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Short Communication

Transcriptional analysis of *Myceliophthora thermophila* on soluble starch and role of regulator AmyR on polysaccharide degradation

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ABSTRACT

Thermophilic fungus *Myceliophthora thermophila* has great capacity for biomass degradation and is an attractive option for use as cell factory to produce chemicals directly from renewable polysaccharides, such as starch, rather than monomer glucose. To date, there has been no transcriptomic analysis of this thermophilic fungus on starch. This study determined the transcriptomic profile of *M. thermophila* responding to soluble starch and a 342-gene set was identified as a "starch regulon", including the major amylolytic enzyme (Mycth_72393). Its overexpression led to increased amylase activities on starch by 35%. Furthermore, overexpressing the key amylolytic enzyme regulator AmyR in *M. thermophila* significantly increased amylase activity by 30%. Deletion of *amyR* by the CRISPR/Cas9 system led to the relief of carbon catabolite repression and 3-fold increased lignocellulase activities on cellulose. This study will accelerate rational fungal strain engineering for biochemical production from biomass substrates such as raw corn starch and even crop straw.

1. Introduction

In the natural environment, filamentous fungi contribute as essential degraders of complex polysaccharides, and allows storage compounds like starch and plant cell wall biomass to play a key role in the biological carbon cycle (Gao et al., 2011; Li et al., 2017; Samal et al., 2017). Starch is a major stored carbohydrate in green plants and is composed of two major glucose polymers, amylose and amylopectin (Tsukagoshi et al., 2001). Amylose contains α-1,4-glucan chains, while amylopectin contains α-1,6 glycosidic bonds in addition to α-1,4-glucan bonds. Three types of starch-degrading enzymes, including α-amylase, glucoamylase, and α-glucosidases, have received much attention because of their industrial applications (Sindhu et al., 2017). Recent studies of *Neurospora crassa* demonstrated that a new family of lytic polysaccharide monooxygenases (LPMO) together with a biological redox partner oxidatively cleave amylose, amylopectin, and starch (Vu et al., 2014).

Regulation of starch degradation is mainly based on research of *Aspergillus* spp., which have been used for commercial production of starch-hydrolysis enzymes (Tsukagoshi et al., 2001). In *Aspergillus* species, transcriptional levels of amylase genes are coordinately activated by the transcription factor AmyR with a $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motif (Petersen et al., 1999; Tani et al., 2001). This similar function of AmyR orthologs in starch utilization was also found in other cellulolytic fungi such as *N. crassa* (Xiong et al., 2017), *Penicillium oxalicum*, and *Penicillium decumbens* (Li et al., 2017, 2015). In addition, recent reports on *P. oxalicum* (Li et al., 2015) and *Trichoderma reesei* (Nitta et al., 2012) found that AmyR directly represses the transcription of cellulase genes on cellulose and deletion of *amyR* resulted in increased cellulase activity, suggesting a key role of AmyR in the regulatory effect for cellulolytic genes.

Thermophilic filamentous fungus *Myceliophthora thermophila* represents a potential reservoir of novel industrial thermostable enzymes (Dimarogona et al., 2012), and has the potential to produce chemicals and biofuels directly from renewable biomass. In recent studies of *M. thermophila*, transcriptomic approaches were used to examine the utilization of various plant cell wall polysaccharides, and the results provide a comprehensive view of the response to a wide range of biomass sources (Kolbusz et al., 2014). Starch degradation is of great interest not only for its important roles in the ecological recycling of biomass, but also for its industrial applications (Sindhu et al., 2017). The production of amylolytic enzymes by *M. thermophila* was investigated in 1992 (Sadhukhan et al., 1992); however, to our knowledge, a systematic investigation of starch degradation in this thermophile fungus was not reported because of a lack of mature genetic approaches. In this study, transcriptomic analysis was performed for *M. thermophila* on soluble

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starch, phenotypes of Δ*amyR* disruptant were assessed, and the roles of two major starch hydrolases during starch degradation were examined. This study provides fundamental knowledge for genetic engineering in thermophilic fungi to produce chemicals direct from starch without the need for extra enzymes.

2. Materials and methods

2.1. Strains and growth conditions

Myceliophthora thermophila ATCC 42464 was cultivated in Vogel's minimal medium supplemented with 2% sucrose (MM medium) at 45 °C for 10 days to obtain conidia. For flask culture, *M. thermophila* conidia at 10^6/mL were inoculated in 100-mL aliquots (1 × Vogel's salt with 2% starch or Avicel) at 45 °C. For growth phenotype, 1-μL conidia suspension were centrally spotted onto MM plates with selected carbon sources for 3 days.

2.2. Vector construction for genetic engineering

To construct plasmids for overexpressing genes, the open reading frames (ORFs) of starch hydrolase genes (Mycth_2296256 and Mycth_72393) were amplified and cloned into pAN52-MtgpdA-TtrpC using the NEB Gibson assembly kit to generate plasmids pOE-Mycth_2296256 and pOE-Mycth_72393, respectively. The sgRNA expression plasmids U6-*amyR*-sgRNA, U6-Mycth_2296256-sgRNA, and U6-Mycth_72393-sgRNA were constructed as described previously (Liu et al., 2017). For construction of gene deletion substrates, 5′ and 3′ flanking fragments of *amyR*, Mycth_2296256, and Mycth_72393 were amplified and fused with the P*trpC-neo* cassette from a p0380-*neo* to generate donor DNA sequences.

2.3. Transformation of M. thermophila protoplasts

Transformation of *M. thermophila* protoplasts was performed according to a previously described procedure (Wang et al., 2015). For target gene expression, 10 μg of ligated plasmid was added to the fungal protoplasts. Colonies grown were screened for *neo* gene resistance using G418 (80 μg/mL). Gene disruption mediated by CRISPR/Cas9 system was performed as described previously (Liu et al., 2017).

2.4. Transcriptional profiling and data analysis

Myceliophthora thermophila strains were inoculated into 1 × Vogel's salt with 2% glucose and cultivated at 45 °C for 16 h. Mycelia were collected and washed three times with 1 × Vogel's salt, and then transferred into the medium with 2% starch, Avicel, or no carbon media for 4 h of cultivation. Mycelia were harvested, homogenized in liquid nitrogen, and stored at −80 °C. Total RNA from frozen samples was isolated as previously described (Xiong et al., 2017). Two biological duplicates were sequenced for each of the conditions via the Illumina HiSeq™ 2000 platform and the data were analyzed according to the method described previously (Wang et al., 2017). Genes with reads per kilobase million (RPKM) value > 20, fold change > 2.0, and DESeq Padj value < 0.05 were considered to be expressed with significant difference between growth conditions. The sequence data can be accessed at the Gene Expression Omnibus [GEO: GSE110062]. The qRT-PCR was performed using the method as that described previously (Liu et al., 2017).

2.5. Protein and enzyme activity measurements

Total extracellular protein was determined with a Bio-Rad DC protein assay kit (Bio-Rad) based on absorbance at 595 nm, using bovine serum albumin as the standard. Azo-CM-cellulose assay kit (Megazyme) and Azo-xylan kit (Megazyme) were used to determine endoglucanase and endo-1,4-β-xylanase activities, respectively. Amylase activity was determined as previously described (Li et al., 2015). Results were expressed as the average of at least three independent experiments.

3. Results and discussion

3.1. Transcriptional profiling of M. thermophila on soluble starch

Myceliophthora thermophila is capable of utilizing polysaccharides like starch (Sadhukhan et al., 1992), and potentially can directly ferment starch to produce bio-chemicals and fuels. However, the taste of starch to *M. thermophila* has not been previously investigated. Herein, transcriptomic profiles from *M. thermophila* cultures exposed to 2% starch or 2% glucose were analyzed, using a no-carbon condition as control. Principal component analysis (PCA) revealed high reproducibility for two biological replicate samples from the same condition. PCA plotting showed distinct expression patterns for cultures exposed to no carbon or starch. Transcriptional profiles on starch were distant from those on glucose in the PCA analysis, suggesting that polysaccharide induced substantial transcriptional changes. Statistically significant decreased (1122 genes) and increased (1259 genes) expression levels were found when exposed to starch, using gene expression on no carbon as a reference. Hierarchical clustering of expression patterns for 1259 genes on starch, glucose, and no-carbon conditions resulted in three groups with similar transcriptional patterns (Fig. 1A).

Group 1 consisted of 90 genes that exhibited higher expression levels on glucose. Function category (FunCat) analysis showed that genes involved in metabolism were the most enriched. In this set, Mycth_112491 ortholog (NCU01633) in *N. crassa* showed high affinity glucose transport activity while Mycth_108924 ortholog (NCU05627) mediated the uptake of xylose (Li et al., 2014).

Group 2 contained 342 genes with higher expression levels on starch, designated as a "starch regulon". Genes involved in transport facilities and protein folding and stabilization were most enriched (Fig. 1B). Thirty-five genes encoding carbohydrate active enzymes (CAZyme) were grouped in this set, including two α-mannosidase genes (Mycth_2294863 and Mycth_2306973), four LPMO genes (Mycth_112089, Mycth_80312, Mycth_111088, and Mycth_110651), and two cellobiose dehydrogenase genes (Mycth_81925 and Mycth_111388). It has been reported that the starch-active LPMOs together with a biological redox partner, cellobiose dehydrogenase, oxidatively cleave α-glycosidic bonds of starch (Vu et al., 2014). As expected, expression levels of glucoamylase gene (Mycth_72393) were increased 290-fold on starch, for which the role on starch utilization was further investigated. Eight other enzymes were involved in protein synthesis and secretion, including three ER chaperones (Mycth_2300500, Mycth_2302852, and Mycth_2312968), and five translocation proteins EC62 (Mycth_2310016), SEC63 (Mycth_2303597), SEC66 (Mycth_2313775), ERV41 (Mycth_23S02675), and ERV46 (Mycth_2295135), which were prerequisite for the secretion of carbohydrate hydrolase. Three sugar transporter genes (Mycth_2312740, Mycth_2296480, and Mycth_2308157) were also greatly induced by starch. In *N. crassa*, Mycth_2308157 ortholog (NCU10021), a high affinity glucose transporter, played a critical role in carbon sensing and cellulose degradation (Wang et al., 2017).

Group 3 was the largest group and contained 827 genes that exhibited high expression levels on both starch and glucose. These genes were mainly involved in growth and proliferation, such as amino acid metabolism, protein synthesis, and biogenesis of cellular components (Fig. 1C). This gene set also included a transcription regulator (Mycth_46530) with 31-fold and 41-fold increases in expression levels on glucose and starch, respectively. In *N. crassa*, Mycth_46530 ortholog (Vib-1) is required for extracellular protease secretion in response to both carbon and nitrogen starvation and for cellulose utilization (Xiong et al., 2014).