FUNGAL BIOLOGY 117 (2013) 776–782

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Expression of *Arabidopsis thaliana* xylose isomerase gene and its effect on ethanol production in *Flammulina velutipes*

Tomoko MAEHARA[a], *Koji TAKABATAKE*[b], *Satoshi KANEKO*[a,*]

[a]*Food Biotechnology Division, National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan*

[b]*Forest Institute, Toyama Prefectural Agricultural, Forestry and Fisheries Research Center, 3 Yoshimine, Tateyama, Toyama 930-1362, Japan*

ARTICLE INFO

Article history:
Received 18 March 2013
Received in revised form
4 September 2013
Accepted 30 September 2013
Available online 12 October 2013
Corresponding Editor:
Daniel Eastwood

Keywords:
Basidiomycete fungi
Ethanol fermentation
Flammulina velutipes
Heterologous gene expression
Xylose metabolism

ABSTRACT

To improve the pentose fermentation rate in *Flammulina velutipes*, the putative xylose isomerase (XI) gene from *Arabidopsis thaliana* was cloned and introduced into *F. velutipes* and the gene expression was evaluated in transformants. mRNA expression of the putative XI gene and XI activity were observed in two transformants, indicating that the putative gene from *A. thaliana* was successfully expressed in *F. velutipes* as a xylose isomerase. In addition, ethanol production from xylose was increased in the recombinant strains. This is the first report demonstrating the possibility of using plant genes as candidates for improving the characteristics of *F. velutipes*.

Introduction

To achieve an economic bioethanol production from lignocellulosic biomass, consolidated bioprocessing (CBP) that combines enzyme production, enzymatic saccharification and ethanol fermentation in one step was proposed by Lynd *et al.* (2005). Recently, several studies of CBP capable of high yield conversion of biomass to ethanol were reported using the combined abilities of two organisms such as a cellulolytic enzyme-producing organism and an ethanol-producing organism (Zhang & Zhang 2010; van Zyl *et al.* 2011). However, no single organism suitable for CBP has been developed.

Flammulina velutipes is a wood-rotting fungus that can completely degrade lignocellulose. Because the *F. velutipes* Fv-1 strain produces both ethanol and cellulolytic enzymes, we investigated CBP bioethanol production using *F. velutipes* and found that this strain was a good candidate for ethanol production from lignocelluloses by CBP (Mizuno *et al.* 2009a, 2009b; Maehara *et al.* 2013). Furthermore, we have developed gene transformation methods for *F. velutipes* (Maehara *et al.* 2010a, 2010b). The Fv-1 strain was able to ferment D-glucose, D-fructose, D-mannose, sucrose, maltose, cellobiose and cellulose; however, it can hardly produce ethanol from pentoses (Maehara *et al.* 2013). Because lignocellulosic biomass contains

* *Corresponding author*. Tel.: +81 29 838 8063; fax: +81 29 838 7996.
E-mail address: sakaneko@affrc.go.jp (S. Kaneko).
1878-6146/$ – see front matter
http://dx.doi.org/10.1016/j.funbio.2013.09.005

up to approximately 30–40% pentoses, utilisation of pentoses is necessary for effective ethanol production. Therefore, improvement of pentose fermentation by *F. velutipes* would be required.

A major bioethanol producer, *Saccharomyces cerevisiae* is not able to ferment pentoses. Thus, many studies have been conducted for the genetic engineering of this yeast directed at improving pentose fermentation (Jeffries & Jin 2004; Matsushika *et al.* 2009). There have been two general approaches to this work. The first approach has been the expression of heterologous xylose reductase (XR) and xylitol dehydrogenase (XDH) genes to improve the endogenous pentose-metabolising pathway. Recombinant *S. cerevisiae* strains that expressed XR and XDH showed improved ethanol production from D-xylose. However, xylitol accumulation caused by an NADPH/NAD cofactor imbalance under anaerobic condition was observed; thus, it would be desirable to introduce additional enzyme-encoding genes to address this problem. The second approach has been the expression of a heterologous xylose isomerase (XI) gene to produce a new bypass pathway for pentose metabolism to convert D-xylose to D-xylulose directly. Because XI does not require redox cofactors, it does not cause xylitol accumulation and cofactor imbalances; however, in general, XI is involved in the pentose pathway of bacteria and is difficult to express in eukaryotes.

Because *F. velutipes* is expected to have the same sugar metabolism pathway as *S. cerevisiae* (Barnett 1976; Maehara *et al.* 2013) and because the introduction of multiple enzyme-encoding genes into *F. velutipes* would be difficult, we aimed to introduce a heterologous XI gene from a eukaryotic organism into *F. velutipes*.

In this study, we focused on the gene encoding a putative XI from the plant *Arabidopsis thaliana* (*AtXI*). This putative gene was transformed into the *F. velutipes* Fv-1 strain, and its expression and effect on ethanol production from D-xylose were evaluated in *F. velutipes*. To our knowledge, there is no report on the use of a plant *XI* gene for improving pentose fermentation and our study is the first report on improving the characteristics of *F. velutipes* by genetic engineering.

Materials and methods

Strains, media and culture conditions

The wood-rotting fungus *Flammulina velutipes* Fv-1 strain was used as the transformation host. The strain was grown at 25 °C with shaking at 120 rpm in B medium [1% yeast extract, 1% peptone, 1% D-glucose, 0.1% KH_2PO_4 and 0.01% $MgSO_4 \cdot 7H_2O$ (pH 5.5)]. Hygromycin B (30 μg ml^{-1}) was added to the cultivation medium if required. The bacterial strain *Escherichia coli* DH5α was used for cloning *AtXI*. The *E. coli* strain was grown at 37 °C in Luria–Bertani (LB) medium. Ampicillin (100 μg ml^{-1}) was added to the medium when required. The seeds of *Arabidopsis thaliana* were cultivated at 20 °C on Murashige–Skoog medium supplemented with 2% sucrose, vitamin solution (0.3% thiamine HCl, 0.5% nicotinic acid and 0.05% pyridoxine HCl) and 0.3% agar.

Plasmid construction

The plasmid used in this study is shown in Fig 1. The plasmid designated pFvGX carried a 1368-bp XI gene fragment isolated from *Arabidopsis thaliana*. Total RNA of *A. thaliana* was prepared using RNeasy Plant Mini Kit (QIAGEN, CA, USA), and cDNA was prepared by reverse transcriptase (RT) reactions using ReverTra Ace-α (Toyobo, Osaka, Japan) with 0.1 ng of total RNA as the template. The gene encoding XI from *A. thaliana* was amplified by PCR with KOD-plus- ver.2 (Toyobo, Osaka, Japan) using the primer pairs 5′-GATCCACCAACATGTCCTGCTGATTTGG-3′ and 5′-TTACATTGCAGATTGGAAAATCATCTCAGCGAGT-3′ from *A. thaliana* cDNA. The PCR product was inserted into blunt-ended NcoI and HindIII sites of pFvG as described previously (Maehara *et al.* 2010b). The inserted nucleotide sequence for XI was confirmed using an ABI PRISM 310 genetic analyser (Applied Biosystems, Foster City, CA, USA).

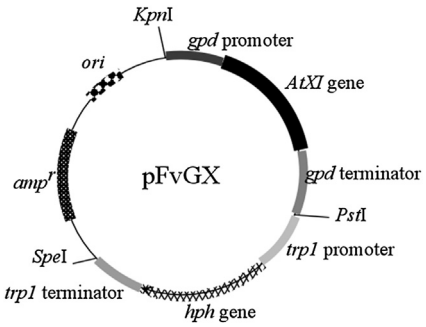

Fig 1 – Structures of the plasmid used in this study. pFvGX (7828 bp) was derived from pFvG (Maehara *et al.* 2010a) by cloning the *AtXI* gene without a signal sequence into the multi-cloning sites (MCS) of pFvG. *hph* is a hygromycin B phosphotransferase gene and *amp*^{r} is an ampicillin resistance gene. *gpd* is glyceraldehydes-3-phosphate dehydrogenase and *trp1* is the tryptophan synthetase gene.

Construction of XI transformants

Flammulina velutipes Fv-1 strain was transformed with pFvGX as described previously (Maehara *et al.* 2010a). Transformants were selected on MYGS medium (0.4% malt extract, 0.4% yeast extract, 1% glucose and 0.5 M sucrose) containing 30 μg ml^{-1} hygromycin B, and expression of the XI gene was confirmed by RT-PCR. cDNAs from each transformant were prepared as described above. Total DNA from each transformant was prepared using DNeasy Plant Maxi Kit (QIAGEN, CA, USA), and plasmid integration was confirmed by Southern hybridisation (Sambrook & Russell 2001), using the digoxigenin-labelled *XI* gene from *Arabidopsis thaliana* as a probe.

XI activity assays

Flammulina velutipes mycelia were cultivated for 6 d and collected by centrifugation at 3000 ×*g* for 10 min, washed twice with 0.85% NaCl, collected by filtering through Miracloth (Calbiochem, California, USA) and squeezed to remove the water. The mycelia were frozen in liquid nitrogen and ground to fine powder. The fine powder was suspended in 100 mM Tris–HCl (pH 7.5) and the suspension was centrifuged at 12000 ×*g* for 10 min. The supernatant was used as the cell extract. XI activity in the cell extract was determined at 30 °C according to previously described methods (Kersters-Hilderson *et al.* 1987;