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Regular paper

**Improvement of ethanol production by recombinant expression of pyruvate
decarboxylase in the white-rot fungus *Phanerochaete sordida* YK-624**

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Abstract

To improve ethanol production by *Phanerochaete sordida* YK-624, the pyruvate decarboxylase (PDC) gene was cloned from and reintroduced into this hyper lignin-degrading fungus; the gene encodes a key enzyme in alcoholic fermentation. We screened 16 transformant *P. sordida* YK-624 strains that each expressed a second, recombinant PDC gene (*pdc*) and then identified the transformant strain (designated GP7) with the highest ethanol production. Direct ethanol production from hardwood was 1.41 higher with GP7 than with wild-type *P. sordida* YK-624. RT-PCR analysis indicated that the increased PDC activity was caused by elevated recombinant *pdc* expression. Taken together, these results suggested that ethanol production by *P. sordida* YK-624 can be improved by the stable expression of an additional, recombinant *pdc*.

Introduction

Production of ethanol fuel from biomass has attracted worldwide interest as an alternative to conventional, fossil fuels (1, 2). A single microorganism that can hydrolyze cellulose and then ferment the monosaccharides subunits to ethanol could potentially increase ethanol yields and reduce production costs (3). Researchers

discovered that white-rot fungi produce enzymes that can be used to pretreat lignocellulosic biomass before downstream enzymatic saccharification (4, 5). In addition, Okamoto et al. (2010; 2011) demonstrated that several species of white-rot fungi could produce ethanol directly from hexoses, starch, wheat bran, or rice straw (6, 7). Since white-rot fungi can hydrolyze cellulose (8, 9), white-rot fungi could be used for one-step (direct) alcohol fermentation of lignocellulosic biomass.

Pyruvate decarboxylase (PDC), which catalyzes the non-oxidative decarboxylation of pyruvate to acetaldehyde with the release of carbon dioxide, is a key enzyme in ethanol fermentation (10). Overexpression of the PDC gene in the yeast *Hansenula polymorpha* was recently shown to increase PDC activity, resulting in elevated ethanol production (11). This finding suggests that overexpression of the PDC gene in white-rot fungi will increase the rate of alcoholic fermentation.

Among white-rot fungi, including the well-characterized species *Phanerochaete chrysosporium* and *Trametes versicolor*, *P. sordida* YK-624 is particularly suited for the alcoholic fermentation of lignocellulose due to its high ligninolytic activity and selectivity (12). Here, we generated high *pdv*-expressing transformants of *P. sordida* YK-624 and evaluated their capacity for direct ethanol production from hardwood.

Materials and methods

Fungal strain

P. sordida YK-624 (ATCC 90872) and uracil auxotrophic strain UV-64 (13) were used in this study. The fungus was maintained on potato dextrose agar (PDA) slants at 4 °C.

Cloning of a full-length gene encoding PDC

P. sordida YK-624 was incubated on PDA plates at 30 °C for 3 days, and 10-mm diameter disks then were punched out from the growing edge of the mycelia using a sterile cork borer. Two mycelial disks were placed into a 100-mL Erlenmeyer flask containing 10 mL potato dextrose broth (PDB) medium (Becton, Dickinson and Company). Genomic DNA of *P. sordida* YK-624 was extracted from mycelia using an ISOPLANT II kit (Nippon Gene, Tokyo). All primers used in this process are listed in Table 1. The conserved region of the PDC-encoding gene was amplified using the primer set PcPDCF-PcPDCR, which was designed based on complementary to the *P. chrysosporium* PDC gene archived in the Joint Genome Institute database. The 5'-coding region of the PDC gene from *P. sordida* YK-624 was cloned from genomic DNA using an inverse PCR method (14) with the primer sets InverseF1-InverseR1, InverseF2-InverseR2, InverseF3-InverseR3, InverseF4-InverseR4, InverseF5-InverseR5

and InverseF6-InverseR6. The primer sets InverseF7-InverseR7 and InverseF8-InverseR8 were used to clone the 3'-coding region. The primer set PDCF1-PDCR1, which was designed based on sequences obtained from the inverse PCR, was used to determine the sequence of the full-length gene encoding PDC of *P. sordida* YK-624.

Construction of a PDC gene expression vector, co-transformation of UV-64, and screening of regenerated clones

The full-length genomic PDC gene (2124 bp) was amplified using primers PDCF1-PDCR1 (Table 1). The resulting PCR product was ligated into the cloning vector pMD20-T (Takara Bio, Shiga, Japan) and introduced into *Escherichia coli* DH5 α for sequencing. Primers PDCF2 and PDCR2 were designed to introduce an *Xba* I site into the *pd*c; these primers were also used to amplify the *pd*c with the *Xba* I from the recombinant plasmid, which was used as a template for sequencing. The plasmid *pPsGPD-pro*, which was generated in our previous study (15), was used to construct a *pd*c-expression plasmid. The DNA fragment amplified with primers PDCF2 and PDCR2 was digested with *Xba* I and cloned into *Xba* I digested-*pPsGPD-pro*, yielding plasmid *pPsGPD-PDC*. The *pPsGPD-PDC* was sequenced to verify the absence of PCR errors. UV-64 protoplasts were prepared via a standard technique involving cellulases

and were then co-transformed with *pPsURA5* and *pPsGPD-PDC* using a polyethylene glycol (PEG) method, as previously described (13). The co-transformed strains (GP strains) were selected by PCR using the primers *gpdF1* and *PDCR3* (Table 1), which were designed to amplify the entire recombinant *pdc* gene.

Assessment of pdc expression transformants based on ethanol production and fermentative capacity for various saccharides

The effect of stable *pdc* expression on ethanol production by the *pdc*-expressing transformants (GP strains) was investigated. Briefly, *P. sordida* YK-624 (WT) and 16 GP strains each were incubated on separate PDA plates at 30 °C for 3 days; 10-mm diameter disks then were punched out from the growing edge of mycelia using a sterile cork borer. For each strain, two mycelial disks were placed into a 100-mL Erlenmeyer flask containing 10 mL basal liquid medium (20 g L⁻¹ glucose, 10 g L⁻¹ yeast extract, 10 g L⁻¹ KH₂PO₄, 2 g L⁻¹ (NH₄)₂SO₄, and 0.5 g L⁻¹ MgSO₄·7H₂O, pH 4.5). After sealing each flask with a silicone plug stopper (semi-aerobic conditions), each culture was statically incubated at 30 °C for 4 days (16). Culture samples were separated by high-performance liquid chromatography (HPLC) using a Shodex SH1821 column (8.0 mm × 300 mm, Showa Denko K.K., Tokyo, Japan) at 75 °C with 0.5 mM H₂SO₄ as the mobile phase at a flow rate of 0.6 mL min⁻¹, and ethanol and glucose concentrations in

the cultures were measured using an online Refractive Index Detector.

The GP strain with the highest ethanol fermentation efficiency (GP7) was also examined for its ability to produce ethanol from each of eight saccharides— D-glucose, D-mannose, D-galactose, D-fructose, D-xylose, L-arabinose, cellobiose, and maltose (Wako Pure Chemical Industries, Osaka, Japan)—using the above-described fermentation method.

Enzyme assay

WT and GP7 were each incubated at 30 °C for 2, 4, 6, or 8 days in basal liquid medium, as described above. A mycelial sample from each strain was added to 2 ml of 400 mM Tris-HCl buffer (pH 6.0) and was then homogenized on ice using a Polytron PT1200E (Kinematica, Canada). The homogenate was centrifuged (4 °C, 10,000 x g, 10 min); 100 µL 10 mM thiamine diphosphate and 100 µL 10 mM MgCl₂ were added to each obtained supernatant, which was then incubated at 60 °C for 30 min. For each culture, this solution was used as a cell-free extract to measure PDC activity, which was determined by monitoring the oxidation of NADH to NAD⁺ ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) as previously described (17). Each reaction mixture (1 mL) consisted of 50 µL cell-free extract, 100 µL 10 mM pyruvate, 150 µL distilled water, 100 µL 6 U mL⁻¹ alcohol dehydrogenase solution (Sigma-Aldrich, USA), and 100 µL 0.4 mM NADH and 500 µL

400 mM Tris-HCl buffer (pH 6.0).

Transcriptional analysis of strain GP7

WT and GP7 were cultured as described in the *enzyme assay section*, and were each incubated at 30 °C for 2, 4, 6, or 8 days. Mycelia were then collected and stored at -80 °C. Under liquid nitrogen and using a mortar and pestle, 100 mg of mycelium mat was ground to a fine powder, and total RNA was then isolated from the mycelial powder using a Qiagen RNeasy Mini Kit (Hilden, Germany). Reverse transcription (RT)-PCR was performed using 200 ng of total RNA with a PrimeScript RT-PCR Kit and the gene-specific primer sets PDCF4-PDCR4 (recombinant *pdc*), PDCF5-PDCR5 (native *pdc*) and ActinF-ActinR (*actin*, Table 1).

Determination of ligninolytic properties and fermentation with wood meal as a carbon source

Mycelial discs of WT and GP7 were added to 50-mL Erlenmeyer flasks containing 0.5 g extractive-free beech wood meal (80–100 mesh) and 1.25 mL distilled water; each resulting culture was incubated at 30 °C for 28 days. After the incubation period, weight loss, Klason lignin content, and acid-soluble lignin content in the fungal-treated wood meal were determined, as previously described (12).

After this 28 day-incubation of wood meal culture inoculated with either WT or

GP7, 10 mL of basal liquid medium lacking saccharides was added to each culture, which was then homogenized using a Polytron PT1200E. To investigate the effect of saccharification on ethanol fermentation, 36 paper units (FPU) of Cellulose Onozuka RS/g wood meal were then added to each culture. Each culture flask was sealed with a silicon plug stopper (semi-aerobic conditions) and then further incubated with shaking at 30 °C for 3, 6, or 9 days. After fermentation, the wood meal cultures were centrifuged (10,000 x g, 5 min), and the amount of ethanol in the obtained supernatant was analyzed by HPLC, as described in above.

Nucleotide sequence accession number

The nucleotide sequence of *pdc* derived from *P. sordida* YK-624 has been deposited in the DDBJ database (<http://www.ddbj.nig.ac.jp/>) under accession no. LC057149.

Results and discussion

Generating pdc-expression transformants and screening for ethanol productivity

Full-length genomic *pdc* (2124 bp) was obtained from *P. sordida* YK-624 using PCR, inverse PCR, and genomic DNA template. Amino acid sequence alignment of theoretical translations of *pdc* from *P. chrysosporium* (PcPDC) and *P. sordida* YK-624

(PsPDC) indicated that the sequence identity of the two predicted proteins was 89% (Fig. 1). The procedure for constructing the *pdc* expression plasmid, *pPsGPD-PDC*, is shown in Fig. 2. The *pPsGPD-PDC* expression plasmid was introduced into strain UV-64 together with *pPsURA5* as a marker plasmid. We obtained 16 GP strains co-transformed with *pPsGPD-PDC* and *pPsURA5*.

Ethanol production by each GP transformant strain was then investigated. Ethanol production by all 16 recombinant GP strains was higher than that by the WT strain (data not shown). Transformant GP7 showed the highest ethanol production and glucose consumption among the 16 screened transformants (data not shown) and was therefore selected for further analyses of PDC activity and gene expression.

PDC activity and transcriptional analysis of recombinant pdc in GP7

The intracellular activity of PDC in strain GP7 was compared with that in WT. GP7 showed the highest PDC activity after 4 days of incubation, with 1.3-fold higher PDC activity relative to that of the WT strain (Fig. 3a).

We next used RT-PCR to analyze the temporal transcription profile of *pdc* in GP7 and WT (Fig. 3b). The highest levels of recombinant *pdc* expression were detected after 4 days of incubation, after which the expression levels of *pdc* decreased. Although the native *pdc* was detected in WT by RT-PCR, the expression level was markedly lower

than expression of recombinant *pdc* in GP7 (data not shown). Thus, these results suggested that the higher production of PDC was due to the higher expression of recombinant *pdc* in GP7.

Ethanol production from saccharides

For WT and GP7, the time course of glucose fermentation is shown in Fig. 4. GP7 produced the maximum ethanol concentration (3.8 g L^{-1}) from glucose after 8 days of incubation, and this maximum was higher than that for WT (3.1 g L^{-1}). The ability of GP7 to ferment each of several monosaccharides and disaccharides as a carbon source was determined (Table 2). In the cultures containing mannose or fructose as the sole carbon source, maximum ethanol concentrations were 2.5 and 1.5 g L^{-1} , respectively (Table 2). However, only low levels of ethanol production were detected in the cultures containing galactose, suggesting that the production of galactose-1-phosphate uridylyltransferase and phosphoglucomutase by *P. sordida* YK-624 was not sufficient for galactose to be metabolized to glucose-6-phosphate (18). In the cultures supplemented with xylose or arabinose, a slight decrease in the concentration of each saccharide was observed; notably, ethanol production from xylose was relatively low, and no ethanol was produced from arabinose (Table 2).

Hexose sugars are generally considered to be easily fermented to ethanol, while pentose sugars are not fermented by most fungi, including yeast and alcohol-fermenting fungi (7, 19). These findings, together with our present results, suggest that white-rot fungi fermented hexoses to ethanol more effectively than pentoses. Additionally, when GP7 was cultured with the disaccharide cellobiose or maltose, ethanol concentrations of 2.4 and 1.8 g L⁻¹ were produced, respectively, and WT also converted either disaccharide to ethanol (Table 2).

After incubation for 5 days, the white-rot fungus *Phlebia* sp. MG-60 exhibits ethanol production above 5 g L⁻¹ with glucose, mannose, galactose, fructose, xylose, cellobiose, or maltose. *Phlebia* sp. MG-60, like *P. sordida* YK-624, does not produce ethanol from arabinose. When *Phlebia* sp. MG-60 was incubated with 20 g L⁻¹ of each individual sugar, these sugars were completely assimilated after 5 d of incubation (16). However, the decrease amount of each sugar could not reach 100% even after 8 d of incubation by *P. sordida* YK-624 in this study. These results suggested that effective consumption of sugars might improve ethanol production by *P. sordida* YK-624.

Direct ethanol production by GP7 with wood meal

P. sordida YK-624 exhibits greater ligninolytic activity and selectivity than either

P. chrysosporium or *T. versicolor* (12). Here, we examined whether direct ethanol production from wood meal was better in the *pdc*-overexpressing strain GP7 than in WT because GP7 showed higher sugar-based ethanol-producing activity than WT. GP7 displayed the same ligninolytic activity as the WT strain, degrading 46.6% of the lignin in beech wood meal during a 28-day incubation. This result indicated that the expression of *pdc* did not affect the ligninolytic activity of this strain.

Wood meal inoculated with strain GP7 was transferred to semi-aerobic conditions after a 28-day pretreatment period, followed by the addition of a basal liquid medium for ethanol production. In contrast to the ethanol production by WT or GP7 in cultures without added cellulase, significantly higher ethanol production was observed in either WT or GP7 cultures containing cellulase (Fig. 5). Moreover, after 6 days of semi-aerobic incubation with 30 FPU of added cellulase, strain GP7 showed 1.41-fold higher ethanol production than WT (Fig. 5). As the carbon source for the ethanol production, some holocellulose—0.328 g (2.02 mmol hexose) or 0.276 g (1.70 mmol hexose)—remained in wood meal treated with GP7 or WT, respectively. The highest ethanol conversion rate was 7.42% by GP7 in 6-day cultures with cellulase, and this rate was 1.18-fold higher than that by WT (6.28%). These results indicated that expression of an additional, recombinant *pdc* in *P. sordida* YK-624 improved direct ethanol

fermentation from wood. However, the secretion of cellulases (exoglucanase, endoglucanase and β -glucosidase) by *P. sordida* YK-624 appeared to be insufficient for the enzymatic saccharification of lignocellulosic biomass. In a future study, we plan to simultaneously express genes encoding cellulase and PDC to further improve the efficiency of one-step ethanol fermentation by this strain. To our knowledge, this is the first report describing an improvement in ethanol production in a white-rot fungus because of expression of recombinant *pdv*.

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Fig. 1 Alignment of predicted PDC amino acid sequences from *P. chrysosporium* and *P. sordida*. Identical and similar residues are shown in white with black shading.

Fig. 2 Plasmid map of the *pdv*-expression vector, pGPD-*pdv*.

Fig. 3 Production of PDC and expression of *pdv* by strain GP7. (A) Time course of PDC activity in strain GP7 (closed square) and WT (closed diamond). (B) RT-PCR analysis of *pdv* expression in strain GP7. The expression of actin was monitored as an internal control.

Fig. 4 Time course of glucose consumption and ethanol production by strain GP7 (closed square) and WT (closed diamond). Values are presented as the mean \pm SD of triplicate samples. Asterisks indicate values that were determined by the Student's t-test to be significantly different compared to WT (* $P < 0.05$, ** $P < 0.01$).

Fig. 5 Ethanol production on beech wood meal after 28 days of pretreatment with WT or GP7. Cellulase (30 FPU/g wood) was also added to some (indicated) WT and GP7 cultures. Values are presented as the mean \pm SD of triplicate samples. Asterisks indicate values that were determined by the Student's t-test to be significantly different compared to WT (* $P < 0.05$, ** $P < 0.01$).

Table 1 Oligonucleotide primers used in this study.

Table 2 Ethanol production by GP7 and WT with various saccharides as the carbon source.

Table 1 Oligonucleotide primers used in this study.

Primer name	Nucleotide sequence (5'-sequence-3')
PcPDCF	ACTTCCCGGTATATGCTGC
PcPDCR	AGAGGTGAGTGATCGTCTC
InverseF1	GTCTTTGCGTCTGTCTAACGC
InverseR1	GTAGCGGTCATAGTCTTCGG
InverseF2	GCCTAGATTTACGTCGGAACG
InverseR2	TATCGCCGTCTTGCCCATTG
InverseF3	TCAAGGAAGGAAGCCTCG
InverseR3	ATATGCTGCGTTCAGCTCG
InverseF4	CAGAAGAGCAAGCCCATG
InverseR4	GGAGCTTGATTCTATGTAGTCC
InverseF5	ACTCTGACCACACTCGCATC
InverseR5	AGCTACAAAGCGTGGGTCAG
InverseF6	ATCGGCATGAAGCGTCTGC
InverseR6	AGCGAAAATGCTGGAGCGTAC
InverseF7	CTGACGTTATCCTAGGCTTC
InverseR7	CAGGCACGCCAAAGATTTC
InverseF8	ATACAGCCGTCACTCATGTG
InverseR8	AGTAGGCGTAGATAGAAGTCG
PDCF1	ATGCAGGTCGCCGACCAG
PDCR1	TCACAGCGTGTCGCTCGC
PDCF2	AATCTAGAATGCAGGTCGCCGACCCG
PDCR2	AATCTAGATCACAGCGTGTCGCTCGC
gpdF1	AAGCAGCGAGGATTGTACC
PDCR3	GGAGCTTGATTCTATGTAGTCC
PDCF4	GTCATTCTCTTCGTTGGAGAC
PDCR4	GGTACTTGTACAATCCTCC
PDCF5	TTGTCAAGCTGATCGAGGAG
PDCR5	CTCCTTCTTGACGTTATGCC
ActinF	AGCACGGTATCGTCACCAAC
ActinR	AGCGAAACCCTCGTAGATGG

Table 2 The ethanol production from various saccharides by GP7 and WT.

Saccharides	GP7		WT	
	Maximum ethanol productivity (g L ⁻¹)	Incubation time (day)	Maximum ethanol productivity (g L ⁻¹)	Incubation time (day)
Glucose	3.8	8	3.1	8
Mannose	2.5	6	1.6	8
Galactose	0.3	4	0.1	4
Fructose	1.5	8	1.6	8
Xylose	0.3	4	0.1	4
Arabinose	N. D.		N. D.	
Cellobiose	2.4	6	1.9	6
Maltose	1.8	6	2.5	8

N. D.: not detected

Pc FDC **M**EVADQIP**S**AG**E**CT**I**ADYLL**A**RLVQL**N**VT**N**IFGV**P**GD**F**N**L**G**F**LD**Y**IES**H**PK**L**EWAG**N**C**N**EL**N**AA**Y**AAD**G**YAR**V**KE**G**SL**G**VL**V**TT**F**GV**G**EL**S**AT**N**GI**A**GA 100
 Ps FDC **M**EVADQIP**S**AG**E**CT**I**ADYLL**A**RLVQL**N**VT**N**IFGV**P**GD**F**N**L**G**F**LD**Y**IES**S**PK**L**EWAG**N**C**N**EL**N**AA**Y**AAD**G**YAR**V**KE**G**SL**G**VL**V**TT**F**GV**G**EL**S**AT**N**GI**A**GA 100

Pc FDC **F**SE**R**VP**I**L**H**IV**G**VP**S**T**A**Q**Q**K**S**K**P**ML**H**HT**L**GD**G**RY**D**AY**L**KA**A**Q**Q**F**V**I**S**H**A**SL**Q**D**K**ST**A**AE**I**DR**L**IT**E**CV**V**M**A**RP**V**Y**L**ML**P**T**D**LV**H**E**Q**I**F**SS**R**RL**T**PL**N**VE 200
 Ps FDC **F**SE**R**VP**V**L**H**IV**G**VP**S**T**A**Q**Q**K**S**K**P**ML**H**HT**L**GD**G**RY**D**AY**L**KA**A**Q**Q**F**A**I**S**T**A**SL**Q**D**K**ST**A**PA**E**ID**R**L**I**T**E**CV**V**T**A**RP**V**Y**L**ML**P**T**D**LV**H**E**Q**I**S**SS**R**RL**T**PL**N**VE 200

Pc FDC **P**PEN**D**AE**I**EN**F**VL**D**E**I**V**K**L**I**EE**A**DR**D**V**V**L**V**D**A**CA**I**R**H**N**V**KE**L**L**E**F**V**E**K**V**H**FP**V**Y**A**AP**M**G**K**T**A**I**P**E**D**Y**D**R**Y**G**G**I**Y**V**G**S**I**S**R**PE**V**E**C**K**V**E**S**A**K**L**L**L**S**I**G**G 300
 Ps FDC **P**PEN**D**FE**I**EN**F**VL**D**E**I**V**K**L**I**EE**A**DR**D**V**V**L**V**D**A**CA**I**R**H**N**V**KE**V**FE**F**AE**R**TH**F**FP**V**Y**S**AP**M**G**K**T**A**I**P**E**D**Y**D**R**Y**G**G**I**Y**V**G**T**I**S**R**PE**I**E**C**K**V**E**S**A**K**L**L**L**S**I**G**G 300

Pc FDC **L**K**S**D**F**NT**G**N**F**T**Y**E**I**FA**T**R**I**VEL**H**S**D**E**V**E**V**Y**A**A**Y**H**G**I**G**N**K**R**L**L**P**K**L**TH**R**M**P**F**F**E**D**A**K**A**I**E**V**P**K**F**T**S**V**P**Q**E**D**N**E**T**I**S**H**L**W**L**W**P**R**V**G**Q**F**F**E**K**G**D**V**I**V**A**E**T 400
 Ps FDC **L**K**S**D**F**NT**G**N**F**T**Y**Q**I**P**T**T**R**T**I**EL**H**S**D**E**T**R**I**FA**A**Y**H**G**I**G**N**K**R**L**L**P**K**L**TH**R**M**Q**P**F**F**E**D**A**K**A**L**T**V**P**K**F**T**L**P**V**P**Q**E**D**N**E**T**I**S**H**L**W**L**W**P**R**V**G**Q**F**F**E**K**G**D**V**I**V**A**E**T** 400

Pc FDC **G**T**S**S**F**G**I**L**D**V**P**L**P**G**G**A**V**F**V**S**Q**I**L**W**G**S**I**G**W**T**V**G**S**T**L**G**A**A**I**A**A**R**D**I**G**L**P**RV**I**L**F**V**G**D**G**S**I**Q**L**T**V**Q**E**L**S**T**M**M**R**S**G**V**E**P**I**I**F**L**L**N**S**G**Y**T**I**E**R**Y**L**H**G**E**K**A**R**V**N**D 500
 Ps FDC **G**T**S**S**F**G**I**L**D**V**P**L**P**G**G**S**V**F**V**S**Q**I**L**W**G**S**I**G**W**T**V**G**S**T**L**G**A**A**I**A**A**R**D**I**G**L**N**RV**I**L**F**V**G**D**G**S**I**Q**L**T**V**Q**E**L**S**T**M**M**R**T**G**V**E**P**I**I**F**L**L**N**S**G**Y**T**I**E**R**Y**L**H**G**E**K**A**R**V**N**D 500

Pc FDC **I**V**N**W**Y**ESS**L**LE**V**MG**G**VE**G**ET**C**Q**S**Y**T**V**R**TE**S**EL**L**D**N**PE**F**A**K**AD**K**I**Q**L**V**E**M**IMP**M**H**D**A**P**E**G**LE**I**Q**A**E**L**S**G**R**A**N**K**Y**V**S**V**G**I**K**T**A**S**D**T**F 589
 Ps FDC **I**V**N**W**Y**AAP**F**LE**L**EM**G**G**V**E**G**ET**C**Q**S**Y**T**V**R**TE**S**EL**L**D**D**AA**F**A**R**AD**K**I**Q**L**V**E**M**IMP**M**H**D**A**P**RA**L**EV**Q**A**E**L**S**G**R**A**N**K**Y**V**S**V**G**V**E**K**T**A**S**D**T**L 589

Fig. 1

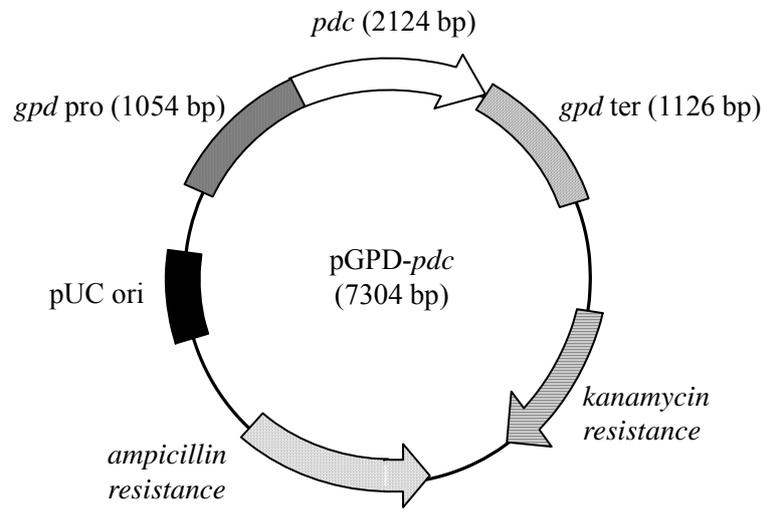


Fig. 2

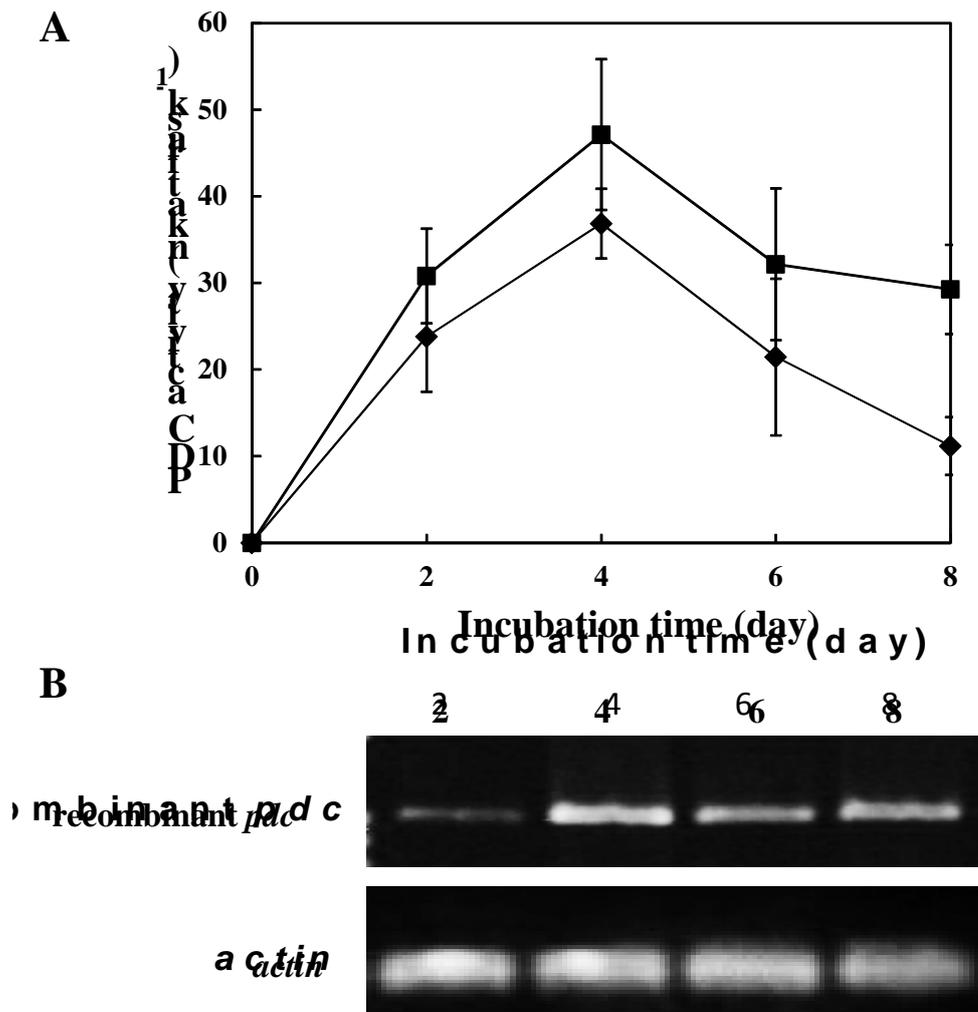


Fig. 3

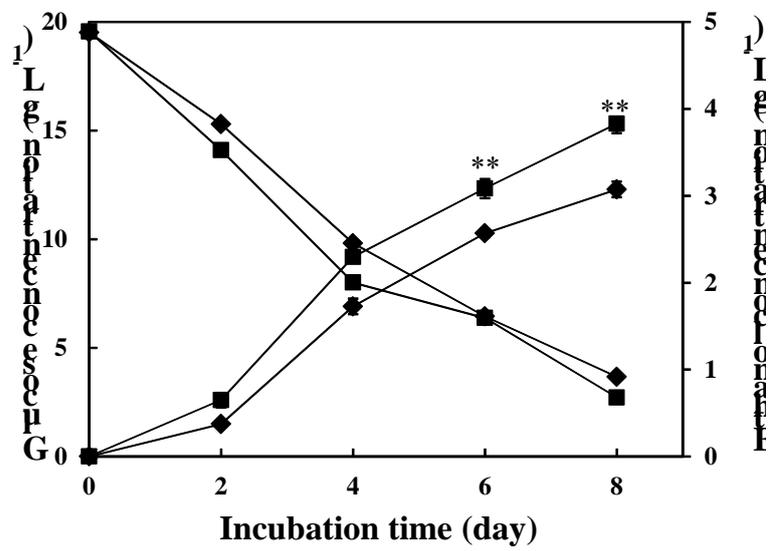


Fig. 4

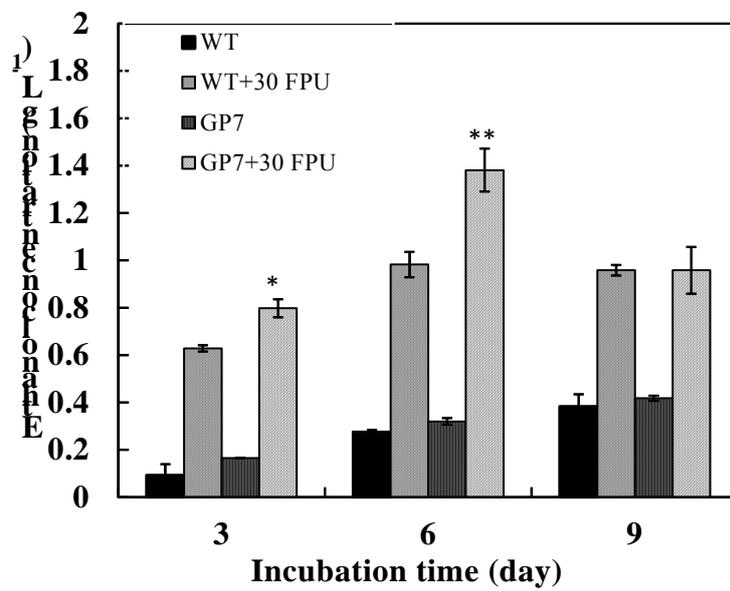


Fig. 5

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Ps PDC -----NQVADQIPFAGKTCIADYLLERLVQLKVTIEIFGVPGDFNIGFLDYIES 49
Pc PDC -----MKVADQIPFAGETCTIADYLLARLVQLNVTNIFGVPGDFNIGFLDYIES 49
At PDC MDTKIGSIDACNPTNHDIGGPPNGGVSTVQNTSPLHSTTVSPCDATLGRYLARRIVEIGVTDVFSVPGDFNLTLLDHLIA 80
Sp PDC -----MSSEKVLVGEYLFTRRLQLGKISILGVPGDFNLLALLDLEIK 41

Ps PDC S--SKLEWAGNCNELNAAAYAADGYARVEEGLGVLVTTTCVGELSAANGIAGAFSEHVEVLHIVGVFSTAQQKSKPMLHH 127
Pc PDC H--PKLEWAGNCNELNAAAYAADGYARVEEGLGVLVTTTCVGELSAANGIAGAFSEHVEVLHIVGVFSTAQQKSKPMLHH 127
At PDC EP--NLKLIIGCCNELNAGYAADGYARSRG--VGACVVTFTVGGLEVLNAIAGAYSBNLPLICIVGGFNSNDYGTNRILHH 156
Sp PDC VGEETFRHWVGNENELNGAYAADAYARVEG--ISAIWTTTGVGELBALNGFAGAYSERIFVVIHIVGVFNTKAQATRPLHH 119

Ps PDC TTCDGRYDAYLKAEEQFAISTASLQDESTAPAEIDRLITECVVTARVVYLMLPTDLVHEQISSKRLLLFLNVEPPENDPE 207
Pc PDC TTCDGRYDAYLKAEEQFVISHASLQDESTAAAEIDRLITECVVMARVYLMLPTDLVHEQIPSSRLLFLNVEPPENDAE 207
At PDC TTCGLPDTQLELRCFQAVTCFQAVINNLEESHELIDTAISTALKESKVYYISISCNLPAIPLFTFSRHVPFMLPMKVSNQ 236
Sp PDC TTCNGDFKVFQRMSSELSADVAFLDSGDSGRLDINLETCVRTSRVYLAVPSDAGYFYTDASFLKTFLVFVPENKE 199

Ps PDC IENFVLDEIVKLIEEADRDVVLVDACAIRHNVKEEVFEFAERTHFPVYSAFMGRTAIFEEDYDRYGGIVVGTISRPEIKE 287
Pc PDC IENFVLDEIVKLIEEADRDVVLVDACAIRHNVKEELLEFVEKVHFPVYAAFMGRTAIFEEDYDRYGGIVVGSISRPEVKQ 287
At PDC IGLDAAVEAAAEFLNKAVEVLVGGPEMRVAKAADAFVELADASGYGLAVMSAEGQVPEHHHFIGIVVGAVSTAFCAE 316
Sp PDC IEHEVVESEILELIEKS-KNPSILVDACVSRFHIQETQDFIDATHFPTYVTEMGTAINSSPFYFDGVIGLSTEPSIKE 278

Ps PDC KVESAKLLLSIGGLKSDFNTGNFTYQIPTTRTIELHSDHTRIRFA-AYHGICMRRLLPKLTHRMQPFREDAKALTVPKFT 366
Pc PDC KVESAKLLLSIGGLKSDFNTGNFTYEIPATRTVELHSDHVRRYA-AYHGICMRRLLPKLTHRMHPPFREDAKAIEVPKFT 366
At PDC IVESADAYLFAGPIFNDYSSVGYSLLLEKEKAIIVQPDRVTIGNGFAFGCVLMEDFLSELAKRIKHN--NTSYENYHRIY 394
Sp PDC RAESTDLLLLIIGGLRSDFNSGTFTYATFASQTIEPHSDYTKIRSG-VYEGISMEHLLPKLTAAIDKKSVQAKARPV-HFE 356

Ps PDC LF--VPQE--DNETISHLWLWPRVGQFFKKGDVIVAETGTSSFGILDVPLFGGSVFVSQILWGSIGWIVGSTIGAAIAAR 442
Pc PDC SP--VPQE--DNETITHLWLWPRVGQFFRRKGDVIVAETGTSSFGILDVPLFGGAVFVSQILWGSIGWIVGSTIGAAIAAR 442
At PDC VEGKPLRDNFNESLVNVLFQHIQNMLSSESAVLAETGSWFNCQKLLEPGCGYEFQNYGSIGWVGATIGYAQAMP 474
Sp PDC PEKAAAEGYAEGTITHWFWPTFASFLRESDVVTETETSNFGILDCIFFRGCQNLSQVLWGSIGWVGAMFGATLGIK 436

Ps PDC DIGLN--EVILFVGDGSIQLTVQELSTMMRSGVKPIIFLNNSGYTIERYLHGEKARYNDINWAAP-FLETMGGVEGE 519
Pc PDC DIGLP--EVILFVGDGSIQLTVQELSTMMRSGVKPIIFLNNSGYTIERYLHGEKARYNDIVNWYESSLLEVMGGVEGE 520
At PDC N-----RRVIACIGDGSFQVTAGDVSTMIRGGQKTIIFLNNGGYTIERVEIE--DGPNVYIKNNYTAFVEAIHNG--EG 545
Sp PDC DSDAPHRSLIIVGDGSLHLTVQEISATIRNGLTPIIFVINNGYTIERLIHGLHAVYNDINTEWDYQNLLEGYG---AK 513

Ps PDC TCQSYTVRTKELSALLDDAAFARADEIQLVEMMFMMDAPRAIKVQAELSGEMKYVSVGVKTASDTL 588
Pc PDC TCSYTVKTKNELSELLDNFEFAKADEIQLVEMIMFMMDAPRGIKLQAELSGEKANKYVSVGIKTASDTF 589
At PDC KCWTAKVRCEEELVKAINTATNEEESFCFIEVIVHKDTSKELEWGSRVSAANSRFPNFQ----- 607
Sp PDC NSRSYNIHSEELLDLFKDEEFGKADVIQLVEVHMFMVDAPRVIEQAKLTASLIKQ----- 570

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Fig. S1 Amino acid sequence alignment of Ps PDC, Pc PDC, *Arabidopsis thaliana* (At) PDC and *Schizosaccharomyces pombe* 972h- (Sp) PDC. Identical and similar residues are shown in white with black shading.

The GenBank accession numbers for At PDC and Sp PDC are NC_003076 and NC_003424.