The use of *Agrobacterium*-mediated insertional mutagenesis sequencing to identify novel genes of *Humicola insolens* involved in cellulase production

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Abstract

A transfer DNA (T-DNA)-tagged mutant library of *Humicola insolens* was screened for mutants with altered cellulase production using the plate-clearing zone assay. Three selected mutants (5-A7, 5-C6, and 13-B7) exhibited significantly depressed FPase, CMCase and xylanase activities compared with the wild-type strain upon shake-flask fermentation, while the pNPCase and pNPGase activities of the three mutants were relatively higher than those of the parental strain. Combined with the results of SDS-PAGE and mass spectrometry, we suggest that expression of the CMCases Cel6B, Cel7B, CMC3, and XynA/B/C was reduced in the mutant strains. Twelve putative T-DNA insertion sites were identified in the three mutants via *Agrobacterium*-mediated insertional mutagenesis sequencing (AIM-Seq). Bioinformatics analysis suggested that a putative dolichyl pyrophosphate phosphatase, two hypothetical proteins encoding genes of unknown function, and/or nine intergenic fragments may be involved in cellulase and hemicellulase production by *H. insolens*. This provides promising new candidate genes relevant to cellulase production by the fungus, which will be crucial not only for our understanding of the molecular mechanism underlying cellulase production, but also for strain improvement.

Keywords AIM-Seq · Cellulase · *Humicola insolens* · T-DNA insertional mutagenesis

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Introduction

Humicola species are endowed with a powerful ability to degrade polysaccharide constituents of biomass, such as cellulose and hemicellulose, and are potential sources of cellulolytic enzymes of scientific and commercial interest. Many of the cellulolytic enzymes of *Humicola insolens* have been heterologously expressed, and their catalytic properties are well investigated (Davies et al. 2000; Du et al. 2013; Meleiro et al. 2014; Schulein 1997; Yang et al. 2014). Unlike the enzymes of *Trichoderma*, *Aspergillus*, and *Penicillium* spp., the cellulolytic enzymes of *H. insolens* are pH-neutral active, alkali tolerant, and thermostable (Du et al. 2013; Schulein 1997). Such valuable properties make them commercial applications in the textile and brewing industries, including EGV (Carezyme; Novozymes) and a β -glucanase preparation (Ultraflo L; Novozymes). Despite the extensive characterization of various cellulolytic enzymes, the mechanism of regulation of cellulase production in *H. insolens* remains poorly understood. Identification of crucial regulators of cellulase production in *H. insolens* is essential for genetic engineering of the strain and to reveal the molecular

mechanisms by which the strain efficiently decomposes lignocellulose.

T-DNA element, which can be transformed into fungi though *Agrobacterium tumefaciens*-mediated transformation and insert randomly within chromosomes (Tzfira et al. 2004), has proven to be one of the most powerful insertional mutagens to create mutant library of fungi (Michielse et al. 2005; Mullins et al. 2001). Since the DNA sequence of the inserted fragment is known, the genomic flanking sequences of T-DNA insertions can be recovered by PCR-based strategies such as thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). Therefore, T-DNA insertion mutagenesis has been extensively used to clone functional genes and other regulatory sequences from a wide variety of fungi (Michielse et al. 2005), including genes involved in cell wall synthesis of the opportunistic pathogen *Cryptococcus neoformans* (Esher et al. 2015), genes participating in cellular differentiation and cephalosporin production in the important industrial fungus *Acremonium chrysogenum* (Long et al. 2013), and genes related to cellulase production in *Trichoderma reesei* (Zhong et al. 2012). AIM-Seq (Agrobacterium-mediated insertional mutagenesis sequencing) is a new high-throughput genomic sequencing and analysis approach recently developed by Esher et al., which is much more rapid and economical than the traditional TAIL-PCR method. (Esher et al. 2015). It allows investigators to identify the insertion sites of multiple mutants related to specific bioprocess or exhibiting similar phenotype generated by ATMT through batch sampling, whole genome resequencing, and followed bioinformatic analysis by the Agrobacterium-mediated Insertional Mutagenesis High-throughput Insert Identification (AIM-HII) program as long as the insert sequence and genome sequence of the target organism are known.

In our previous work, we established an efficient *Agrobacterium*-mediated transformation method (ATMT) for *H. insolens* and used the T-DNA insertional mutation technique to generate a random mutant library (Xu et al. 2016). In the present study, three mutant strains exhibiting altered cellulase production were selected and characterized in terms of cellulose hydrolysis. Furthermore, the T-DNA insertion sites were identified via AIM-Seq (Esher et al. 2015) and new candidate genes possibly involved in cellulase production were thus revealed.

Materials and methods

Strains, media, and growth conditions

The *H. insolens* Y1 (CGMCC 4573) used in this study was isolated from forest soil in Hebei, China. *H. insolens* Y1 was grown on potato dextrose agar for 7 days at 42 °C to induce

sporulation. Screening medium (all components/l: 10 g sodium carboxymethyl cellulose, 20 g tryptone, 1 g yeast extract, 0.6 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g sodium deoxycholate, and 16 g agar) was used to identify fungi expressing cellulase. MNN medium (all components/l: 20 g tryptone, 1 g yeast extract, 0.6 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 20 g Avicel) was used for shake-flask fermentation.

Screening of mutants

Primary screening was performed using the clearing zone method. *H. insolens* Y1 wild-type and mutants were grown on screening medium for 5 days at 42 °C, followed by the addition of 1 g Congo Red l^{-1} for 30 min, and washed with 1 M NaCl for 30 min.

Enzyme production and hydrolysis experiments

Humicola insolens Y1 wild-type and mutant strains were fermented in 40-ml amounts of MNN medium at 42 °C for 6 days. The cellulase activities of supernatants were assayed every 24 h. Filter paper (FPase), carboxymethyl cellulase activity (CMCase), and xylanase activities were measured using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). The enzyme preparations were appropriately diluted in 100 mM Na_2HPO_4 /citric acid (pH 6.0). Whatman No. 1 filter papers (6×1 cm in area) were immersed in 2 mL of appropriately diluted enzyme solutions and held at 60 °C for 30 min. Then, 3 ml amounts of DNS were added to terminate the reaction, followed by boiling for 5 min, and reading of absorbance at 540 nm.

The standard assay for CMCase was as follows: 100 μl of appropriately diluted enzyme solution was added to 900 μl of 100 mM Na_2HPO_4 /citric acid (pH 6.0) containing 10 g CMC-Na l^{-1} , and the mixture was held at 60 °C for 10 min. DNS solution (1.5 ml) was added to each mixture to terminate the reaction, followed by boiling for 5 min and measurement of absorbance at 540 nm. Xylanase was assayed as was CMCase, except that birchwood xylan served as the substrate.

pNPGase activity was assayed as follows: 250 μl of appropriately diluted enzyme solution was added to 250 μl 4 mM *p*-nitrophenyl- β -D-glucopyranoside (pNPG) in 100 mM Na_2HPO_4 /citric acid (pH 6.0), and the mixture was held at 60 °C for 10 min. Then, 1.5 ml of 1.0 M Na_2CO_3 was added to terminate the reaction, and the absorbance was read at 420 nm. pNPCase activity was measured in the same manner using *p*-nitrophenyl- β -D-cellobioside (pNPC) as a substrate.

Control assay was performed with the same procedure as the reaction system, except that the enzyme solutions were added to the reaction mixture followed by the termination reagent. One unit of enzyme activity was defined as the

amount of enzyme required to release 1 μmol of reduced sugar (for FPase, CMCase, and xylanase activity) or 1 μmol of *p*-nitrophenyl (for pNPGase and pNPCase) per min. Glucose, xylose, and *p*-nitrophenyl served as standards, respectively.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein assay

SDS-PAGE was performed on 12% (w/v) polyacrylamide gels. Proteins were stained by Coomassie Brilliant Blue G-250. Protein concentration was measured by the Bradford method using a protein assay kit (Bio-Rad). For protein identification, the target band was excised from the SDS-PAGE gel, digested with trypsin, and sequenced by liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) at the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences.

Molecular analysis of the T-DNA insertion mutants

Molecular analysis of the T-DNA insertion mutants was performed as described previously (Esher et al. 2015). Genomic DNA from 5-A7, 5-C6, and 13-B7 were pooled and sequenced on an Illumina HiSeq X Ten platform running the 150-bp paired-end module. *Agrobacterium*-mediated Insertional Mutagenesis High-Throughput Insert Identification (AIM-HII; <https://github.com/granek/aimhii>) software was used to analyze the insertion sites.

Nucleotide sequence accession numbers

The nucleotide sequences of all insertion sites of *H. insolens* Y1 mutants were deposited in GenBank under accession nos. MF966970–MF966977, respectively.

Results

Insertional mutagenesis screening for *H. insolens* cellulase mutants

A previously constructed library of *H. insolens* T-DNA random insertional mutants (Xu et al. 2016) was screened for strains with altered cellulose production using the plate-clearing zone assay. After growth on screening plates using CMC-Na as the carbon source, and dying with Congo Red, three putative mutants that exhibited significantly smaller clearing zones (5-A7, 5-C6, and 13-B7) than the wild-type strain were selected for the study (Fig. 1). The ratio of the diameter of the clearing zone to the colony of the wild-type strain, 5-A7, 5-C6, and 13-B7, were 2.3, 2.1, 2.0, and 2.0, respectively.

Fig. 1 Plate-clearing zone assay of wild-type and mutant *Humicola insolens* Y1. *H. insolens* was grown for 5 days on screening plates containing sodium carboxymethyl cellulose (CMC-Na) and the production of cellulolytic enzymes was evaluated. Zones of CMC hydrolysis were clearly visible after staining with Congo Red. WT, the wild-type strain of *H. insolens* Y1

Cellulase production and hydrolysis during shake-flask fermentation

Strains 5-A7, 5-C6, and 13-B7 were evaluated for production of extracellular cellulase during shake-flask fermentation. The time course of dry biomass accumulation and cellulase production was recorded over 6 days. No obvious difference was evident in terms of the dry biomass of the wild-type *H. insolens* Y1 and the three mutant strains (Fig. 2a). Compared with the wild-type strain, all three mutants exhibited relatively reduced FPase activities after 2 days of cultivation. After 6 days of fermentation, the maximum FPase activity of the wild-type strain was 11.19 U/ml, whereas those of 5-A7, 5-C6, and 13-B7 were 7.70, 8.14, and 7.51 U/ml, respectively (Fig. 2b).

The extracellular CMCase, pNPCase, pNPGase, and xylanase activities were also determined. As shown in Fig. 2c, the maximum CMCase activities of 5-A7, 5-C6, and 13-B7 were dramatically reduced to 14.92, 15.87, and 14.09% that of the parental strain, respectively. However, the three mutants expressed relatively higher pNPCase and pNPGase activities than the parental strain. The pNPCase activities of 5-A7, 5-C6, and 13-B7 were 85.46, 117.96, and 62.67% greater, respectively, than that of the wild-type strain at 6 d (Fig. 2d). The pNPGase activities of 5-A7, 5-C6, and 13-B7 were 53.56, 63.38, and 40.26% higher, respectively, than that of the wild-type strain at 6 d (Fig. 2e). Strains 5-A7, 5-C6,

Fig. 2 Dry biomass accumulation and cellulase production profiles of 5-A7, 5-C6, 13-B7 and the wild-type *H. insolens* Y1. Time course of dry weight (a), FPase (b), CMCase (c), pNPCase (d), pNPGase (e),

and xylanase (f) activities in shake-flask-fermentation culture using 2.0% (w/v) Avicel as the carbon source

Table 1 Primary proteins in culture supernatant of wild-type *H. insolens* Y1

Protein	Describe	References
Cel6A	GH6, cellobiohydrolase	Annabelle Varrot et al. (1999)
Cel6B	GH6, CMCase	Davies et al. (2000)
Cel7A	GH7, cellobiohydrolase	Schulein (1997)
Cel7B	GH7, CMCase	Davies et al. (1997)
CMC3	GH6, CMCase	Dalbøge and HeldtHansen (1994)
XynA	GH2, xylanases	Du et al. (2013)
XynB	GH2, xylanases	Du et al. (2013)
XynC	GH2, xylanases	Du et al. (2013)
Xyl43A	GH43, bifunctional β -xylosidase/ α -arabinosidases	Yang et al. (2014)
HIGAL	GH2, β -1,4-galactanases	Le Nours et al. (2003)

Fig. 3 Extracellular protein profiles of 5-A7, 5-C6, 13-B7, and the wild-type *H. insolens* Y1. SDS-PAGE of the extracellular protein profiles of the three mutants and the wild-type strain (WT) after growth for 6 days. M, Pageruler prestained protein ladder

and 13-B7 also exhibited less xylanase activity compared with the parental strain (Fig. 2f).

SDS-PAGE was used to examine extracellular protein secretion profiles. As shown in Fig. 3, the primary band at

around 40 kDa of the wild-type strain was much weaker in the three mutants. To identify the proteins in the primary band, peptides obtained via LC-ESI-MS/MS were submitted to the UniProt database. Ten previously reported proteins yielded matches: Cel6A, Cel6B, Cel7A, Cel7B, CMC3, XynA, XynB, XynC, Xyl43A, and HIGAL (Table 1).

Identification of mutational insertion sites using AIM-Seq

To locate the T-DNA insertion sites in the mutant strains, genomic DNA from 5-A7, 5-C6, and 13-B7 were re-sequenced and analyzed with the aid of the AIM-HII software (Esher et al. 2015). Twelve putative insertion sites were identified in the three mutants (Table 2). Of these, only two exhibited typical pair-type insertions, with genomic DNA sequences flanking both sides of the insertion fragment. In addition, both typical insertions exhibited short deletions of 19 and 14 bp. The remaining ten insertion sites were of the atypical singleton type, which may reflect the fact that T-DNA integration triggered chromosomal rearrangements.

Bioinformatics analysis revealed that three of the insertions were in genes encoding a putative dolichyl pyrophosphate phosphatase (HiInsert03-orf1) and two hypothetical proteins of unknown function (HiInsert04-orf1, HiInsert05-orf2); the other nine insertion sites were in the intergenic fragments (Table 2).

Discussion

Humicola insolens is one of the most well-known filamentous fungi that produces a complete set of cellulolytic enzymes and thus exhibits immense potential for the commercial production of neutral cellulase. Identification of genes involved in cellulase expression is crucial not only to allow us to understand the regulatory mechanisms underlying cellulase production but also to allow for strain improvement.

Genetic screens based on random insertional mutagenesis have been used widely to elucidate the functions of many genes in a variety of organisms (Chacko et al. 2015; Long et al. 2013; Michielse et al. 2005). In this work, we

screened a T-DNA-tagged mutant library of *H. insolens* for mutants with altered cellulase production and obtained three putative mutants: 5-A7, 5-C6, and 13-B7. Compared with the wild-type strain, the maximum FPase and CMCase activities of the three mutants decreased dramatically; however, the pNPCase and pNPGase activities were relatively higher. These results indicated that the reduction in cellulose hydrolysis levels exhibited by the mutant strains were attributable principally to reduced endoglucanase expression and/or secretion. Combined with the results of SDS-PAGE and mass spectrometry, we suggest that expression of the endoglucanase Cel6B, Cel7B, and CMC3 was reduced in the mutant strains. Strains 5-A7, 5-C6, and 13-B7 also exhibited lower xylanase activities than the parental strain, suggesting that the insertions may affect XynA/B/C production as well as that of cellulases.

To explore the molecular mechanism responsible for alterations in cellulase production, we re-sequenced and analyzed the genomic DNA of 5-A7, 5-C6, and 13-B7 using the AIM-Seq method, a high-throughput genomic sequencing and analysis approach developed by Esher et al. to allow investigators to identify the insertion sites of mutants generated by ATMT much more rapidly and economically than the traditional thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) method (Esher et al. 2015). In this study, we identified 12 insertion sites in 5-A7, 5-C6, and 13-B7. Similar to what has been reported in other fungi (Esher et al. 2015; Kemski et al. 2013; Li et al. 2007), the putative insertion sites in the three mutants exhibited truncations of the T-DNA insertion fragment, atypical singleton clusters, and typical insertions.

Molecular characterization of the mutants identified new promising candidate genes controlling cellulase production by *H. insolens*. Our results suggest that a putative dolichyl pyrophosphate phosphatase, two hypothetical genes encoding proteins of unknown function, and/or nine intergenic

Table 2 AIM-HII identified insert locations

Ref_chrom	Position	Insert location	Insert type	Describe
HiInsert01	2412–2761	Intergenic	Pair	–
HiInsert02	1771–2271	Intergenic	Pair	–
HiInsert03	369–495	CDS	Singleton	Putative dolichyldiphosphatase
HiInsert04	488–734	CDS	Singleton	Hypothetical protein
HiInsert04	7083–7335	Intergenic	Singleton	–
HiInsert05	1634–1869	Intergenic	Singleton	–
HiInsert05	1938–2163	CDS	Singleton	Hypothetical protein
HiInsert06	2053–2294	Intergenic	Singleton	–
HiInsert07	5492–5535	Intergenic	Singleton	–
HiInsert07	7323–7517	Intergenic	Singleton	–
HiInsert07	7791–8018	Intergenic	Singleton	–
HiInsert08	7661–7904	Intergenic	Singleton	–

CDS encoding DNA sequence

fragments may be involved in cellulase and hemicellulase production. Dolichyl pyrophosphate phosphatase-mediated N-glycosylation plays an important role in the lipid homeostasis of *Saccharomyces cerevisiae* (James et al. 2016). However, no study has yet found that such genes play roles in cellulase production. According to previous reports, intergenic fragments may be transcribed into functional long non-coding RNAs (Chacko et al. 2015). We identified many more insertion sites than the number of mutant strains; it is thus likely that not all mutated sites contribute to the mutant phenotypes. Further experiments including creation of independent mutant strains and analysis of their phenotypes will confirm the relevance of DNA disrupted by various insertions in cellulase production by *H. insolens*.

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Author contributions Conceived and designed the experiments: XX, CF, PS, and WZ. Performed the experiments: CF, XX, and JL. Analyzed the data: CF, XX, LS, WG, and BL. Contributed reagents/materials/analysis tools: WZ. Wrote the paper: XX and CF.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

References

- Chacko N, Zhao Y, Yang E, Wang L, Cai JJ, Lin X (2015) The lncRNA RZE1 Controls *Cryptococcal* morphological transition. *PLoS Genet* 11:e1005692
- Dalbøge H, HeldtHansen HP (1994) A novel method for efficient expression cloning of fungal enzyme genes. *Mol Gen Genet* 243:253–260
- Davies GJ, Ducros V, Lewis RJ, Borchert TV, Schülein M (1997) Oligosaccharide specificity of a family 7 CMCase: insertion of potential sugar-binding subsites. *J Biotechnol* 57:91–100
- Davies GJ, Brzozowski AM, Dauter M, Varrot A, Schülein M (2000) Structure and function of *Humicola insolens* family 6 cellulases: structure of the CMCase, Cel6B, at 1.6 Å resolution. *Biochem J* 348:201–207
- Du Y, Shi P, Huang H, Zhang X, Luo H, Wang Y, Yao B (2013) Characterization of three novel thermophilic xylanases from *Humicola insolens* Y1 with application potentials in the brewing industry. *Bioresour Technol* 130:161–167
- Esher SK, Granek JA, Alspaugh JA (2015) Rapid mapping of insertional mutations to probe cell wall regulation in *Cryptococcus neoformans*. *Fungal Genet Biol* 82:9–21
- James AW, Gowsalya R, Nachiappan V (2016) Dolichyl pyrophosphate phosphatase-mediated N-glycosylation defect dysregulates lipid homeostasis in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1861:1705–1718
- Kemski MM, Stevens B, Rappleye CA (2013) Spectrum of T-DNA integrations for insertional mutagenesis of *Histoplasma capsulatum*. *Fungal Biol* 117:41–51
- Le Nours J, Ryttersgaard C, Lo Leggio L, Østergaard PR, Borchert TV, Christensen LLH, Larsen S (2003) Structure of two fungal β -1,4-galactanases: searching for the basis for temperature and pH optimum. *Protein Sci* 12:1195–1204
- Li G, Zhou Z, Liu G, Zheng F, He C (2007) Characterization of T-DNA insertion patterns in the genome of rice blast fungus *Magnaporthe oryzae*. *Curr Genet* 51:233–243
- Long L, Wang Y, Yang J, Xu X, Liu G (2013) A septation related gene AcsepH in *Acremonium chrysogenum* is involved in the cellular differentiation and cephalosporin production. *Fungal Genet Biol* 50:11–20
- Meleiro LP, Zimbardi AL, Souza FH, Masui DC, Silva TM, Jorge JA, Furriel RP (2014) A novel pNPGase from *Humicola insolens* with high potential for untreated waste paper conversion to sugars. *Appl Biochem Biotechnol* 173:391–408
- Michielse CB, Hooykaas PJ, van den Hondel CA, Ram AF (2005) *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Curr Genet* 48:1–17
- Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31:426–428
- Mullins ED, Chen X, Romaine P, Raina R, Geiser DM, Kang S (2001) *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. *Phytopathology* 91:173–180. <https://doi.org/10.1094/phyto.2001.91.2.173>
- Schülein M (1997) Enzymatic properties of cellulases from *Humicola insolens*. *J Biotechnol* 57:71–81
- Tzfira T, Li J, Lacroix B, Citovsky V (2004) *Agrobacterium* T-DNA integration: molecules and models. *Trends Genet* 20:375
- Varrot Annabelle, Schülein Martin, Davies Gideon J (1999) Structural changes of the active site tunnel of *Humicola insolens* cellobiohydrolase Cel6A upon oligosaccharide binding. *Biochemistry US* 38:8884–8891
- Xu X, Li J, Shi P, Ji W, Liu B, Zhang Y, Yao B, Fan L, Zhang W (2016) The use of T-DNA insertional mutagenesis to improve cellulase production by the thermophilic fungus *Humicola insolens* Y1. *Sci Rep UK* 6:31108
- Yang X, Shi P, Huang H, Luo H, Wang Y, Zhang W, Yao B (2014) Two xylose-tolerant GH43 bifunctional β -xylosidase/ α -arabinosidases and one GH11 xylanase from *Humicola insolens* and their synergy in the degradation of xylan. *Food Chem* 148:381–387
- Zhong Y, Wang X, Yu H, Liang S, Wang T (2012) Application of T-DNA insertional mutagenesis for improving cellulase production in the filamentous fungus *Trichoderma reesei*. *Bioresour Technol* 110:572–577