Pyritthiamine Resistance Gene (ptrA) of Aspergillus oryzae: Cloning, Characterization and Application as a Dominant Selectable Marker for Transformation

Takafumi Kubodera, Nobuo Yamashita, and Akira Nishimura

Research & Development Department, Hakutsuru Sake Brewing Co. Ltd., 4-5-5, Sumiyoshinimami-machi, Higashinada-ku, Kobe 658-0041, Japan

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A pyritthiamine (PT) resistance gene (ptrA) was cloned from a genomic DNA library prepared from a PT resistant mutant of Aspergillus oryzae. It conferred high resistance to PT on an A. oryzae industrial strain as well as A. nidulans. Nucleotide sequence analysis showed that the ptrA gene contained one intron (58-bp) and encodes 327 amino acid (aa) residues. Additionally, the deduced aa sequence has 72% and 63% identity to Fusarium solani sti35 encoding a stress-inducible protein and Saccharomyces cerevisiae THI4 encoding an enzyme involved in thiamine biosynthesis, respectively, indicating that ptrA is a mutated allele of a gene belonging to the THI4 family. The mutation point was identified in the conserved motif in 5′-flanking region of these three THI4 homologous genes (ptrA, sti35, and THI4). The introduction of the ptrA gene allowed an A. oryzae industrial strain to grow on the minimum medium containing PT (0.1 mg/l) on which an untransformed strain did not grow. This result indicates that the ptrA is applicable as a dominant selectable marker for transformation of A. oryzae.

Key words: drug resistance gene; pyritthiamine; A. oryzae; dominant selectable marker

A. oryzae is an important filamentous fungus in the Japanese fermentation industry, such as sake, soy sauce, and miso manufacture, as well as in commercial enzyme production. A. oryzae is also considered to be a favorable host for heterologous protein production because of its ability to secrete a large amount of proteins. So the development of a more useful transformation system for A. oryzae is required. The transformation of A. oryzae has been mainly alone using host-vector systems with auxotrophic and nutrition use deficient mutants. In these transformation systems, a conventional mutation procedure is mostly necessary before screening of host strains with genetic markers. As the only exception, Gomi et al. reported transformation of an A. oryzae wild type strain using amdS gene as a dominant selectable marker. Various mutation procedures frequently confer unfavorable damage on a parent strain at the DNA level, so it is desirable to use a wild type strain as host for molecular breeding of industrial use strains. In various filamentous fungi, transformation systems based on drug resistance genes as dominant selectable markers have been reported: an oligomycin resistance gene for Aspergillus niger, the G418 phosphotransferase gene for Fusarium solani, the bleomycin resistance gene for Neurospora crassa, the hygromycin B phosphotransferase gene for Penicillium citrinum, a benomyl resistance gene (beta-tubulin gene) for Acremonium chrysogenum, and the aureobasidin A resistance gene for Aspergillus nidulans, etc. These transformation systems have the advantage of availability of a wild type strain as a host. On the other hand, A. oryzae is insensitive to most available antifungal antibiotics as described above. The early literature contained an only one report of a transformation system based on a phleomycin resistance gene for a protease non-producing strain of A. oryzae.

Pyritthiamine (PT) is a potent antagonist of the thiamine. Exposure to PT is lethal to yeasts. Although PT reduces the thiamine transport ability, sufficient amounts enter the cells to inhibit thiamine pyrophosphokinase (TPK). TPK is required for synthesis of thiamine pyrophosphate (TPP) from thiamine. Since TPK is a necessary cofactor for several critical enzymes, lack of its production is lethal. PT-resistant mutants have been described for Saccharomyces cerevisiae and Schizosaccharomyces pombe and, in each case, were shown to be severely deficient in thiamine transport. Moreover the PT-resistant phenotype was recessive to the wild sensitive phenotype by mating analysis. In this study, we found that PT also inhibited the growth of Aspergillus oryzae effectively and PT-resistant mutants were obtained at a fairly high frequency. So on the basis of

To whom correspondence should be addressed. Takafumi Kubodera, FAX: +81-78-822-4832; E-mail: kubodera@hakutsuru.co.jp

Abbreviations: PT, pyritthiamine; ptrA, pyritthiamine resistance gene; A, Aspergillus; aa, amino acid
the speculation that the PT resistance phenotype may be conferred by a single locus dominant mutation, we attempted to isolate the gene conferring PT resistance on \textit{A. oryzae} in order to use it as a dominant selectable marker for transformation. This is the first report describing the cloning of a gene conferring drug resistance on an \textit{A. oryzae} wild type strain.

\section*{Materials and Methods}

\textbf{Strains, plasmids, and media.} The pyrithiamine (PT) resistant mutant, \textit{A. oryzae} PTR26, derived from \textit{A. oryzae} HL1034 (industrial strain for sake brewing), was used as a source of DNA. \textit{A. nidulans} FGSC89 (\textit{bioA1; argB2}), \textit{A. oryzae} M-2-3 (\textit{argB}), were kindly provided by Fungal Genetics Stock Center and National Research Institute of Brewing, respectively, and HL1034 were used as host strains for transformation. M-2-3 was also used for protoplast fusion experiment. \textit{Escherichia coli} strains, DH5\textalpha{} and JM109, were used as hosts. The plasmid vector, pDHG25,\textsuperscript{19} which carries the \textit{A. nidulans} \textit{argB} gene as a selective marker and the \textit{AMA1} sequence as a replication origin, was used for constructing a genomic library. pBluescript II was used for DNA manipulation. Czapek-Dox (CD) medium consisting of 0.3\% NaNO\textsubscript{3}, 0.2\% KCl, 0.1\% KH\textsubscript{2}PO\textsubscript{4}, 0.05\% MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O, and 2\% glucose adjusted to pH 6.5, and DPY medium containing 2\% dextrin, 1\% Polypepton, 0.5\% yeast extract, 0.5\% KH\textsubscript{2}PO\textsubscript{4}, and 0.05\% MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O were used as minimal medium and complete medium, respectively.

\textbf{Measurement of minimum inhibitory concentration (MIC).} After precultivation of \textit{A. oryzae} HL1034 on CD-plates at 30\textdegree{}C for 5 days, the conidia suspension prepared was spread on CD-plates containing various concentrations of PT (0.001 \textendash{} 100 mg/l). Then, the MIC of PT on \textit{A. oryzae} was identified. In the case of \textit{A. nidulans} FGSC89, it was measured in the same manner.

\textbf{Isolation of PT-resistant mutant.} \textit{A. oryzae} HL1034 conidia was incubated in the phosphate buffer (pH 7.0) containing 4 g/l N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at 30\textdegree{}C for 10 min and spread on CD-plate containing 2 mg/l PT. The clones grown were isolated and confirmed for their resistance to PT on the same plate.

\textbf{Protoplast fusion.} Protoplast fusion was done as described by Ushijima et al.,\textsuperscript{19} except for using 0.8 M NaCl as the osmotic stabilizer.

\textbf{Construction of the \textit{A. oryzae} genomic DNA library.} Standard molecular cloning procedures were used as described by Sambrook et al.,\textsuperscript{19} To construct a genomic library of \textit{A. oryzae}, the genomic DNA was purified from the PT-resistant mutant PTR26 according to Tsuchiya et al.,\textsuperscript{20} The DNA was partially digested with \textit{Sau3A1} and fragments ranging from 3 to 15-kb were collected by agarose gel electrophoresis. The DNA fragments were ligated into the \textit{BamH}I site of pDHG25 and used to transform \textit{E. coli} DH5\textalpha{}.

\textbf{Transformation experiment.} \textit{E. coli} transformation was done by the method of Hanahan et al.,\textsuperscript{21} The transformation of \textit{A. oryzae} and \textit{A. nidulans} was done as described by Gomi et al.,\textsuperscript{13} except that recipient strains were cultivated first with thiamine-free minimal medium with suitable nutrients.

\textbf{Cloning of the \textit{ptrA} gene.} The genomic DNA library constructed from \textit{A. oryzae} PTR26 was used to transform \textit{A. nidulans} FGSC89. The transformants grown on the CD regeneration plate containing 0.1 mg/l PT were isolated and selected again on the CD-plate containing 2 mg/l PT. Then a plasmid rescued from the selected transformant was used to transform \textit{A. oryzae} M-2-3. Plasmids were recovered again from \textit{A. oryzae} transformants and their inserts were analyzed.

\textbf{Nucleotide sequencing.} A series of unidirectional deletions of the cloned gene (\textit{ptrA}) were introduced using a Kilo-sequencing kit (Takara, Kyoto). The wild allele of \textit{ptrA} was cloned by PCR amplification using DNAs of \textit{A. oryzae} HL1034 as templates and sequenced by direct sequencing. These nucleotide sequencings were done by the dideoxynucleotide chain termination method using an automated DNA sequencer, ALF express (Amersham Pharmacia Biotech). Both direction strands were completely analyzed by overlapping at every junction.

\textbf{Southern blot analysis.} The purified genomic DNA was digested with \textit{HindIII} and separated by agarose gel electrophoresis. The genomic DNA fragments were transferred onto a positively charged nylon membrane, Hybond-N+ (Amersham Pharmacia Biotech), using 0.4 M NaOH as the transfer solution. A 1.2-kb PCR-amplified fragment containing the entire ORF (open reading frame) of \textit{ptrA} was used as a probe. Hybridization and detection were done using the enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech) according to the supplier’s instructions.

\textbf{Northern blot analysis.} Total RNA was prepared by the method of Catahala et al.,\textsuperscript{22} with minor modifications. Approximately 20 \mu{}g of total RNA was separated on a formaldehyde/agarose gel, then transferred onto a nylon membrane Hybond-N+. A
0.37-kb fragment corresponding to the ptrA gene (nucleotide position 396 to 771 with respect to translation start site) amplified by PCR was used as a probe for hybridization. A band of mRNA for ptrA gene was detected by the same method as in the Southern blot analysis described above.

Results and Discussions

Minimum inhibitory concentration (MIC) of PT on A. oryzae and isolation of PT-resistant mutant

MIC of PT on A. oryzae HL1034 was approximately 0.01 mg/l. In A. nidulans FGSC89, it was almost the same level. So after NTG treatment, resistant mutants were selected twice on the CD plate containing 2 mg/l PT. PT-resistant mutants were obtained at a frequency of about 10⁻⁶. Out of them, PTR26 was selected because of its significantly good growth.

Examination on PT-resistance phenotype of PTR26

To verify that PT-resistance of PTR26 was conferred by a dominant mutation, a protoplast fusion experiment was done. The lysine-auxotrophic mutant, PTR26-l (PT⁺, lys⁻) was obtained from PTR26. Its reversion frequency was less than 10⁻⁷. PTR26-l was fused with M-2-3 (PT⁻, argB⁻). Resultant fusants were isolated on a CD-regeneration plate. Eight fusants selected at random were all prototrophic and PT-resistant, indicating that the PT-resistance phenotype of PTR26 was dominant. Therefore we used PTR26 as a DNA donor.

Isolation of a mutant gene (ptrA) that confers resistance to PT on A. oryzae

To isolate the PT-resistance gene, a genomic DNA library (approximately 10⁷ clones) from PTR26 was constructed in the A. nidulans-E. coli shuttle vector pDHG25 containing the argB gene, and introduced into A. nidulans FGSC 89 (argB). Transformants that were arginine-prototrophic and were PT-resistant were further selected on CD plates containing PT. The DNA prepared from the one A. nidulans transformant was directly used to transform E. coli JM109. The rescued plasmid was introduced into A. oryzae M-2-3. Transformants were selected on CD plates containing 0.1 mg/l PT and the recombinant plasmids were recovered from six A. oryzae transformants. The plasmids from six transformants contained insert DNAs, the length and restriction patterns of which were identical. This plasmid, containing a 2.0-kb insert, was designated pDHG-PTR.

Nucleotide and deduced amino acid sequences of the ptrA

To sequence the region containing the ptrA gene, a 3.0-kb restriction fragment including the 2.0-kb in-
sert was excised from the pDHG-PTR and subcloned into pBluescript II, to create pPTR and analyze the restriction map of insert (Fig. 1). Then nucleotide sequencing was done, showing a single ORF, encoding a 327-aa, interrupted by a putative 58-bp intron (Fig. 2). The predicted intron was confirmed by sequencing of its cDNA clone. A blast sequence similarity search showed that the ptra-encoded protein was related to the stil35-encoded protein of *Fusarium solani* and the THI4-encoded protein (Thi4p) of *S. cerevisiae*, showing 72% and 63% aa identity, respectively. Thi4p was identified as a key enzyme in the pathway leading to the biosynthesis of the thiazole moiety of thiamine. The aa sequence alignments are shown in Fig. 3. To identify the mutation point of *ptra*, its wild allele was cloned by PCR amplification with the genomic DNA of HL1034 as a template and the nucleotide sequence was analyzed using the direct sequencing method. When the nucleotide sequence of *ptra* was compared with that of the wild allele, there was no mutation point in the coding region but in the 5′-flanking region G replaced A at position –68 from the translation start site. Comparing of the 5′-flanking regions on three *THI4* homologous genes, a significant conserved motif (region A), 5′-GAAA(A/T-/)-GGATCATG-3′, was found (Fig. 4). The *ptra*’s nucleotide replacement occurred in region A (A→G). So we guess that region A is an important for the transcription of these genes similar to *THI4* and the mutation in region A results in significant changes in the regulatory mechanism of gene transcription (described below).

**Transformation of *A. oryzae* industrial strain with the ptra gene as a dominant selectable marker**

To confirm the *ptra* gene is available as a dominant selectable marker for an *A. oryzae* wild type strain, we examined the transformation frequency using an integrative plasmid, pPTR, and *A. oryzae* HL1034 as a host. In the presence of 0.1 mg/l PT, four PT-resistant transformants was obtained per μg DNA, while no colonies were regenerated in the control experiments with no plasmid (Fig. 5). Five transformants were chosen at random for Southern blot analysis. As shown in Fig. 6, in the each transformant, an additional one or two bands were observed besides the native band (3.2-kb) detected in the host strain, indicating that *ptra* was successfully integrated into the chromosome of PT-resistant transformants. All the transformants could grow on CD plates containing 10 mg/l PT in the same way as DNA donor strain PTR-26. These results indicate that the introduction of single *ptra* gene confers PT-resistance on *A. oryzae*. Therefore *ptra* gene is considered to be useful as a dominant selectable marker for transformation of an *A. oryzae* industrial strain. Furthermore this transformation system is assumed to be applicable for other fungi sensitive to PT besides *A. oryzae*. We are now con-

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**A. oryzae ptra** 1: WSPFAIYEPVTVAATGLKVGVVSETVVP-VGASQTELDDHFGKMDPFXFAPKRESQV 59

**F. solani stil35** 1: WSPFAAV-SSPASEASASAPAVKLVGLPSKNSAAATVEEMEGKMDPFXFAPKRESQV 59

**S. cerevisiae THI4** 1: WSP-A-ATSTATTSASQKLNSTEPVTCHLSDIVEKEDDPFXFAPKRESQV 49

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60: RANTRYFEDLKDSTVVGAGOSCLSTAVLAKARFDLKGTVVANSFPGGKMGW 119
60: RANTRYFEDLKDSTVVGAGOSCLSTAVLAKARFDLKGTVVANSFPGGKMGW 119
50: RANTCFEDLSLSLVDIEGTVVGAGOSCLSTAVLAKARFDLKGTVVANSFPGGKMGW 109
120: DQLFSAMVMKRAPKLVNLGVPYEDANPHVYVVKHASLPISTLSKVSFPNPVNLFA 179
120: DQLFSAMVMKRAPKLVNLGVPYEDANPHVYVVKHASLPISTLSKVSFPNPVNLFA 177
110: DQLFSAMVMKRAPKLVNLGVPYEDANPHVYVVKHASLPISTLSKVSFPNPVNLFA 167
180: TAVELDPF-PTEGKMFAGVHVWTLVNLHDDHSCMDFNPI- -N-A-P- 225
178: TAVELDPF-PTEGKMFAGVHVWTLVNLHDDHSCMDFNPI- -N-A-P- 222
168: TAVELDPF-PTEGKMFAGVHVWTLVNLHDDHSCMDFNPI- -N-A-P- 222
226: VVEETTTCHDDDFGACFARKLXMSGDVGLMGMRDLNQAE-DAIXVKT-ETVXGLI 281
223: LVLVTTCHDDDFGACFARKLXMSGDVGLMGMRDLNQAE-DAIXVKT-ETVXGLI 278
228: HGVHSTTCDDFGACFARKLXMSGDVGLMGMRDLNQAE-DAIXVKT-ETVXGLI 267
282: IGMELSDDGLRAORPTFGAMVLGKABEXEXALEVDERQRECAE 327
279: IGMELSDDGLRAORPTFGAMVLGKABEXEXALEVDERQRECAE 324
288: FADHHYELDDGLRAORPTFGAMVLGKABEXEXALEVDERQRECAE 326

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**Fig. 3.** Amino Acid Sequence Alignments for *A. oryzae* PTRA, *F. solani* ST135 and *S. cerevisiae* THI4.

Identical amino acid residues among them are boxed.
A. oryzae ptrA mutant allele -75 GAAAAAGTCATG
A. oryzae ptrA wild allele -75 GAAAAAGATCATG
F. solani sti35 -63 GAAA-GGATCATG
S. cerevisiae THI4 -166 GAATGGATCATG

Fig. 4. Conserved Motif in 5’-flanking regions of ptrA, F. solani sti35 and S. cerevisiae THI4.
Nucleotide replaced point of ptrA (A -68 G) is shown in bold.

No plasmid  pPTR

Fig. 5. Appearance of Transformed Strains on the Regeneration Medium.
Protoplasts of A. oryzae HL1034 transformed with pPTR (right) and with no plasmid (left) were regenerated for 7 days at 30°C. Regeneration media contain 0.1 mg/l PT.

Fig. 6. Southern Blot Analyses of the Transformants.
Genomic DNA were digested with HindIII and separated by electrophoresis on a 0.8% agarose. A part of ptrA (1.2-kb) was amplified by PCR and used as a probe. Hybridization and detection were performed using ECL system. Lane 1, 3HindIII; 2, 1.2-kb probe; 3, HL1034 (host); 4-8, transformants.

Fig. 7. Northern Blot Analyses of the Transformants.
Total RNAs were prepared from mycelium cultured in Cz-Dox medium (a), Cz-Dox medium containing 0.1 mg/l PT (b), Cz-Dox medium containing 10 µM thiamine (c). Lanes: 1, HL1034; 2, PTR26; 3-6, transformants. PCR-amplified fragment, corresponding to ptrA coding region (0.37-kb) was used as a probe. Hybridization and detection were done using the ECL system. (A): Ethidium bromide staining of rRNA. (B): The detection of the mRNA for ptrA gene.

transcription level of ptrA, we prepared total RNA from the mycelium of each transformant and PTR26 harvested in different culture conditions (CD broth, CD broth + 0.1 mg/l PT, CD broth + 10 µM thiamine) and compared the ptrA transcription patterns of the transformants with that of the host strain by Northern blot analysis. This analysis showed that PT-resistant transformants had increased mRNA levels for ptrA compared with the host strain did in the basal condition without PT or thiamine. Additionally, in the case of transformants and PTR26, their ptrA transcriptions were not repressed in the presence of thiamine as well as PT. On the contrary, in the case of the host strain, its transcription is repressed completely in the same condition (Fig. 7). PTR26 and transformants showed the same transcription pattern, strongly suggesting that the thiamine non-repressible transcription pattern of transformants are not caused by a gene dosage effect but by introduction of a single copy of the ptrA gene. This result of PTR26 indicates that a single copy of ptrA can confer PT-resistance on A. oryzae. In yeast, the enzymes involved in thiamine biosynthesis are negatively regulated all together as increasing of thiamine or TPP in the cell. The THI4 also is regulated in the same manner. Therefore the ptrA is strongly suggested to be a mutant allele of the gene having the same function as S. cerevisiae THI4 because of its significant identity with Thi4p in aa level and being thiamine-repressible in its wild allele. So we speculate that the increased expression of ptrA results in thiamine overproduction and removing the antagonism by PT. This speculated resistance mechanism is different from the one previously reported on yeast. However, further examination is needed to discover the resistance mechanism to PT in A. oryzae. This study is now in progress.

structuring a plasmid vector convenient in handling for Aspergillus transformation.

The transcription pattern of the ptrA gene in the PT-resistant transformant
In the PT-resistant transformants with the introduced ptrA gene, their gene expressions were presumed to be improved, compared with that of the host strain. Therefore, to clarify the changes in the
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References


