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Magnaporthe oryzae Chloroplast Targeting Endo- β -1,4-Xylanase I *MoXYL1A* Regulates Conidiation, Appressorium Maturation and Virulence of the Rice Blast Fungus

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Abstract

Endo- β -1,4-Xylanases are a group of extracellular enzymes that catalyze the hydrolysis of xylan, a principal constituent of the plant primary cell wall. The contribution of Endo- β -1,4-Xylanase I to both physiology and pathogenesis of the rice blast fungus *M. oryzae* is unknown. Here, we characterized the biological function of two endoxylanase I (*MoXYL1A* and *MoXYL1B*) genes in the development of *M. oryzae* using targeted gene deletion, biochemical analysis, and fluorescence microscopy. Phenotypic analysis of $\Delta Moxy1A$ strains showed that *MoXYL1A* is required for the full virulence of *M. oryzae* but is dispensable for the vegetative growth of the rice blast fungus. *MoXYL1B*, in contrast, did not have a clear role in the infectious cycle but has a critical function in asexual reproduction of the fungus. The double deletion mutant was severely impaired in pathogenicity and virulence as well as asexual development. We found that *MoXYL1A* deletion compromised appressorium morphogenesis and function, leading to failure to penetrate host cells. Fluorescently tagged *MoXYL1A* and *MoXYL1B* displayed cytoplasmic localization in *M. oryzae*, while analysis of *MoXYL1A*-GFP and *MoXYL1B*-GFP *in-planta* revealed translocation and accumulation of these effector proteins into host cells. Meanwhile, sequence feature analysis showed that *MoXYL1A* possesses a transient chloroplast targeting signal peptide, and results from an *Agrobacterium* infiltration assay confirmed co-localization of *MoXYL1A*-GFP with ChCPN10C-RFP in the chloroplasts of host cells. *MoXYL1B*, accumulated to the cytoplasm of the host. Taken together, we conclude that *MoXYL1A* is a secreted effector protein that likely promotes the virulence of *M. oryzae* by interfering in the proper functioning of the host chloroplast, while the related xylanase *MoXYL1B* does not have a major role in virulence of *M. oryzae*.

Keywords: Xylanases, *Magnaporthe oryzae*, Chloroplast targeting peptide, Pathogenesis, Rice blast disease

Background

The plant cell wall, composed of celluloses, hemicelluloses, and pectin, is the first obstacle a pathogen encounters in plant-pathogen interaction (Kubicek et al. 2014). For this reason, pathogens produce and secrete an array of plant cell wall degrading enzymes to weaken and overcome this initial barrier (Brito et al. 2006; Kubicek et al. 2014; Mori et al. 2008; Win et al. 2012). Cell wall degrading enzymes (CWDEs) are key virulence factors for pathogens as they help not only in host cell invasion but

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also facilitate the depolymerization of plant macromolecules to small molecules that can be acquired as nutrient resources by the pathogen (Fernandez et al. 2014). CWDEs can also act as elicitors of the plant defense response (Ramonell et al. 2002; Ryan and Farmer 1991). CWDEs play vital roles in promoting the successful invasion and colonization of host tissues by phytopathogenic fungi during early and late stages of pathogen-host interaction (Gibson et al. 2011). The first CWDE found to be necessary for virulence was pectate lyase in *Erwinia chrysanthemi* (Roeder and Collmer 1985), followed by endo polygalacturonase in *Aspergillus flavus* (Shieh et al. 1997) and ethylene inducing xylanase in *Trichoderma* spp. (Beliën et al. 2006).

Xylan is the principal polysaccharide component of hemicellulose, which, with cellulose and lignin, makes up the majority of plant cell wall biomass, including that of plants of the *Gramineae* family (Collins et al. 2005). It consists of a 1,4-linked D-Xylp backbone with side branches of AraF and GlcpA (Scheller and Ulvskov 2010). Glycoside hydrolases (GHs) are the broad category of enzymes capable of breaking glycosidic bonds in oligosaccharides and polysaccharides. Most fungal xylanases belong to the GH10 family of high molecular mass endoxylanases (> 30 kDa) and the GH11 family of lower molecular mass endoxylanases (< 30 kDa) (Biely et al. 1997; Lagaert et al. 2009).

Endo-1,4- β -xylanases (EC 3.2.1.8) cleave β -1,4-linkages between xylose units and play a significant role in fungal penetration and colonization (Beliën et al. 2006; Dornez et al. 2010), (Walton 1994) and induce necrosis in host tissues. The xylanase encoding genes in *C. carbonum* (Apel-Birkhold and Walton 1996), *F. oxysporum* (Gómez-Gómez et al. 2002) and *F. graminearum* (Sella et al. 2013) plays non-essential role in virulence. However, endo- β -1,4-xylanase encoding genes in other plant pathogens have been shown to have key roles in virulence, including *xynB* in *Xanthomonas oryzae* pv. *Oryzae* (Rajeshwari et al. 2005), *xyn11A* in *Botrytis cinerea* (Brito et al. 2006), *SsXyl1* in *Sclerotinia sclerotiorum* (Yu et al. 2016) and *VmXyl1* in *Valsa mali* (Yu et al. 2018). Two xylanases belonging to the GH10 family, *ppxyn1* and *ppxyn2* were sufficient to impart virulence to an oomycete *Phytophthora parasitica* for infection of tomato and tobacco plants (Lai and Liou 2018).

The blast fungus *Magnaporthe oryzae* (syn *Pyricularia oryzae*) is a hemibiotrophic filamentous ascomycete threatening worldwide rice and wheat production (Dean et al. 2012; Ebbole 2007). The life cycle of the fungus starts with a three-celled conidium adhering to the leaf surface and undergoing various morphological changes to form a dome-shaped appressorium (Talbot 2003). A turgor pressure of 8 MPa is built up inside the

appressorium that is translated into a mechanical force used to form a penetration peg to breach the leaf cuticle and enter the host (Raman et al. 2013; Wang et al. 2011). The genome of *M. oryzae* contains 20 xylanase genes that encode six glycoside hydrolases in the GH10 family, five in the GH11 family, and nine in the GH43 family (Dean et al. 2005). This prevalence of xylanase genes suggests that they may serve important roles in the life cycle of the blast fungus. In a previous study, Endo- β -1,4-Xylanases in *Magnaporthe oryzae* were silenced to reveal their potential roles in fungal virulence (Nguyen et al. 2011). In this study, the authors characterized xylanases that were specifically upregulated during wheat infection and reported *MGG_07955* (GH11) and *MGG_08424* (GH11), referred to as MoXYL1A and MoXYL1B, to be non-expressed xylanases. We hypothesized that these two xylanases likely play roles that are directly related to the pathogenicity, or virulence of the rice blast fungus. We therefore investigated the contribution of these two Endo- β -1,4-Xylanases in the virulence of *M. oryzae* as well as their intrinsic function as secreted effector proteins.

Materials and Methods

Strains and Culture Conditions

Magnaporthe oryzae isolate, Guy11 protoplast, was used for generating gene deletion mutant strains for functional characterization of *MoXYL1*.

The strains were cultured on Complete Medium (CM; Yeast extract 6 g/L, Casamino acid 6 g/L, Sucrose 10 g/L, and Agar 20 g, dissolved in double distilled water), supplemented with antibiotic (streptomycin 100 μ g/100 mL), under standard incubation conditions of 28 °C (Chen et al. 2008).

For sporulation assays, Rice Bran Media (RBM; Rice bran 40 g/L and agar 15 g, dissolved in dd water with pH adjusted to 6) (Zhang et al. 2019), Straw Decoction and Corn media (SDC; Rice straw 200 g, corn agar 40 g/L, 15 g agar in 1 L double distilled water) (Chen et al. 2010) and CM-II medium (50 mL 20 \times nitrate salts, 1 mL trace elements, 10 g glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acids, 1 mL vitamin solution, 15 g agar in 1 L distilled water) (Chen 2018) were used. Culture plates were kept in dark conditions for 7-days, followed by scratching hyphae and exposing the plates for 3-days to fluorescent light at 28 °C (Aliyu et al. 2019; Zhang et al. 2017).

For generation of competent cells, the *Escherichia coli* strain DH5 α was cultured on lysogeny broth (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl in 800 mL dH₂O), with pH adjusted to 7 by adding 1 M NaOH, before adding up more deionized water to make up 1 L with distilled water (Abdul et al. 2018).

Microscopy Assays to Confirm Protein Secretion

MoXYL1 protein secretion was observed by inoculating strains expressing GFP fusion constructs on barley leaves (Chen 2018). Mycelial plugs were prepared by shaking small pieces of the strains in liquid CM in a 28 °C shaking incubator at 120 rpm for 3-days. Barley leaves placed on moistened filter papers were inoculated with the mycelial plugs. The inoculated leaves were incubated under dark conditions at 28 °C. The leaf sheath was excised with a sterilized fork and observed under a laser scanning confocal microscope at different time intervals: 24 h, 36 h, 48 h, 72 h and 96-hpi. The exposed leaf was also observed at the same time intervals to assess protein secretion and accumulation of GFP signal in plant organelles. An analogous experiment was carried out using spores of the MoXYL1A-GFP strain rather than mycelia.

Transient Expression of Proteins by Agroinfiltration on Tobacco Plants

Tobacco plants were grown in a chamber with conditions as follows: 8/16 h night/day at 22 °C. The effector protein genes *MoXYL1A/B* (amplified with the primer pairs pGDG-F/R given in Additional file 1: Table S1, using Guy11 cDNA as a template), were cloned into the pGDG vector and transformed into *Agrobacterium tumefaciens* GV3101 competent cells. The transformed bacterial strains were grown in LB media supplemented with antibiotics (200 µL/100 mL Rifampicin and 100 µL/100 mL Kanamycin) and incubated at 28 °C and 200 rpm in a shaking incubator. Independently, the rice chloroplast protein ChCPN10C (amplified with the primer pairs Os-CH-pGDR-F/R given in Additional file 1: Table S1, using rice protoplast cDNA as a template), was identified, and cloned into the pGDR vector and transformed in GV3101 competent cells following the same protocol. The pGDG and pGDR cultures at an OD₆₀₀ of 0.5 were centrifuged at 5000 rpm for 5 min. The pellets were suspended in agroinfiltration buffer (prepared by mixing 10 mM MES, 10 mM MgCl₂, and 150 µM acetosyringone in sterilized double distilled water). The pGDG and pGDR strains were combined and incubated at room temperature for 2–3-h. The strain suspension was inoculated on 6-week-old tobacco plants following the standard protocol of infection (Wang et al. 2011, 2017). The infected plants were kept in the dark for 48 h, and then the expression of GFP and RFP fluorescent proteins was observed under a confocal microscope at 488 nm and 561 nm wavelengths, respectively (Martin et al. 2009).

Generation of Mutant and Complement Strains

The Split-marker approach was used to generate gene disruption mutants. Flanking regions 1.1 kb upstream (A

fragment) and 1.1 kb downstream (B fragment) of both *MoXYL1A* and *MoXYL1B* were amplified and cloned in pCX62 vector to flank the hph cassette. The primer pairs MoXYL1A-AF/AR and MoXYL1A-BF/BR to amplify the A and B fragments and YG/F and HY/R for hph used with MoXYL1A-BR and MoXYL1A-AF, to obtain A-fragment/hygromycin, and B-fragment/hygromycin (BH and AH) fusion constructs, are given in Additional file 1: Table S1. The same approach was used for MoXYL1B using the respective primers provided in Additional file 1: Table S1. PEG-mediated fungal transformation was carried out using the Guy11 protoplast (Sweigard et al. 1992). The hygromycin-resistant transformants were screened with ORF and UAH primers (Additional file 1: Table S1) to identify candidate mutant strains. Southern blotting was performed to confirm gene replacement following the protocol given by (Norvinyeku et al. 2017). To generate double deletion mutants, the protocol given by Lin et al. (2019) was followed.

For complementation, 3 kb upstream and the full-length ORF, excluding the stop codons were amplified with the primer pair MoXYL1A and MoXYL1B-Comp-F and R (Additional file 1: Table S1) and cloned into pKNTG to generate GFP-fusion vectors. The GFP vectors were transformed into Guy11 protoplast and the protoplast of confirmed mutants and screened with ORF and UAH primers to identify the G418-resistant GFP-fusion and exact complements, respectively. GFP-fusion candidates were selected on the basis of PCR and GFP signal intensity.

Infection Assays

One-week old Golden Promise Cultivar barley plants were used to conduct the virulence assay. The wild type strain Guy11, mutant strains (Δ *Moxy1A* and Δ *Moxy1B*), and the complemented strains were cultured in liquid CM, for 3-days at 28 °C in a 120 rpm shaking incubator. The isolates were drained, washed with sterile dd water, and media plugs were removed. Mycelia with moderate moisture were used to inoculate intact and abraded leaves of barley placed on moistened filter papers following established inoculation methods (Chen 2018) with slight modification. Incubation conditions were: 24 h in the dark, followed by 6-days in 12 h dark/12 h light at 28 °C. Disease severity was assessed, and photographs were taken on day 7 post-inoculation.

Two-week-old susceptible rice cultivar CO-39 was used to assess the disease development of mutants, wild type, and complemented strains. Strains were grown on RBM media for 7-days. Hyphae were scratched off, and plates were exposed to fluorescent light for 3-days to produce conidia. Conidia were then harvested from the plates and diluted with sterile double distilled water. Conidia count

was conducted using a hemocytometer. An equal inoculum (based on conidium number) with 0.2% Tween-20 was prepared and sprayed on rice (Akagi et al. 2015). The inoculated rice plants were kept in a humid, dark growth room for the first 24 h. Later they were shifted in the light growth room. Disease phenotype was assessed at 7-days post-infection.

Penetration Assays

Barley leaves were kept upside down in the moistened filter papers. Fungal spores were adjusted to the desired volume (5×10^4 mL⁻¹), with 0.2% Tween 20 and a 20 µL was used to inoculate leaves with the spores of each strain (Akagi et al. 2015). The inoculated barley plates were incubated following the conditions used in virulence trials (see above). The leaf sheath was peeled, and invasive hyphae were observed at 24 h, 36 h, 48 h, and 72 h under the optical microscope.

Appressorium Formation Assays

20 µL conidial suspension (5×10^4 mL⁻¹) from wild type, mutants (Δ *MoxylIA-3* and Δ *MoxylIA-13*) and complemented strains were placed on hydrophobic Thermo Fisher Scientific coverslips to induce appressorium formation (Abdul et al. 2018; Aliyu et al. 2019). The incubation was done at 28 °C in a dark incubator and observed under a Nikon TiE system (Nikon, Japan) at 2 h, 4 h, 6 h, and 8 h, respectively.

Conidiophoregenesis Assays

Asexual reproduction of the wild type, mutants, and complemented strains was assessed upon growth on RBM, SDC, and CM-II media. Plates were scratched on day 7 post-inoculation and kept in a light incubator at 28 °C. Conidiophore formation was observed at 12 h, 24 h, 36 h, and 48 h. To quantify spore production, conidia were washed off the plates and counted on a hemocytometer under a microscope.

Cell Wall Stress Response

For cell wall sensitivity assays, CM media was supplemented with cell wall perturbing agents: sodium dodecyl sulphate (SDS, 0.01%), Calcofluor White (CFW, 200 µg/mL) or Congo Red (CR, 200 µg/mL) and cultured in the dark for 10-days at 28 °C. The colony diameter was measured on day 10 after inoculation. The inhibition rate was calculated as previously described (Zhang et al. 2014).

Genomic DNA Extraction

For the Southern blot assay, total genomic DNA was extracted from the mutants, wild type and complemented strains grown in liquid CM shaken for 3-days at 120 rpm and 28 °C, using the SDS-CTAB DNA Extraction method

(Aliyu et al. 2019). The resultant DNA suspension was then digested with Ste I Restriction enzyme for MoXYL1A and HindIII enzyme for MoXYL1B, and Southern blotting was performed as previously described (Norviyenye et al. 2017).

Real-Time RT-PCR Assay

Total RNA extraction was carried out following the previously described protocol (Lin et al. 2019). To check the expression of *MoXYL1A* and *MoXYL1B in-planta* and in individual deletion strains with quantitative real-time PCR (qRT-PCR), RNA extracted from the wild type strain Guy11 and the mutant strains (Δ *MoxylIA* and Δ *MoxylIB*) were subjected to reverse transcription using SYBR[®] Premix Ex. Taq[™] (TliRNaseH Plus). A reaction mixture of 25 µL was formulated using 12.5 µL of Premix Ex-Taq and 1 µL of each 10 µM primer (Additional file 1: Table S1) and 1 µL of cDNA template and incubated in the Eppendorf Realplex2 master cycler (Eppendorf AG 223341, Hamburg). Actin was used as positive control. The delta delta-CT method ($2^{-\Delta\Delta CT}$) was used for data analysis (Aliyu et al. 2019).

Yeast-Two-Hybrid Assay

The pGBKT7 (AD) and pGBKT7 (BD) vectors were used for the construction of bait and prey constructs by In-fusion HD Cloning Kit (Clontech, USA). The CDS of respective genes were cloned and co-transformed into the AH109 yeast strain after sequencing. The Matchmaker Gal4 Two-Hybrid System 3 (Clontech, USA) was employed following the manufacturer's guidelines. The positive transformants on SD-Trp-Leu medium were tested on SD-Trp-Leu-His-Ade medium, using the positive and negative controls from the Kit. The rich (YPD), lactate (YPL; 1% yeast extract, 2% peptone, 2% lactate), galactose (YPGal; 1% yeast extract, 2% peptone, 2% galactose), synthetic minimal with glucose (SMD; 0.67% yeast nitrogen base, 2% glucose, amino acids, and vitamins), synthetic minimal with lactate (SML; 0.67% yeast nitrogen base, 2% lactate, amino acids, and vitamins) or synthetic minimal with galactose (SMGal; 0.67% yeast nitrogen base, 2% galactose, amino acids, and vitamins) media were used for growth of yeast cells.

Results

Identification of *M. oryzae* Endo-1,4-Beta-Xylanase I and Generation of Δ *Moxyl1* Strains

Domain-specific BLASTp search for the *Neurospora crassa* glycoside hydrolase family 11 domain amino acid sequence identified two GH11 family domain-containing proteins in *Magnaporthe oryzae* (*MoXYL1A*), encoded by *MGG_07955*, and *MoXYL1B* encoded by *MGG_08424*. To elucidate the physiological and pathological functions

of *MoXYL1A* and *MoXYL1B* in *M. oryzae*, we generated targeted gene knock-out strains by replacing the coding region of *MoXYL1A* and *MoXYL1B* with the hygromycin phosphotransferase resistance (*hph*) gene using established homologous recombination techniques (Catlett et al. 2003). Putative *MoXYL1A* and *MoXYL1B* gene deletion transformants were selected on double layered TB3 agar containing 300 µg/mL (bottom layer) and 600 µg/mL (upper layer) hygromycin B and screened by PCR. Two successful knock-out strains each for *MoXYL1A* ($\Delta Moxy1A-3$ and $\Delta Moxy1A-13$), and *MoXYL1B* ($\Delta Moxy1B-5$ and $\Delta Moxy1B-7$) identified by PCR screening were checked using qRT-PCR and Southern Blotting (Additional file 1: Fig. S1). These assays confirmed the successful replacement of the *MoXYL1A* and *MoXYL1B* genes with *hph* in these strains (Additional file 1: Fig. S1). Our ability to recover deletion mutants indicates that survival of the rice blast fungus is independent of *MoXYL1A* and *MoXYL1B* function under standard conditions.

Influence of *MoXYL1A* and *MoXYL1B* Gene Deletion on Vegetative and Asexual Growth of *M. oryzae*

To investigate the role of *MoXYL1* gene deletion on the growth of *M. oryzae*, mycelial plugs of single and double $\Delta Moxy1A$ and $\Delta Moxy1B$ mutants, wild type (Guy11) and the complemented mutant strains were inoculated on Complete Medium (CM) and incubated under dark conditions at 28°C for 10-days. Growth measurements (mm) were taken on day 10 post-inoculation and plates were photographed. This assay showed no strong adverse effects on growth for all strains tested (Fig. 1a, b, Additional file 1: Fig. S2a, b). However, a noticeable reduction in aerial hyphae and minimal but statistically significant difference in colony diameter was observed for $\Delta Moxy1A$ compared to WT. In contrast, there was no significant difference between $\Delta Moxy1B$ and WT. The double deletion strain (DKO) was obtained via HR-based deletion of *MoXYL1B* on the $\Delta Moxy1A$ background. Colony morphology and size of the double mutant was not significantly different from either single knockout. We conclude that *MoXYL1A* and *MoXYL1B* do not have specific morphogenesis-related functions in blast fungus under standard conditions.

A conidiophoregenesis assay was conducted to ascertain the impact of these mutants on asexual reproduction in *M. oryzae*, as conidiation plays a vital role in the survival and dissemination of the fungus (He et al. 2015). To quantify conidia production, conidia were harvested after 10-days, diluted with an optimized volume of sterile distilled water and then counted using a hemocytometer. The results showed that the $\Delta Moxy1A$ and $\Delta Moxy1A/\Delta Moxy1B$ strains were severely impaired in conidiophore production, with almost no conidia produced,

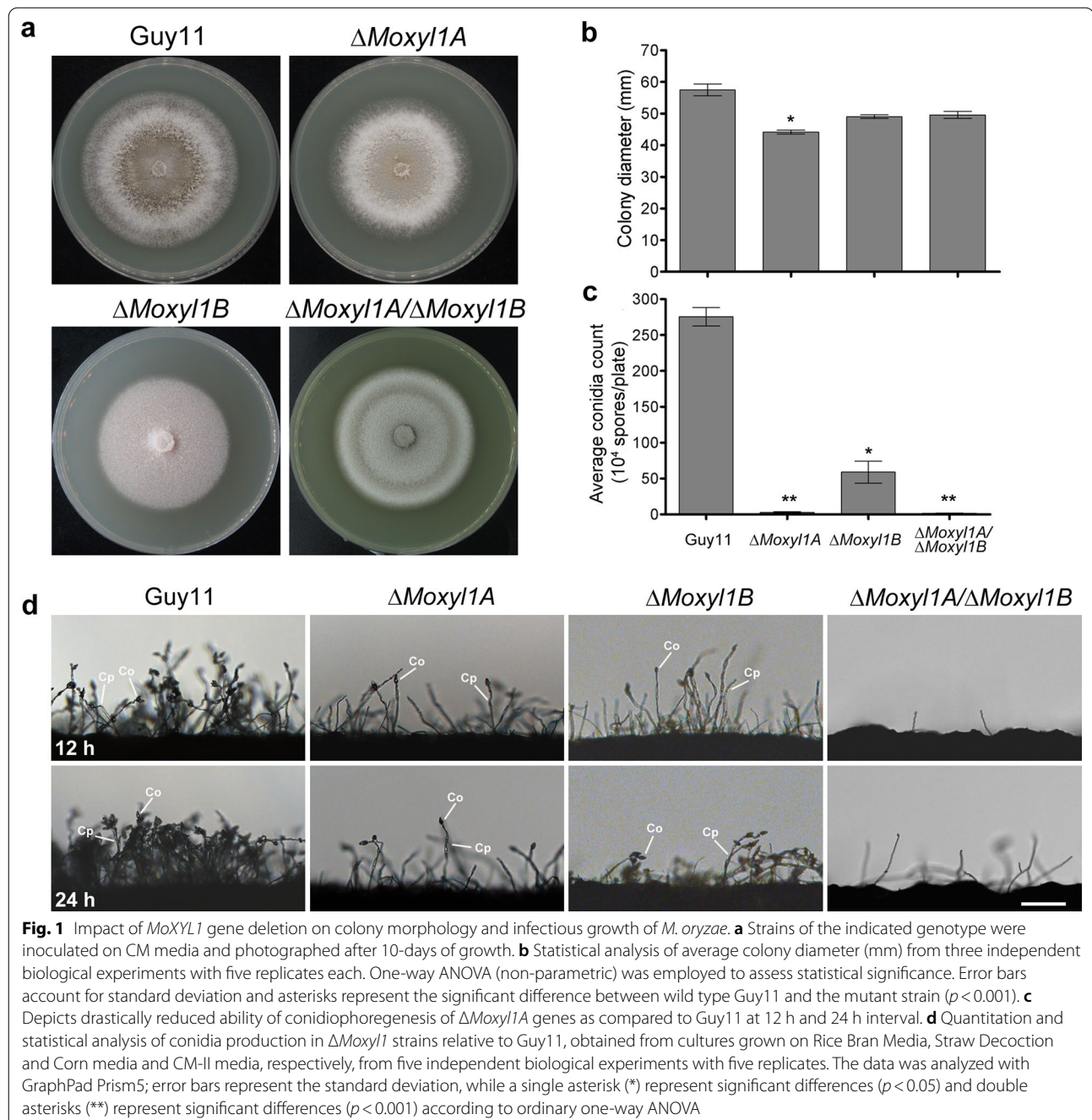
while $\Delta Moxy1B$ produced conidiophores of WT shape but in reduced number relative to WT (Fig. 1c). To further corroborate this defect, mutant and wild type strains were also grown on SDC and CM-II media and conidia were counted (Fig. 1d, Additional file 1: Fig. S2). The results confirmed a significant reduction in spore production in the deletion mutants, with a complete lack of conidiation in the double mutant, suggesting that there is clear contribution of these genes to the asexual development of *M. oryzae*, with *MoXYL1A* having an essential role and *MoXYL1B* a partial role in this growth phase. The conidiation defect of $\Delta Moxy1A$ was partially rescued in the complemented strain; however, although it produced conidia that are morphologically indistinguishable from the wild type, the overall number was reduced. The defective conidiation was fully restored in the *MoXYL1B*-complemented strain (Additional file 1: Fig. S2).

MoXYL1A is Required for Complete Virulence of *M. oryzae*

A susceptible rice cultivar CO39 and leaves detached from the Golden Promise cultivar of barley were used to conduct pathogenicity assays to assess the role of *MoXYL1* genes in the pathogenesis of rice blast fungus. These examinations revealed an impairment in the virulence characteristics displayed by the $\Delta Moxy1A$ and $\Delta Moxy1A/\Delta Moxy1B$ strains, which were unable to produce proficient blast lesions, while $\Delta Moxy1B$ produced typical blast lesions (Fig. 2a). These results suggest *MoXYL1B* plays a dispensable role in pathogenicity while *MoXYL1A* plays a significant role in imparting virulence to *M. oryzae*. A comparable experiment was done using a spore suspension inoculated onto intact and abraded barley leaves. We infected with conidia of WT, single mutants, and complemented strains and observed similar pathogenicity defects for $\Delta Moxy1A$ conidia as with mycelia. Virulence defects were rescued in the complemented strain (Fig. 2b). These results revealed the critical role of *MoXYL1A* in the pathogenicity of rice blast disease on barley.

We further conducted inoculation trials with spore suspensions (1×10^5 conidia per mL in an aqueous solution of 0.2% Tween 20) on rice (cultivar CO39). Spore suspensions of wild-type and *MoXYL1A* or *MoXYL1B* mutant strains were independently and evenly sprayed on rice leaves. The inoculated seedlings were kept under proper incubation conditions (see Methods) for 7-days. This rice pathogenicity trial showed consistent results with the barley experiments, with $\Delta Moxy1A$ and $\Delta Moxy1A/\Delta Moxy1B$ strains completely lacking virulence compared to wild-type and the complemented strains (Fig. 2c).

To unravel the factors responsible for the impairment in pathogenicity of the *MoXYL1A* deletion mutants, we performed a penetration bioassay using barley as the



host plant. We inoculated barley leaves obtained from one-week-old barley plants with conidia harvested from $\Delta Moxy1A$ and wild type Guy11 to examine the penetration ability and colonization efficiency of the fungus. The results showed that the targeted gene replacement of *MoXYL1A* had a profound impact on the penetration and likely colonization abilities of *M. oryzae* as compared to wild-type. At 48hpi, for $\Delta Moxy1A$, no invasive hyphae were visualized inside the barley leaf when its sheath was

excised and observed under the microscope, while wild-type micrographs showed pronounced invasive hyphae that were branched and colonizing adjacent cells. These results confirmed the inability of *MoXYL1A* mutants to invade host plants and cause blast disease. Consistent with earlier results, no penetration defects were observed for the *MoXYL1B* deletion (Fig. 2d).

To further investigate the reason for pathogenicity defect observed in the $\Delta Moxy1A$ strain, we performed

an appressorium formation assay to assess the efficiency of pathogenic dirrentiation in the $\Delta Moxy1A$, and $\Delta Moxy1B$ strains compared to the wild-type and the complementation strain. The $\Delta Moxy1A$ strain was unable to form a normal appressorium at 8 h of incubation on hydrophobic coverslips. The mutant produced an abnormal appressorium with a long germ tube and no melanin-ring, suggesting that it was a non-functional appressorium that could not penetrate and colonize the barley leaves (Fig. 2e). This phenotype was rescued by complementation of *MoXYL1A*. The *MoXYL1B* deletion mutant strains also had delayed appressorium formation but their appressoria were morphologically normal. As the double deletion mutants are unable to form conidia, we could not assess their appressorium development. Furthermore, we demonstrated that targeted replacement of *MoXYL1A* gene significantly attenuated the generation of turgor in the appressorium (Fig. 2f, g). These observations showed that *MoXYL1A* positively regulate appressorium integrity in the rice blast fungus possibly by modulating cellular parameters associated with the generation and accumulation turgor pressure.

$\Delta Moxy1A$ and $\Delta Moxy1B$ are Sensitive to Cell Wall Stress

Fungal cell wall integrity is crucial for infection of host cells, as the fungal cell wall maintains shape and facilitates exchange between the environment and fungus (Cabib et al. 2001). For proper growth and development, the cell wall requires repeated remodeling (Jeon et al. 2008). Therefore, we set out to assess the impact of cell wall-perturbing reagents on the growth of $\Delta Moxy1I$ strains. Calcofluor White (CFW) was used to test whether fungal strains are defective in cell wall assembly or have a defect in cell wall integrity (Lussier et al.

1997; Ram et al. 1994). Sodium dodecyl sulphate (SDS) is a detergent that compromises membrane stability, since cell wall defects increase the vulnerability of the plasma membrane to SDS, sensitivity can indicate problems with the cell wall (Bickle et al. 1998; Igual et al. 1996; Shimizu et al. 1994). CR, Congo Red (CR) is an additional cell wall stress reagent (Wood and Fulcher 1983). We supplemented CM culture media with Calcofluor White (200 $\mu\text{g}/\text{mL}$ CFW), Congo Red (200 $\mu\text{g}/\text{mL}$ CR), or sodium dodecyl sulphate (0.01% SDS) prior to inoculation with WT and mutant strains. Quantification of the growth inhibition rate, based on colony size, showed that the $\Delta Moxy1B$ strain was more sensitive to cell wall stress reagents than $\Delta Moxy1A$, suggesting a possible role for this gene in cell wall integrity. Interestingly, we observed that double gene deletion, however, rescued the *MoXYL1B* phenotype to approximate that of the *MoXYL1A* single mutant, suggesting that the absence of *MoXYL1A* improves stress tolerance of $\Delta Moxy1B$ strains (Fig. 3). From these observations, we speculated that *MoXYL1A* and *MoXYL1B* possibly modulates stress homeostasis in *M. oryzae* by counter regulating either expression, or enzymatic activities of each other.

MoXYL1A and *MoXYL1B* Localize to the Cytoplasm in *M. oryzae*

The subcellular localization of the *MoXYL1A* and *MoXYL1B* proteins in *M. oryzae* was investigated by transforming GFP fusion constructs of both proteins under their respective native promoters into the protoplast of the Guy11 strain (Dr. Didier Tharreau, CIRAD, Montpellier, France). The cultured strains harboring the fluorescence signals were observed with a Nikon laser confocal and laser excitation epifluorescence microscope, showing

(See figure on next page.)

Fig. 2 Targeted gene replacement of *MoXYL1A* compromised turgor-mediated appressorium integrity and impaired the virulence of *M. oryzae*.

a Showed hyphae-mediated virulence characteristics of the individual strains on intact and injured leaves of one-week-old, barley seedlings. Induction of blast lesion was assessed at 7-dpi. Images are representative of three independent assays, each assay with three replicates. **b** Virulence bioassay conducted on 21-days-old rice seedlings using spore-drop inoculation method. Conidial suspensions were prepared as 1×10^5 conidia mL^{-1} in 0.2% Tween 20, for both the mutants and wild-type 20 μL . **c** Conidia-mediated pathogenicity/virulence characteristics $\Delta Moxy1A$, $\Delta Moxy1B$, and the wild-type on 21-days-old rice seedlings, through spray-inoculation with conidial suspensions (1×10^5 conidia mL^{-1} in 0.2% Tween 20). **d** The micrograph portrays the inability of $\Delta Moxy1A$ and double deletion mutants to invade and colonize barley tissue at 48hpi. The absence of invasive hyphae were evident in barley cells inoculated with $\Delta Moxy1A$ or the double deletion strain. In contrast, numerous invasive hyphae were seen in the leaves inoculated with either $\Delta Moxy1B$ or Guy11. Images are representative of $n = 2$ independent biological replicates. Scale bar, 20 μm . **e** Appressoria were produced artificially on Thermo-fisher hydrophobic coverslips and observed at 8 hpi. Images show non-functional appressoria lacking melanin lining for the $\Delta Moxy1A$ mutant. **f** Showed results from incipient cytorrhysis assays performed to evaluate the turgidity of appressorium form by conidia from the $\Delta Moxy1A$, $\Delta Moxy1B$, $\Delta Moxy1A_Com.$, $\Delta Moxy1B_Com.$, and the wild-type hydrophobic coverslips for 8-h appressorium formed were treated with 2 M glycerol solutions. Collapse appressorium were counted using the Olympus DP80 light microscope. Scale bar = 20 μm . **g** The bar graph showed results from the statistical evaluation of the proportion of collapsed appressorium recorded in the $\Delta Moxy1A$, $\Delta Moxy1B$, $\Delta Moxy1A_Com.$, $\Delta Moxy1B_Com.$, and the wild-type strains on hydrophobic coverslips for 8-h and treated independently with varying concentrations (1 M, 2 M, 3 M, and 4 M) of glycerol solutions during incipient cytorrhysis assays. Consistent results from three independent biological experiments with each consisting of five technical replicates were used for statistical analyses. For each independent biological experiment, 100 appressoria were counted ($n = 100 \times 3$). treatments yielding significant difference ($P \leq 0.05$) are denoted with asterisks***

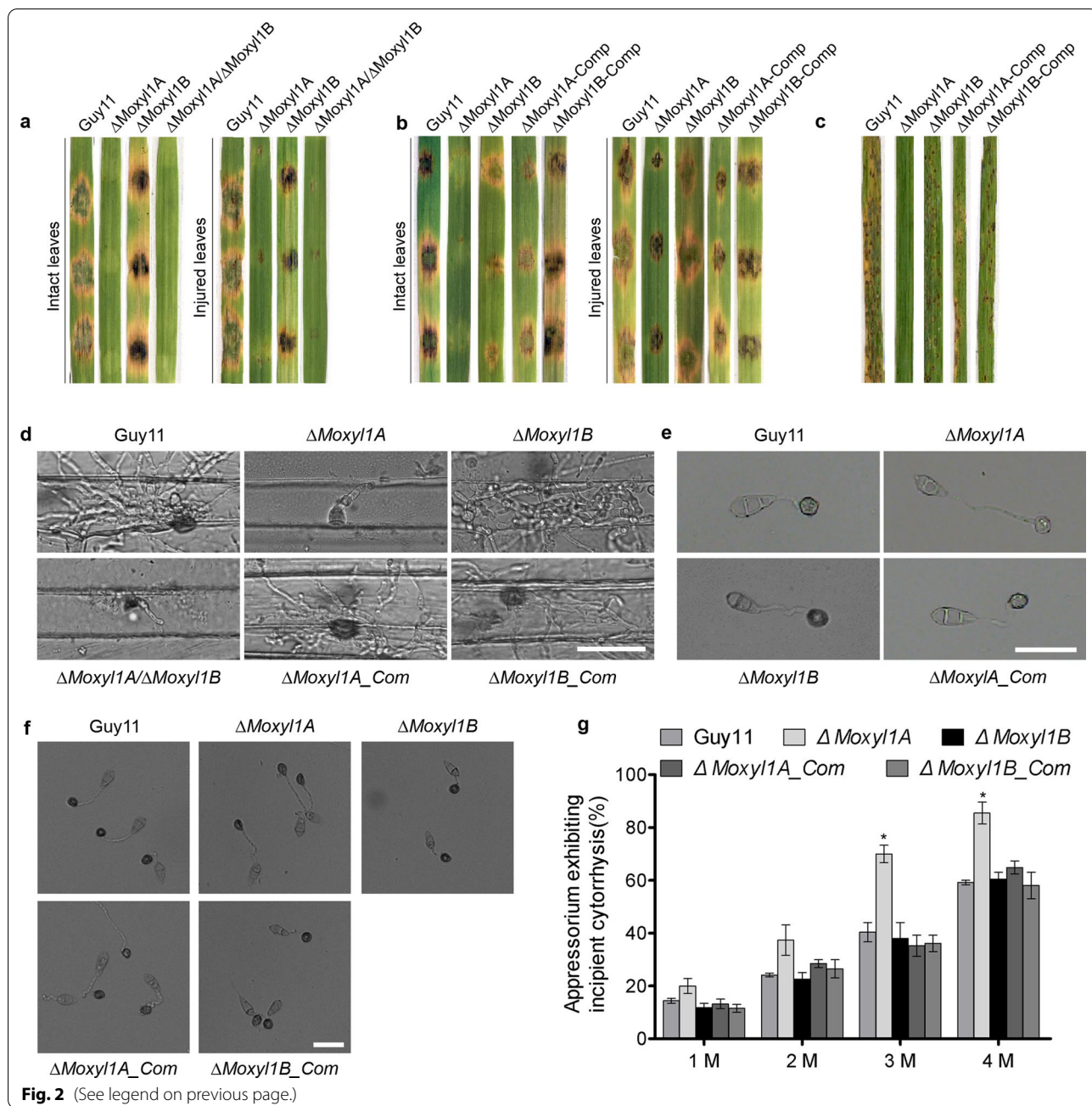
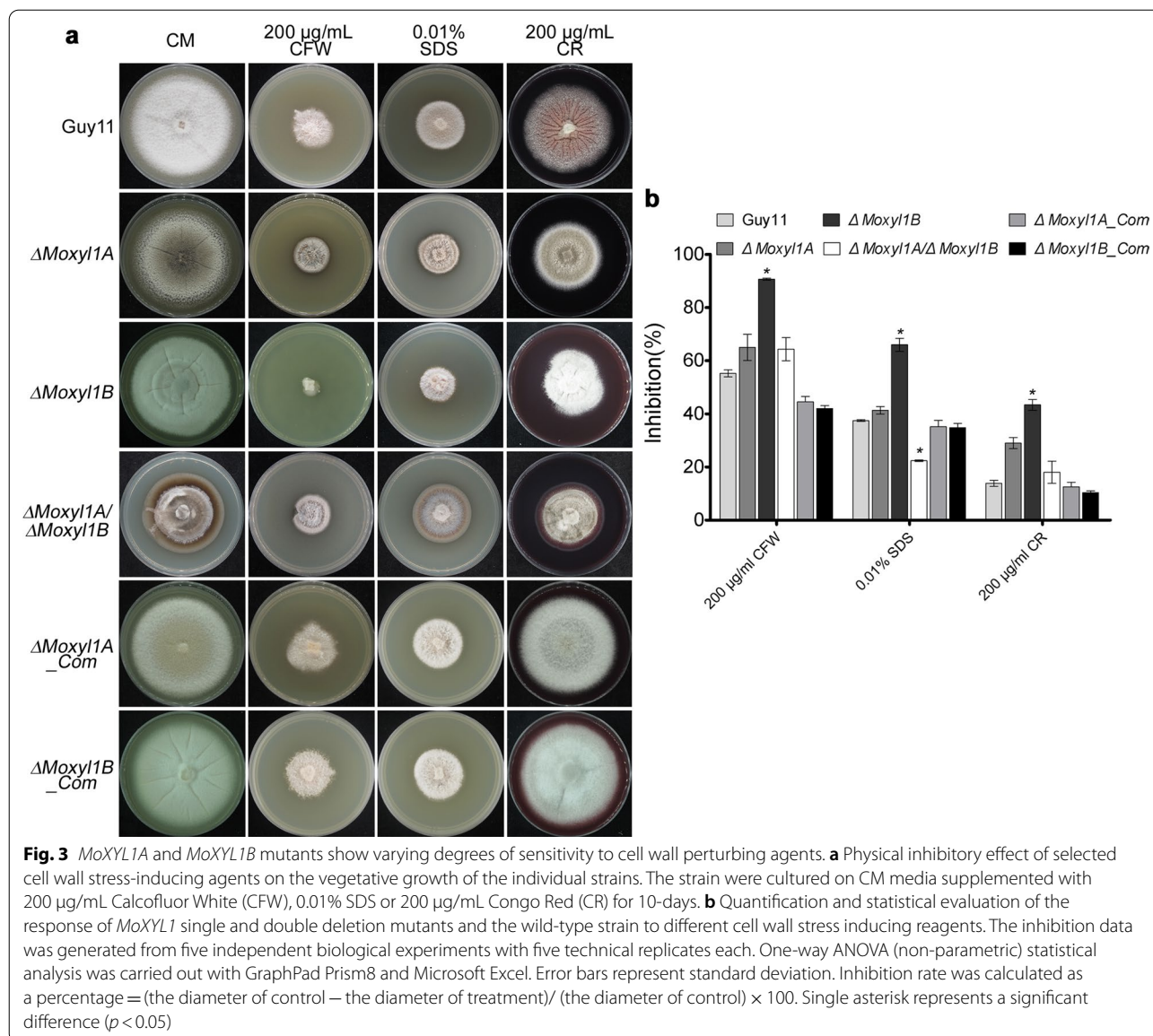


Fig. 2 (See legend on previous page.)

that both fusion proteins were mainly localized in the cytoplasm during vegetative and infectious development of the rice blast fungus (Fig. 4a, b). However, there was a weak GFP signal observed in conidia and the appressorium for MoXYL1B (Fig. 4b). To assess expression dynamics of these genes, the transcript levels of *MoXYL1A* and *MoXYL1B* were measured during host-plant interaction at varying intervals of infection. 6-week-old rice seedlings were infected with a spore suspension of WT *M. oryzae* and RNA was extracted from the infected

plants at 12 h, 24 h, 36 h, 72 h and 96 h post inoculation for qRT-PCR assessment of *MoXYL1A* and *MoXYL1B*. Results showed that both *MoXYL1A* and *MoXYL1B* were not expressed at the hyphal stage, since control mycelia did not have detectable transcripts and we infer therefore that the genes are expressed below the limit of detection. In early infection stages, the expression of *MoXYL1A* and *MoXYL1B* was down-regulated, suggesting that these genes do not play any key role in initiation of the infection cycle (Fig. 4c). However, *MoXYL1A* expression

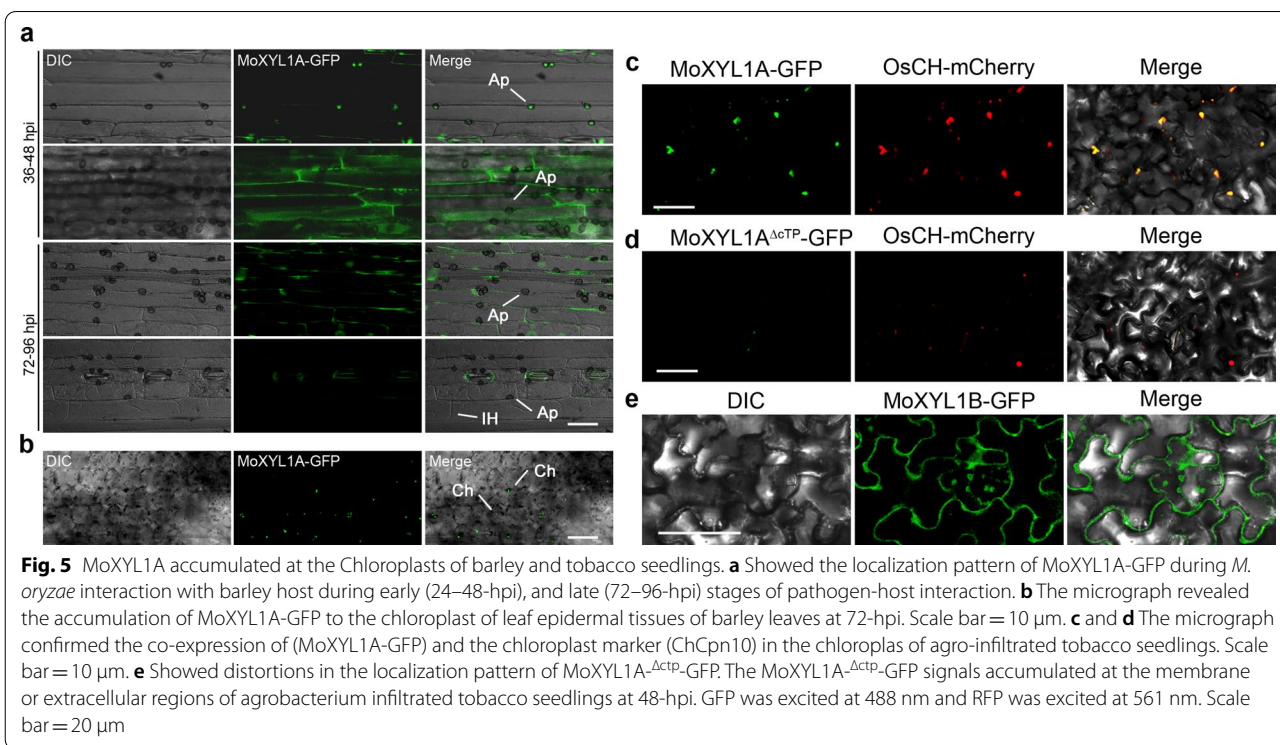
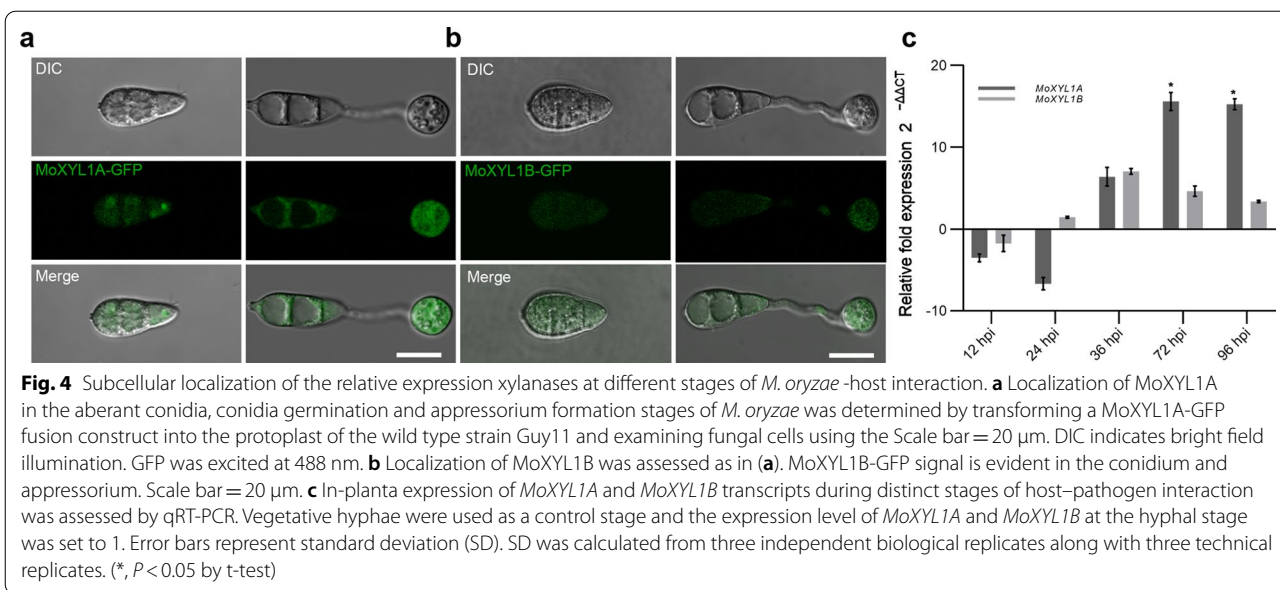


was significantly upregulated at 72-hpi, suggesting that *MoXYL1A* has some regulatory role in the later infection stages of the disease cycle. The expression profile of *MoXYL1B* was not highly dynamic, suggesting that it is unlikely to play a major role in the infection process and may instead have some other regulatory roles in the fungus independent of pathogenicity.

***M. oryzae* Likely Deploys *MoXYL1A* as Putative Cytoplasmic Effector Protein Targeting Host Chloroplast**

Magnaporthe oryzae mediates blast infection using appressorium-like structures produced on hyphal-tips (Kong et al. 2013). As noted earlier, the *MoXYL1* genes were annotated as non-expressed xylanases in a prior

study (Nguyen et al. 2011), which we posit was due to their potential secretion. To assess host localization of this effector protein, mycelial plugs from *M. oryzae* expressing *MoXYL1A*-GFP under its native promoter were used to inoculate barley plants and observed under a confocal microscope at different stages of disease development. Barley leaf sheath was peeled off to see the localization of the effector protein in host leaf cells. As fungal disease progressed through early stages, the invasive hyphae displayed GFP signal, and the effector protein was secreted out of hyphae at 72-hpi (Fig. 5a). At this time, the barley leaf was examined to track the translocation of effector proteins within the host, at which point it was trafficked to the chloroplast (Fig. 5b). The same



chloroplast localization was observed upon inoculation with spore suspension.

Furthermore, we endeavored to verify the localization of MoXYL1A to rice chloroplasts. An *Agrobacterium tumefaciens*-based MoXYL1A-GFP construct driven by the CaMV35s promoter was generated and transiently co-expressed with the rice chloroplast marker protein ChCPN10C-RFP, in *Nicotiana benthamiana*. Using

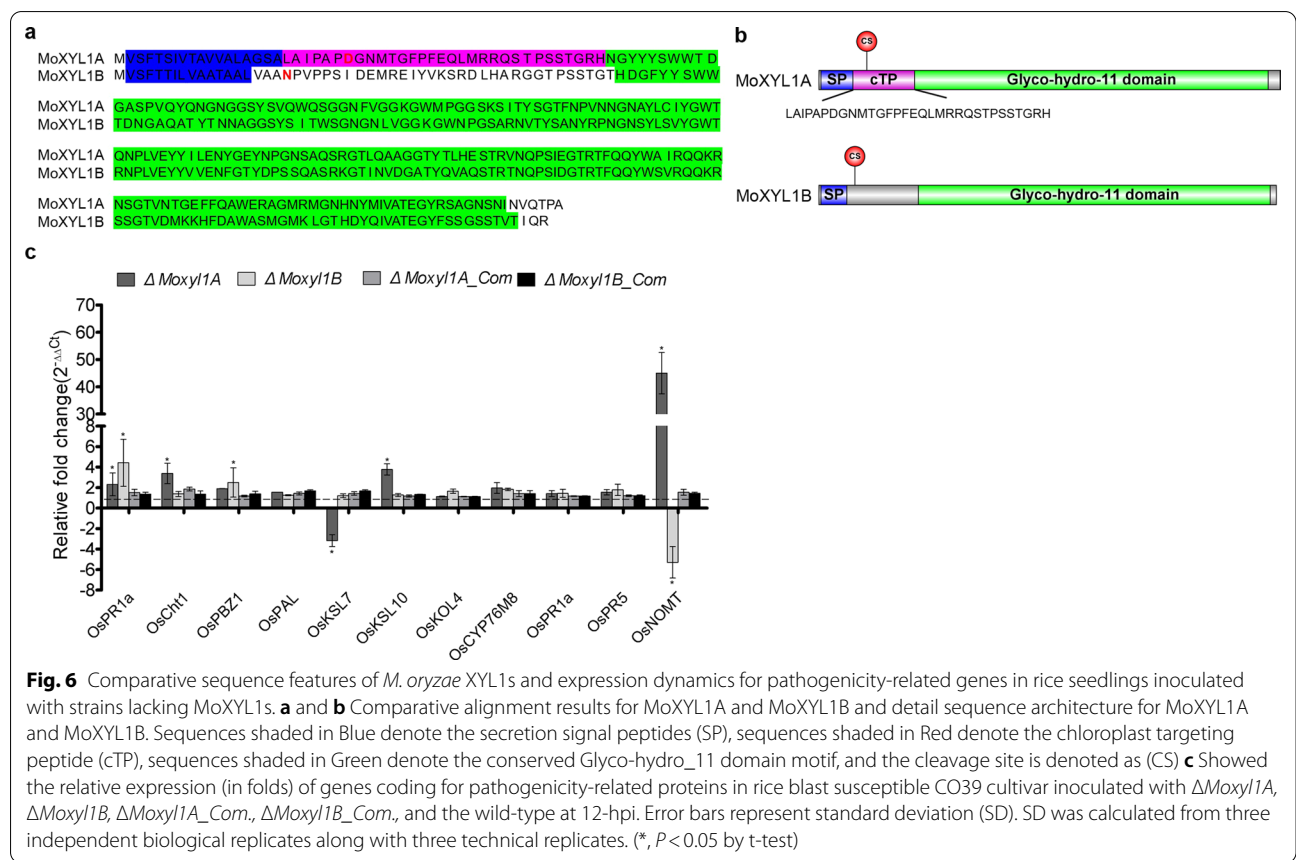
confocal microscopy to assess protein localization, at 48 hpi MoXYL1A-GFP and Ch-CPN10C-RFP were found to be co-localized in transfected tobacco cells, confirming the localization to the chloroplast of the effector protein (Fig. 5c). To ascertain the role of the chloroplast transit peptide in the chloroplast localization of the effector protein, we constructed GFP vectors with MoXYL1A lacking its chloroplast transit sequence (cTP) and co-expressed

MoXYL1A- Δ cTP-GFP with Os-CH-RFP (a rice chloroplast marker protein) in tobacco plants. The deletion of the 42-amino acid cTP from MoXYL1A-GFP resulted in no observable GFP signal, confirming the requirement of the transit peptide for proper localization or targeting of MoXYL1A to the chloroplast (Fig. 5d). Consistent with results obtained from bioinformatic analyses, microscopy examination of the localization of MoXYL1B in leaves of tobacco seedlings transfected with *Agrobacterium* strains harbouring the MoXYL1B-GFP constructs confirmed that MoXYL1B does not target any specific host organelle but instead accumulated at the periphery (extracellular region) of the host cells (Fig. 5e). We inferred that besides the promotion of vegetative growth, sporulation, and stress tolerance, MoXYL1A additionally functions as cytoplasmic effector protein that targets and possibly compromise the integrity of the chloroplast during pathogen-host interaction.

The Impact of MoXYL1 Genes Deletion on the Expression of Pathogenicity-Related Genes During *M. oryzae*-Rice Interaction

Finally, comparative analyses of protein sequences of MoXYL1A and MoXYL1B with SignalP 5.0 (Teufel et al.

2022) confirmed both MoXYL1A and MoXYL1B possess the N-terminal secretion signal peptide. Meanwhile, TargetP-assisted analysis (Armenteros et al. 2019) identified a chloroplast targeting peptide (cTP) exclusively in MoXYL1A (Fig. 6a, b). From these observations, we posited that beyond secretion, *M. oryzae* deploys XYL1A as an effector to target and possibly compromise the defense capabilities of the chloroplast during pathogen-host interaction. Furthermore, we examined the expression pattern of pathogenicity-related genes, including Probenazole-inducible protein PBZ1/PR10B (Os12t0555200), pathogenesis-related protein1/PR1A (Os07g0129200), Thaumatin-like pathogenesis-related protein3 precursor/PR5 (Os12g0628600), KAURENE SYNTHASE-LIKE_7/KSL7 (Os02g0570400), KAURENE SYNTHASE-LIKE_10/KSL10 (Os12g0491800), syn-pimaradiene 3-monooxygenase/KOL4 (Os06g0569500), CytochromeP450/CYP76M8 (Os02g0569400), NARINGENIN_7-O-METHYLTRANSFERASE/NOMT (Os12g0240900), Phenylalanine ammonia-lyase/ (Os05g0427400), and chitinase1/Cht1 (Os06g0726200) (Jeon et al. 2020; Nie et al. 2019) in 14–21-days old CO39 rice seedlings independently challenged with the individual strains at 12-hpi. These



examinations revealed a significant upregulation in the expression level of putative chloroplast localized PR genes (Additional file 1: Table S2), particularly, OsNOMT and OsKSL10 in rice seedlings inoculated with $\Delta Moxy1A$ (Fig. 6c). We inferred that deploying MoXYLs, especially MoXYL1A, possibly functions as a cytoplasmic effector that subverts host immunity by suppressing the expression of PR genes during pathogen-host interaction.

Discussion

MoXYL1A and *MoXYL1B* belong to the glycosyl hydrolase family GH11 (Wu et al. 2006). The GH11 family is the pathogen-encoded GH group encoding xylanases with high substrate specificity (Paës et al. 2012). Many phytopathogenic fungi employ cell wall degrading enzymes to colonize their host (Annis and Goodwin 1997; Reignault et al. 2008; Have et al. 2002; Mary Wanjiru et al. 2002). However, not all genes encoding xylan-degrading enzymes play a role in the pathogenesis of the fungi that encode them (Gómez-Gómez et al. 2002; Wu et al. 1997). Given this discrepancy, we sought to characterize two *M. oryzae* cell wall degrading enzymes in the current work: Endo β -1,4-xylanases I *MoXYL1A* and *MoXYL1B*.

Barley plants infected with *M. oryzae* expressing MoXYL1A-GFP were used to determine if this effector protein is secreted into host cells. Transfer of GFP signal from invasive hyphae to plant cells was evident at 72-hpi. Given bioinformatic predictions, we further confirmed that the protein is released into host plant chloroplasts. In contrast, the related effector MoXYL1B-GFP did not traffic to host chloroplasts and was found to remain cytoplasmic. Plant chloroplasts act as integrators of disease and defense responses (Stael et al. 2015), yet very few effector proteins have been reported to target chloroplasts (Jelenska et al. 2010; Petre et al. 2016). As most parasitic microbes feed on host plant carbon compounds and thereby increase demand for photosynthesis, plant chloroplasts represent a crucial target of pathogens (Chen et al. 2010). In future, it will be of great interest to assess the role of MoXYL1A in host chloroplasts to better understand the pathogenesis mechanisms of blast fungus.

Previous transcriptomic profiling results revealed a substantial reduction in the expression patterns of MoXYL1A and MoXYL1B (Endo- β 1,4-xylanases) during early invasive growth *M. oryzae in-planta* (Nguyen et al. 2011). This study, we observed that MoXYL1A and MoXYL1B possess secretion signal peptide. Further transcriptomic analyses of the expression pattern of *MoXYL1A* and *MoXYL1B* at different stages of *M. oryzae*-host interaction revealed about 3-folds increase in the expression

pattern of *MoXYL1A* at 72-hpi (late stages of invasive growth), meanwhile, there was no visible changes in the expression of *MoXYL1B* during vegetative and invasive growth of the rice blast fungus. Also, we demonstrated that, while the deletion of *MoXYL1B* has no adverse effects on the pathogenicity or virulence attributes of the defective strains, the deletion of *MoXYL1A* severely compromised the virulence of the rice blast fungus. From these results, we speculated that the expression, particularly during late stages of infection and possibly the secretion of MoXYL1A is likely crucial in the pathogenesis *M. oryzae*.

Individual knockout of two endoxylanase I, particularly XYL1B genes has no obvious adverse effects on the vegetative growth of *M. oryzae*. *MoXYL1A* deletion had a mild negative effect on fungal growth, while *MoXYL1B* deletion had no effect. However, the sexual spores of this pathogen (conidia) are known to be a key determinant of fungal virulence, asexual spores are readily transported from sporulation sites (blast lesions) diseased plants nearby onto healthy host by wind current or water drops resulting in the rapid dissemination blast infection. The disease severity of blast fungus is therefore proportional to the number of conidia produced in blast lesions (Teng et al. 1991). Although *MoXYL1A* and *MoXYL1B* both have important conidiogenesis-related roles in rice blast fungus, with *MoXYL1A* particularly being indispensable for the asexual process, with mutant strains forming both fewer and deformed conidia across multiple conidiation-inducing media. Results from Yeast-two-Hybrid assays (Y2H) suggest the absence of physical interaction the two xylanases in *M. oryzae* (Additional file 1: Fig. S3) indicating that MoXYL1A and MoXYL1B influence sporogenesis in the rice blast fungus possibly by modulating independent pathways.

Successful penetration into and colonization of the host are two main factors contributing to the virulence of a fungal pathogen. For *M. oryzae*, penetration occurs within the first 24 h post-infection (Lim et al. 2018; Sun et al. 2017). The virulence of *MoXYL1A* mutants was severely compromised on both barley and rice plants, with mutant strains unable to penetrate the host cell. We speculated that the defects in pathogenicity of the mutant strains were caused by the inability to form a functional appressorium. Appressorium formation in *M. oryzae* is triggered by various stimuli emanating from both the environment and the host. The formation of functional appressoria is an essential infection parameter in the disease cycle of rice blast fungus. Of the fewer conidia produced by *MoXYL1A* mutants, many (4/5) could not develop a normal appressorium. Also, the appressoria produced by these mutants failed to cause disease lesions on barley and rice plants. To further investigate the

deficiency in appressorium formation, we used artificial induction of appressorium on hydrophobic coverslips and found that the mutant appressorium lacked the characteristic melanin layer. This layer is involved in cell wall assembly (Howard et al. 1991). The deposition of melanin is an essential cellular phenomenon that facilitates the generation of optimum turgor pressure required to support the formation of penetration peg used to breach the leaf cuticle and lead to fungal colonization (Chumley and Valent 1990; Howard and Valent 1996). We demonstrated that the *MoXYL1A* mutant strains could not develop a proper host invasion machinery to enter and proliferate in the plant.

Fungal cell walls composed of a network of polysaccharides that play crucial roles in regulating the exchange of molecules between the cell and their environment (Jeon et al. 2008; Lipke and Ovalle 1998). Therefore, the sensitivity of *MoXYL1* defective strains to different types of cell-wall perturbing osmolytes indicated that both genes are vital for intact cell wall integrity. Also, the lack of melanin deposition in the cell wall of the *MoXYL1A* mutant strains likely accounted for the pronounced sensitivity of the defective strains to multiple stress-inducing agents. Also, the chloroplast contributes significantly to the enforcement of plant defense by facilitating the generation of diverse molecules, hormones, and proteins with antimicrobial or anti-parasitic properties (Kuzniak and Kopczewski 2020; Lu and Yao 2018). The observed up-regulations in the expression pattern of putative chloroplast destined PR genes, particularly in rice seedlings challenged with Δ *Moxy11A*, suggest *MoXYL1A*, likely mitigates the survival of *M. oryzae in-planta* partly by suppressing the expression PR genes during pathogen-host interaction. Also, from the observed accumulation of *MoXYL1B* apparently to the plasma membrane or apoplast region of *Agrobacterium* transfected leaf cells of tobacco seedlings, coupled with the absence of organelle targeting motif and the almost intact virulence characteristics recorded in the Δ *Moxy11B* strains, we intimated that the functions of *MoXYL1B* as a secreted hydrolytic enzyme does not impact directly on the infection or virulence characteristics of *M. oryzae*. Pathogenic microbes met with hostile and stress endowed environments that threaten their survival and influence their ability to invade and colonize host tissues. Invading pathogens deploy a vast array of strategies to counter resistance posed by the potential host plants. Here, we showed that xylanases, particularly *MoXYL1A*, do not only contribute significantly to the stress tolerance of *M. oryzae* during physiological development but also support the survival of the rice blast fungus in-planta either targeting and subverting chloroplast integrity as a whole or partly by suppressing the expression of chloroplast associated PR

genes during pathogen-host interaction. These observations underscore the potential significance of xylanases, especially *MoXYL1A* developing anti-blast strategies.

Conclusion

In conclusion, we identified and cloned two endoxylanase-encoding genes, *MoXYL1A* and *MoXYL1B*, and found that endoxylanase I has a critical role in the asexual reproduction of the blast fungus *M. oryzae*. *MoXYL1A* but not *MoXYL1B*, is required for full virulence of the fungus. Deletion of endoxylanase I also compromises the cell wall integrity of *M. oryzae*. Moreover, the putative effector protein *MoXYL1A* is translocated to plant chloroplasts, though *MoXYL1B* does not target any plant organelle and instead accumulates in the plant cytoplasm. It is still unclear what the molecular role of *MoXYL1A* is in host chloroplasts and further insight into its roles in plant defense remain to be addressed. We further suggest that the chloroplast transit peptide sequence of *MoXYL1A* is important for the pathogenicity of rice blast fungus. We therefore propose that there might be some chloroplast protein essential for the effector to function appropriately in fungal virulence.

Abbreviations

CM: Complete Medium; CR: Congo Red; CW: Calcofluor White; CWDEs: Cell wall degrading enzymes; GHs: Glycoside hydrolases; GH10: Glycoside hydrolase 10; GH11: Glycoside hydrolase 11; HPH: Hygromycin phosphotransferase; MM: Minimum media; DKO: Double deletion strain; WT: Wild-type; ANOVA: Analysis of variance; CFW: Calcofluor white; SDS: Sodium dodecyl sulphate; GFP: Green fluorescent protein; SD: Standard deviation; qRT-PCR: Quantitative real-time polymerase chain reaction; cTP: Chloroplast transit peptide; RFP: Red fluorescent protein; Y2H: Yeast-two-hybrid assays; RBM: Rice Bran Media; SDC: Straw Decoction and Corn Media; LB: Lysogeny Broth; BH and AH: A-fragment/hygromycin, and B-fragment/hygromycin; ORF: Open reading frame; UAH: Upstream sequence fused to hygromycin; SMD: Synthetic minimal with glucose; SML: Synthetic minimal with lactate; SMGal: Synthetic minimal with galactose; PR: Pathogenicity-related.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12284-022-00584-2>.

Additional file 1. Table S1. List of primers used in this study. **Table S2.** Predicted location of pathogenicity related protein in rice. **Figure S1.** Single copy insertion confirmed by Southern Blot. **Figure S2.** Complementation of *MoXYL1A* and *MoXYL1B* rescued the defects exhibited by the mutant strains. **Figures S3.** Y2H-mediated interaction pattern between putative *M. oryzae* xylanases A1 and 1B.

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Author contributions

JN, and ZW, conceived, designed and sourced for funding for the research, AS, WB, DY, LL, QA, CX, and SY performed the experiments. AS, WB, DY, and HG analysed the data. AS, WB, DY drafted the manuscript. SM, ZW, and NJ revised the manuscript. All authors contributed to the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval and Consent to Participate

This study complied with the ethical standards of China, where this research work was carried out.

Consent for Publication

All authors are consent for publication.

Competing interests

The authors declare that they have no competing interests.

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References

- Abdul W, Aliyu SR, Lin L, Sekete M, Chen X, Otieno FJ, Yang T, Lin Y, Norvi-nyeku J, Wang Z (2018) Family-four aldehyde dehydrogenases play an indispensable role in the pathogenesis of *Magnaporthe oryzae*. *Front Plant Sci* 9:980
- Akagi A, Jiang C-J, Takatsuji H (2015) *Magnaporthe oryzae* inoculation of rice seedlings by spraying with a spore suspension. *Bio-Protoc* 5(11):e1486–e1486
- Aliyu SR, Lin L, Chen X, Abdul W, Lin Y, Otieno FJ, Shabbir A, Batool W, Zhang Y, Tang W, Tang W (2019) Disruption of putative short-chain acyl-CoA dehydrogenases compromised free radical scavenging, conidiogenesis, and pathogenesis of *Magnaporthe oryzae*. *Fungal Genet Biol* 127:23–34
- Annis SL, Goodwin PH (1997) Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *Eur J Plant Pathol* 103(1):1–14
- Apel-Birkhold PC, Walton JD (1996) Cloning, disruption, and expression of two endo-beta 1, 4-xylanase genes, XYL2 and XYL3, from *Cochliobolus carbonum*. *Appl Environ Microbiol* 62(11):4129–4135
- Armenteros JJA, Salvatore M, Emanuelsson O, Winther O, Von Heijne G, Elofsson A, Nielsen H (2019) Detecting sequence signals in targeting peptides using deep learning. *Life Sci Alliance* 2(5):e201900429
- Beliën T, Van Campenhout S, Robben J, Volckaert G (2006) Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? *Mol Plant Microbe Interact* 19(10):1072–1081
- Bickle M, Delley P-A, Schmidt A, Hall MN (1998) Cell wall integrity modulates RHO1 activity via the exchange factor ROM2. *EMBO J* 17(8):2235–2245
- Biely P, Vršanská M, Tenkanen M, Kluepfel D (1997) Endo- β -1, 4-xylanase families: differences in catalytic properties. *J Biotechnol* 57(1–3):151–166
- Brito N, Espino JJ, González C (2006) The endo- β -1, 4-xylanase Xyn11A is required for virulence in *Botrytis cinerea*. *Mol Plant Microbe Interact* 19(1):25–32
- Cabib E, Roh D-H, Schmidt M, Crotti LB, Varma A (2001) The yeast cell wall and septum as paradigms of cell growth and morphogenesis. *J Biol Chem* 276(23):19679–19682
- Catlett NL, Lee B-N, Yoder O, Turgeon BG (2003) Split-marker recombination for efficient targeted deletion of fungal genes. *Fungal Genet Rep* 50(1):9–11
- Chen X-L (2018) Infection process observation of *Magnaporthe oryzae* on barley leaves. *Bio-Protoc* 8(9):1360–1376
- Chen J, Zheng W, Zheng S, Zhang D, Sang W, Chen X, Wang ZJPP (2008) Rac1 is required for pathogenicity and Chm1-dependent conidiogenesis in rice fungal pathogen *Magnaporthe grisea*. *4(11):e1000202*
- Chen L-Q, Hou B-H, Lalonde S, Takanaga H, Hartung ML, Qu X-Q, Chaudhuri B (2010) Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* 468(7323):527–532
- Chumley FG, Valent B (1990) Genetic analysis of melanin-deficient, non-pathogenic mutants of *Magnaporthe grisea*. *Mol Plant-Microbe Interact* 3(3):135–143
- Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol Rev* 29(1):3–23
- Dean RA, Talbot NJ, Ebbole DJ, Farman ML, Mitchell TK, Orbach MJ, Thon M, Kulkarni R, Xu JR, Pan H, Read ND (2005) The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* 434(7036):980–986
- Dean R, Van Kan JA, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD (2012) The Top 10 fungal pathogens in molecular plant pathology. *Mol Plant Pathol* 13(4):414–430
- Dornez E, Croes E, Gebruers K, De Coninck B, Cammue BP, Delcour JA, Courtin CM (2010) Accumulated evidence substantiates a role for three classes of wheat xylanase inhibitors in plant defense. *Crit Rev Plant Sci* 29(4):244–264
- Ebbole DJ (2007) *Magnaporthe* as a model for understanding host-pathogen interactions. *Annu Rev Phytopathol* 45:437–456
- Fernandez J, Marroquin-Guzman M, Wilson RA (2014) Mechanisms of nutrient acquisition and utilization during fungal infections of leaves. *Annu Rev Phytopathol* 52:155–174
- Gibson DM, King BC, Hayes ML, Bergstrom GC (2011) Plant pathogens as a source of diverse enzymes for lignocellulose digestion. *Curr Opin Microbiol* 14(3):264–270
- Gómez-Gómez E, Ruiz-Roldan M, Di Pietro A, Roncero M, Hera C (2002) Role in pathogenesis of two endo- β -1, 4-xylanase genes from the vascular wilt fungus *Fusarium oxysporum*. *Fungal Genet Biol* 35(3):213–222
- Have A, Tenberge KB, Benen JA, Tudzynski P, Visser J, van Kan JA (2002) The contribution of cell wall degrading enzymes to pathogenesis of fungal plant pathogens *Agricultural Applications*. Springer, Berlin, pp 341–358
- He P-H, Wang X-X, Chu X-L, Feng M-G, Ying S-H (2015) RNA sequencing analysis identifies the metabolic and developmental genes regulated by BbSNF1 during conidiation of the entomopathogenic fungus *Beauveria bassiana*. *Curr Genet* 61(2):143–152
- Howard RJ, Valent B (1996) Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annu Rev Microbiol* 50(1):491–512
- Howard RJ, Ferrari MA, Roach DH, Money NP (1991) Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc Natl Acad Sci* 88(24):11281–11284
- Igual J, Johnson AL, Johnston L (1996) Coordinated regulation of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity. *EMBO J* 15(18):5001–5013
- Jelenska J, Van Hal JA, Greenberg JT (2010) *Pseudomonas syringae* hijacks plant stress chaperone machinery for virulence. *Proc Natl Acad Sci* 107(29):13177–13182
- Jeon J, Goh J, Yoo S, Chi MH, Choi J, Rho HS, Park J, Han SS, Kim BR, Park SY, Kim S (2008) A putative MAP kinase kinase, MCK1, is required for cell wall integrity and pathogenicity of the rice blast fungus, *Magnaporthe Oryzae*. *Mol Plant-Microbe Interact* 21(5):525–534
- Jeon J, Lee GW, Kim KT, Park SY, Kim S, Kwon S, Huh A, Chung H, Lee DY, Kim CY, Lee YH (2020) Transcriptome profiling of the rice blast fungus *Magnaporthe oryzae* and its host *Oryza sativa* during infection. *Mol Plant Microbe Interact* 33(2):141–144
- Kong LA, Li GT, Liu Y, Liu MG, Zhang SJ, Yang J, Zhou XY, Peng YL, Xu JR (2013) Differences between appressoria formed by germ tubes and appressorium-like structures developed by hyphal tips in *Magnaporthe oryzae*. *Fungal Genet Biol* 56:33–41

- Kubicek CP, Starr TL, Glass NL (2014) Plant cell wall-degrading enzymes and their secretion in plant-pathogenic fungi. *Annu Rev Phytopathol* 52:427–451
- Kuźniak E, Kopczyński T (2020) The chloroplast reactive oxygen species-redox system in plant immunity and disease. *Front Plant Sci* 11:1798
- Lagaert S, Beliën T, Volckaert G (2009) Plant cell walls: protecting the barrier from degradation by microbial enzymes. Paper presented at the seminars in cell & developmental biology
- Lai M-W, Liou R-F (2018) Two genes encoding GH10 xylanases are essential for the virulence of the oomycete plant pathogen *Phytophthora parasitica*. *Curr Genet* 64(4):931–943
- Lim YJ, Kim KT, Lee YH (2018) SUMOylation is required for fungal development and pathogenicity in the rice blast fungus *Magnaporthe oryzae*. *Mol Plant Pathol* 19(9):2134–2148
- Lin L, Chen X, Shabbir A, Chen S, Chen X, Wang Z, Norviyenyku J (2019) A putative N-BAR-domain protein is crucially required for the development of hyphae tip appressorium-like structure and its plant infection in *Magnaporthe oryzae*. *Phytopathol Res* 1(1):1–15
- Lipke PN, Ovalle R (1998) Cell wall architecture in yeast: new structure and new challenges. *J Bacteriol* 180(15):3735–3740
- Lu Y, Yao J (2018) Chloroplasts at the crossroad of photosynthesis, pathogen infection and plant defense. *Int J Mol Sci* 19(12):3900
- Lussier M, White A-M, Sheraton J, di Paolo T, Treadwell J, Southard SB, Horenstein CI, Chen-Weiner J, Ram AF, Kapteyn JC, Roemer TW (1997) Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. *Genetics* 147(2):435–450
- Martin K, Kopperud K, Chakrabarty R, Banerjee R, Brooks R, Goodin MM (2009) Transient expression in *Nicotiana benthamiana* fluorescent marker lines provides enhanced definition of protein localization, movement and interactions in planta. *Plant J* 59(1):150–162
- Mary Wanjiru W, Zhensheng K, Buchenauer H (2002) Importance of cell wall degrading enzymes produced by *Fusarium graminearum* during infection of wheat heads. *Eur J Plant Pathol* 108(8):803–810
- Mori T, Jung H-Y, Maejima K, Hirata H, Himeno M, Hamamoto H, Namba S (2008) *Magnaporthe oryzae* endopolygalacturonase homolog correlates with density-dependent conidial germination. *FEMS Microbiol Lett* 280(2):182–188
- Nguyen QB, Itoh K, Van Vu B, Tosa Y, Nakayashiki H (2011) Simultaneous silencing of endo- β -1, 4 xylanase genes reveals their roles in the virulence of *Magnaporthe oryzae*. *Mol Microbiol* 81(4):1008–1019
- Nie H-Z, Zhang L, Zhuang H-Q, Shi W-J, Yang X-F, Qiu D-W, Zeng H-M (2019) The secreted protein MoHrip1 is necessary for the virulence of *Magnaporthe oryzae*. *Int J Mol Sci* 20(7):1643
- Norviyenyku J, Zhong Z, Lin L, Dang X, Chen M, Lin X, Wang Z, Abdul W (2017) Methylmalonate-semialdehyde dehydrogenase mediated metabolite homeostasis essentially regulate conidiation, polarized germination and pathogenesis in *Magnaporthe oryzae*. *Environ Microbiol* 19(10):4256–4277
- Paës G, Berrin J-G, Beaupré J (2012) GH11 xylanases: structure/function/properties relationships and applications. *Biotechnol Adv* 30(3):564–592
- Petre B, Lorrain C, Saunders DG, Win J, Sklenar J, Duplessis S, Kamoun S (2016) Rust fungal effectors mimic host transit peptides to translocate into chloroplasts. *Cell Microbiol* 18(4):453–465
- Rajeshwari, R., Jha, G., & Sonti, R. V. (2005). Role of an in planta-expressed xylanase of *Xanthomonas oryzae* pv. *oryzae* in promoting virulence on rice. *Molecular plant-microbe interactions*, 18(8), 830–837.
- Ram AF, Wolters A, Hoopen RT, Klis FM (1994) A new approach for isolating cell wall mutants in *Saccharomyces cerevisiae* by screening for hypersensitivity to calcofluor white. *Yeast* 10(8):1019–1030
- Raman V, Simon SA, Romag A, Demirci F, Mathioni SM, Zhai J, Meyers BC, Donofrio NM (2013) Physiological stressors and invasive plant infections alter the small RNA transcriptome of the rice blast fungus, *Magnaporthe oryzae*. *BMC Genomics* 14(1):1–18
- Ramonell KM, Zhang B, Ewing RM, Chen Y, Xu D, Stacey G, Somerville S (2002) Microarray analysis of chitin elicitation in *Arabidopsis thaliana*. *Mol Plant Pathol* 3(5):301–311
- Reignault P, Valette-Collet O, Boccara M (2008) The importance of fungal pectinolytic enzymes in plant invasion, host adaptability and symptom type. *Eur J Plant Pathol* 120(1):1–11
- Roeder DL, Collmer A (1985) Marker-exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. *J Bacteriol* 164(1):51–56
- Ryan CA, Farmer EE (1991) Oligosaccharide signals in plants: a current assessment. *Annu Rev Plant Biol* 42(1):651–674
- Scheller HV, Ulvskov P (2010) Hemicelluloses. *Annu Rev Plant Biol* 61:263–289
- Sella L, Gazzetti K, Faoro F, Odorizzi S, D'Ovidio R, Schäfer W, Favaron F (2013) A *Fusarium graminearum* xylanase expressed during wheat infection is a necrotizing factor but is not essential for virulence. *Plant Physiol Biochem* 64:1–10
- Shieh M-T, Brown RL, Whitehead MP, Cary JW, Cotty PJ, Cleveland TE, Dean RA (1997) Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. *Appl Environ Microbiol* 63(9):3548–3552
- Shimizu J, Yoda K, Yamasaki M (1994) The hypo-osmolarity-sensitive phenotype of the *Saccharomyces cerevisiae* hpo2 mutant is due to a mutation in PKC1, which regulates expression of β -glucanase. *Mol Gen Genet* 242(6):641–648
- Stael S, Kmiciek P, Willems P, Van Der Kelen K, Coll NS, Teige M, Van Breusegem F (2015) Plant innate immunity—sunny side up? *Trends Plant Sci* 20(1):3–11
- Sun D, Cao H, Shi Y, Huang P, Dong B, Liu X, Lin F, Lu J (2017) The regulatory factor X protein MoRfx1 is required for development and pathogenicity in the rice blast fungus *Magnaporthe oryzae*. *Mol Plant Pathol* 18(8):1075–1088
- Sweigard JA, Chumley FG, Valent B (1992) Disruption of a *Maanaporthe arisea* cutinase gene. *Mol Gen Genet* 232(2):183–190
- Talbot NJ (2003) On the trail of a cereal killer: exploring the biology of *Magnaporthe grisea*. *Annu Rev Microbiol* 57(1):177–202
- Teng P, Klein-Gebbinck H, Pinnschmidt H (1991) An analysis of the blast pathosystem to guide modeling and forecasting. Paper presented at the Rice blast modeling and forecasting. Selected papers from the international rice research conference, 27–31 Aug 1990, Seoul, Korea Republic
- Teufel F, Almagro Armenteros JJ, Johansen AR, Gislason MH, Pihl SI, Tsirigos KD, Winther O, Brunak S, von Heijne G, Nielsen H (2022) SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nat Biotechnol* 1–3
- Walton JD (1994) Deconstructing the cell wall. *Plant Physiol* 104(4):1113
- Wang Q, Han C, Ferreira AO, Yu X, Ye W, Tripathy S, Kale SD, Gu B, Sheng Y, Sui Y, Wang X (2011) Transcriptional programming and functional interactions within the *Phytophthora sojae* RXLR effector repertoire. *Plant Cell* 23(6):2064–2086
- Wang L, Ding X, Xiao J, Jiménez-Góngora T, Liu R, Lozano-Durán R (2017) Inference of a geminivirus–host protein–protein interaction network through affinity purification and mass spectrometry analysis. *Viruses* 9(10):275
- Win J, Chaparro-García A, Belhaj K, Saunders D, Yoshida K, Dong S, Schornack S, Zupfel C, Robatzek S, Hogenhout SA, Kamoun S (2012) Effector biology of plant-associated organisms: concepts and perspectives. Paper presented at the Cold Spring Harbor symposia on quantitative biology
- Wood PJ, Fulcher R (1983) Dye interactions. A basis for specific detection and histochemistry of polysaccharides. *J Histochem Cytochem* 31(6):823–826
- Wu S-C, Ham K-S, Darvill AG, Albersheim P (1997) Deletion of two endo- β -1, 4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. *Mol Plant Microbe Interact* 10(6):700–708
- Wu S-C, Halley JE, Luttig C, Fernekes LM, Gutiérrez-Sánchez G, Darvill AG, Albersheim P (2006) Identification of an endo- β -1, 4-D-xylanase from *Magnaporthe grisea* by gene knockout analysis, purification, and heterologous expression. *Appl Environ Microbiol* 72(2):986–993
- Yu Y, Xiao J, Du J, Yang Y, Bi C, Qing L (2016) Disruption of the gene encoding endo- β -1, 4-xylanase affects the growth and virulence of *Sclerotinia sclerotiorum*. *Front Microbiol* 7:1787
- Yu C, Li T, Shi X, Saleem M, Li B, Liang W, Wang C (2018) Deletion of endo- β -1, 4-xylanase VmXyl1 impacts the virulence of *Valsa mali* in apple tree. *Front Plant Sci* 9:663
- Zhang H, Zhao Q, Guo X, Guo M, Qi Z, Tang W, Dong Y, Ye W, Zheng X, Wang P, Zhang Z (2014) Pleiotropic function of the putative zinc-finger protein MoMsn2 in *Magnaporthe oryzae*. *Mol Plant Microbe Interact* 27(5):446–460
- Zhang X, Wang G, Yang C, Huang J, Chen X, Zhou J, Li G, Norviyenyku J, Wang Z (2017) A HOPS protein, MoVps41, is crucially important for vacuolar morphogenesis, vegetative growth, reproduction and virulence in *Magnaporthe oryzae*. *Front Plant Sci* 8:1091

Zhang L, Zhang D, Chen Y, Ye W, Lin Q, Lu G, Ebbole DJ, Olsson S, Wang Z (2019) *Magnaporthe oryzae* CK2 Accumulates in Nuclei, Nucleoli, at Septal Pores and Forms a Large Ring Structure in Appressoria, and Is Involved in Rice Blast Pathogenesis. *Front Cell Infect Microbiol* 9:113

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