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Transcriptome profiling uncovers the involvement of CmXyn1, a glycosyl hydrolase 11, in *Cochliobolus miyabeanus* pathogenicity

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Abstract

Necrotrophic pathogen *Cochliobolus miyabeanus* (*C. miyabeanus*) causes rice brown leaf spot disease and drastically affects the yield and quality of rice grains. However, the molecular mechanism of rice-*C. miyabeanus* remains poorly understood due to the limited research conducted on this pathosystem. To elucidate the molecular mechanism of rice-*C. miyabeanus*, a transcriptome analysis was conducted from in vitro and in planta grown *C. miyabeanus*. This analysis led to the identification of a total of 24,060 genes of which 426 in vitro and 57 in planta expressed genes were predicted to encode for secretory proteins. As these 57 genes were specifically expressed in planta and were predicted to be secretory in nature, these were consider as putative effectors, highlighting their possible roles in the fungal pathogenicity. Notably, among these putative effectors, *CmXyn1* which encodes a glycosyl hydrolase 11 displayed the highest expression level under in planta conditions and was thus selected for further functional characterization. Interestingly, the extracellular expression of *CmXyn1* transiently induced cell death in *Nicotiana benthamiana* leaves, while intracellular expression was comparatively lesser effective. In addition, transcriptome analysis on rice leaves during *C. miyabeanus* infection and comparing it to the rice leaf transcriptome data obtained during hemibiotrophic pathogen *Magnaporthe oryzae* infection led to the discovery of 18 receptors/receptor-like kinases that were commonly expressed in response to both pathogens, indicating their key roles in rice defense response. Taken together, our findings provide new insights into rice-*C. miyabeanus* interaction as well as the unique and common defense responses of rice against hemibiotroph and necrotroph model systems.

Keywords *Cochliobolus miyabeanus*, Rice, Transcriptomics, *Magnaporthe oryzae*, Abscisic acid, Receptors

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Introduction

Cochliobolus miyabeanus (=anamorph *Bipolaris oryzae*) is a devastating Dothideomycetes pathogen that causes brown leaf spot (BLS) in rice, wheat, and maize [1]. This necrotrophic pathogen is known to reduce the kernel weight and quality and the number of grains per panicle. In 1943, *C. miyabeanus* caused an epidemic in the West Bengal region of India, resulting in the death of approximately three million people [1]. The mineral-leached fields with minimum nutrient retaining capacity provide a favorable ground for *C. miyabeanus* infection which progressively infects the leaves, leaf sheath, panicle branches, glumes, and ultimately the spikelets, causing plant death [2, 3].

Although fewer reports are available on *C. miyabeanus* as compared to other rice pathosystems, constant genomic efforts have led to the identification of several QTLs responsible for BLS disease resistance in rice [4, 5]. Moreover, several proteins along with biochemical factors and phytohormones have also been identified that play crucial roles in rice-*C. miyabeanus* interaction [6]. For instance, the involvement of silicon (Si) uptake in modulating rice resistance to *C. miyabeanus* through the inactivation of the fungal ethylene pathway was reported [7]. Further, essential nutrients like nitrogen and potassium might also contribute to the development of resistance against BLS disease [8]. Volz and coworkers validated distinctness in the defense response of dicots from monocots when subjected to BLS infection [9]. The *Arabidopsis* mutants displayed the involvement of conjunctive ethylene (ET) and salicylic acid (SA) signaling mechanism in contrast to an independent SA mechanism in BLS-infected rice [9]. In addition, a previous report highlighted the involvement of abscisic acid (ABA) in improving resistance against BLS disease [10]. It was shown that ABA plays a unique role in the defense response against necrotrophic and biotrophic pathogens [11–13]. ABA has been observed to stimulate defense responses against the necrotrophic pathogen, *C. miyabeanus* [10], but it has also been found to have a negative effect on resistance against certain biotrophs [14].

Ahn and coworkers reported distinct patterns of defense mechanisms operative during *Magnaporthe oryzae* (*M. oryzae*) and *C. miyabeanus* infections in rice, which can be expected because of the differences in their mode of nutrition [15]. Necrotrophic pathogens are distinguished by their repertoire of effectors, which consists of secreted toxins and cell-wall degrading enzymes, as supported by various cytological, genomic, and functional investigations [16]. A previous study reported the expression analysis of BLS pathogen effector during wild rice (*Zizania palustris* L.) and *C. miyabeanus* interaction [17]. However, a detailed comparison between the hemibiotrophic and necrotrophic fungi is required to get an in-depth understanding of their mode of infection and nutrition. Moreover, rice responses against these pathogens also need to be investigated to understand the molecular mechanism of their interaction in more detail.

During the past two decades, extensive transcriptome investigations have been performed in several crops deciphering constitutive and induced host–pathogen interactions [18]. However, to date, no report provides transcriptome profiles of rice-*C. miyabeanus* interaction which is crucial to understand the molecular mechanism of their interaction. Thus, the present study aims to elucidate the transcriptomic changes in vitro and in planta conditions of rice infected with *C. miyabeanus*, with the goal of comprehending the plant defense response to necrotrophic fungi.

Materials and methods

Plant materials

Rice seeds (*O. sativa* L. cv. Dongjin) were surface sterilized using 0.05% Spotak solution (Bayer Crop Science, Korea) for 2 h. Sterilized seeds were washed with distilled water five times and then placed on the moist filter papers for germination at 28 °C. Germinated seeds were planted in sterilized soil and allowed to grow at 25 ± 2 °C and 70% humidity with a light and dark cycle of 16 and 8 h, respectively, for 4 weeks [6].

Fungal growth conditions and inoculation on rice seedlings

Cochliobolus miyabeanus strain Cm36 was grown for sporulation on potato dextrose agar medium. Conidia of *C. miyabeanus* were collected from cultured plates after transferring to the incubator in dark condition at 25 °C for 3 days. Inoculated plates were moved from dark to light conditions for additional growth of conidia at 25 °C for 2 weeks. Upon sporulation, conidia were harvested in distilled water containing 0.02% Tween-20, and filtered through Wypall L25 Kimtowels (Kimberly-Clark, USA) to remove mycelia. Harvested conidia were washed two or three times using distilled water containing 0.02% Tween-20 and were used for inoculation on 4 weeks-old rice leaves with 5×10^5 conidia/mL using a sprayer as described previously [15]. Immediately following inoculation, plants were moved into a dew chamber (100% relative humidity) to facilitate fungal penetration and, 20 h later, transferred to greenhouse conditions for disease development. Inoculated leaves were harvested at 36 and 60 h post inoculation (hpi), frozen with liquid nitrogen, and stored at −70 °C until use. For the identification of in vitro *C. miyabeanus* expressed genes analysis, the fungus was cultured in complete medium (CM), minimal

medium (MM), nitrogen starvation (NS), and carbon starvation (CS) [19, 20].

Exogenous ABA treatment

ABA was purchased from Sigma (Aldrich, St. Louis, MO, USA). ABA was first dissolved in a few drops of ethanol. For exogenous ABA treatment (100 μM) of rice leaves, intact seedlings (4 weeks-old rice leaves) were sprayed until near runoff with a fine mist of either compound at the indicated concentrations described previously [10]. Control plants were sprayed evenly with a 0.02% (v/v) Tween 20 solution only. One day post application, ABA-treated rice leaves were challenged with *C. miyabeanus* as described above. Inoculated leaves were harvested at 36 hpi, frozen with liquid nitrogen, and stored at −70 °C until use.

Tiling DNA microarray analysis

Tiling DNA microarray was performed as described previously [21, 22]. Tiling arrays are a subtype of microarray chips with an average size of 60 nucleotides probe covering whole rice and *C. miyabeanus* genes. The probes were designed with *C. miyabeanus* whole genome downloaded from the fungal genome resource and the rice whole genome, respectively [23].

Total RNA extraction and qRT-PCR

Total RNA was isolated from 4-weeks old rice seedlings (infected for 36 and 60 hpi), the mycelium of *C. miyabeanus* cultured with different nutrient conditions, CM, MM, NS, and CS, and *Nicotiana benthamiana* (*N. benthamiana*) using Trizol reagent (Invitrogen, Madison, WI, USA). The cDNA was synthesized according to the manufacturer's instruction by Superscript III First-Strand Synthesis System (Invitrogen, Massachusetts, USA). qRT-PCR was conducted with three replicates on a Rotor-Gene Q instrument (Qiagen, Hilden, Germany) using Prime Q-Master Mix reagents (Genetbio, South Korea) following the manufacturer's protocol. Primers used are listed in Additional file 1: Table S1. The relative fold differences in template abundance for each sample were determined by normalizing the threshold cycle (Ct) value of each gene-specific gene to the Ct value of *OsUbi* and calculating its relative value to a calibrator using the 2-ΔCT algorithm.

Comparative transcriptome analysis and functional annotation

The *C. miyabeanus* and rice transcripts were analyzed for differential expression at least twofold and statistically significant at a p-value less than 0.05. The log-transformed fold values were utilized for data analysis. The *C. miyabeanus* secreted proteins were selected using Signal P 5.0, Secretome P, and subjected to functional annotation and putative effector prediction using Gene Ontology and Effector P [24–26]. The enzyme pathways were enriched using KEGG BlastKOALA tool [27]. The biological processes associated with each effector were manually enriched using available literature. Subsequently, hierarchical clustering was performed using gene expression profiles using MultiExperimentViewer Version 4.9.0 software (MEV, Version 4.9.0).

Plasmid construction

The full-length coding sequence of *CmXyn1* (719 bp) without native signal sequence was synthesized based on *Bipolaris oryzae* ATCC 44560 glycoside hydrolase family 11 protein partial mRNA, using Invitrogen GeneArt™ Strings™ DNA Fragments. The *CmXyn1* sequence was concatenated with a rice *OsGlu1* signal sequence fragment (60 bp) [20], and cloned using the gateway cloning system. *CmXyn1* was cloned into the pDONR221 vector using the Gateway cloning system, resulting in the generation of two constructs: full-length *CmXyn1* (FL-*CmXyn1*) and *CmXyn1* without the signal peptide (NS-*CmXyn1*). To generate the FL-*CmXyn1* construct, the entire construct was introduced into the pGWB517 binary vector with C-terminal MYC fusion tags. The NS-*CmXyn1* construct was derived from the FL-*CmXyn1* construct by amplifying only the *CmXyn1* sequence without the N-terminal signal peptide.

Agro-infiltration assay and DAB staining

For in planta transient expression, an *Agrobacterium tumefaciens* strain GV3101, carrying an appropriate construct was inoculated overnight in yeast extract peptone medium containing selective antibiotics at 28 °C. *CmXyn1* was expressed under the control of the CaMV 35S promoter. The incubated culture was centrifuged at 5000 rpm for 10 min and resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, and 200 μM acetosyringone) and diluted to a final concentration at an optical density at 600 nm of 1. For efficient transformation, the suspension was incubated at room temperature for 2 h and co-infiltrated with P19 into approximately 4- to 5-week-old *N. benthamiana* using a needleless syringe. *N. benthamiana* was grown in a controlled environmental chamber at 23 °C under a 16 h light photoperiod. The infiltrated regions in leaves were harvested after infiltration for 48 h and used for the study. Accumulation of H_2O_2 in leaves was detected by staining them with 3,3-diaminobenzidine tetrahydrochloride hydrate solution (1 mg/mL, pH 3.8; Sigma-Aldrich, St. Louis, MO, USA) in the dark for 1 h, followed by destaining with 95% ethanol, and visualization.

Western blot validation

Nicotiana benthamiana leaf samples were collected 72 h post agro-infiltration and stored in liquid nitrogen. Total protein isolation from *N. benthamiana* leaves was performed according to previously described methods [6]. Briefly, 1 g of fine-ground powder with 5 mL of Tris-MG/NP-40 buffer and centrifuging at 14,000×*g* for 10 min at 4 °C. The resulting pellets were washed with chilled 80% acetone, dissolved in 80% acetone, and stored at −20 °C for SDS-PAGE. Western blots were carried out by transferring proteins to polyvinylidene difluoride membranes, blocking with 1% skimmed milk in PBS, and probing with primary anti-Myc (Santa Cruz, Dallas, Texas, USA) and secondary antibodies [28]. The peroxidase was detected with ECL Western Blotting Substrate Kit (Thermo Fisher, Waltham, US).

Results

Processing and filtering of transcriptome data

In planta and in vitro cultured *C. miyabeanus* samples and *C. miyabeanus* infected rice leaf samples were used for transcriptome analysis using the Agilent Custom Gene Expression 8×60 K chip platform [22]. For the identification of in planta *C. miyabeanus* expressed genes, the fungus was inoculated on rice leaves and samples were harvested at 0, 36, and 60 hpi while for in vitro analysis, the fungus was cultured in CM, MM, NS, and CS medium for 24 h. Following the harvesting of samples, RNA was isolated, followed by amplification and labeling using Agilent's Low RNA Input Linear Amplification kit PLUS. Microarray hybridization was carried out using Agilent's Gene Expression Hybridization Kit and scanning and image analysis was carried out using Agilent's DNA microarray scanner and Feature Extraction Software. Normalization of the obtained data was carried out using Agilent's GeneSpring Software. A total of 36,009 probes showed positive signals of which 32,935 were considered reliable after removing the spots showing signal intensity values lower than the background value, or the standard deviation of each pixel value. Of these reliable 32,935 genes detected, 26,852 showed more than two-fold change as compared to normalized ratio values and were used for further analysis (Fig. 1A). A total of 8749 and 10,750 genes were identified from in planta grown *C. miyabeanus* at 36 and 60 hpi, respectively. In vitro growth conditions resulted in the detection of 24,060 genes at CS, 23,610 genes at MM, and 24,022 genes at NS. Among all the identified genes, 12,560 unique genes were identified under in planta growth conditions of which 6939 were detected in both the time points, 1810 were detected only at 36 hpi and 3811 were detected only at 60 hpi (Fig. 1B). In the case of in vitro, a total of 25,324 genes were identified of which 22,634 were commonly identified in all the samples (Fig. 1C). A total of 6378 genes were found to be present in all the samples based on the Venn diagram analysis (Fig. 1D). Moreover, out of the 627 in planta-specific genes, 269 were identified in both the time points, 165 were identified at 36 hpi and 193 were specific to 60 hpi (Fig. 1D). In the case of 36 hpi and 60 hpi, a total of 165 and 193 unique genes were identified while 269 were common between these two samples. Since these 627 genes were only expressed during in planta growth conditions, these are likely to be involved in the fungal pathogenicity and plant-fungal interactions in the early post-infection stage. Moreover, the majority of the fungal genes exhibited higher expression during in planta culture conditions as compared to that of in vitro.

Expression profiling of *C. miyabeanus* secretory genes

Effector proteins are crucial components of fungal pathogenicity. Since these effector proteins are secretory in nature, the in planta and in vitro specific genes were screened for the presence of secretory proteins by SignalP and SecretomeP tools. These analyses led to the identification of a total of 426 and 57 secreted proteins from in vitro and in planta conditions, respectively (Fig. 2A, B). Of the 57 secreted proteins identified from in planta sample, 23 were found to be cytoplasmic or apoplastic effectors based on screening using the Effector 3.0 database (Additional file 2: Table S2). The identification of necrotrophic effectors can be challenging due to their diverse structures and the lack of clear sequence motifs that distinguish them from non-effectors [29]. Therefore, we additionally selected four genes based on their putative functions and confirmed their expression using qRT-PCR. Among the four selected genes, three including endo-1,4-beta-xylanase I and two hypothetical proteins showed increased expression in the later stages of infection under in planta conditions (Fig. 2C). In particular, endo-1,4-beta-xylanase I showed approximately 2000-fold higher expression at 60 hpi. Therefore, this endo-1,4-β-xylanase (subsequently termed as *CmXyn1* in the manuscript) was chosen for further functional analysis based on its highest expression and high apoplastic effector score of 0.89 (Fig. 2D).

Apoplastic effector *CmXyn1* triggered cell death

To investigate the potential role of *CmXyn1* as an apoplastic effector, Xyn1 was fused with the N-terminal signal sequence of *OsGlu1* (endo-1,4-β glucanase 1), and the construct was transiently expressed in *N. benthamiana* leaves (Fig. 3A). The FL-*CmXyn1* transiently expressed in *N. benthamiana* induced cell death at 2–3 days post-infiltration and triggered the accumulation of H_2O_2 as observed by the DAB (3,3′-diaminobenzidine)

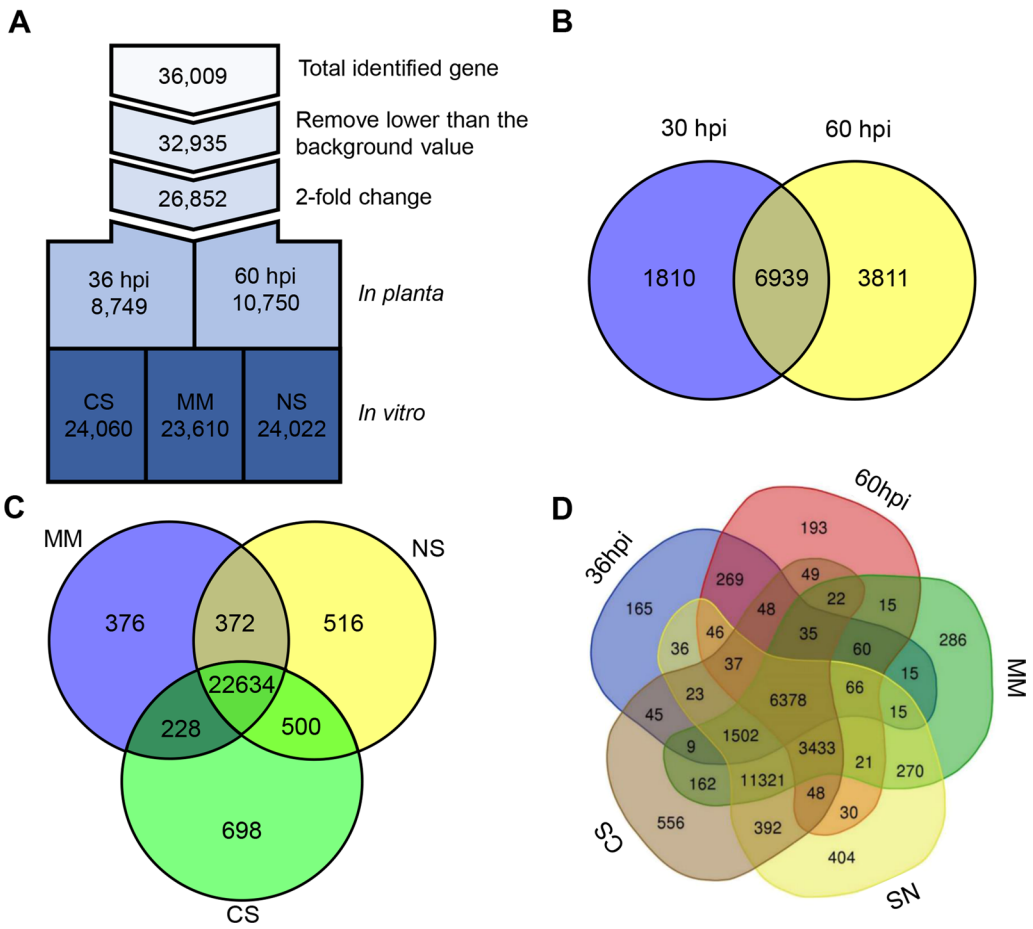

Fig. 1 Transcriptome profiling of *C. miyabeanus* genes during in planta and in vitro growth conditions. **A** Image showing an overview of the identified transcripts in each sample. **B** Venn diagram showing unique and commonly expressed genes between 36 h post inoculation (hpi) and 60 hpi under in planta growth conditions. **C** Venn diagram showing unique and commonly expressed genes among complete medium (CM), minimal medium (MM), and nitrogen starvation (NS) conditions under in vitro growth conditions. **D** Venn diagram showing unique and commonly expressed genes among different samples of in planta and in vitro growth conditions

staining. In contrast, transient expression of a cytoplasmic *CmXyn1*, NS-*CmXyn1*, was relatively ineffective in inducing cell death and H_2O_2 accumulation (Fig. 3B, C). The protein expression analysis of *CmXyn1* in the infiltrated *N. benthamiana* leaves confirmed the accumulation of both FL-*CmXyn1* and NS- *CmXyn1*, however, the protein expression of FL-*CmXyn1* was substantially higher than that of NS-*CmXyn1* (Fig. 3D). Moreover, transient expression of both FL-*CmXyn1* and NS-*CmXyn1* induced the expression of PR family genes including PR1 and PR5. However, the induction was over 20-fold higher when FL-*CmXyn1* was secreted in the apoplastic space, compared to the cytoplasmic expression of NS-*CmXyn1* (Fig. 3E). The phylogenetic analysis of *CmXyn1* was carried out which showed its proximity with glycoside hydrolase 11 (GH11) proteins of other phytopathogenic fungi including *Bipolaris maydis* and *M. oryzae* (Additional file 4: Fig. S1).

Expression profiles and clustering of pathogen-responsive rice genes

Microarray analysis using isolated RNA from rice leaves during *C. miyabeanus* infection revealed differential expression of 367 rice genes. Phytohormone ABA was described as a susceptibility factor, however, De Vleesschauwer and co-workers reported that ABA activates plant defense response against *C. miyabeanus* [10]. Therefore, rice-*C. miyabeanus* interaction in susceptible variety was investigated in response to the exogenous application of ABA. A comparison of gene expression (fold change) among three sets (*C. miyabeanus*, ABA, and ABA + *C. miyabeanus*) revealed the upregulation of various genes during *C. miyabeanus* infection. However, pretreatment with ABA (ABA + *C. miyabeanus*) exhibited a prevalency of downregulated genes in comparison with that in presence of *C. miyabeanus* infection. Interestingly, ABA pretreatment is likely to trigger induced-defense

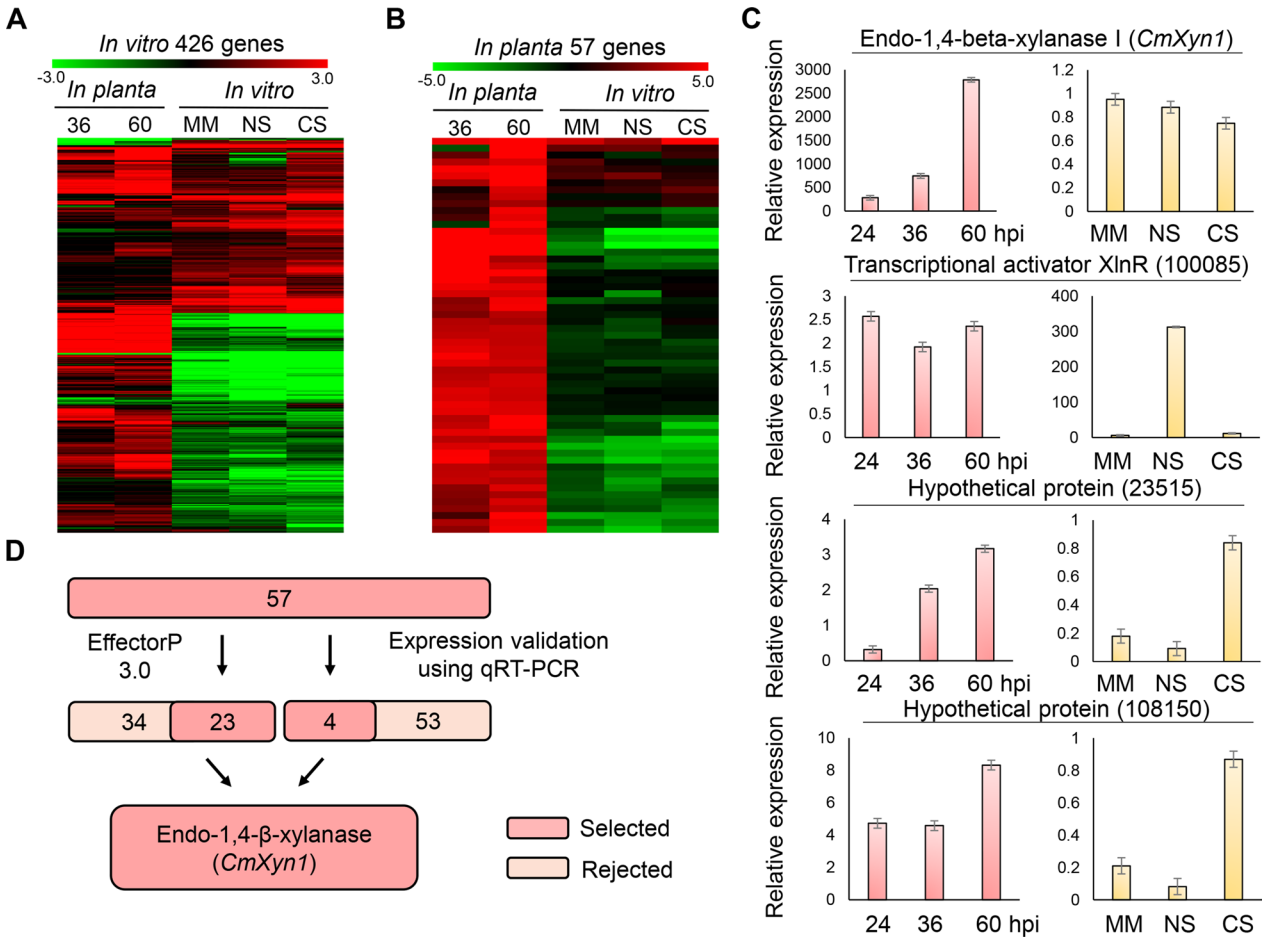

Fig. 2 Comparative profiles of the differentially expressed genes during in planta and in vitro culture conditions derived from *C. miyabeanus* by microarray analysis. **A** Hierarchical clustering analysis (HCA) of significantly expressed genes. 426 genes were grouped based on in vitro growth conditions (MM, NS, CS). **B** HCA of significantly expressed 57 genes that were significantly expressed *in planta* (36 and 60 hpi). **C** Validation of putative effector gene expression patterns using qRT-PCR in both in planta and in vitro conditions. **D** Flow diagram summarizing identification of putative effector genes

response which is manifested by a prevalent downregulation of pathogenesis-related protein (PR)-genes and various genes for cell wall hydrolyzing enzymes. Moreover, to obtain significant insights into the similarities and differences in the rice defense response to necrotrophic and hemibiotrophic pathogens, we compared the obtained rice transcriptome data with a previously reported transcriptome rice data during *M. oryzae* infection [21]. This comparison led to the identification of a total of 72 common upregulated genes in rice during both *M. oryzae* and *C. miyabeanus* infections. Out of these total 72 differential genes, ABA pretreatment showed downregulation of 60 genes majorly including thaumatin, chitinase family proteins, secretory, and other metabolic proteins (Additional file 3: Table S3). Interestingly, 11 genes exhibited upregulation in presence of ABA pretreatment (ABA + *C. miyabeanus*), which include cysteine-rich receptor-like protein kinase, alpha-galactosidase, osmotin, G-protein coupled receptor family protein (GPR)176, transposon protein and dehydration stress-induced protein (Additional file 3: Table S3).

Receptor-like genes induced during infection

To identify the rice receptors and receptor-like kinases (RLKs) that participate in the defense against both necrotrophic and hemibiotrophic pathogenic fungi, 72 commonly upregulated genes were screened for the receptor and RLKs. A total of 18 receptors/RLKs were identified including OsWAK71, Ser/Thr protein kinase, Brassinosteroid insensitive associated receptor kinase (BAK1), S-locus-like receptor protein kinase, cysteine-rich repeat secretory protein, expressed protein-related gene, and morphogenesis of root hair 1 (MRH1) (Fig. 4A). Comparative expression analysis of these

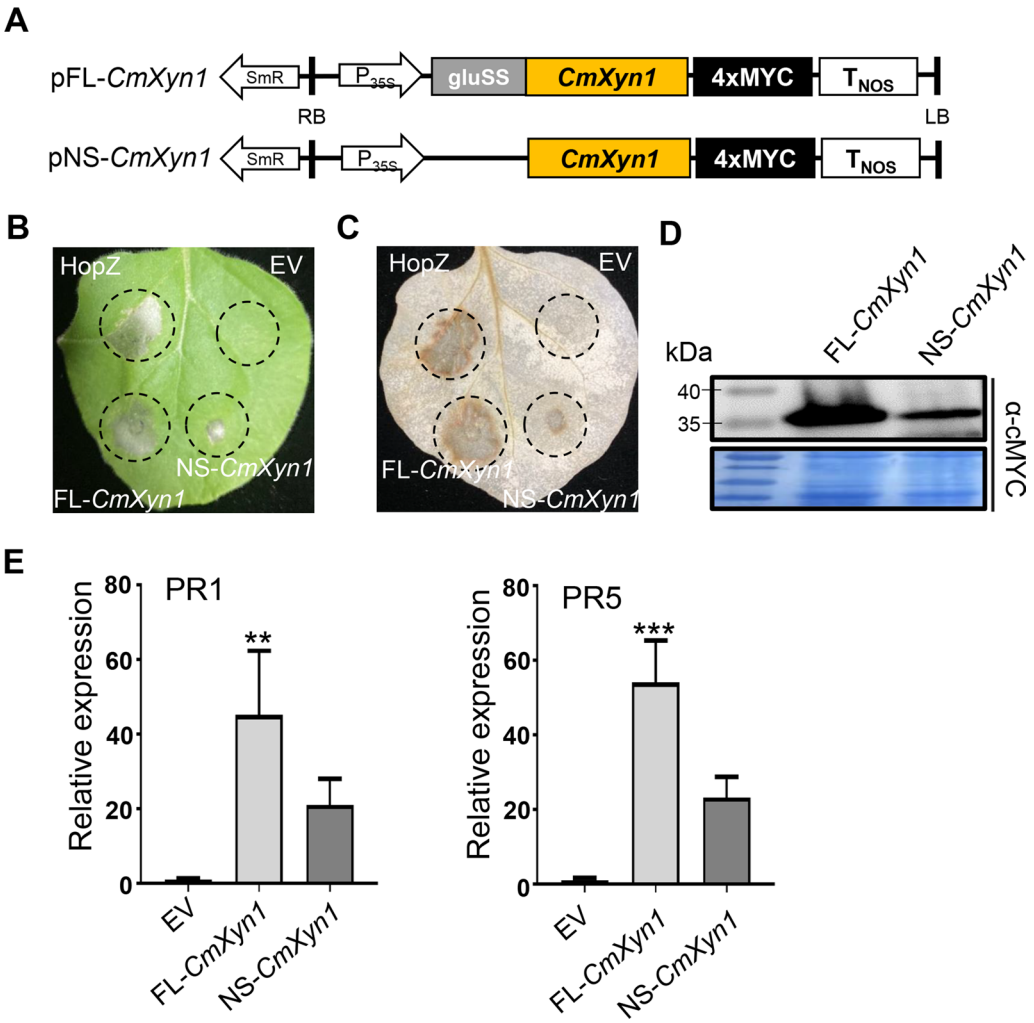

Fig. 3 Characterization of a putative effector of *C. miyabeanus*, glycoside hydrolase11 (*CmXyn1*). **A** Schematic presentation of the vector constructs used, including those containing the signal sequence (pFL-*CmXyn1*) and those without it (pNS-*CmXyn1*). **B** A positive control (HopZ), an empty vector control (pGWB517, EV), constructs with FL/NS-*CmXyn1* used for agro-infiltration. **C** DAB-staining of *N. benthamiana* leaves showed an accumulation of ROS. **D** Validation of anti-α-MYC specific GH11 expression levels in *N. benthamiana* leaves infiltrated with FL/NS-*CmXyn1* using Western blotting, with a loading control. **E** Validation of pathogenesis-related genes (PR1 and PR5) in *N. benthamiana* leaves transiently infected with FL/NS-*CmXyn1* using qRT-PCR (**$p < 0.01$, ***$p < 0.001$)

selected four receptor/RLK genes with the transcriptome data of rice leaves in response to *Xanthomonas oryzae* showed that these were specifically induced in response to fungal pathogens (Fig. 4B). Domain architecture analysis showed the presence of an N-terminal signal peptide in all four receptor/RLK genes while the transmembrane domain was only observed in the three genes (Fig. 4C). To validate the expression of these genes, rice actin 1 was used as an internal control to normalize the cDNA concentration in each sample. Our results revealed that domain unknown protein 26 (DUF26) (LOC_Os03g16950) and S-locus-like receptor protein kinase (LOC_Os12g03640) exhibited approximately 100-fold and 16-fold higher expression in 60 hpi, respectively (Fig. 4D).

Discussion

Cochliobolus miyabeanus is emerging as one of the most devastating diseases of rice, and changes in global climate are further increasing the severity of this disease throughout the globe. Despite the completion of genome sequencing for both partners, information regarding the secreted proteins of *C. miyabeanus* and the corresponding responses of rice to this virulent pathogen remain largely obscure. Furthermore, the absence of any identified *C. miyabeanus*-resistant rice cultivar in Korea has

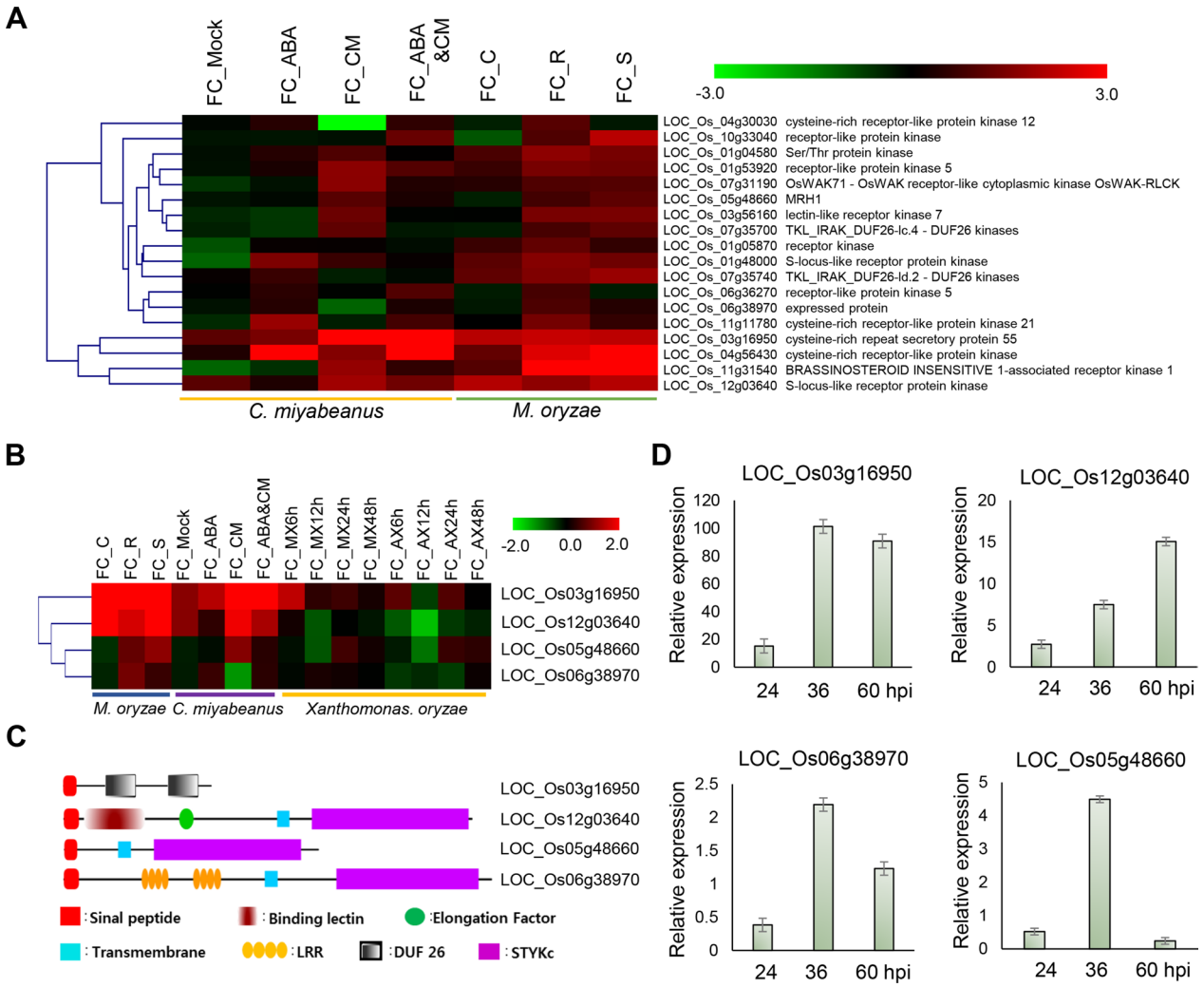

Fig. 4 Rice receptor-like genes induced during rice-*M. oryzae* and *C. miyabeanus* interaction. **A** HCA of 18 receptor/receptor-like kinase (RLK) genes commonly induced in response to *C. miyabeanus* and *M. oryzae* inoculation (C-control, R-resistance, and S-susceptible). **B** Heatmap showing the expression pattern of the selected receptor/RLK genes in response to *M. oryzae*, *C. miyabeanus,* and *Xanthomonas oryzae* inoculation (MX- treated *Xanthomonas* to mock, AX-treated both of ABA and *Xanthomonas*). **C** Domain architecture analysis of receptor/RLKs showing the presence of signal peptides and different domains in different genes. **D** qPCR of selected rice genes showing their expression pattern at different time points of *C. miyabeanus* infection

driven us to explore the possible genes involved in the fungal virulence and rice defense. This will help us to devise effective strategies for controlling this destructive pathogen that targets rice plants. For the identification of potential virulence factors, in planta expressed *C. miyabeanus* genes were compared with those expressed in vitro as it can be presumed that in vitro expressed genes could be majorly associated with fungal survival and reproduction while in planta expressed genes could be associated with pathogenicity. Likewise, rice genes potentially involved in the resistance were identified by comparison with *C. miyabeanus* and ABA pretreatment followed by *C. miyabeanus* inoculation as ABA is proposed to be associated with the rice resistance against *C. miyabeanus* [10]. Furthermore, to identify rice defense genes against both hemibiotrophic and necrotrophic fungi, the transcriptome data obtained during rice—*C. miyabeanus* interaction was compared with the transcriptome data associated with *M. oryzae* interaction [21]. The goal of this analysis was to identify potential genes that can be targeted in future research for developing resistant rice cultivars against a broad spectrum of phytopathogens. Thus, the present work provides an insight into the transcriptome profile of rice-*C. miyabeanus* interaction (Fig. 5). Comparative analysis of defense mechanisms in susceptible rice infected by *M.*

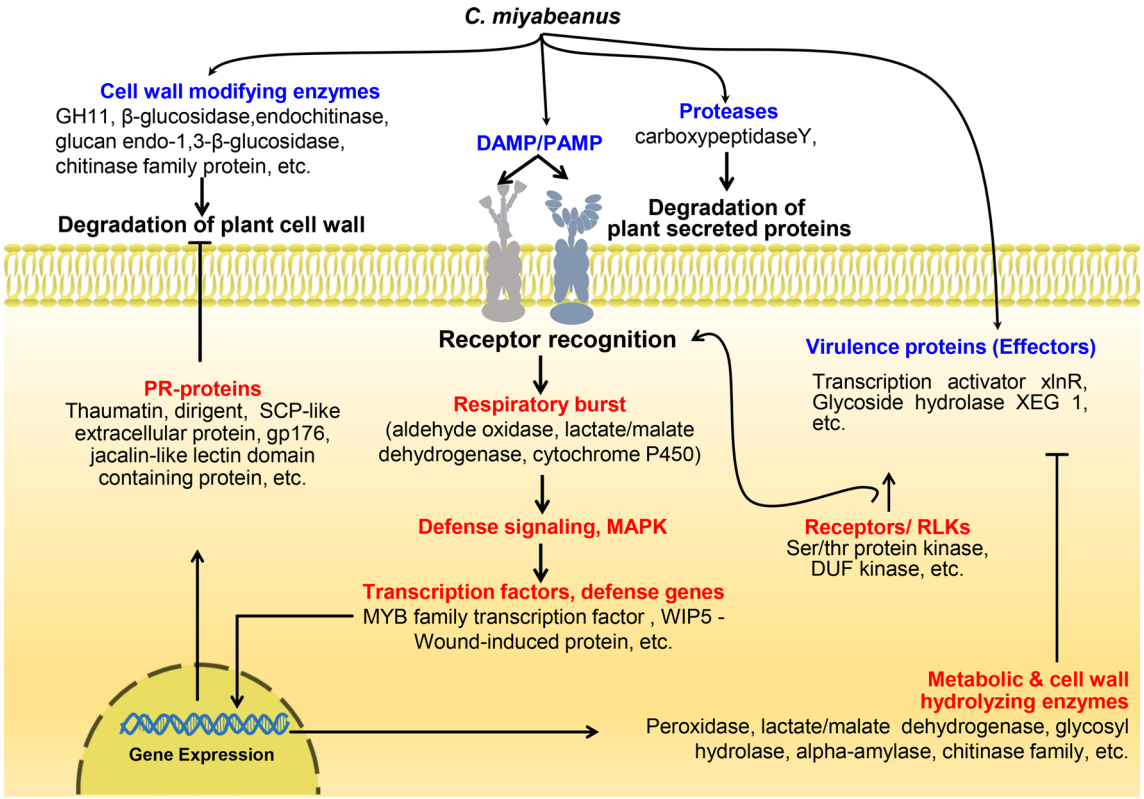

Fig. 5 Rice-*C. miyabeanus* interaction and molecular signaling mechanism in rice plants is mediated by effector-triggered immunity (ETI) and receptor signaling. Transcriptome analysis in *C. miyabeanus* (*in planta*) reveals the expression of genes pertaining to cell wall hydrolyzing and proteolytic enzymes expressed at 36 and 60 hpi. Plant defense against *C. miyabeanus* mediates through gene expression for PR protein, metabolic enzymes, and cell wall hydrolyzing enzymes. various genes for Receptor-Like Kinases (RLK) exhibit upregulation in response to *C. miyabeanus* interaction in rice plants. (PAMP—pathogen-associated molecular pattern, DAMP—damage-associated molecular patterns). The following are the genes that are classified under each category. Cell wall modifying enzymes: chitin deacetylase, endo-1,4-β-xylanase I. Transcription factors, defense genes: thaumatin, MLO domain-containing protein. PR-proteins: dirigent, osmotin, WIP3—wound-induced protein, nepenthesin. Virulence proteins (Effectors): SnodProt 1, candidapepsin, peptidyl-prolyl cis–trans isomerase B. Metabolic & cell wall hydrolyzing enzymes: glutamate dehydrogenase protein, RIPER1-Ripening-related family protein, citrate-binding protein. Receptors/RLKs: brassinosteroid insensitive 1-associated receptor kinase 1, gp176, cytoplasmic kinase MRH 1

oryzae and *C. miyabeanus* reveals conserved signaling mechanisms mediated by PR proteins, cell wall hydrolyzing, and redox enzyme genes that exhibited common expression during infection with both pathogens. The list of commonly upregulated RLKs reported here could be targeted in the future to develop rice varieties resistant to a number of hemibiotrophic and necrotrophic pathogens.

Cochliobolus miyabeanus secretes proteases and cell wall hydrolysis enzymes during early phases of infection

Expression analysis of *C. miyabeanus* genes revealed that the early inducible genes exhibiting higher levels of expression at 36 hpi as compared to 60 hpi included those involved in the hydrolysis of host cell wall such as beta-glucosidase 1, endochitinase 1, glucan 1,3-beta-glucosidase and mannan endo-1,6-alpha-mannosidase DCW1, among others. Glucanase, chitinases, and cellulose hydrolyzing enzymes in fungi are commonly known to trigger the infection in host plants [30]. Interestingly, *Cochliobolus carbonum* has been reported to produce extracellular beta-glucanases necessary for degrading cell wall components in host plants among cereal members [31]. Since 1,3–1,4-beta-glucan is an important component of the cell wall in *Poaceae* members, glucan hydrolyzing enzymes in *M. oryzae* were shown to be crucial for cell wall degradation during rice infection [32]. Moreover, chitinase secreted by *M. oryzae* has been shown to interact with jacalin-related lectins proteins of rice to promote infection [33]. Further, enhanced expression of a xylanolytic transcriptional activator (XlnR) gene was also observed under in planta conditions. XlnR proteins function as transcription factors and although a direct role of these proteins in cell wall hydrolysis has not been reported, it is established that these proteins mediate the expression of target genes

which mostly include hydrolytic enzymes necessary for the dissolution of 1,4-xylan, arabinoxylan, cellulose, and xyloglucan components in the cell wall in *Aspergillus* and other phytopathogenic fungi [34]. As we observed elevated expressions of *C. miyabeanus* genes encoding for cell wall degrading enzymes at 36 hpi, it can be speculated that early signaling events of the fungus are associated with cell wall degradation accompanied by infection in host plants.

Apart from the cell wall hydrolyzing enzymes, early inducible *C. miyabeanus* genes also included various proteases such as carboxypeptidase-Y and candidapepsins, among several others. In the case of *M. oryzae*, the carboxypeptidase-Y gene encodes for a vacuolar serine protease that plays a crucial role in the appressorium formation during *M. oryzae* infection in rice [28]. In addition, the function of these proteases has also been detected in the apoplastic region where these proteins are involved in the degradation of host proteins. Although the role of carboxypeptidase-Y in *C. miyabeanus* has not been investigated so far, its higher expression observed in this study indicates a potential role of this protein in *C. miyabeanus* pathogenicity. Besides, carboxypeptidase-Y, another serine protease inhibitor Alp1 also showed elevated expressions under in planta conditions. Alp1 encodes for an alkaline serine protease and is known to function as an effector associated with necrotrophy in *Sclerotinia sclerotiorum* [35], suggesting a similar role of this protein in *C. miyabeanus*. Further, a candidapepsin 3 also exhibited higher expression during in planta growth conditions. Candidapepsins are extracellular aspartic proteinases that are known to be virulence factors in the *Candida* spp. [36]. In the case of *Candida dubliniensis* genome, eight genes encoding for candidapepsins have been identified suggesting their crucial roles in fungal survival on the host plants. Since these proteins can function only at the acidic pH which is usually present in the apoplastic space of the plants, it can be presumed further that these proteins may also participate in the *C. miyabeanus* pathogenicity and function by degrading rice defense proteins which are usually secreted in the apoplastic region [36].

In addition to these cell wall hydrolysis enzymes and proteases, genes encoding for a peptidyl-prolyl cis–trans isomerase B and a phthalate transporter also showed higher gene expression at 36 hpi. Earlier investigations in *M. grisea* revealed the role of cyclophilins (Peptidyl-prolyl cis–trans isomerase) as a virulent determinant during plant infection [33]. Despite a lack of previous investigations into the role of phthalate transporter in fungal pathogenicity, the higher expression observed in this study suggests its potential involvement in the virulence and establishment of infection in the *C. miyabeanus*-rice interaction. Nevertheless, additional investigations are necessary to determine the potential role of phthalate transporters during the early stages of interaction between *C. miyabeanus* and the rice plant. Currently, it remains plausible that the secretion of phthalic acid by pathogens could have toxic effects on host cells.

CmXyn1 plays a key role in *C. miyabeanus* pathogenicity

GH11 proteins, also known as endo-1,4-β-xylanase named as CmXyn1, are another class of cell wall degrading enzymes that are involved in the degradation of xylans, a major hemicellulose component in the plant cell walls [37]. A substantial number of investigations have shown that the expression of these genes is increased during the infection, indicating their crucial roles in the pathogenicity [37–39]. Their role in fungal pathogenicity could be due to their capacity to degrade the plant cell wall, which serves as a key structural barrier for pathogens. As the transient expression of FL-*CmXyn1* induced high levels of cell death in *N. benthamiana*, a possible role of *CmXyn1* in *C. miyabeanus* pathogenicity could be speculated potentially through degradation of the rice cell wall. A recent report has shown that the hemibiotrophic phytopathogen *Verticillium dahliae* genome encodes for six xylanases having a GH11 domain and all of these showed elevated expressions during *V. dahliae* infection to cotton roots [39]. Among all of these four genes, xylanase 4 (*VdXyn4*), in particular, was reported to be involved in fungal virulence. It was shown that *VdXyn4* exhibits cytotoxic activity and induces necrosis during the late stages of infection [39]. Similarly, glycoside hydrolase family protein (XEG 1) has also been shown to function as an important virulence factor in the soybean pathogen *Phytophthora sojae* and has been recognized as a pathogen-associated molecular pattern (PAMP) [40]. Further, the GH11 domain containing endoxylanases of *Trichoderma, Fusarium*, and *Botrytis* has also been shown to elicit the defense responses in the host plant. Recently, a 25 amino acid long peptide (Xyn25), derived from the *Botrytis cinerea* xylanase (*BcXyn11A*) has been shown to function as an elicitor and cause leaf necrosis, cell death, activation of reactive oxygen species (ROS) burst, and expression of pathogenesis-related (PR)-proteins, among others [41]. Moreover, the activity of a GH11 domain containing the xylanase protein of *B. cinerea* was shown to be required for the pathogenicity and contribute to fungal virulence [37, 38]. Thus, in the present work, we showed CmXyn1 as an apoplastic effector responsible for inducing cell death. In conclusion, this study not only identified unique effectors of *C. miyabeanus* but also revealed a distinct transcriptional response of rice against fungal pathogens, establishing

the role of CmXyn1 apoplastic effector in inducing cell death in rice.

Magnaporthe oryzae and *C. miyabeanus* infection triggers common expression of defense-related genes in rice

Microarray analysis of rice genes upon *C. miyabeanus* infection revealed higher expression of genes encoding for PR-proteins and other proteins involved in plant-pathogen interactions. For instance, thaumatin gene expression was observed to be higher in presence of *C. miyabeanus*, however, it was decreased in the presence of ABA. A previous report utilizing a proteomic approach to investigate the changes in the leaf proteome of susceptible rice plants also revealed the upregulation of thaumatin-like proteins necessary for the induction of whole plant-specific resistance against *M. oryzae* [42]. Thus, in support of earlier investigations, the present work reveals the possible role of thaumatin in early defense signaling during rice-*C. miyabeanus* interaction. Similarly, Cysteine-rich secretory protein (SCP)-like secretory protein genes were also observed to be overexpressed in the presence of *C. miyabeanus* and are known to be expressed in response to *M. oryzae* infection in rice [43]. SCPs likely function as endopeptidases and help in regulating the plant defense responses. However, ABA treatment seems to reduce the expression of SCP-like proteins in the host plant. Likewise, upregulated expressions of genes encoding for jacalin-related lectins (JRLs) and dirigent proteins were observed during both rice-*M. oryzae* and rice-*C. miyabeanus* interactions. The upregulation of JRLs genes in rice has previously been observed in response to *M. oryzae* infection, however, no such observations have been reported in the case of *C. miyabeanus* infection [33]. Previous findings have shown that the JRL protein domain in fusion with dirigent elicits early or late plant defense response in rice [33]. However, ABA treatment resulted in a downregulation of both these genes. A similar expression pattern was also observed in the case of a WIP3 (Wound-Induced Protein precursor) gene which showed decreased expression after ABA treatment and increased expression in both resistant and susceptible varieties of rice infected by *M. oryzae* and *C. miyabeanus* samples. These WIN proteins were originally thought to be induced in response to wounds only, however, subsequent reports suggested their crucial roles in plant defense. It was reported that these WIN proteins belong to the PR-4 protein family and exhibit antifungal and RNase activities [44] and upregulation of this gene during *C. miyabeanus* and *M. oryzae* infections indicate its key role in plant defense. In addition to these genes involved in plant-pathogen interaction and plant defense, the expression of bifunctional monodehydroascorbate reductase (MDHAR) and carbonic anhydrase nectarin-3 were markedly increased upon infection with *C. miyabeanus*. However, the two proteins were suppressed when treated with ABA. Of these, MDHAR plays a role in detoxifying ROS generated during stress responses. ROS-burst is a crucial feature of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) signaling and ROS so produced needs to be subsequently detoxified to prevent the ROS-induced membrane damage and oxidation of biomolecules [45, 46]. Upregulation of MDHAR following *C. miyabeanus* and *M. oryzae* infection thus highlights the plant's strategy to detoxify the stress-induced ROS.

Expression profiling of rice genes showed that the majority of the *C. miyabeanus* and *M. oryzae*-induced genes encoding for PR-proteins and other proteins involved in plant defense showed downregulation or no change after ABA pretreatment. This observation, at first seems contradictory to already known rice defense response which is majorly manifested by an increase in the PR-proteins, however, a previous report has shown similar results in ABA-induced resistance on the expression of PR genes [10]. Expression analysis of *OsPR1b* and *PBZ1* showed ABA pretreatment did not induce, and decreased the expression of these genes, indicating that ABA-induced *C. miyabeanus* resistance was independent of PR-genes induction [10]. Subsequent analysis showed that ABA-induced resistance against the *C. miyabeanus* was independent of SA dependent defense mechanism which employs the upregulation of PR-proteins [10]. Moreover, in the case of ABA-induced resistance, ROS (H_2O_2) production was limited to the site of infection only and no ROS was detected in the adjacent cells, suggesting a localized and lower production of ROS in the case of ABA-induced defense. Therefore, the downregulation of genes involved in ROS detoxification, such as MDHAR, identified in this study after ABA treatment, can be justified as compared to the *C. miyabeanus* and *M. oryzae* infected samples.

In contrast to the above-mentioned genes which were mainly downregulated in response to ABA pretreatment, genes encoding for the osmotin proteins exhibited enhanced expression after ABA treatment, suggesting its key role in ABA-induced defense response in rice. Osmotins are plant-derived antifungal proteins known to trigger pathogen defense and induce cell wall lysis [47]. It has been shown that overexpression of osmotin *OsOSM1* in rice induced resistance to sheath blight infection caused by *Rhizoctonia solani* [48]. In the present work, a significant upregulation of osmotin gene was observed by ABA treatment increased further in response to *C. miyabeanus* inoculation. Thus, ABA-induced defense pathways are likely to trigger osmotin expression during *C. miyabeanus* infection in rice. Likewise, enhanced

expression of a GPR176 family protein was also observed in the ABA pretreatment sample. GPRs are important cell surface receptors associated with hormone signaling in plants and animals and a similar observation has also been reported previously where it was shown that ABA-induced resistance is mediated by the activity of an α-subunit of heterotrimeric G-protein [10]. Therefore, the upregulation of GPR176 observed here further supports the function of G-protein signaling in ABA-induced defense responses against *C. miyabeanus* infection.

Defense mechanism in rice involves RLK-mediated signaling

Plant genomes code for a diverse group of receptors, receptor-like genes, and RLKs which function in plant defense signaling during pathogen invasion [49]. Protein kinases amplify the extracellular signals through the phosphorylation of key proteins involved in pathogen-induced signal transduction. Most of these kinases are situated on the cell surface with active cytoplasmic domains and are activated during pathogen-triggered immunity (PTI) signaling upon the perception of PAMP by the receptors [50, 51]. Here we observed the upregulation of a cysteine-rich receptor-like kinase gene (CRK) or DUF26 in ABA-induced defense against *C. miyabeanus* infection. These CRKs have critical roles against fungal pathogens and earlier evidence suggests cysteine-rich receptor-like protein kinase functions as an early inducible receptor in response to *M. oryzae* infection in rice [51]. OsWAK71—an OsWAK receptor-like cytoplasmic kinase (OsWAK-RLCK) was expressed during *C. miyabeanus* infection. OsWAK71 has been known to induce innate immunity by damage-associated molecular patterns (DAMPs) in incompatible interaction of rice-*M. oryzae* [21]. The BAK1 gene, on the other hand, was upregulated in all the treatments, however, decreased in the ABA pretreated sample. This might indicate the lack of involvement of brassinosteroids (BRs) in ABA-conferred stress tolerance in *C. miyabeanus* treatment.

Taken together, the results reported here increase our understanding of rice interaction with necrotrophic and hemibiotrophic pathogens. The list of differential genes identified here could be used in the future to alter the fungus pathogenicity while the identified rice genes can be targeted in the future to generate rice cultivars with improved resistance to hemibiotrophic and necrotrophic pathogens that are the major concern for the sustainable rice productivity across the globe.

Abbreviations

BLS	Brown leaf spot
ET	Ethylene
SA	Salicylic acid
ABA	Abscisic acid
MM	Minimal medium
NS	Nitrogen starvation
CS	Carbon starvation
RLP	Receptor-like protein
RLK	Receptor-like kinase
ROS	Reactive oxygen species
CRK	Cysteine-rich receptor-like kinase gene
GPR	G-protein coupled receptor family protein
PTI	Pathogen triggered immunity
PAMP	Pathogen-associated molecular pattern
DAMP	Damage-associated molecular pattern

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00789-z.

Additional file 1: Table S1. List of primers used for qRT-PCR.

Additional file 2: Table S2. Expression profile of *C. miyabeanus* differentially expressed under in vitro and in planta cultured condition.

Additional file 3:Table S3. Expression profiles of rice genes in response to *C. miyabeanus* and *M. oryzae* infections.

Additional file 4: Figure S1. The phylogenetic analysis for *CmXyn1* shows a relationship with the *Bipolaris maydis and Magnaporthe oryzae*.

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Author contributions

GHL and JSY performed the experiments and GHL, HRO, RG, CWM, and JWJ analyzed the data. RG, KHJ, YJK, YM, and STK conceived of and supervised the study. GHL, JSY, SM, RG and STK wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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