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Toshikazu Irie · Yoichi Honda · Takuro Matsuyama
Takashi Watanabe · Masaaki Kuwahara

Isolation and sequence analysis of the promoter and an allelic sequence of the iron–sulfur protein subunit gene from the white-rot fungus *Pleurotus ostreatus*

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Abstract We have isolated a structural gene of *sdi1*, which encodes the iron-sulfur protein (Ip) subunit of succinate dehydrogenase (EC 1.3.99.1), from a white-rot basidiomycete, *Pleurotus ostreatus*. Here we report isolation of the promoter region of *sdi1* and an allelic sequence encoding the second-type cDNA fragment isolated in the former experiments. The nucleotide sequence analysis of the promoter region revealed the existence of putative CAAT and TATA boxes, which permits us to develop an expression system in this species. The Southern blot analysis and the restriction fragment length polymorphism assay using monokaryotic strains demonstrated that no family genes to *sdi1* exist in the haploid genome of *P. ostreatus*. Moreover, a genetic analysis to detect a linkage between the *sdi1* genotypes and flutolanil resistance in the mutant *P. ostreatus* strains was also developed.

Key words Succinate dehydrogenase · Iron–sulfur subunit · Gene cloning · White-rot basidiomycete · *Pleurotus ostreatus*

Introduction

Pleurotus ostreatus is a white-rot basidiomycete fungus that is considered to be a good model for understanding the biochemical processes involved in lignin degradation.[1] It is, moreover, a commercially important edible mushroom. We are interested in developing techniques for transformation and gene expression in this species. The techniques can be used for breeding and permit us to combine molecular and biochemical analysis of the enzymes involved in lignin biodegradation. In generally, promoters are reported to be less compatible in the basidiomycetes.[2] In this context, isolation of a promoter sequence from *P. ostreatus* has been desired because it can provide us effective expressions of various genes, including selectable markers and genes encoding industrially useful enzymes in this species.

A nuclear gene, *sdi1* encodes the iron-sulfur protein (Ip) subunit of succinate dehydrogenate, which is a component of mitochondrial complex II. In the plant pathogenic homobasidomycete *Ustilago maydis*, a mutated Ip subunit gene, *Cbx*^{r}, has been shown to confer a dominant resistance to the fungicide carboxin.[3,4] Carboxin is one of the carboxanilides, which are potent inhibitors of complex II from mitochondria of fungi and animal tissues and from plasma membranes of bacteria.[5–8] A *P. ostreatus* mutated Ip subunit gene such as *Cbx*^{r} would provide a potentially valuable selectable marker for the development of transformation vectors. We have shown that dominant resistant mutations to a carboxanilide, flutolanil, which is frequently used in Japan, can occur in *P. ostreatus*[9] and cloned the structural gene of *sdi1* and its transcript from a wild-type *P. ostreatus* strain.[10] During the course of this experiment, a second-type cDNA sequence was also isolated. The second-type cDNA was different from *sdi1* cDNA in eight nucleotide positions, but the deduced amino acid sequence was identical to that of *sdi1*. We report here the isolation and sequence analyses of the *sdi1* promoter region and the genomic clone of the allelic sequence.

Materials and methods

Strains

A dikaryotic strain of *Pleurotus ostreatus*, #261 (ATCC 66376), was used through this study. Several monokaryotic progenies isolated from basidiospores from #261 were used in the Southern blot and genetic analyses of *sdi1*. The flutolanil-resistant dikaryotic mutant MA206 was isolated from a selective culture of ultraviolet (UV)-irradiated basidiospores from #261 and was characterized in our laboratory.[9]

T. Irie · Y. Honda · T. Matsuyama · T. Watanabe
M. Kuwahara (✉)
Wood Research Institute, Kyoto University, Uji 611-0011, Japan
Tel. +81-774-38-3640; Fax +81-774-38-3600
e-mail: mkuwahar@kuwri.kyoto-u.ac.jp

Fig. 1. Nucleotide sequence of the promoter region of *Pleurotus ostreatus sdi1*. Small letters indicate the coding sequence, and the first ATG is in boldface. Putative CAAT and TATA *boxes* are indicated by boxes. Motifs of the binding site for *Saccharomyces cerevisiae* Adr1p are *underlined*

Polymerase chain reaction amplification and cloning of *sdi1* promoter

We attempted to clone the promoter region of *sdi1* by a cassette-primer polymerase chain reaction (PCR) technique[11] using the TaKaRa LA PCR in vitro cloning kit (Takara Shuzo Co.). The method involves digestion of genomic DNA with suitable restriction enzymes followed by ligation of the restriction fragments with adapters, which can be amplified by PCR. Using the first-specific primer that anneals to a complement sequence and primer C1 in this kit, which hybridizes to the adapter, an unknown flanking genomic sequence can be amplified. To improve specification, the second PCRs were carried out using the second-specific primer, primer C2 in this kit, which anneals to the adapter, and the previous PCR product as a template. For cloning the promoter region of *sdi1* using this kit, we prepared the *Xho* I-digested genomic DNA of *P. ostreatus* #261 as a template and the first-specific primer S3R (5′-GACGAGGTGGAGCAAGTCCGAATCGAGCGAGATGA-3′), which corresponds to the region from 68 to 34 of *sdi1*, and the second-specific primer S4R (5′-GGTGAGCG-CCTGCATGGTTCAATGATTTGTGTGTT-3′), which corresponds to the region from 15 to −20 of *sdi1*. [The A of the first methionine is numbered as "1" (Fig. 1).] The manipulations were done according to the manufacturer's instructions. As a result, a 1.5-kb fragment was amplified, and we cloned and sequenced it. Because the sequence of the overlapping region with *sdi1* is identical to that of the second-type sequence, we could not know which genes acted as the template. To solve this problem, we synthesized an oligo nucleotide primer, R4 (5′-TCCGATGACACTG-CCAACGAC-3′), which is at the 1.3-kb upstream region from the first ATG in the 1.5-kb fragment. The PCR amplification was performed with R4, S2R (5′-TGGCGTTGCGGTCAATTCGGCACAGGCAAG-3′), which corresponds to the region from 426 to 455 of *sdi1*, and the genomic DNA of *P. ostreatus* #261 as a template using TaKaRa Ex Taq DNA polymerase (Takara Shuzo Co.). This polymerase ensures high sequence fidelity during the polymerase reaction[12] and was used for PCR. The 1.8-kb fragments of 5′-flanking sequences of *sdi1* and the second-type sequence were amplified, cloned into pGEM-T plasmid, Promega, and sequenced. Judging the sequences, the first 1.5-kb fragment is derived from the second-type sequence. The nucleotide sequence of the fragment was determined by the dideoxy chain termination method with ABI model 377 DNA sequencer.

PCR amplification and cloning of another Ip subunit gene

The structural gene for the second-type cDNA sequence was amplified from the genomic DNA using primers, R1

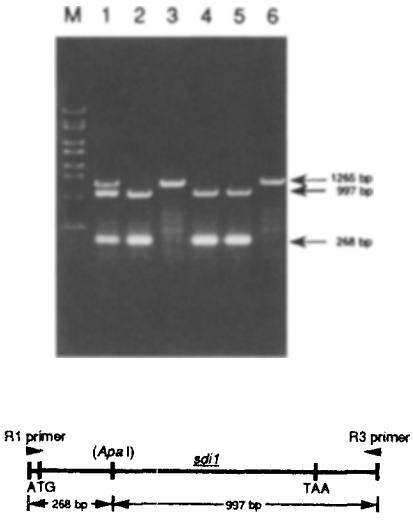

Fig. 2. *Apa* I assay and confirmation of the allelic sequence in *P. ostreatus* strains. PCR fragments containing *sdi1* sequence from each strain amplified with primers, R1 and R3 (see Materials and Methods) were digested with *Apa* I and electrophoresed on a 0.7% agarose gel. Lane 1, parental dikaryon, no. 261; lanes 2–6, monokaryotic progenies from strain 261. Lane M: λ/*Eco*T14 as a marker DNA

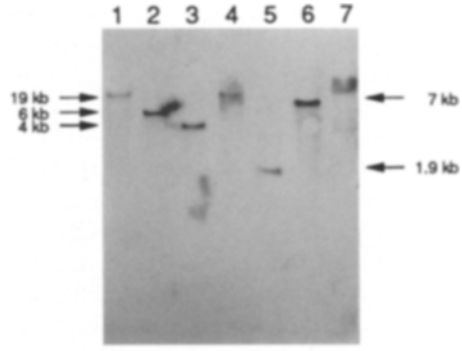

Fig. 3. Southern blot analysis of genomic DNA extracted from a monokaryotic progeny of strain 261. DNA samples (5 μg) applied in each lane were probed with polymerase chain reaction (PCR) product containing *sdi1*. The DNA samples were digested with: *Eco*RI (lane 1), *Sac* I (lane 2); *Kpn* I (lane 3), *Apa* I (lane 4); *Sal* I (lane 5), and *Sph* I (lane 6). Undigested DNA was applied in lane 7

(5′-CACACAAATCATTGAACCATGC-3′) and R3 (5′-AGCATCGCAAGTGAAACCGA-3′) (Fig. 2). TaKaRa Ex Taq DNA polymerase was used for PCR. The amplified PCR products were cloned and sequenced as previously described. Determined nucleotide sequences appear in DDBJ, EMBL, and GenBank databases with accession number AB009845 and AB009846.

Southern blot analysis

Southern blot analysis of the *P. ostreatus* haploid genome DNA digested with restriction enzymes *Eco*R I, *Sac* I, *Kpn* I, *Apa* I, *Sal* I, or *Sph* I was performed using the PCR amplified partial sequence of *sdi1* as a probe (Fig. 3). The labeling, hybridization, and signal detection were done using DIG DNA Labeling and Detection Kit (Boehringer Mannheim), according to Nomura and Inazawa.[13] Hybridization was done in hybridization buffer (500 mM sodium phosphate buffer pH 7.2, 7% SDS, 1 mM EDTA) for 48 h at 65°C. Hybridized membranes were rinsed three times in the wash buffer (40 mM sodium phosphate buffer pH 7.2, 1% SDS) for 20 min at 65°C.

Results

Cloning and sequence analysis of the *sdi1* promoter region

The sequence of the promoter region of *sdi1* was determined as described in Materials and Methods. It is shown in Fig. 1. The promoter region contained putative CAAT and TATA boxes and a binding site motif for transcription factor Adr1p, which is required for maximal expression of *ADR2* encoding an isozyme of alcohol dehydrogenase, ADHII, in the yeast *Saccharomyces cerevisiae*.[14,15] Although the function of these putative expression signals must be confirmed by further experiments, it is plausible that they should participate in the regulation of *sdi1* gene expression. The isolated promoter region of *sdi1* provides us a useful component for developing an expression system for recombinant DNAs in *P. ostreatus*.

Characterization of the second-type Ip subunit gene

The PCR amplification of *P. ostreatus* #261 genomic DNA using specific primers (see Materials and Methods) resulted in isolation of a novel sequence similar to *sdi1*. A comparison between the nucleotide sequence of this PCR fragment and the previously cloned second-type cDNA revealed that there was no difference in the coding sequences.

The second-type gene was interrupted by five introns at the positions same as *sdi1*.

All of the introns found in this gene abode by GT-AG splicing rule and nucleotide sequences of the introns differed from *sdi1* intron sequences at 11 positions. A stretch of ATTAAA that was similar to the consensus sequence of the polyadenylation signal AATAAA is also present 20 nucleotides downstream of the stop codon.

Southern blot analysis and *Apa* I assay

To determine the copy number of Ip subunit genes, Southern blot analysis was performed using monokaryotic progenies derived from basidiospores of #261. When genomic DNA fragments digested with various restriction enzymes were probed with *sdi1* sequence, a single hybridization signal was detected in every digest of every monokaryon's DNA. The representative result for a monokaryotic progeny of #261 is shown in Fig. 3. The monokaryons were divided into two groups according to the hybridization pat-

terns; and the hybridization patterns of parental strain #261 contained the patterns of both groups (data not shown). This finding suggested that *sdi1* and the second-type gene were allelic in #261, and that no similar sequences other than them exist. To confirm that these hybridized bands were *sdi1* and the second-type sequence, further experiment's were performed as described below.

The second-type gene contained an *Apa* I site that did not exist in *sdi1*. Therefore *Apa* I digests of the PCR-amplified fragment of *sdi1* could be used to distinguish the type(s) of gene contained in a certain *P. ostreatus* strain. This restriction fragment length polymorphism (RFLP) assay was designated as an *Apa* I assay (Fig. 2). Using progenies derived from basidiospores of #261, a series of *Apa* I assays demonstrated that the monokaryotic strains contained only one of the two genes, whereas the parental dikaryon contained both of them. The classification of monokaryons by *Apa* I assay consisted of the grouping of the monokayons by the Southern blot analysis, which confirmed that *sdi1* and the second-type gene were allelic and not distinct members of a gene family.

Using an *Apa* I assay, linkage between the *sdi1* genotypes and other characteristics can be analyzed easily. Especially, analysis for a linkage between the *sdi1* genotypes and the flutolanil-resistance phenotype is useful for investigating whether the drug-resistance links to *sdi1* in a particular mutant strain. Such an assay was performed using monokaryotic progenies of a flutolanil-resistant mutant MA206. No linkage between drug resistance and the *sdi1* genotypes was observed among the progenies of this mutant strain (data not shown).

Discussion

This report is the first one on cloning a promoter sequence of the *P. ostreatus* genes expressed during the primary metabolic phase. It was shown that *sdi1* has no homologous genes in the haploid genome of *P. ostreatus*. Hence it is unlikely that *P. ostreatus* contains more than one isoform of Ip subunit protein for complex II, which is the case in some other organisms, such as *Haemonchus contortus*.[16] In this context, it is conceivable that *sdi1* is expressed constitutively through the aerobic growth of this organism. The promoter region should be useful for constructing selectable marker genes for transformation and for overexpressing various enzymes during the primary metabolic phase in *P. ostreatus*. Accordingly, we have constructed selectable markers consisting of the *sdi1* promoter and terminator and heterologous drug-resistance genes. Examination of *P. ostreatus* transformation with these constructs are in progress.

In a flutolanil-resistant mutant strain, MA206, it was shown that drug resistance was not linked to the *sdi1* genotypes classified by *Apa* I digestion. It is conceivable that in this strain the mutation leading to drug resistance occurred in some other genes, such as structural genes for remaining complex II-subunit proteins. In fact, existence of the another nuclear locus, *oxr*-2, conferring carboxin resistance, has been reported in *Ustilago maydis*.[3] The *Apa* I assay provides a useful method for screening a *P. ostreatus* mutant strain with the flutolanil-resistant phenotype linked to an *sdi1* genotype. Furthermore, an attempt to transform *P. ostreatus* with a modified *sdi1* gene containing the same amino acid substitution as in *U. maydis* Cbx^r is also being undertaken.

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