar transporter (c20529_g1_i1), hexose transporter (CL5845Contig1), and drug resistance transporter (CL7221Contig1) were induced exclusively during necrotrophic phase (Fig. 3). In addition, we observed an MFS general substrate transporter (CL3034Contig1) being upregulated at both establishment and necrotrophic phases of *R. solani* infection.

Effectors

The putative secreted effector proteins of *R. solani* induced during pathogenesis in rice were predicted by different computational tools (SignalP, Phobius, and EffectorP). The common ($n = 12$) amongst them were selected and most of them were uncharacterized (Online Resource 9). We also investigated the conservation of these genes across different sequenced strains of *R. solani*. In general, most of them were having low sequence similarity; however, a few of the necrotrophic phase-specific effectors encoding putative cell wall degrading enzymes were relatively more conserved (Online Resource 9). Furthermore, the expression profiles of five selected effector encoding genes of *R. solani* were studied through qPCR and their expression patterns were found to be comparable to that obtained through RNAseq analysis (Fig. 4).

PHI-base homologs

We identified 63 PHI-base orthologs to be upregulated during *R. solani* infection process (Online Resource 10).

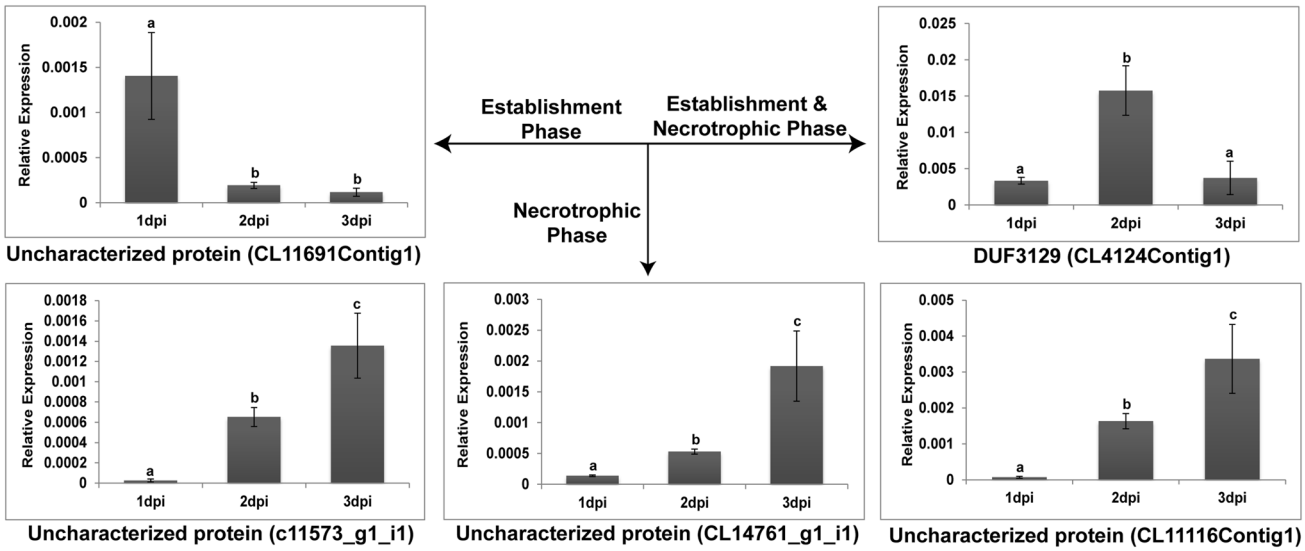


Fig. 4 Expression dynamics of putative secreted effectors of *R. solani* during different phases of pathogenesis in PB1 rice. The relative expression of selected secreted effector encoding genes with respect to reference gene (18s rRNA) was quantified through qPCR and are summarized as bar graphs. Standard error bars are represented as vertical lines and different letters represent significant difference at p value < 0.05 (estimated by one-way ANOVA)

Several of them showed homology to virulence determinants of important fungal pathogens such as *Fusarium graminearum, Magnaporthe oryzae, Aspergillus fumigatus, Candida albicans*, and *Cryptococcus neoformans*. Nine of PHI-base orthologs were found induced during establishment phase of *R. solani* pathogenesis. Amongst them, seven were associated with reduced virulence, while two were related to loss of pathogenicity phenotype (Fig. 1). We also detected 44 PHI-base orthologs being induced during necrotrophic phase wherein 33 genes were associated with reduced virulence, 2 genes with loss of pathogenicity, 5 genes having lethal phenotype, and 4 genes to encode potential effectors (Online Resource 10). 10 genes having reduced virulence phenotype were found to be upregulated during both establishment and necrotrophic phases of *R. solani* pathogenesis on rice (Fig. 1). We studied their conservation across different *R. solani* strains and found most of them to have high sequence similarity with *i* strains belonging to different anastomosis groups (Online Resource 10).

Validation of expression dynamics of putative pathogenicity determinants of *R. solani* through qPCR

We selected few establishment and necrotrophic-specific genes to validate their expression dynamics through qPCR. Reduced virulence phenotype-associated genes, i.e., *Mpr1* (c22975_g3_i1), transcription factor *GzZC236* (CL7543Contig1), and loss of pathogenicity phenotype-associated ABC3 transporter (c19012_g1_i2) were selected amongst establishment phase specific genes. Similarly, GH43 family (CL10447Contig1), GT34 family (CL16Contig3), MFS general substrate transporter (CL978Contig1) along with reduced virulence phenotype *AOX1* (CL4725Contig1), *MEP1* (CL8460Contig1), and *SPM1* (c23749_g6_i2) were selected amongst necrotrophic phase-specific genes. We could observe strong correlation between RNAseq and qPCR based expression data (Fig. 3; Online Resource 11).

Interestingly, several genes were depicting higher induction (> twofold change difference) during pathogenesis in susceptible rice genotypes (PB1 and TP309) than in partially resistant rice genotype (Tetep) (Online Resource 7). The qPCR-based expression analysis of few such genes like CAZymes (chitin deacetylase; CL8196Contig1 and endoglucanase; CL6520Contig1), PHI-base category encoding potential effector (c26016_g1_i1; CL3589Contig1 and CL5400Contig1), and transcription factors (c18866_g1_i2) were carried out in *R. solani* infected PB1 and Tetep rice genotypes (Fig. 5). Both RNAseq and qPCR analyses correlated higher fold induction of these genes during pathogenesis on PB1 than on Tetep (Fig. 5; Online Resource 12).

Discussion

In this study, using an Indian isolate of *R. solani* AG1-IA (strain BRS1), we identify various candidate pathogenicity determinants during establishment and necrotrophic phases of its pathogenesis on rice. The transcriptome

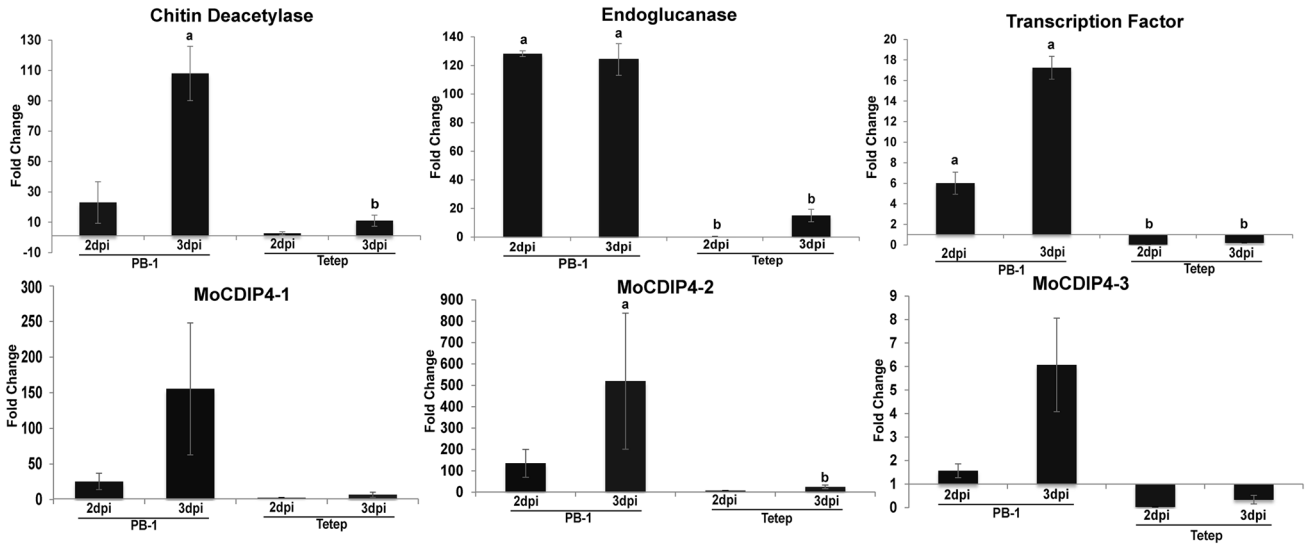


Fig. 5 qPCR-based expression profile of *R. solani* genes during pathogenesis in PB1 (susceptible) and Tetep (partially resistant) rice genotype. The fold change in the expression of CL8196Contig1 (chitin deacetylase), CL6520Contig1 (endoglucanase), c18866_g1_i2 (transcription factor), and c26016_g1_i1, CL3589Contig1, CL5400Contig1 (orthologs of *MoCDIP4*; effector) genes at 2 and 3 dpi with respect to 1 dpi of pathogenesis is represented as bar graphs. 18s rRNA gene of *R. solani* was used as reference for normalization. The *Y*-axis represents mean fold change observed in three biological replicates. Standard error bars are represented as vertical lines. Different letters indicate significant difference at p value < 0.05 (estimated by one-way ANOVA)

analysis suggested that BRS1 is quite diverse from the other *R. solani* strains whose genome sequences are publically available. Only 47% of the BRS1 transcripts showed best sequence similarity to AG1-IA strain (Chinese isolate), while 23 and 28% of them showed BLASTX hits with AG1-IB and AG3 strains, respectively. Previously, gene expression studies in *R. solani* were explored by detached leaf assay using mycelial plugs or inoculum toothpicks (Zhao et al. 2008; Zheng et al. 2013). However, in this study, we have used sclerotia (natural infection propagules of *R. solani*) of BRS1 to infect intact rice tillers. Furthermore, we particularly selected *R. solani* transcripts that were found upregulated in three different rice genotypes (having variable degree of disease susceptibility) and identified those which were associated with establishment and necrotrophic phase of *R. solani* pathogenesis. The upregulation of these *R. solani* genes in each of the rice genotypes highlights their ubiquitous and indispensable role during sheath blight disease in rice. Many of them showed sequence homology with PHI-base genes that were associated with reduced virulence, loss of pathogenicity, lethal, and effectors in various other pathosystems. Based upon in-planta induction pattern and conservation amongst different *R. solani* strains (Online Resource 8), it is tempting to speculate that these genes might be playing crucial role during pathogenesis of *R. solani*. We observed ABC (ATP-binding cassette) transporter, transcription factors, and extracellular metalloprotease (*Mpr1*) to be induced exclusively during establishment phase. The ABC3 transporters of *M. oryzae* are known to be involved in the efflux of cytotoxic compounds such as phytoalexins produced by the host (Sun et al. 2006; Patkar et al. 2012). The *Mpr1* orthologs in *F. verticillioides* encode fungalysins (zinc metalloproteases) and they protect the pathogen from the action of host chitinases produced as a defense strategy (Naumann et al. 2011; Vu et al. 2014). Taken together, this suggests that during establishment phase, *R. solani* upregulates its arsenals to protect itself from cell wall degrading enzymes and cytotoxic compounds produced by the host (Fig. 3). Furthermore, upregulation of transcriptional factor encoding genes such as *CRZ1* ("CRaZy," calcineurin-responsive zinc finger transcription factor) and *GzZC236* highlights their importance in regulating expression of pathogenicity-associated genes during host colonization. It is noteworthy that the *CRZ1* homolog in *M. oryzae* is involved in modulating virulence functions and regulating delivery of effectors (Schumacher et al. 2008; Kim et al. 2010).

The PHI-base orthologs encoding cytochrome P450s (*CYP52X1*), appressorial penetration-associated proteins (*GAS1*), and phosphate utilization-related proteins (*PHO84*) were found induced during both establishment and necrotrophic phases of pathogenesis (Fig. 3). Interestingly, the *GAS1* (encoding β-1,3-glucanosyltransferase) mutants of *M. oryzae* and *F. oxysporum* were defective in appressorium penetration and causing disease (Xue et al. 2002; Caracuel et al. 2005). As during pathogenesis, *R. solani* forms infection cushions or lobate appressoria (Basu et al. 2016;

Ghosh et al. 2017), it would be interesting to test the potential involvement of the *GAS1* gene (CL4124Contig1 and CL5792Contig1) in this context.

Our study suggests that progression of *R. solani* into necrotrophic phase is associated with induction of large suite of cell wall degrading enzymes, effector proteins, and ability to manage oxidative stress. The induction of two orthologs of *AOX1* (encoding alternate oxidase) genes was observed during necrotrophic phase. *AOX1* is involved in alternative oxidative pathway, and it provides resistance against oxidative stress during pathogenesis of *Cladosporium fulvum* and *C. neoformans*, etc (Segers et al. 2001; Akhter et al. 2003). We also observed induction of two orthologs of *SidH* (Anoyl-CoA hydratase) and an ortholog of *DHOD* (fumarate reductase) during necrotrophic phase of *R. solani*. *SidH* is known to be involved in production of siderophore in *A. fumigatus*, which in turn assists the pathogen to survive under iron starved oxidative stress conditions. On the other hand, *DHOD* is involved in maintaining cellular redox homeostasis in *Trypanosoma cruzi* (human pathogen) to survive in anaerobic host conditions (Annoura et al. 2005; Yasmin et al. 2012). This affirms that *R. solani* has adapted its arsenals to survive under hostile oxidative and anaerobic stress conditions inside host plants (Fig. 3). It is worth noting that recent transcriptome studies had also suggested that *R. solani* adopts strategies to combat oxidative stress to infect diverse host plants such as soybean, turf grass, rice, maize, and lettuce etc (Zheng et al. 2013; Zhu et al. 2016; Copley et al. 2017).

A few secreted effector encoding genes of *R. solani* were found induced during pathogenesis on rice (Online Resource 9). The limited sequence similarities of these effectors amongst various *R. solani* strains suggest that they might be evolving rapidly. It is known that phytopathogens diversify effector proteins to escape from host recognition (Spanu et al. 2010; De Jonge et al. 2011; de Wit et al. 2012), although we observed some of the necrotrophy-specific effector genes encoding plant cell wall damaging enzymes to be relatively more conserved amongst different *R. solani* strains. It is worth noting that effectors involved in necrosis-associated host cell death have been often described in other pathosystems (Oliver et al. 2012; Lo Presti et al. 2015). For example, *M. oryzae* utilizes *MoCDIP4*, encoding secreted effector proteins to induce necrotrophic phase-specific cell death response in rice (Chen et al. 2013; Shirke et al. 2016). Notably, three orthologs of *MoCDIP4* in *R. solani* were showing more than sixfold higher induction in susceptible rice genotypes (PB1 and TP309) than in partially resistant (Tetep) rice (Fig. 5). Not only *MoCDIP4* orthologs, several other necrotrophy associated cell wall degrading enzymes were also having several fold induction in susceptible than partially resistant interactions. Overall, this suggests that induction level of these genes might be associated with extent of host cell wall damage which in turn might influence the disease severity of *R. solani* on different rice genotypes. It is possible that the expression level of these genes can be explored as markers of disease susceptibility or resistance in the conventional breeding programs. In general, rapid induction of host cell wall damage is known to activate plant defense response (Jha et al. 2007; Schwessinger and Zipfel 2008; Dodds and Rathjen 2010; Sinha et al. 2013; Tayi et al. 2016). As Tetep is known to harbor several major disease resistance QTLs against various pathogens (Barman et al. 2004; Channamallikarjuna et al. 2009), thus to avoid induction of plant defense, it is possible that *R. solani* might be restricting higher fold induction of these genes during pathogenesis on Tetep.

In conclusion, our study has identified candidate pathogenicity determinants of *R. solani* that might play a crucial role during establishment of rice sheath blight disease. In general, homologous recombination, insertional mutagenesis, RNAi-based gene silencing, etc had been explored to functionally characterize pathogenicity-associated genes of fungi (Jeon et al. 2007; Li et al. 2015; Zhang et al. 2015). However, as it is difficult to genetically transform *R. solani*, establishing the role of identified pathogenicity determinants remains a challenge. Exploring host induced gene silencing approach (Nowara et al. 2010; Jahan et al. 2015) to knockdown the *R. solani* genes during its pathogenesis process might turn out to be an alternate strategy.

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