



Disruption of the Gene Encoding Endo- β -1, 4-Xylanase Affects the Growth and Virulence of *Sclerotinia sclerotiorum*

Yang Yu*, Jifen Xiao, Jiao Du, Yuheng Yang, Chaowei Bi and Ling Qing

College of Plant Protection, Southwest University, Chongqing, China

Sclerotinia sclerotiorum (Lib.) de Bary is a devastating fungal pathogen with worldwide distribution. *S. sclerotiorum* is a necrotrophic fungus that secretes many cell wall-degrading enzymes (CWDEs) that destroy plant's cell-wall components. Functional analyses of the genes that encode CWDEs will help explain the mechanisms of growth and pathogenicity of *S. sclerotiorum*. Here, we isolated and characterized a gene *SsXyl1* that encoded an endo- β -1, 4-xylanase in *S. sclerotiorum*. The *SsXyl1* expression showed a slight increase during the development and germination stages of sclerotia and a dramatic increase during infection. The expression of *SsXyl1* was induced by xylan. The *SsXyl1* deletion strains produce aberrant sclerotia that could not germinate to form apothecia. The *SsXyl1* deletion strains also lost virulence to the hosts. This study demonstrates the important roles of endo- β -1, 4-xylanase in the growth and virulence of *S. sclerotiorum*.

Keywords: *Sclerotinia sclerotiorum*, endo- β -1, 4-xylanase, sclerotia, carpogenic germination, virulence, gene deletion

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*Correspondence:

Yang Yu
yuyang2011@foxmail.com

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INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a ubiquitous necrotrophic fungal pathogen that infects more than 400 plant species worldwide resulting in significant losses in many cultivated crops such as oilseed, sunflower, soybean, and common bean (Purdy, 1979; Boland and Hall, 1994; Bolton et al., 2006).

Sclerotinia sclerotiorum produces sclerotia—the primary long-term structures composed of compact vegetative hyphal cells. In a suitable environment, the sclerotia can germinate to form apothecia, which release a huge number of ascospores that cause crop sclerotioses (Bolton et al., 2006). The carpogenic germination of sclerotia is a key process underlying diseases caused by *S. sclerotiorum*. It is highly significant in an economical context. Sclerotial germination is a complex biological process that is controlled by both external environmental conditions and internal natural factors. Many factors have been shown to play important roles in sclerotial germination including temperature (Clarkson et al., 2003, 2007), soil humidity, ventilation condition and the depth of burial (Wu and Subbarao, 2008).

Some cellular signal transduction pathways have been shown to regulate sclerotial germination. Disruption of adenylate cyclase gene *Sac1* in *S. sclerotiorum* decreases cAMP and impairs carpogenic germination of sclerotia suggesting that the cAMP signal pathway regulates the sclerotial germination (Jurick and Rollins, 2007). The process was also influenced by glutathione

accumulation because the sclerotia produced by the strain that lost the gamma-glutamyl transpeptidase gene *Ss-Ggt1* failed to produce apothecia (Li et al., 2012). Furthermore, some secreted proteins located at the cell wall were identified as being associated with the sclerotial germination of *S. sclerotiorum* (Zhu et al., 2013; Xiao et al., 2014). However, analysis of the molecular mechanisms underlying the carpogenic germination of sclerotia is still in its infancy.

As a necrotrophic fungus, *S. sclerotiorum* secretes many cell wall-degrading enzymes (CWDEs) to degrade the plant cell wall components and feed on the tissues (Lumsden, 1969; Riou et al., 1991; Alghisi and Favaron, 1995). The fungus produces several forms of pectinases such as polygalacturonases (PGs) to degrade pectin—the major constituent of the plant cell wall (Waksman et al., 1991; Reymond et al., 1994; Cotton et al., 2003; Favaron et al., 2004; Li et al., 2004; Kasza et al., 2004). PGs genes encode important virulence factors in many important fungal pathogens indicating a universal role in the pathogenicity in *S. sclerotiorum* (Wagner et al., 2000; Garcia-Maceira et al., 2001; Kars et al., 2005). In addition to the PGs, Yajima et al. (2009) reported that an arabinofuranosidase/ β -xylosidase precursor gene disruption mutant of *S. sclerotiorum* showed reduced virulence on canola tissue.

Xylan is the major constituent of hemicelluloses in plant cell walls. It consists principally of xylose and arabinose (Bastawde, 1992). The main chain of xylan is composed of β -(1–4) linked β -xylopyranose. The degradation of xylan requires the incorporation of several hydrolytic enzymes. Of those, endo- β -1, 4-xylanase (EC 3.2.1.8) is the most crucial enzyme (Collins et al., 2005). Xylanases are mainly divided into two families of glycosyl hydrolases: family F or GH10 and family G or GH11 based on amino acid sequences (Jeffries, 1996; Zhou et al., 2008). Due to the important roles of xylanases in the infection processes in pathogens, many genes in fungal pathogens that encode xylanases have been identified and characterized. Most xylanase genes are not essential for pathogenicity (Apel et al., 1993; Gómez-Gómez et al., 2001; Wu et al., 2006; Sella et al., 2013). However, Brito et al. (2006) reported that an endo- β -1, 4-xylanase encoding gene *xyn11A* is required for virulence in *Botrytis cinerea*. Until now, functional characterization of the xylanase gene in *S. sclerotiorum* at the molecular biology level remains rare.

In this research, a gene named *SsXyl1* (Accession No. XM_001591074, SS1G_07749) was predicted to encode an endo- β -1, 4-xylanase in *S. sclerotiorum*. The gene was characterized with reverse-genetic methods, and its function in pathogenicity and development was analyzed. The results will assist in developing our understanding of the mechanism underlying its pathogenicity and development of *S. sclerotiorum*.

MATERIALS AND METHODS

Fungal Strains and Culture Conditions

In this study, *S. sclerotiorum* isolate 1980 (Godoy et al., 1990) was used as the wild-type strain. Strains were routinely cultured at 20°C on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). Gene deletion transformants were cultured on PDA

with hygromycin B at 100 μ g/ml (Calbiochem, San Diego, CA, USA). Gene complemented strains were cultured on PDA with hygromycin B at 100 μ g/ml and G-418 at 30 μ g/ml (Sigma, St. Louis, MO, USA).

Sequence Analysis and Alignment

SsXyl1 gene was obtained through the BLAST searches for homologous sequences in *S. sclerotiorum* genome using reported xylanases gene in *B. cinere* (Brito et al., 2006) and some other plant pathogenic fungi. The signal peptide sequence and conserved domain were predicted using the SignalP 4.1 Server¹ and PFAM², respectively. The sequence alignments were carried out using DNAMAN software (Lynnon BioSoft, Vaudreuil, QC, Canada) and displayed with GeneDoc software (Nicholas and Nicholas, 1997). Conserved amino acids were shown with a shaded background. The phylogenetic tree was constructed using maximum likelihood analysis in MEGA (Tamura et al., 2011).

Xylanase Activity Assays

To assay the xylanase activity of *SsXyl1*, the cDNA fragment encoding the amino acid T²² to S²²² of *SsXyl1* (without signal peptide) was artificially synthesized (Shengong, Shanghai, China) and inserted into pPICZ α A. The resulting vector was transformed into *Pichia pastoris* X33 strain via electroporation. The strain was cultured in BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate, 0.00004% biotin, and 0.5% methanol) at 28°C under shaking (220 rpm) for 48 h. The culture filtrate was collected and the xylanase activity was measured via the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959; Karaoglan et al., 2014). To measure the xylanase activity, 900 μ l 1.0% beechwood xylan (Sigma, St. Louis, MO, USA) in 50 mM sodium citrate buffer (pH 5.0) were incubated 5 min at 50°C. One hundred microliters of samples (culture filtrate) were added in the xylan solution followed by incubation 5 min at 50°C. Next, 9 ml DNS solution was added to the reaction mixture. The mixture was then boiled for 5 min, and the absorbance was measured at 540 nm. Xylose (0–6 μ mol) was used to create a standard curve. One unit of xylanase activity was defined as the amount of enzyme catalyzing the formation of 1.0 μ mol of xylose per minute at pH 5.0 at 50°C. The culture filtrate of the strain transformed with pPICZ α A as the control.

RT-PCR and Real-Time RT-PCR

A reverse-transcriptase polymerase chain reaction (RT-PCR) was applied to determine the relative expression levels of *SsXyl1* during the different development stages and the infection processes of the *S. sclerotiorum*. The wild-type strains were inoculated on the PDA medium and *Arabidopsis thaliana* as described by Yu et al. (2015) followed by mycelia harvest. To compare the expression level of *SsXyl1* on different carbon sources, the wild-type strains were cultured on different minimum medium in which the glucose was replaced with xylan or tomato leaf extract.

¹<http://www.cbs.dtu.dk/services/SignalP/>

²<http://pfam.xfam.org/>

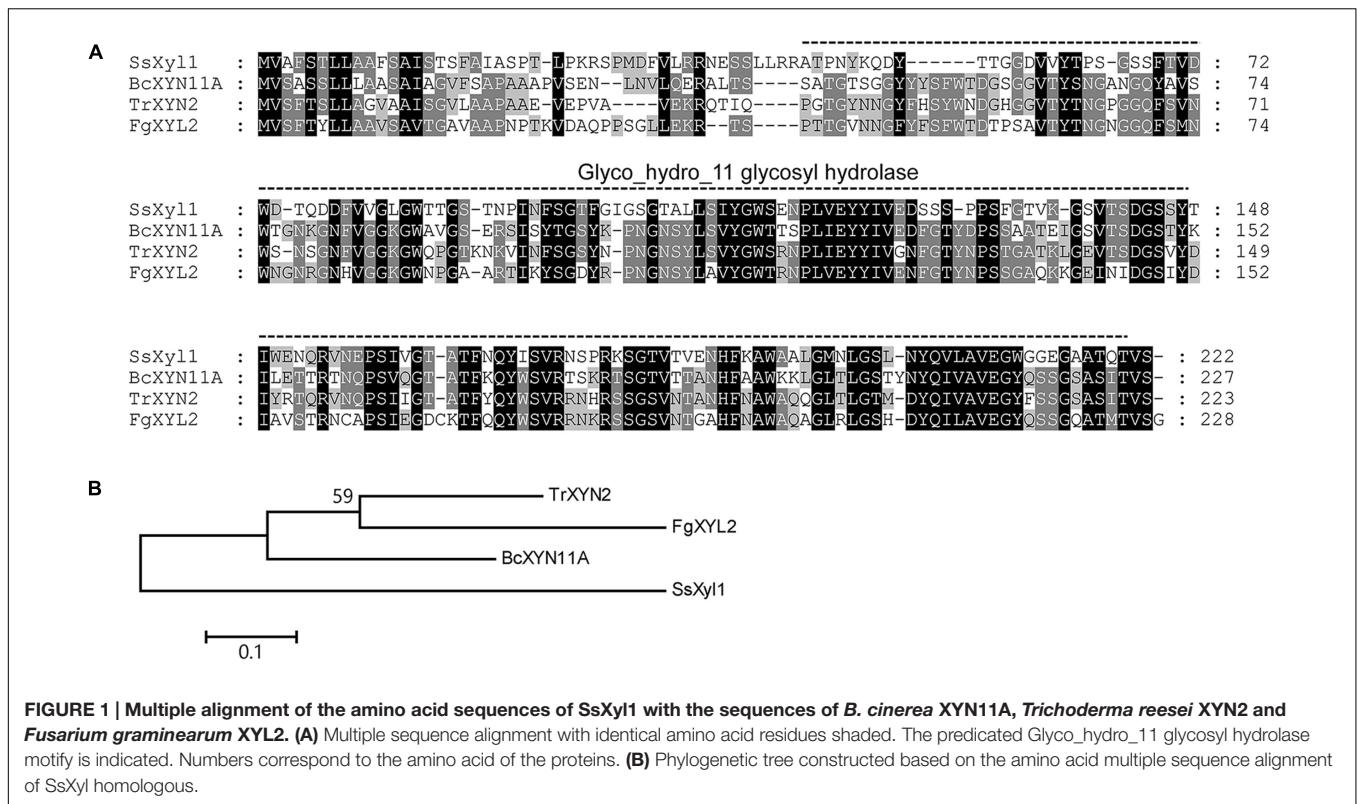


FIGURE 1 | Multiple alignment of the amino acid sequences of SsXyl1 with the sequences of *B. cinerea* XYN11A, *Trichoderma reesei* XYN2 and *Fusarium graminearum* XYL2. (A) Multiple sequence alignment with identical amino acid residues shaded. The predicated Glyco_hydro_11 glycosyl hydrolase motif is indicated. Numbers correspond to the amino acid of the proteins. (B) Phylogenetic tree constructed based on the amino acid multiple sequence alignment of SsXyl homologous.

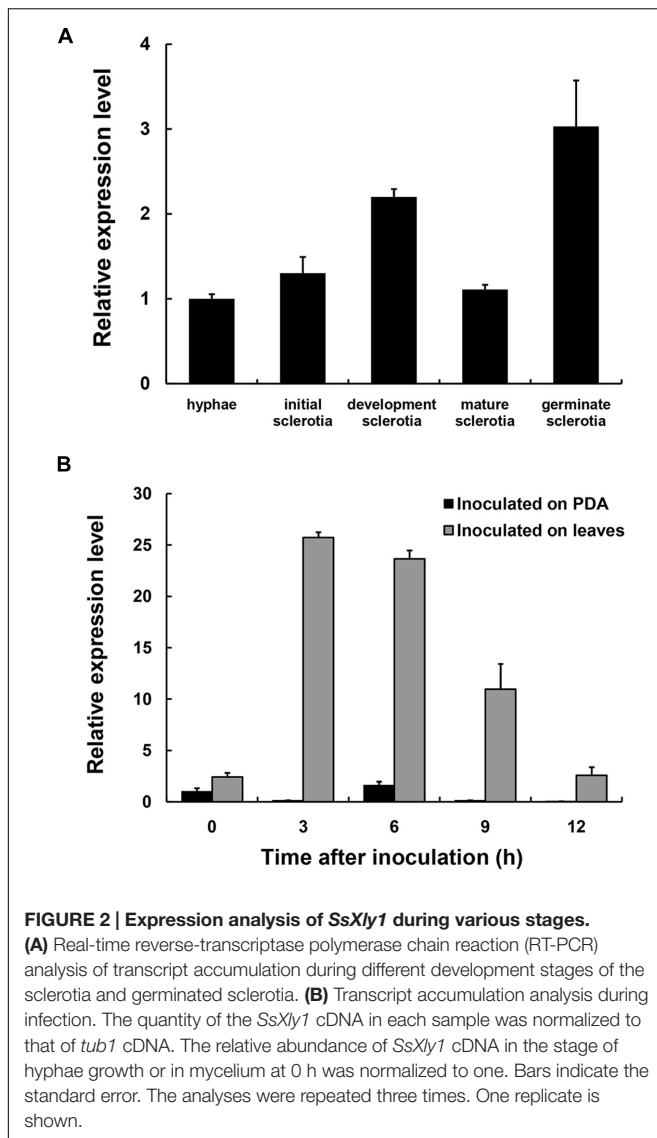
The total RNA in each sample was extracted with Trizol (Huashun, China). The first-strand of cDNA synthesis was performed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The real-time RT-PCR was applied on a Bio-Rad CFX96™ Realtime System (Hercules, CA, USA). The *SsXyl1* cDNA abundance was normalized using the β-tubulin gene (*tub1*, SS1G_04652) as an internal control. The primer pairs qRT-Xyl1fp (TTCTCGGC AATTCTACATCC)/qRT-Xyl1rp (CCCAATCTACAGTGAAG GAGC) and qRT-Tubfp (GTGAGGCTGAGGGCTGTGA)/qRT-Tubrp (CCTTTGGCGATGGGACG) were designed with Primer Premier 6.0 (Premier, Canada) and used to amplify the cDNA of *SsXyl1* and *tub1*. The amplification mixtures were composed of 10 μl of SYBR Green Realtime PCR Master Mix (Toyobo, Japan), with 4 pM primer, 1 μl of cDNA, and ddH₂O water to a final volume of 20 μl. Amplification steps were as follows: 95°C for 2 min (1 cycle) followed by 95°C for 20 s, 58°C for 15 s and 72°C for 20 s (40 cycles). Each sample was analyzed over three biological replicates and each real-time RT-PCR analysis was repeated three times.

The RT-PCR was used to analyze *SsXyl1* expressions in the gene deletion and complemented strains. The transforms were cultured on PDA supplemented with different antibiotics for 4 days, and the mycelia were harvested. The total RNA extraction and cDNA synthesis were performed as mentioned before. The primer pairs RT-Xyl1fp (CTTCACTGTAGATTGGGACACC)/RT-Xyl1rp (ACTAACCGTTTGGCTAGCAG) were amplified a 460-bp fragment of *SsXyl1* cDNA. The *tub* expressions amplified with qRT-Tubfp/qRT-Tubrp served as the control.

Generation of Gene Deletion and Complementation Strains

The gene deletion vector was constructed based on vector pSKH (Hamid et al., 2013). A primer pair Xyl1DF (C GCAAGCTTGGGGAGAGAGATATGCAAATGT) and Xyl1DR (CCGCTCGAGAGAGGCTTTTAGTTTACAAATG) were designed to amplify a 1000-bp of 3' untranslated region (UTR) of *SsXyl1* gene. The fragment was digested with *Hind*III and *Xho*I and then inserted into pSKH to produce the pSKHXYL1. The primer pairs Xyl1UF (GCCGAGCTCA TGGAAATAACACGCCTAGC) and Xyl1UR (GCCGCTCGAC CCACGCATGCTTTATATACT) were amplified a 1000-bp of 5' UTR of *SsXyl1* gene. The fragment was then digested with *Sac*I and *Sal*I and inserted into pSKHXYL1 to generate the pSKHXYL2. The construct was used as a PCR template to generate split-marker fragments. The 5'UTR::N-HY was amplified using primers Xyl1U (CATGGAATAAACACGCCT AGC) and HY (AAATTGCCGTCAACCAAGCTC). The 3'UTR:: C-YG was generated using YG (TTTCAGCTTCGATGTAG GAGG) and Xyl1D (GAGAGGCTTTTAGTTTACAAATG). The overlap between the two hygromycin resistance gene (*hph*) fragments was 741 bp. The two fragments were used to transform into *S. sclerotiorum* wild-type protoplasts as described by Rollins (2003).

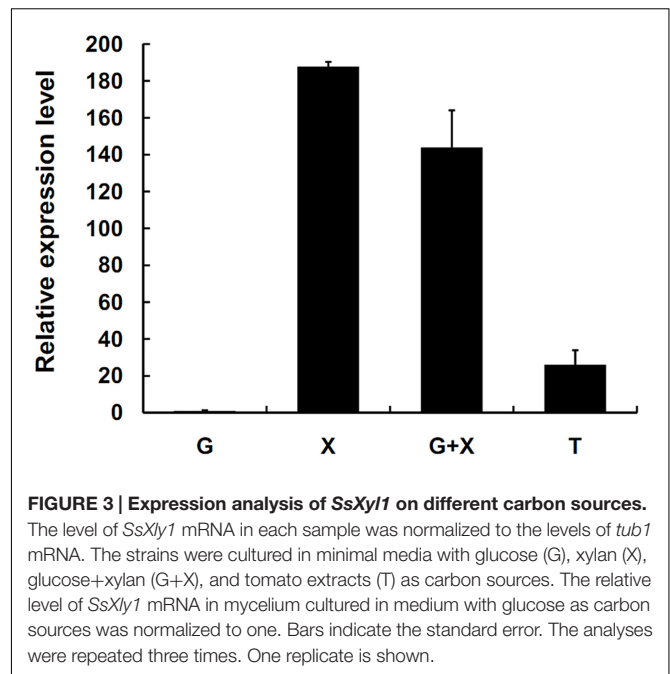
To construct the gene complement vector, a neomycin-resistance gene was inserted into pCAMBIA3300 at the *Xba*I site to produce pCAMBIA3300NEO. A pair of primers 07749FP (CCGGAATTCTTCTATGCACGCAGACTTATTG) and 0774 9RP (CCGCTCGAGGTCAAATACAACCTGCCACCT) were



designed to amplify the genome DNA from the wild-type strain and yield 2.8-kb fragments that contained the *SsXyl1* coding sequence and 1 kb of 5' and 1 kb of 3' untranslated region. The fragment was digested with *EcoRI* and *XhoI* and then inserted into pCAMBIA3300NEO to produce the pCXYL1. The vector was then linearized with *XhoI* and transformed into the KO52 protoplasts with PEG methods (Rollins, 2003).

Pathogenicity Assays

The pathogenicity assays were performed on leaves of *Brassica napus* Zhongshuang 9 and *A. thaliana* Columbia-0. *B. napus* were grown in a greenhouse at 25°C to 35°C for about 10 weeks. *A. thaliana* were cultivated in a growth chamber at 25°C with a 12 h light/12 h dark cycle for 5 weeks. The leaves of *B. napus* and *A. thaliana* were inoculated with mycelia-colonized agars ($\varphi = 6$ mm) obtained from actively growing colony margins of the wild-type, gene deletion, and



complemented strains. The inoculated plants were maintained at 90% relative humidity. Photographs were taken at 72 h post inoculation (hpi) for rapeseed leaves and at 120 hpi for *A. thaliana*. The experiment was repeated three times and five plants were inoculated with each strain for each repeat.

RESULTS

SsXyl1 Characterization

The *SsXyl1* gene contains an ORF of 669 bp and encodes a 222-amino-acid polypeptide. The N-terminal of *SsXyl1* was predicted to contain a typical peptide with SignalP 4.1 Server (Petersen et al., 2011). The predicted cleavage site was between amino acid position 21 and 22. This generates a putative mature protein with a calculated molecular mass of 21.69 kDa and an isoelectric point (pI) of 4.82. Residues 46–220 in the protein were predicted by PFAM (Finn et al., 2015) to be a Glyco_hydro_11 glycosyl hydrolase motif. Sequence comparison showed that *SsXyl1* exhibited high sequence similarity with endo- β -1, 4-xylanase of *B. cinerea* XYN11A (53% identities, *E*-value: $5e-50$) (Brito et al., 2006), *Trichoderma reesei* XYN2 (45% identities, *E*-value: $2e-55$) (Enkerli et al., 1999) and *Fusarium graminearum* XYL2 (41% identities, *E*-value: $3e-46$) (Dong et al., 2012). The sequence alignment and phylogenetic tree are shown in **Figure 1**.

To confirm the xylanase activity of *SsXyl1*, the *SsXyl1* cDNA (without signal peptide) was linked into pZICZ α A. The resulting vector was transformed into the yeast *P. pastoris* X33 strain. The strains were cultured with shaking, and the xylanase activity of culture filtrate was determined. The results showed that the enzyme activity for the strain carrying *SsXyl1*

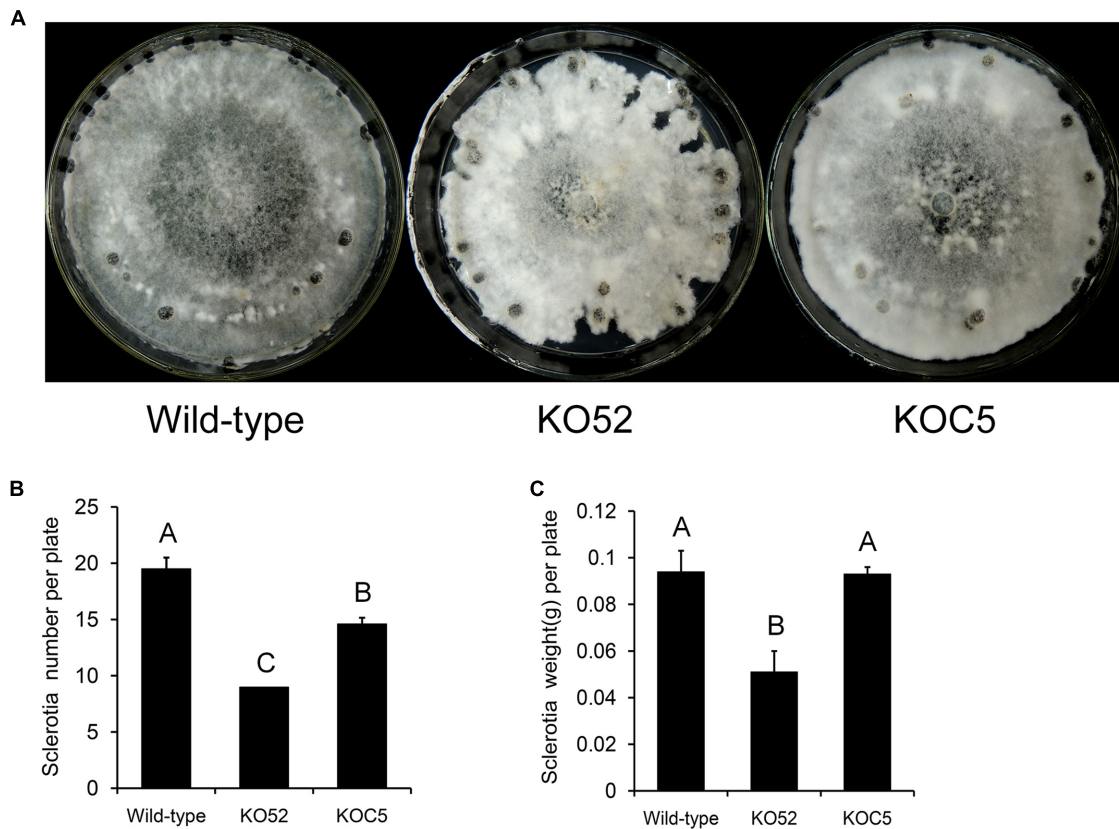


FIGURE 4 | Deletion of *SsXyl1* has significant effects on colony morphology and sclerotial development. (A) Phenotype of wild-type strain, *SsXyl1* deletion (KO52) and complemented (KOC5) strains on potato dextrose agar (PDA) plates for 15 days. **(B)** The number of sclerotia produced in 9 cm per petri plates. **(C)** Sclerotia mass per plate. Different letters on a graph indicate significant differences, $P < 0.05$.

was 39.17 U/ml, and the wild-type strain was 0.47 U/ml. These results indicate that *SsXyl1* encodes a xylanase in *S. sclerotiorum*.

SsXyl1 Expression Patterns

The expression levels of *SsXyl1* during the different development stages and the infection process were determined with real-time RT-PCR. **Figure 2A** shows that the *SsXyl1* expression was slightly increased during the development and carpogenic germination stages of sclerotia. When inoculated on the *A. thaliana*, the *SsXyl1* expression was dramatically increased at 3 hpi and then decreased gradually. The expression was still higher than at 0 dpi (**Figure 2B**). The results suggest that *SsXyl1* expression is strongly induced during infection.

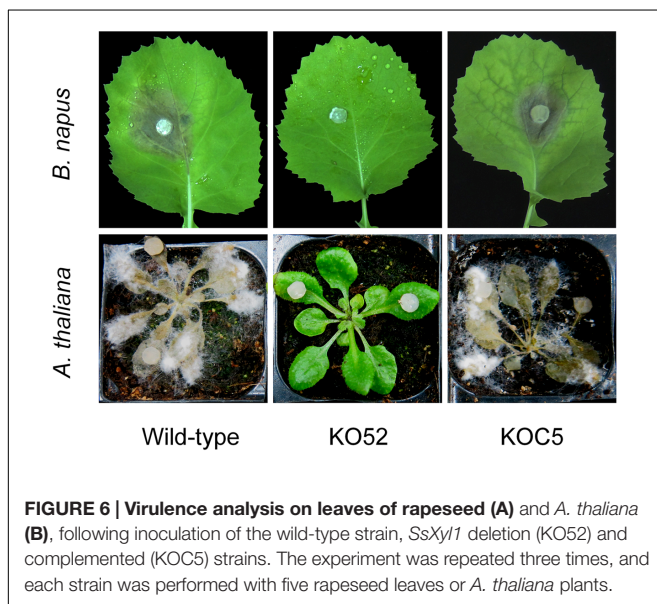
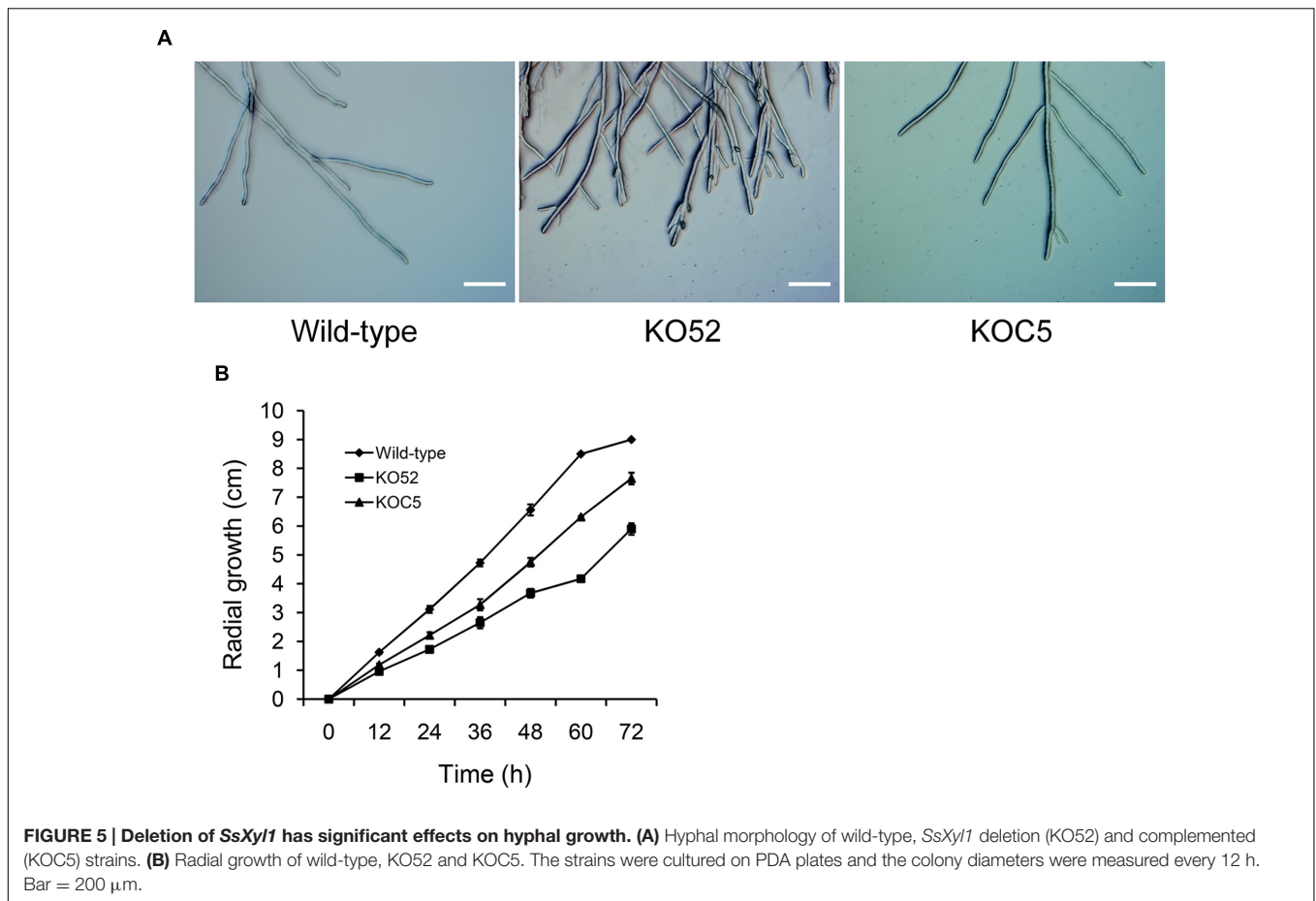
SsXyl1 expression was also determined in the mycelia of *S. sclerotiorum* grown on different carbon sources. **Figure 3** shows that the expression level of *SsXyl1* for the mycelia grown in xylan was significantly higher than that grown in glucose. The level was maximal when xylan was the only carbon source. The expression pattern indicates that *SsXyl1* expression was induced by xylan. The *SsXyl1* expression is

also induced when the tomato leaf extracts were the carbon source.

Role of *SsXyl1* in Fungal Growth and Development

To functional analysis the *SsXyl1* in *S. sclerotiorum*, *SsXyl1* gene deletion vector was constructed based on the vector pSKH as described in the Section "Materials and Methods." The construct was used as the PCR template to generate split-marker fragments, which transformed the protoplasts of the wild-type strain (**Supplementary Figure S1A**). Several transformants were obtained and confirmed by amplifying of the hygromycin resistance gene. Some strains were chosen randomly and the *SsXyl1* expressions were determined with RT-PCR. The results showed that KO52 strains lacked the *SsXyl1* transcript (**Supplementary Figure S1B**). The complementation strain KOC5 was generated via the transformation of the *SsXyl1* complemented vector into the KO52 protoplasts. RT-PCR revealed that the *SsXyl1* transcript was present in KOC5 (**Supplementary Figure S1B**).

Figure 4A shows the colonial morphology of the wild-type, *SsXyl1* deletion (KO52), and complemented (KOC5) strains



that the number and weight of sclerotia for KO52 were approximately half of those of the wild-type strain. Most importantly, sclerotia produced by the KO52 cannot produce apothecia under standard conditions of carpogenic germination. The KO52 exhibited a dense hyphal branch when observed by microscopy (Figure 5A). It has a slower growth rate versus the wild-type strain (Figure 5B).

Role of *SsXyl1* in Virulence

The wild-type, *SsXyl1* deletion, and complemented strains were all inoculated on the detached leaves of oilseed rape and *A. thaliana* plants. Figure 6 shows that the gene deletion mutant KO52 lost the virulence to the hosts, while the re-induction of *SsXyl1* in the deletion mutant almost completely rescued the phenotype. The results suggest that *SsXyl1* plays an important role in the virulence of *S. sclerotiorum*.

DISCUSSION

In this report, we described an endo- β -1, 4-xylanase gene named *SsXyl1* in *S. sclerotiorum*. *SsXyl1* was predicted to contain a GH11

on PDA plates. The gene deletion strain showed abnormal morphology versus the wild-type strain. The strain produced fewer sclerotia than the wild-type strain. Figures 4B,C indicates

glycosyl hydrolase motif with xylanase activities. *SsXyl1* was up-regulated during the development and carpogenic germination of sclerotia. The *SsXyl1* deletion strains produced aberrant sclerotia that were defective in carpogenic germination. *SsXyl1* was also significantly up-regulated during the infection processes; gene deletion strains lost virulence to the hosts. Our results suggest that *SsXyl1* plays an essential role in the growth and virulence of *S. sclerotiorum*.

SsXyl1 is believed to belong to the GH11 group because of the GH11 glycosyl hydrolase motif, its low molecular weight, and the basic pI. The GH11 group was the best characterized GH group with bacterial and fungal members considered to be a true xylanase because of their high substrate specificity (Paës et al., 2012). Here, the xylanase activity of *SsXyl1* was confirmed using the 3, 5-DNS method. Fungal plant pathogens colonize their hosts via the production of different CWDEs including pectinases, cellulases, xylanases, and cutinases (Annis and Goodwin, 1997; ten Have et al., 2002; Wanjiru et al., 2002; D'Ovidio et al., 2004; Reignault et al., 2008). In *S. sclerotiorum*, enzymes such as pectinase, cellulose, proteases, and glucoamylases can facilitate the invasion and colonization of host tissue (Riou et al., 1991; Martel et al., 1998, 2002; Poussereau et al., 2001a,b). Our results indicate a role of xylanases in the pathogenicity of this fungus.

Until now, many genes that encode xylanases in fungal pathogens have been cloned and functionally analyzed, but most have no relationship with the fungal pathogenicity (Wu et al., 1997; Gómez-Gómez et al., 2002). In this research, *SsXyl1* shows a high level of expression during the infection and gene deletion strains lost virulence to many hosts. These findings indicate that the gene is essential for the virulence of *S. sclerotiorum*. In *B. cinerea*, *xyn11A* encodes an endo- β -1, 4-xylanase Xyn11A and deletion of the gene has a more pronounced effect on virulence (Brito et al., 2006). Xyn11A contributes to virulence with its necrotizing activity not with its catalytic activity (Noda et al., 2010). The similarity between the xylanases *SsXyl1* and Xyn11A in the amino acid (53% identity, 67% positive; **Figure 1**) seems to indicate that *SsXyl1* also contributes to the virulence via the necrotizing activity, but more evidence is needed.

Carpogenic germination of sclerotia is a key process in Sclerotinia diseases. Until now, many genes associated with cell signal transduction pathway have been confirmed to control this process in *S. sclerotiorum* (Jurick and Rollins, 2007; Li et al., 2012), but the proteins that worked downstream of the signal transduction pathway to control sclerotial germination have remained largely unknown. In this research, the sclerotia for *SsXyl1* deletion mutants cannot germinate to form apothecia suggesting that *SsXyl1* is related to the carpogenic germination. To the best of our knowledge, this is the first report to show that the xylanase is related to the sclerotial germination in fungi.

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The carpogenic germination of sclerotia can be divided into three stages: (1) carpophores initials form in inner medullary tissues of sclerotia; (2) the carpophores arise through the ruptured rind; and (3) the tips of the carpophores become modified to form apothecia (Coley-Smith and Cooke, 1971). Once a sclerotial rind is broken or damaged, the sclerotia germinate more rapidly (Makkonen and Pohjakallio, 1960). As the resting structure of *S. sclerotiorum*, the sclerotia rind forms a thick cell wall that could maintain internal water and energy. The thick cell wall contains the primary cell wall, cellulose, hemicelluloses as well as some yet-to-be-identified materials surrounding it (Willets and Bullock, 1992; Young and Ashford, 1992). Thus, the sclerotial rind plays an important role in the inhibition of sclerotial germination (Garg et al., 2010). We hypothesized that the sclerotia use enzymes to hydrolyze the thick cell wall and degrade it before germinating. *SsXyl1* may be an important candidate enzyme, but the hydrolytic activity of *SsXyl1* to the cell wall of the sclerotia requires more study.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: YY, JX, JD, YHY, CB, and LQ. Performed the experiments: YY and JX. Analyzed the data: YY and JX. Contributed reagents/materials/analysis tools: JD, YHY, CB, and LQ. Wrote the paper: YY and JX. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.01787/full#supplementary-material>

FIGURE 1 | Disruption of the *SsXyl1* gene function using a split marker strategy. (A) Schematic describing an overlapping hygromycin phosphotransferase gene (*hph*) marker for *SsXyl1* disruption. The N-HY and C-YG imply 741 bp overlapping the 5' and 3' fragments of *hph* gene, respectively. **(B)** RT-PCR analysis the expressions of *SsXyl1* in the wild-type, gene deletion (KO52), and complemented (KOC5) strains. The expression of the *Tub* gene was the internal control.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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