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Gene targeting by RNAi-mediated knockdown of potent DNA ligase IV homologue in the cellulase-producing fungus *Talaromyces cellulolyticus*

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Abstract The genome of the cellulase-producing fungus *Talaromyces cellulolyticus* (formerly *Acremonium cellulolyticus*) was screened for a potent DNA ligase IV gene (*ligD* homologue). Homologous recombination efficiency in *T. cellulolyticus* is very low. Therefore, suppression of a non-homologous end joining system was attempted to enable specific gene knockouts for molecular breeding. The transcript levels of *ligD* homologue were 3.7% of those of the parental YP-4 strain in the Li20 transformant carrying the RNAi construct targeting the *ligD* homologue. Transformation of the hairpin-type RNAi vector into *T. cellulolyticus* could be useful in fungal gene knockdown experiments. Cellulase production and protein secretion were similar in the parental YP-4 strain and the Li20 transformant. Knockout transformation of *ligD* homologue using the Li20 transformant led to 23.1% double crossover gene targeting. Our results suggest that the potent DNA ligase IV gene of *T. cellulolyticus* is related to non-homologous end joining, and that the knockdown of the *ligD* homologue is useful in gene targeting.

Keywords homologous recombination·DNA ligase IV·RNAi·*Acremonium cellulolyticus*·cellulase

High cost of cellulase is one of the major hurdles in the production of bioethanol by using lingo-cellulose. *Hypocrea jecorina* (*Trichoderma reesei*) is a fungus that is widely used for cellulase production [1, 2]. We have previously studied *Acremonium cellulolyticus* in detail as an alternative source of cellulase [3-6]. The wild-type strain Y-94 of *A. cellulolyticus* was isolated from the soil in Japan [7]. Recently, *A. cellulolyticus* was phylogenetically transferred to the genus *Talaromyces* [8]. Further improvements in the cellulase production by the fungus by random mutagenesis and genetic manipulation are necessary to significantly reduce the commercial cost of the enzyme.

The *H. jecorina* and *T. cellulolyticus* strains have been improved by random mutagenesis [9]. Genome sequencing allowed the genetic manipulation of *H. jecorina*

for efficient cellulase and hemicellulase production [10, 11]. Gene targeting (disruption) has been widely used to understand the functions of specific genes. In general, fungi have low homologous recombination efficiencies due to the existence of a non-homologous end joining (NHEJ) system. To overcome this obstacle, deletion of the genes *ku70*, *ku80*, and *ligD* that are related to the NHEJ have been attempted in *Neurospora crassa* [12, 13]. Studies have shown that deletion of DNA ligase IV (*ligD*) improves the efficiency of homologous recombination in *H. jecorina* [14].

RNA interference (RNAi)-mediated gene knockdown is widely used to analyze the functions of specific genes [15, 16]. RNAi can be effectively used for gene knockdown in fungi, which have low homologous recombination efficiencies [6, 17-20]. It has been reported that *ku70* knockdown in *Aspergillus oryzae* by RNAi increases the homologous recombination efficiency [21].

In the current study, we used RNAi to knock down the DNA ligase IV gene (*ligD* homologue) in *T. cellulolyticus*, and generated *ligD*-disrupted transformants.

T. cellulolyticus strain Y-94 (FERM BP-5826, CBS136886) and its uracil auxotrophic mutant YP-4 strain were maintained in potato dextrose agar plates (Difco, USA) in the presence or absence of uracil and uridine [22]. Genome sequences of the *T. cellulolyticus* strain Y-94 were combed to identify the potent DNA ligase IV homologous gene (*ligD*) by performing a DNA-BLAST search using the *ligD* sequence from *Talaromyces marneffei* as a probe sequence [23].

Partially overlapped DNA fragments encoding the potent *ligD* (*g1131*) were amplified using high-fidelity polymerase KOD-plus-neo (Toyobo, Japan) and two primer sets, namely AcLLgpd_F and AcLLgpd_R, and AcLSgpd_F, and AcLSgpd_R (Table 1) on a PCR instrument (TP600; Takara, Japan). The thermal cycling conditions consisted of an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 66°C for 30 s, and extension at 68°C for 30 s. The amplified 592- and 424-base pairs (bp) DNA fragments were inserted into the *EcoRI* site of the pBluescript KS (+) vector (Agilent Technologies, USA) containing

active *pyrF* gene to generate the pligDi vector producing hairpin-type RNA in *T. cellulolyticus* by using In-fusion HD cloning kit (Clontech, USA) (Fig. 1) [5].

The 5′- and 3′-flanking regions (1,170 and 1,250 bp, respectively) of the open reading frame of *ligD* were PCR-amplified using KOD-plus-neo and two primer sets, namely AcLigD_5′_pbs_F and AcLigD_5′_ EcoR1_3′_R, and AcLigD_3′_ EcoR1_F and AcLigD_3′_ pbs_R (Table 1). The *hph* DNA sequence was amplified from a shuttle vector (pLD10) provided by Dr. H. Corby Kistler (University of Minnesota, USA) with the help of KOD-plus-neo and the primers trpCP_ligD5′_2_F and hph_EcoR1_ligD3′_R (Table 1) [4, 24]. These three PCR fragments were inserted into the *EcoRI* site of pBluescript KS (+) using In-fusion HD cloning kit to generate the pΔligD vector. The fΔligD was amplified from the pΔligD vector with the help of KOD-plus-neo and the primers AcLigD_5′_pbs_F and AcLigD_3′_ pbs_R (Table 1).

Transformations of the pligDi vector and the PCR-amplified fragment of fΔligD into the *T. cellulolyticus* were performed according to previously reported methods [5]. Agar plates used for the selection of uracil auxotrophs consisted of minimal medium (MM; 1% glucose, 10 mM NH_4Cl, 10 mM potassium phosphate [pH 6.5], 7 mM KCl, 2 mM $MgSO_4$, and 0.1% trace metals) containing 1 M sucrose. The plates were incubated at 30°C. Initially, the agar plates used for the selection of *hph* transformants contained 10 g/L yeast extract, 10 g/L tryptone, 1 g/L uracil, 1 g/L uridine, and 342.3 g/L sucrose [4]. After 1–2 days of incubation with *hph* transformants at 30°C, 10 mL of 0.5% soft agar containing 24 g/L potato dextrose broth, 500 mg/L hygromycin B, 1 g/L uracil, and 1 g/L uridine was added into the plates and the plates were incubated at 30°C for 1–2 weeks.

Integration of pligDi into the genomic DNA of *T. cellulolyticus* was verified by PCR using the primers PF1, PF2, PR1 (AcLLgpdP _R), PR2, PR3 (AcLSgpdP _R), and PR4 (Table 1). The thermal cycling conditions consisted of initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 98°C for 5 s, annealing at 63°C for 5 s, and extension at 72°C for 20 s. Transformation of fΔligD into *T. cellulolyticus* was

confirmed by PCR using trpCP_ligD5′_2_F and hph_EcoR1_ligD3′_R as primers (Table 1). Homologous integration of fΔligD into the genome DNA was checked by PCR with primers of PFa5, PRa5, PFb3, and PRb3 (Table 1).

Total RNA was extracted using FastRNA pro red kit (MP Biomedicals, US). The levels of *g1131* transcripts were determined using GoTaq 1 step RT-qPCR system (Promega, USA) on a TP900 PCR instrument (Takara, Japan). Primer sets used for reverse transcription quantitative PCR (RT-qPCR) of *g1131* and the β-actin genes were AcLigDRTPCR_F and AcLigDRTPCR_R, and AcB-actinRTPCR_F and AcB-actinRTPCR_R, respectively (Table 1). The thermal cycling conditions consisted of an initial reverse transcription at 42°C for 15 min and initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s. The transcript levels of *g1131* in the parental YP-4 strain and transformants were compared with that of the β-actin gene in the YP-4 strain and transformants [25].

To determine the protein secretion and cellulase activity, the fungal strains were cultured in a conical flask containing 10 mL of cellulose medium (5% Solka Floc, 10 mM NH_4Cl, 10 mM potassium phosphate [pH 6.5], 7 mM KCl, 2 mM $MgSO_4$, 0.1% trace metals, 0.1% uracil, and 0.1% uridine) in a rotary shaker at 150 rpm and 30°C for 7 days. Filter paper assay (FPase, FPU/ml) was used for measuring the cellulase activity according to reported methods [9]. Concentrations of soluble protein in cultures were determined by Bradford's method (Bio-Rad, USA) using bovine serum albumin as standard.

One homologous candidate gene (*g1131*) of the potent DNA ligase IV in the *T. cellulolyticus* Y-94 strain genome was identified, which showed an amino acid sequence similarity of 91% with the sequence of DNA ligase IV from *T. marneffei* (XP_002144840.1). Identities of the amino acid sequence of *ligD* were 89% of *Talaromyces stipitatus* (XP_002340632.1), 71% of *Byssochlamys spectabilis* (GAD98954.1), and 70% of *Aspergillus clavatus* (XP_001274495.1), respectively. The

amino acid sequence of *g1131* was predicted to include an adenylation domain of DNA Ligase IV and an oligonucleotide/oligosaccharide-binding fold domain of ATP-dependent DNA ligase IV. The sequence of *g1131* from the *T. cellulolyticus* Y-94 strain was deposited in the DNA Data Bank of Japan under the accession number AB924384.

The pligDi vector was transformed into the *T. cellulolyticus* YP-4 strain. Approximately 35 transformants that grew on the MM medium were isolated. Integration of the RNAi construct into the fungal cells was confirmed by PCR using the primers PF1, PF2, PR1, PR2, PR3, and PR4 (Fig. 1). The PCR analysis confirmed that the transformants of Li27 and Li31 were carrying only ligD LL sequence in the RNAi construct, a transformant of Li33 was carrying *ligD* LL and 5′-region of *ligD* LS sequences, and the transformants of Li20 and Li24 were carrying *ligD* LL and *ligD* LS sequences (Fig. 1). Relative to that in the parental YP-4 strain, the normalized transcript level of *g1131* in the Li20, Li24, and Li27 transformants were 0.037, 0.50, 0.21, respectively (Fig. 2).

Homologous recombination in Li20 was investigated using the PCR-amplified fragment of fΔligD from the pΔligD vector. Transformation of the fΔligD fragment into *T. cellulolyticus* was confirmed by PCR (Table 1). Thirteen double transformants with the fΔligD fragment integrated into their DNA was recognized from the single transformants of Li20. Homologous recombination of the fΔligD fragment was probed by PCR using the primers PFa5, PFb3, PRa5, and PRb3 (Table 1, Fig. 3). The results of PCR analysis indicated that homologous recombination occurred in three double transformants of LΔ13, LΔ14, and LΔ17 obtained from the single transformants of Li20 (3/13) (Fig. 3).

The cellulase activities in the cultures of the strains YP-4 and Li20 measured by filter paper assay were 2.1 FPU/mL and 2.2 FPU/mL (standard errors 0.3 and 0.1), respectively. The protein concentrations in the cultures of the strains YP-4 and Li20 were 336 μg/mL and 304 μg/mL (standard errors 9.2 and 11), respectively. There were no differences in cellulase production and protein secretion between two strains.

169 The hairpin-type RNAi construct could be useful for gene knockdown in the *T.*
170 *cellulolyticus* YP-4 strain, similar to the manner previously reported for *ade2* and *ura5*
171 in *Histoplasma capsulatum* [10]. A previous study reported reduced transcript levels of
172 a potent sugar-sensing gene in the transformants obtained using similar RNAi methods
173 from the *T. cellulolyticus* YP-4 strain [6]. To our knowledge, this is the second report of
174 a gene knockdown using the hairpin-type RNAi vector in *T. cellulolyticus*. The
175 variations in *g1131* mRNA levels among the transformants were likely due to the
176 differences in the number of integrated RNAi constructs caused by NHEJ and
177 integration position effect.
178 The present experiment using the PCR-amplified fragment of fΔligD from the
179 pΔligD vector suggested that RNAi-mediated knockdown of the DNA ligase IV in *T.*
180 *cellulolyticus* could enable the development of fungal strains capable of homologous
181 recombination. It has been reported that *ku70* knockdown in *Aspergillus oryzae* using
182 RNAi methods leads to an increase in the efficiency of homologous recombination [21].
183 To our knowledge, this is the first report showing the RNAi-mediated knockdown of
184 *ligD* in fungi. The efficiency of homologous recombination in *T. cellulolyticus* strain
185 YP-4 was 0% (0 *creA* disruptants /300 transformants) by using 1-kb homologous
186 flanking regions and 27% (19 *creA* disruptants /71 transformants) by using 2.5-kb
187 homologous flanking regions [26]. Relatively low ratio of homologous recombination
188 could indicate *T. cellulolyticus* has relatively strong NHEJ systems.
189 The uracil auxotroph transformation system is effective in *T. cellulolyticus*, and the
190 marker recycling of auxotroph transformation systems could be useful for further
191 research and development by using the *ligD* knockout transformants [5, 14, 27]. The
192 PCR analysis confirmed that the transformants with the pligDi vector were sometimes
193 carrying shorter constructs (Fig. 1). The NHEJ DNA repair mechanism was seen to
194 intermittently inhibit the whole-length recombination of the RNAi construct.
195 In *N. crassa* lacking *ku70* or *ku80*, the gene-targeting ratio with 2-kb homologous
196 flanking regions was reported to be 100%, compared to the 10–30% observed in wild-

type recipient [12]. In *H. jecorina* lacking *ku70*, the gene-targeting ratio with 1-kb homologous flanking regions was reported to be >95% [28]. Further, in *N. crassa* lacking *ligD*, the gene-targeting ratio with 2-kb homologous flanking regions was reported to be 100% [13], whereas in *Aspergillus oryzae* lacking *ligD*, the *pyrG* targeting ratio with 2 kb homologous flanking regions was reported to be 92%, compared to the 38% observed in wild-type recipient [27]. The gene targeting ratio of 23.1% with 1 kb homologous flanking regions in *T. cellulolyticus* recipient Li20 (present results) suggests that the *ligD* mRNA level of 3.7% led to 76.9% NHEJ.

It was reported that the transformation efficiency in the strain of *N. crassa* lacking *ligD* was 4–5 times lower than that in the wild-type strain [13]. However, our results showed an opposite trend in *T. cellulolyticus*. Further analyses of the double transformants lacking *ligD* with the help of efficient marker systems are necessary to identify the basis of these differences.

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References

1. Ghose, T. K., & Sahai, V. (1979). *Biotechnology and Bioengineering, 21*, 283–296.
2. Kubicek, C. P. (2013). *Journal of Biotechnology, 163*, 133–142.
3. Fujii, T., Fang, X., Inoue, H., Murakami, K., & Sawayama, S. (2009). *Biotechnology for Biofuels, 2 (24)*, 1–8.
4. Kanna, M., Yano, S., Inoue, H., Fujii, T., & Sawayama, S. (2011). *AMB Express, 1 (15)*, 1–8.
5. Fujii, T., Iwata, K., Murakami, K., Yano, S., & Sawayama, S. (2012). *Bioscience, Biotechnology, and Biochemistry, 76*, 245–249.

6. Asada, S., Watanabe, S., Fujii, T., Inoue, H., Ishikawa, K., & Sawayama, S. (2014). *Applied Biochemistry and Biotechnology*, doi: 10.1007/s12010-014-0728-2
7. Yamanobe, T., Mitsuishi, Y., & Takasaki, Y. (1987). *Agricultural and Biological Chemistry, 51*, 65–74.
8. Fujii, T., Hoshino, T., Inoue, H., & Yano, S. (2014). *Federation of European Microbiological Societies Microbiology Letters, 351*, 32–41.
9. Fang, X., Yano, S., Inoue, H., & Sawayama, S. (2009). *Journal of Bioscience and Bioengineering, 107*, 256–261.
10. Martinez, D., Berka, R. M., Henrissat, B. et al. (2008). *Nature Biotechnology, 26*, 553–560.
11. Seidl, V., & Seiboth, B. (2010). *Biofuels, 1*, 343–354.
12. Ninomiya, Y., Suzuki, K., Ishii, C., & Inoue, H. (2004). *Proceedings of the National Academy of Sciences of the United States of America, 101*, 12248–12253.
13. Ishibashi, K., Suzuki, K., Ando, Y., Takakura, C., & Inoue, H. (2006). *Proceedings of the National Academy of Sciences of the United States of America, 103*, 14871–14876.
14. Steiger, M. G., Vitikainen, M., Uskonen, P., Brunner, K., Adam, G., Pakula, T., Penttilä, M., Saloheimo, M., Mach, R. L., & Mach-Aigner, A. R. (2011). *Applied and Environmental Microbiology, 77*, 114–121.
15. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). *Nature, 391*, 806–811.
16. Rahman, M., Ali, I., Husnain, T., & Riazuddin, S. (2008). *Biotechnology Advances, 26*, 202–209.
17. Romano, N., & Macino, G. (1992). *Molecular Microbiology, 6*, 3343–3353.
18. Rappleye, C. A., Engle, J. T., & Goldman, W. E. (2004). *Molecular Microbiology, 53*, 153–165

19. Janus, D., Hoff, B., Hofmann, E., & Kück, U. (2007). *Applied and Environmental Microbiology, 73*, 962–970.
20. Nakayashiki, H., & Nguyen, Q. B. (2008). *Current Opinion in Microbiology, 11*, 494–502.
21. Imamura, K., Tsuyama, Y., Hirata, T., Shiraishi, S., Sakamoto, K., Yamada, O., Akita, O., & Shimoi, H. (2012). *Applied and Environmental Microbiology, 78*, 6996–7002.
22. Inoue, H., Fujii, T., Yoshimi, M., Taylor II, L.E., Decker, S. R., Kishishita, S., Nakabayashi, M., & Ishikawa, K. (2013). *Journal of Industrial Microbiology and Biotechnology, 40*, 823–830.
23. Yuen, K. Y., Pascal, G., Wong, S. S., Glaser, P., Woo, P. C., Kunst, F., Cai, J. J., Cheung, E. Y., Médigue, C., & Danchin, A., (2003). *Archives of Microbiology, 179*, 339–353.
24. Kistler, H. C., & Benny, U. (1992). *Gene, 117*, 81–89.
25. Fujii, T., Murakami, K., Sawayama, S. (2010). *Bioscience, Biotechnology, and Biochemistry, 74*, 419–422.
26. Fujii, T., Inoue, H., and Ishikawa, K. (2013). *AMB Express, 3 (73)*, 1–9.
27. Guangtao, Z., Hartl, L., Schuster, A., Polak, S., Schmoll, M., Wang, T. H., Seidl, V., & Seiboth, B. (2009). *Journal of Biotechnology, 139*, 146–151.
28. Maruyama, J., & Kitamoto, K. (2008). *Biotechnology Letters, 30*, 1811–1817.

Table 1 List of PCR primers used in this study

Name	Sequence (5'-3')	Amplification target
AcLLgpdP_ F	TCACCGCAATGAATTGGTGATGAGCGTGGGGTGTCATTGATGC	*ligD* long sequence
AcLLgpdP _R (PR1)	CGGCCGCGGTAGTAGCTTCTGTGTAC	(LL)
AcLSgpdP _F	CTACTACCGCGGCCGGGCATTGCGACGATCTCGTAGTGTATAGCG	*ligD* short sequence
AcLSgpdP _R (PR3)	GGTTCACGACGAATTGGCAACAGAGGAAGACCCCACGTTCTGG	(LS)
PF1	GCTAGCTACACTTTTTTCCTACAACTATCACCGC	Fig. 1
PF2	GGCTATACGGCCTGTTCATCGCCGATTTG	Fig. 1
PR2	GGACGGTGAAATGATTACATGGGACCCCG	Fig. 1
PR4	GCTTGATATCGAATTACGGCTTGAGATGGAATTTTGGGCTACC	Fig. 1
AcLigDRTPCR_F	GACCCGATGAATGGATTCGACC	*ligD* for RT-qPCR
AcLigDRTPCR_R	CGCGGGAATCGTAGTGTCAATC	
AcB-actinRTPCR_F	CAACTGGGACGACATGGAGA	*β-actin* for RT-qPCR
AcB-actinRTPCR_R	GTTGGACTTGGGGTTGATGG	
AcLigD_5'_pbs_F	CGGGCTGCAGGAATTACCGGATCCATTGTTATTTCGTCTGCTGGG	
AcLigD_5'_ EcoR1_3'_R	ATTTACCCGAGAATTCTGGGTATCTCCGTCCAGCTGACTCATTC	*ligD* 5'
AcLigD_3'_ EcoR1_F	CGGGCTGCAGGAATTCTCGGGTAAATGCAAGGGACGAGGTCAC	l*igD* 3'
AcLigD_3'_ pbs_R	GCTTGATATCGAATTGTATGGCTGCCGGGTGTTCCGATGTAC	
trpCP_ligD5'_2_F	GAGATACCCAGAATTCGTTAACTGATATTGAAGGAGCATTTTTTG	*hph*
hph_EcoR1_ligD3'_R	ATTTACCCGAGAATTCTATTCCTTTGCCCTCGGAC	
PFa5	CCCGCACTTGTTCATGGAGGTCTTGTAGAAG	Fig. 3
PRa5	GTTGACCTCCACTAGCTCCAGCCAAGCC	
PFb3	GTGGAAACCGACGCCCCAGCACTCG	Fig. 3
PRb3	CCACTCACATGCTGGAACCCTAACGCAGC	

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Figure legends

Fig. 1. Verification of the genome integration of *ligD* RNAi construct. A, positions of primers used in the PCR assays performed for identifying the pligDi construct. B & C, the PCR primers used were PF1 and PR1 (Table 1). D, the PCR primers used were PF2, PR2, PR3, and PR4 (Table 1). E, the primers used were PF2 and PR4 (Table 1). M, 200-bp DNA ladder. Numerals, strains transformed with pligDi. P, positive control (pligDi as template). N, negative control (ultra-pure water as template).

Fig. 2. Normalized mRNA levels of *ligD* in the parental strain *T. cellulolyticus* YP-4 and in the transformants (pligDi was used for transformation) relative to that in the parental YP-4 strain. β-actin gene was used as reference gene of *hph*.

Fig. 3. Results of PCR analyses performed to detect homologously integrated transformants. A, positions of primers used in the PCR performed to detect homologously integrated transformants. B, primer sets used were PFa5 and PRa5, and PFb3 and PRb3. M, 200-bp DNA ladder. Numerals, double transformants from the recipient Li20 strain transformed with the fΔligD fragment. N, negative control (ultra-pure water as template).

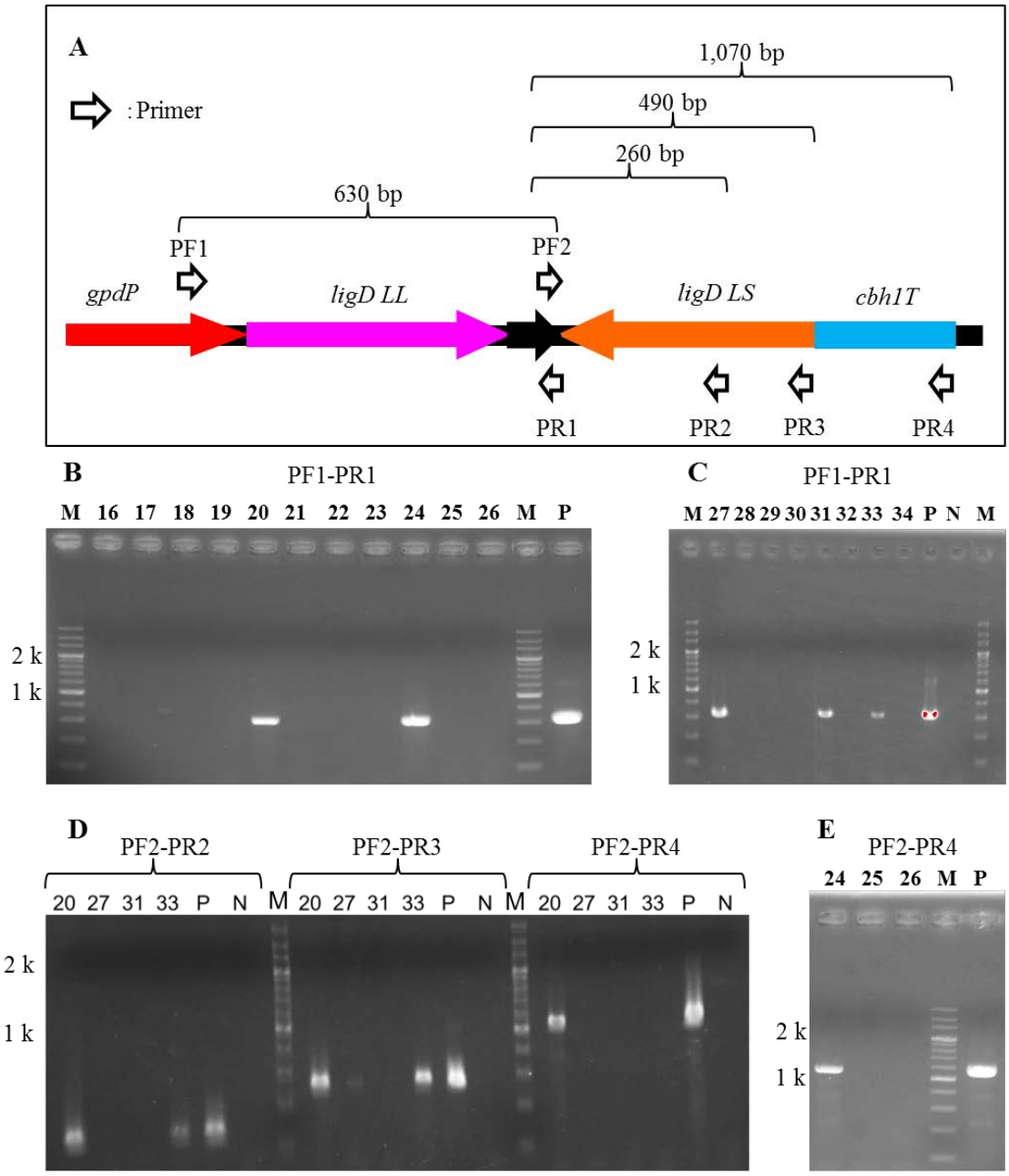

Fig. 1. Hayata et al.

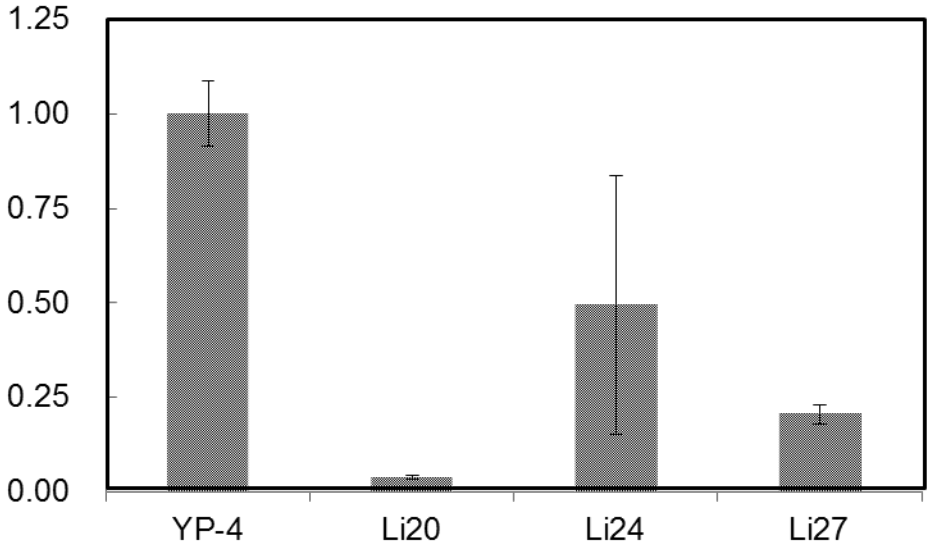

Fig. 2. Hayata et al.

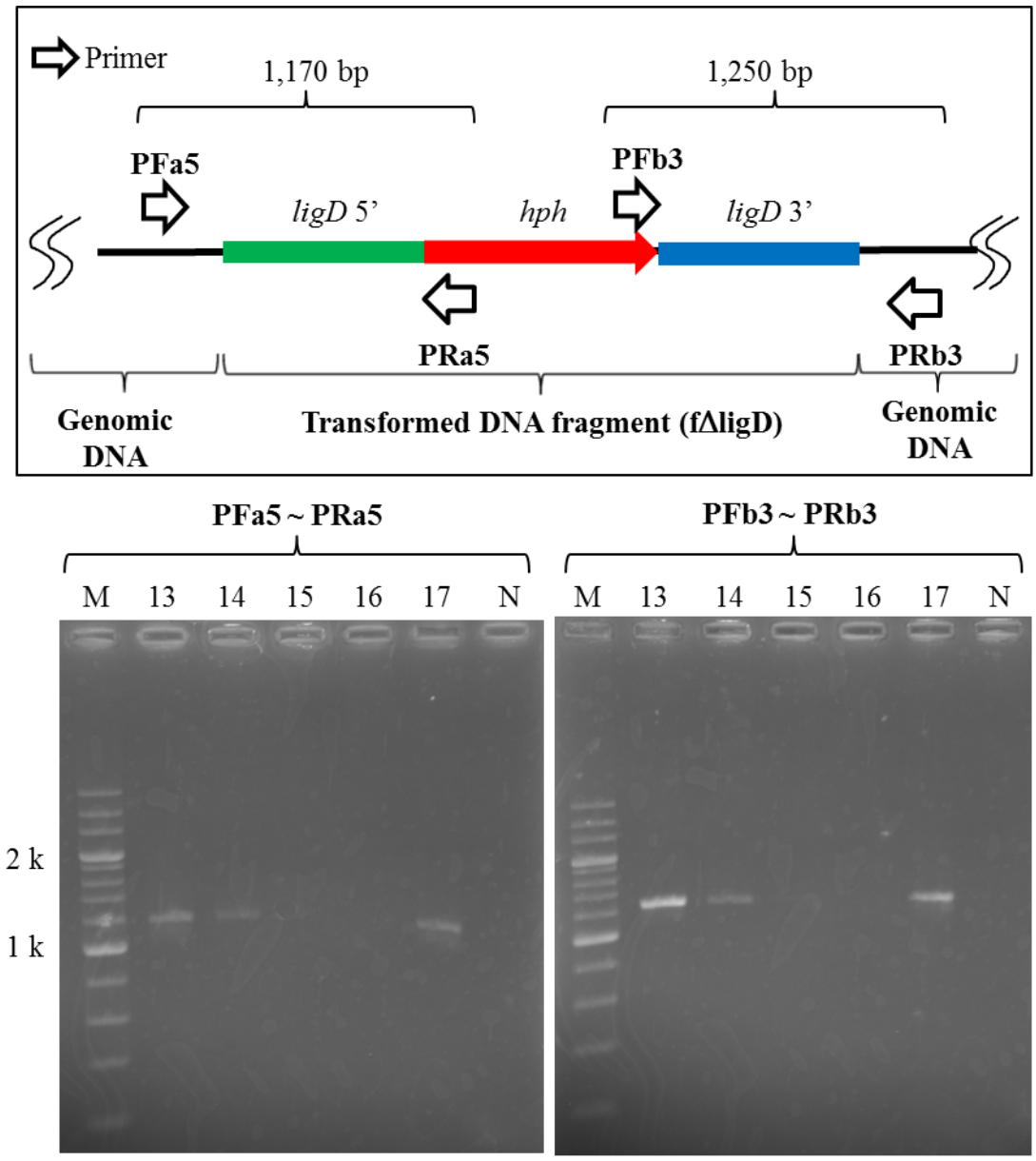

Fig. 3. Hayata et al.