

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 Δ *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*.

Background

The plant cell wall, composed of celluloses, hemicelluloses, and pectin, is the first obstacle a pathogen encounters in plant-pathogen interaction (Kubicek, Starr, & Glass, 2014). For this reason, pathogens produce and secrete an array of plant cell wall degrading enzymes to weaken and overcome this initial barrier (Brito, Espino, & González, 2006; Fernandez, Marroquin-Guzman, & Wilson, 2014; Kubicek et al., 2014; Mori et al., 2008; Nguyen, Itoh, Van Vu, Tosa, & Nakayashiki, 2011; Oeser et al., 2002; Shieh et al., 1997; Skamnioti & Gurr, 2007; Van Vu, Itoh, Nguyen, Tosa, & Nakayashiki, 2012; Win et al., 2012; Wu et al., 2006). Cell wall degrading enzymes (CWDEs) are key virulence factors for pathogens as they help not only in host cell invasion but 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 (Felix, Regenass, & Boller, 1993; Ryan & Farmer, 1991; B. Zhang, Ramonell, Somerville, & Stacey, 2002). CWDEs play an essential role in the disease cycle of fungi that lack special penetration machinery, but all plant pathogenic fungi require these enzymes for later infection stages (Gibson, King, Hayes, & Bergstrom, 2011). The first CWDE found to be necessary for virulence was pectate lyase in *Erwinia chrysanthemi* (Roeder & Collmer, 1985), followed by endo polygalacturonase in *Aspergillus flavus* (Shieh et al., 1997) and ethylene inducing xylanase in *Trichoderma spp.* (Beliën, Van Campenhout, Robben, & Volckaert, 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, Gerday, & Feller, 2005). It consists of a 1,4-linked D-Xylp backbone with side branches of AraF and GlcpA (Scheller & 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, Vršanská, Tenkanen, & Kluepfel, 1997; Collins et al., 2005; Lagaert, Beliën, & Volckaert, 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 (Noda, Brito, & González, 2010). The xylanase encoding genes in *C. carbonum* (Apel-Birkhold & Walton, 1996), *F. oxysporum* (Gómez-Gómez et al., 2002) and *F. graminearum* (Sella et al., 2013) do not have an 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, Jha, & Sonti, 2005), *xyn11A* in *Botrytis cinerea* (Brito et al., 2006), SsXyl1 in *Sclerotinia sclerotiorum* (Y. Yu et al., 2016) and VmXyl1 in *Valsa mali* (C. 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 & Liou, 2018).

The blast fungus *Magnaporthe oryzae* (syn *Pyricularia oryzae*) is a hemibiotrophic filamentous ascomycete threatening worldwide rice and wheat production (R. 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 8MPa 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; Q. 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 (R. A. 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 6g/L, Casamino acid 6g/L, Sucrose 10g/L, and Agar 20g, dissolved in double distilled water), supplemented with antibiotic (streptomycin 100 μ g/100mL), under standard incubation conditions of 28°C (J. Chen et al., 2008).

For sporulation assays, Rice Bran Media (RBM; Rice bran 40g/L and 15g, dissolved in dd water with pH adjusted to 6) (L. Zhang et al., 2019), Straw Decoction and Corn media (SDC; Rice straw 200g, corn agar 40g/L, 15g agar in 1L double distilled water) (Y. Chen et al., 2017) and CM-II medium (50 mL 20 x 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) (Y. Chen et al., 2018) were used. Culture plates were kept in dark conditions for seven days, followed by scratching hyphae and exposing the plates for three days to fluorescent light at 28°C (Abdul et al., 2018; Aliyu et al., 2019; X. 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 (X.-L. Chen, 2018). Mycelial plugs were prepared by shaking small pieces of the strains in liquid CM in a 28° shaking incubator at 120 rpm for three 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: 24h, 36h, 48h, 72h 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: 8/16h night/day at 22°C. The effector protein genes *MoXYL 1A/B* (amplified with the primer pairs pGDG-F/R given in Table 1, 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 (200µl/100ml Rifampicin and 100µl/100ml 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 Table 1, 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 10mM MES, 10mM MgCl₂, and 150 µM acetosyringone in sterilized double distilled water). The pGDG and pGDR strains were combined and incubated at room temperature for 2-3h. The strain suspension was inoculated on 6-week-old tobacco plants following the standard protocol of infection (L. Wang et al., 2017; Q. Wang et al., 2011). The infected plants were kept in the dark for 48h, and then the expression of GFP and RFP fluorescent proteins was observed under a confocal microscope at 488nm 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 1.1kb upstream (A fragment) and 1.1kb downstream (B fragment) of both *MoXYL 1A* and *MoXYL 1B* 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 Table 1. The same approach was used for MoXYL1B using the respective primers provided in Table 1. PEG-mediated fungal transformation was carried out using the Guy11 protoplast (Sweigard, Chumley, & Valent, 1992). The hygromycin-resistant transformants were screened with ORF and UAH primers (Table 1) to identify candidate mutant strains. Southern blotting was performed to confirm gene replacement following the protocol given by (Norvienyeku et al., 2017). To generate double deletion mutants, the protocol given by (Lin et al., 2019) was followed.

For complementation, 3kb upstream and the full-length ORF, excluding the stop codons were amplified with the primer pair MoXYL1A and MoXYL1B-Comp-F and R (Table 1) 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 ($\Delta Moxy/1A$ and $\Delta Moxy/1B$), and the complemented strains were cultured in liquid CM, for three 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 (X.-L. Chen, 2018) with slight modification. Incubation conditions were: 24h in the dark, followed by six days in 12h 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 seven days. Hyphae were scratched off, and plates were exposed to fluorescent light for three 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, Jiang, & Takatsuji, 2015). The inoculated rice plants were kept in a humid, dark growth room for the first 24h. 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 ($5 \times 10^4 \text{ ml}^{-1}$), with 0.2% Tween 20 and a 20 μl micropipette 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 24h, 36h, 48h, and 72h under the optical microscope.

Appressorium formation assays

20 μl conidial suspension ($5 \times 10^4 \text{ ml}^{-1}$) from wild type, mutants ($\Delta Moxy/1A-3$ and $\Delta Moxy/1A-13$) 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 2h, 4h, 6h, and 8h, respectively.

Conidiophorogenesis 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 12h, 24h, 36h, and 48h. 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 $\mu\text{g}/\text{mL}$) or Congo Red (CR, 200 $\mu\text{g}/\text{mL}$) 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 (H. 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 (Norvienyeku 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 ($\Delta Moxy1A$ and $\Delta Moxy1B$) were subjected to reverse transcription using SYBR® Premix Ex. Taq™ (TliRNaseH Plus). A reaction mixture of 25 μ L was formulated using 12.5 μ L of Premix Ex-Taq and 1 μ L of each 10 μ M primer (Table 1) and 1 μ L 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 $\Delta Moxy1$ 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, Lee, Yoder, & Turgeon, 2003). Putative *MoXYL1A* and *MoXYL1B* gene deletion transformants were selected on double layered TB3 agar containing 300 μ g/mL (bottom layer) and 600 μ g/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 (Supplementary Figure 1). These assays confirmed the successful replacement of the *MoXYL1A* and *MoXYL1B* genes with *hph* in these strains (Supplementary Figure 1). 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 $\Delta Moxy11A$ and $\Delta Moxy11B$ 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 (Figure 1a-b, Supplementary Figure 2a-b). However, a noticeable reduction in aerial hyphae and minimal but statistically significant difference in colony diameter was observed for $\Delta Moxy11A$ compared to WT. In contrast, there was no significant difference between $\Delta Moxy11B$ and WT. The double deletion strain (DKO) was obtained via HR-based deletion of *MoXYL1B* on the $\Delta Moxy11A$ 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 conidiophorogenesis 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, Wang, Chu, Feng, & Ying, 2015). Small plugs of the mutant and wild type strains, along with the complemented and double knock out strains were grown on rice bran medium plates and incubated under dark conditions at 28°C for seven days. On day 7, the hyphae were scratched and incubated under light conditions, and conidiophore production was visually observed at 12h, 24h, 36h, and 48h. 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 $\Delta Moxy11A$ and $\Delta Moxy11A/\Delta Moxy11B$ strains were severely impaired in conidiophore production, with almost no conidia produced, while $\Delta Moxy11B$ produced conidiophores of WT shape but in reduced number relative to WT (Figure 1d). To further corroborate this defect, mutant and wild type strains were also grown on SDC and CM-II media and conidia were counted (Supplementary Figure 2). 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 $\Delta Moxy11A$ 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 (Supplementary Figure 2).

MoXYL1A is required for complete virulence of *M. oryzae*

A susceptible rice cultivar CO39 and detached leaves of 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. Fungal mycelia cultured in liquid CM media from wild type, mutants, complementation, and double deletion strains were inoculated on intact and abraded detached leaves of barley and kept under proper humidity conditions at 28°C for 24 h of darkness preceded by six days exposure to light. This assay showed that fungal virulence was impaired in $\Delta Moxy11A$ and DKO strains, which were unable to produce proficient blast lesions, while $\Delta Moxy11B$ produced typical blast lesions (Figure 2a). This suggests that *MoXYL1B* is dispensable for 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 $\Delta Moxy11A$ conidia as were observed with mycelia. Virulence defects were rescued in the complemented strain (Figure 2a). These results support the key 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 Guy11 and *MoXYL 1A* or *MoXYL 1B* mutant strains were independently and evenly sprayed on rice leaves, and plants were kept under proper incubation conditions (see Methods) for seven days. This rice pathogenicity trial showed consistent results with the barley experiments, with $\Delta Moxy1A$ and $\Delta Moxy1A/\Delta Moxy1B$ strains completely lacking virulence as compared to wild type and complemented strains (Figure 2b).

To unravel the factors responsible for the impairment in pathogenicity of the *MoXYL 1A* 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 *MoXYL 1A* 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 WT micrographs showed pronounced invasive hyphae that were branched and colonizing adjacent cells. These results confirmed the inability of *MoXYL 1A* mutants to invade host plants and cause blast disease. Consistent with earlier results, no penetration defects were observed for the *MoXYL 1B* deletion (Figure 2c).

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 differentiation 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 8h 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 (Figure 2d). This phenotype was rescued by complementation of *MoXYL 1A*. The *MoXYL 1B* 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.

$\Delta Moxy1A$ 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, Roh, Schmidt, Crotti, & Varma, 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 $\Delta Moxy1$ strains. Calcofluor White (CFW) is 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, Wolters, Hoopen, & Klis, 1994). Sodium dodecyl sulphate (SDS) is a detergent that compromises membrane stability, and as any cell wall defects increase the vulnerability of the plasma membrane to SDS, sensitivity can indicate problems with the cell wall (Bickle, Delley, Schmidt, & Hall, 1998; Igual, Johnson, & Johnston, 1996; Shimizu, Yoda, & Yamasaki, 1994). Congo Red (CR) is an additional cell wall stress reagent (Wood & Fulcher, 1983). We supplemented CM culture media with Calcofluor White (200 μ g/ml CFW), Congo Red (200 μ g/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 *MoXYL 1B* phenotype to approximate that of the *MoXYL 1A* single mutant, suggesting

that the absence of *MoXYL1A* improves stress tolerance of the *1B* mutant in *M. oryzae* (Figure 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 localization 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 that both fusion proteins were mainly localized in the cytoplasm during vegetative and infectious development of the rice blast fungus (Figure 4a-b). However, there was a weak GFP signal observed in conidia and the appressorium for MoXYL1B (Figure 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 12h, 24h, 36h, 72h and 96h 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 (Figure 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.

Magnaporthe oryzae mediates blast infection using appressorium-like structures produced on hyphal-tips (Kong et al., 2013; Yin et al., 2016). 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 (Figure 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 (Figure 5b). The same chloroplast localization was observed upon inoculation with spore suspension in place of mycelia.

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 (Figure 6a). 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-D_{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 (Figure 6b). In contrast, bioinformatic tools predicted MoXYL1B to be a non-organelle targeting protein,

and our tobacco infection results with MoXYL1B-GFP confirmed that it does not target any specific host organelle but instead localizes to the periphery of the host cells (Figure 6c).

Discussion

MoXYL 1A and *MoXYL 1B* 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, Berrin, & Beaugrand, 2012). Many phytopathogenic fungi employ such cell wall degrading enzymes to colonize their host (Annis & Goodwin, 1997; Reignault, Valette-Collet, & Boccara, 2008; ten Have et al., 2002; Wanjiru, Zhensheng, & Buchenauer, 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, Ham, Darvill, & Albersheim, 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 *MoXYL 1A* and *MoXYL 1B*.

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, Van Hal, & Greenberg, 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 (L.-Q. 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). In this study, we observed that MoXYL1A and MoXYL1B possess secretion signal peptide. Further transcriptomic analyses of the expression pattern of *MoXYL 1A* and *MoXYL 1B* at different stages of *M. oryzae*-host interaction revealed about 3-folds increase in the expression pattern of *MoXYL 1A* at 72 hpi (late stages of invasive growth), meanwhile, there was no visible changes in the expression of *MoXYL 1B* during vegetative and invasive growth of the rice blast fungus. Also, we demonstrated that, while the deletion of *MoXYL 1B* has no adverse effects on the pathogenicity or virulence attributes of the defective strains, the deletion of *MoXYL 1A* 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 mutants of these two endoxylanase I genes in *M. oryzae* were not defective in vegetative growth. *MoXYL 1A* deletion had a mild negative effect on fungal growth, while *MoXYL 1B* deletion had no effect. However, the sexual spores of this pathogen (conidia) are known to be a key determinant of fungal virulence, beginning the infection cycle upon their dissemination from blast lesions to nearby plants and accounting for disease spread with 5-7 days. The disease severity of blast fungus is therefore proportional to the number of conidia produced in blast lesions (Teng, Klein-Gebbinck, & Pinnschmidt, 1991). Although *MoXYL 1A* and *MoXYL 1B* both have important conidiogenesis-related roles in rice blast fungus, with *MoXYL 1A* 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* (Supplementary Fig. 3) 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 hours post infection (Lim, Kim, & Lee, 2018; Sun et al., 2017). The virulence of *MoXYL 1A* 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 response to various stimuli from the environment and the host, is an essential step in the disease cycle of rice blast fungus. Of the fewer conidia produced by *MoXYL 1A* mutants, many (4/5) were unable to develop a normal appressorium and the appressoria produced by these mutants were unable to cause any disease lesions on barley and rice plants. To more deeply investigate this appressorium formation deficiency, 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, Ferrari, Roach, & Money, 1991), as melanin deposition is essential for turgor pressure generation, and this turgor pressure is required for the further formation of the penetration peg used to breach the leaf cuticle and lead to fungal colonization (Chumley & Valent, 1990; Howard & Valent, 1996). Therefore, in sum we found that the *MoXYL 1A* mutant strains could not develop a proper host penetration machinery to enter and proliferate in the plant.

Fungal cell wall is 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 & Ovalle, 1998). Therefore, the sensitivity of *MoXYL 1* defective strains to a plethora of cell-wall perturbing osmolytes indicated that both genes are vital for intact cell wall integrity. Also, the lack of melanin deposition, the cell wall of the *MoXYL 1A* mutant strains likely accounted for the pronounced sensitivity of the defective strains to multiple stress-inducing agents. Since, the survival of pathogenic microbes in the hostile and stress endowed environment of the host cells, these observations suggest xylanases contribute significantly to stress tolerance of *M. oryzae* and underscored their potentials especially *MoXYL 1A* developing anti-blast strategies.

Conclusion

In conclusion, we identified and cloned two endoxylanase-encoding genes, *MoXYL 1A* and *MoXYL 1B*, and found that endoxylanase I has a critical role in the asexual reproduction of the blast fungus *M. oryzae*. *MoXYL 1A* but not *MoXYL 1B*, 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 *MoXYL 1A* is translocated to plant chloroplasts, though *MoXYL 1B* does not target any plant organelle and instead accumulates in the plant cytoplasm. It is still unclear what the molecular role of *MoXYL 1A* 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 *MoXYL 1A* 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

Abbreviations	Definition
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 Sequance fused to Hygromycin
SMD	Synthetic Minimal with Glucose
SML	Synthetic Minimal with Lactate
SMGal	Synthetic Minimal with Galactose

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.

Availability of data and material

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

Competing Interests

The authors declare that they have no competing interests.

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AUTHOR CONTRIBUTIONS

J. N., and Z.W., conceived, designed and sourced for funding for the research, A.S., W.B., D.Y., L.L., A.A., C.X., and S.Y. performed the experiments. A.S., W.B., D.Y., and H.G. analysed the data. A.S., W.B., D.Y. drafted the manuscript. S.M., Z.W., and N.J. revised the manuscript. All authors contributed to the final manuscript.

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Tables

Table 1 is available in the Supplemental Files section

Figures

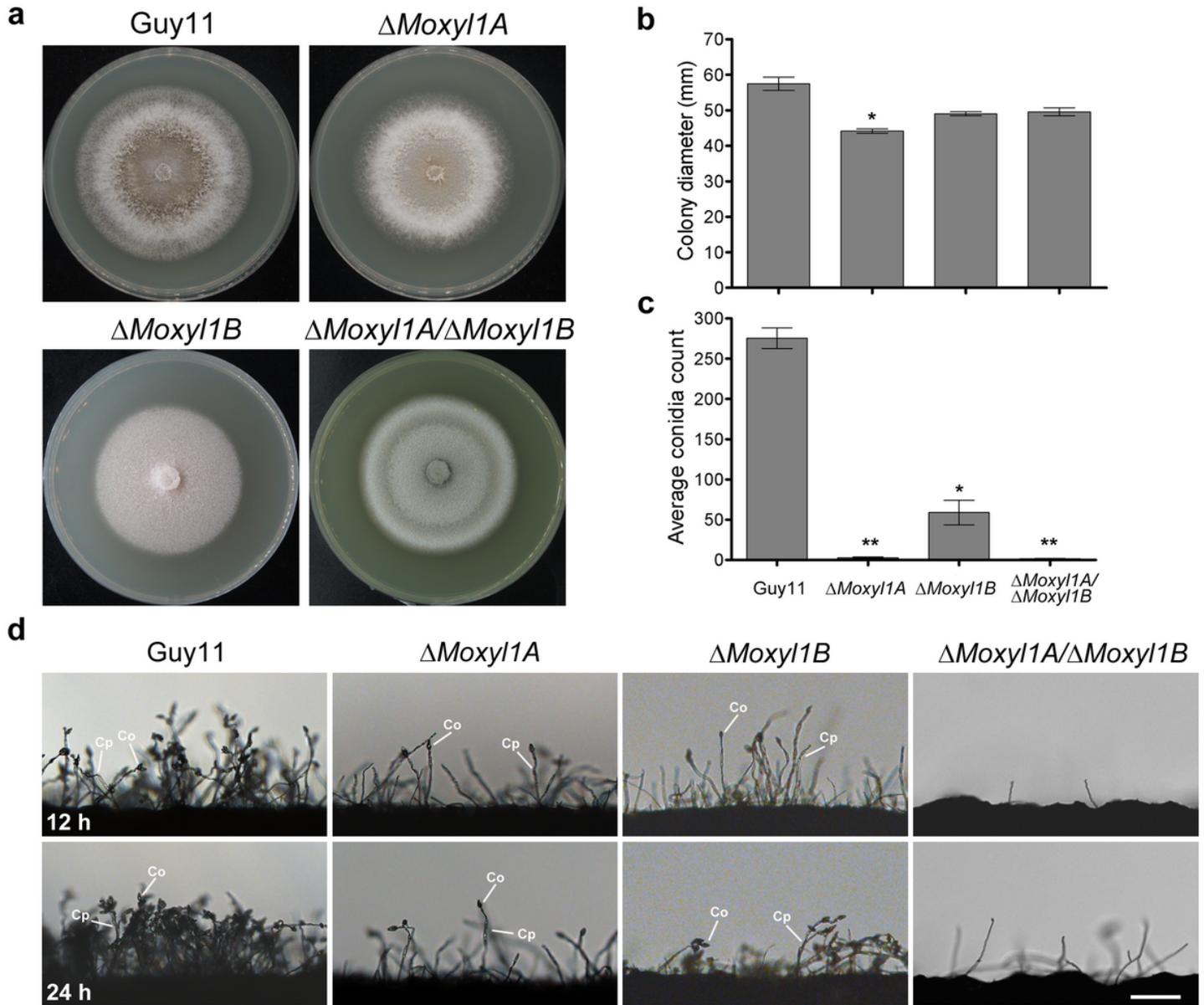


Figure 1

Impact of MoXYL1 gene deletion on colony morphology and infectious growth of *M. oryzae*. (a) Strains of the indicated genotype were inoculated on CM media and photographed after 10 days of growth. (b) Statistical analysis of average colony diameter (mm) from three independent biological experiments with five replicates each. One-way ANOVA (non-parametric) was employed to assess statistical significance. Error bars account for standard deviation and asterisks represent the significant difference between wild type Guy11 and the mutant strain ($p < 0.001$). (c)

Quantitation and statistical analysis of conidia production in Δ Moxy11 strains relative to Guy11, obtained from cultures grown on Rice Bran Media, Straw Decoction and Corn media and CM-II media, respectively, from five independent biological experiments with five replicates. The data was analyzed with GraphPad Prism5; error bars represent the standard deviation,, while a single asterisk (*) represent significant differences ($p < 0.05$) and double asterisks (**) represent significant differences ($p < 0.001$) according to ordinary one-way ANOVA. (d) Depicts drastically reduced ability of conidiophorogenesis of Δ Moxy1A genes as compared to Guy11 at 12h and 24h interval.

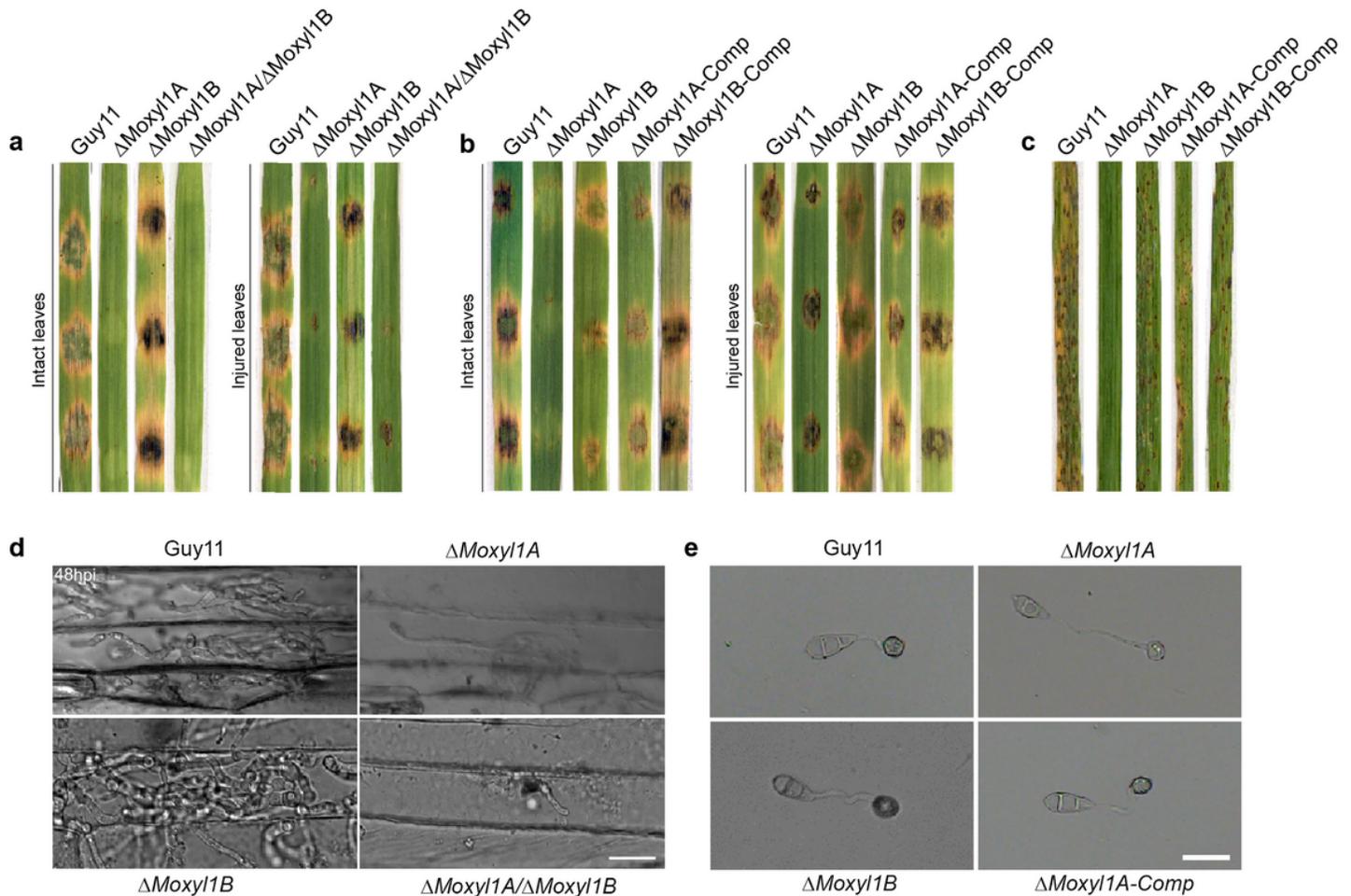


Figure 2

Targeted gene replacement of MoXYL1A severely impaired the virulence. (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 three-week-old rice seedlings by spray 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 Guy11. Photos were taken at the inability of Δ Moxy1A and double deletion mutants to invade and colonize barley tissue at 48hpi. No invasive hyphae were evident inside barley cells inoculated with Δ Moxy1A or the double deletion strain, while prominent invasive hyphae were seen in the leaves inoculated with either Δ Moxy1B or Guy11. Images are representative of $n = 2$ independent biological replicates. Scale bar, 20 μm . (c) Appressoria were produced artificially on Thermo-fisher hydrophobic coverslips and observed at 8 hpi. Images show non-functional appressoria lacking melanin lining for the Δ Moxy1A mutant.

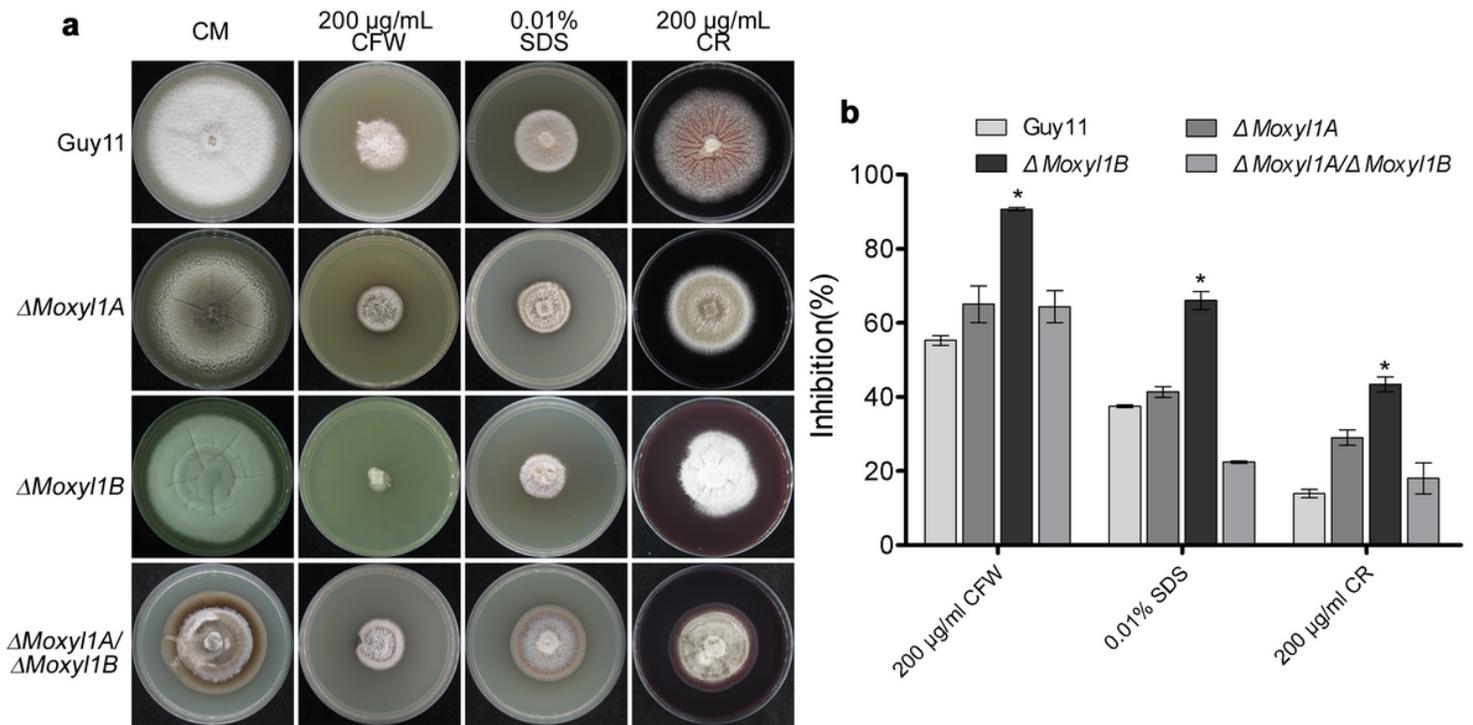


Figure 3

MoXYL1A and MoXYL1B mutants show varying degrees of sensitivity to cell wall perturbing agents (a) Physical inhibitory effect of selected cell wall stress-inducing agents on the vegetative growth of the individual strains. The strain were cultured on CM media supplemented with 200 µg/mL Calcofluor White (CFW), 0.01% SDS or 200 µg/mL Congo Red (CR) for 10 days. (b) Quantification and statistical evaluation of the response of MoXYL1 single and double deletion mutants and the wild-type strain to different cell wall stress inducing reagents. The inhibition data was generated from five independent biological experiments with five technical replicates each. One-way ANOVA (non-parametric) statistical analysis was carried out with GraphPad Prism8 and Microsoft Excel. Error bars represent standard deviation. Inhibition rate was calculated as a percentage = (the diameter of control - the diameter of treatment) / (the diameter of control) × 100. Single asterisk represents a significant difference ($p < 0.05$).

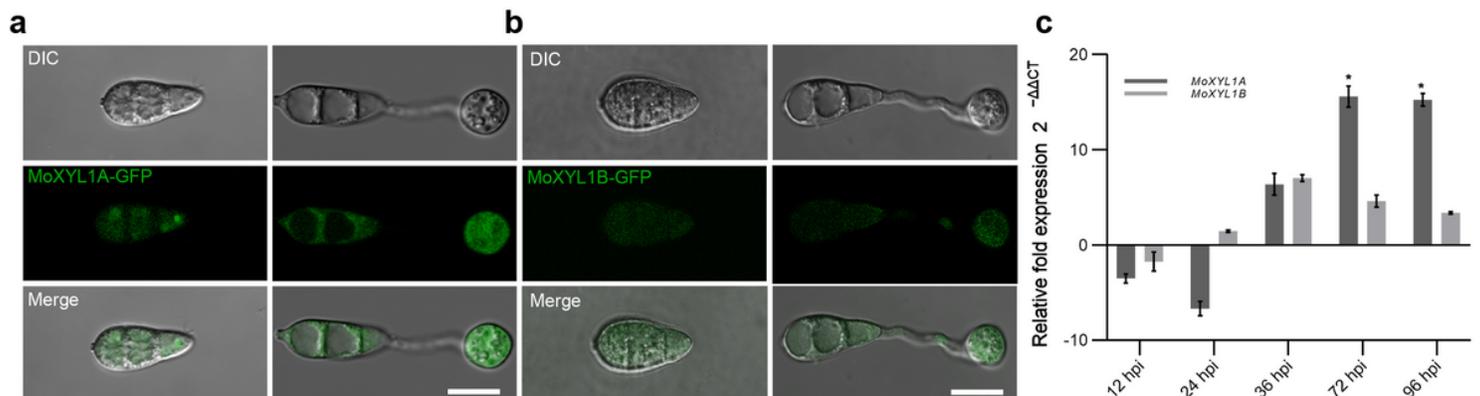


Figure 4

Subcellular localization of the relative expression xylanases at different stages of *M. oryzae*-host interaction. (a) Localization of MoXYL1A in *M. oryzae* at all developmental stages was determined by transforming a MoXYL1A-GFP

fusion construct into the protoplast of the wild type strain Guy11 and examining fungal cells using the Scale bar = 20 μm . DIC indicates bright field illumination. GFP was excited at 488 nm. (b) Localization of MoXYL1B was assessed as in (a). MoXYL1B-GFP signal is evident in the conidium and appressorium. Scale bar=20 μm . (c) In-planta expression of MoXYL1A and MoXYL1B transcripts during distinct stages of host-pathogen interaction was assessed by qRT-PCR. Vegetative hyphae were used as a control stage and the expression level of MoXYL1A and MoXYL1B at the hyphal stage was set to 1. Error bars represent standard deviation (SD). SD was calculated from three independent biological replicates along with three technical replicates. (*, $P < 0.05$ by t-test).

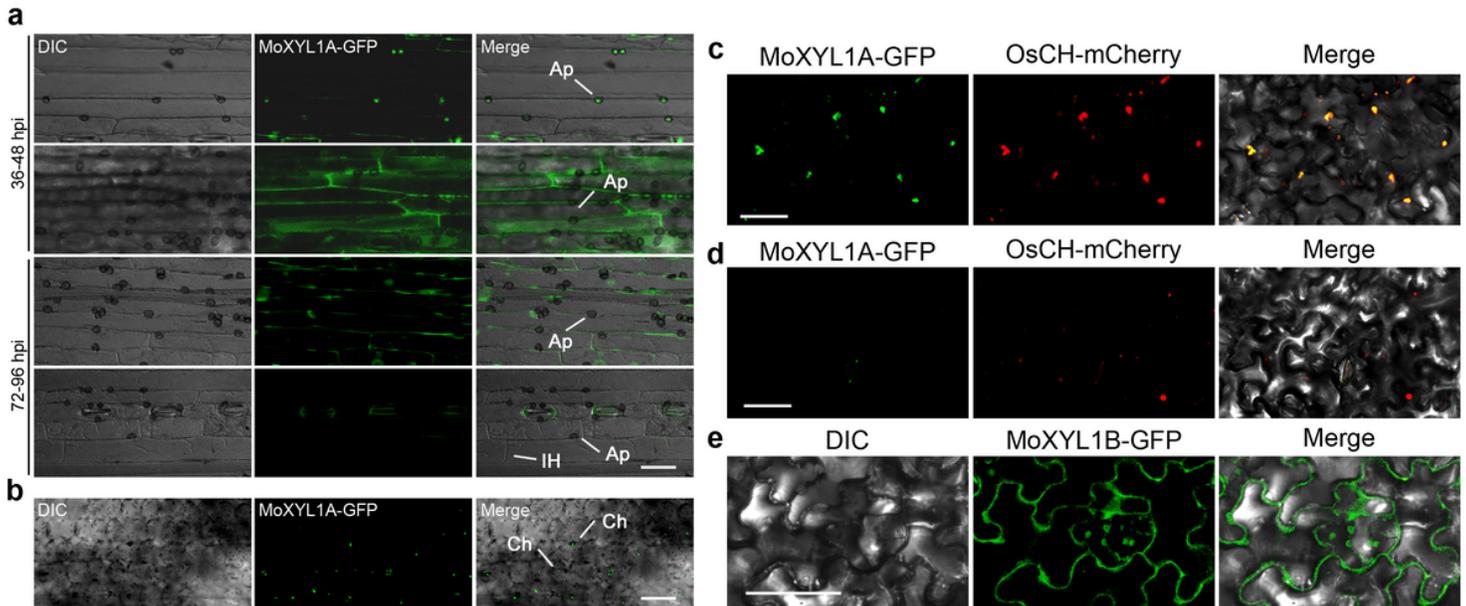


Figure 5

MoXYL1A accumulated at the Chloroplasts of barley and tobacco seedlings. (a) Showed the localization pattern of MoXYL1A-GFP during *M. oryzae* interaction with barley host during early (24-48hpi), and late (72-96hpi) stages of pathogen-host interaction. (b) The micrograph revealed the accumulation of MoXYL1A-GFP to the chloroplast of leaf epidermal tissues of barley leaves at 72 hpi,. Scale bar= 10 μm . (c-d) The micrograph confirmed the co-expression of (MoXYL1A-GFP) and the chloroplast marker (ChCpn10) in the chloroplasts of agro-infiltrated tobacco seedlings. Scale bar = 10 μm . (e) Showed distortions in the localization pattern of MoXYL1A- Δctp -GFP. The MoXYL1A- Δctp -GFP signals accumulated at the membrane of agrobacterium infiltrated tobacco seedlings at 48 hpi. GFP was excited at 488 nm and RFP was excited at 561 nm. Scale bar=20 μm .

Image not available with this version

Figure 6

This image is not available with this version.

Supplementary Files

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