σ_{marY1} is the LTR of the gypsy-Type Retroelement marY1 from the Basidiomycete Tricholoma matsutake, Allows Multicopy DNA Integration in Lentinula edodes

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σ_{marY1} is the LTR of the retroelement marY1 from the homobasidiomycete Tricholoma matsutake. Upon integration through transformation, pLC1-hph carrying a σ_{marY1} derivative, σ_{marY1}*, conferred the hygromycin-resistant phenotype stronger than the vector without σ_{marY1} on Lentinula edodes. Based on the densitometric analysis after Southern hybridization, a copy number of the latter. We conclude that σ_{marY1} allows multicopy DNA integration and will be useful in the genetic research on this fungal group.

Key words: basidiomycetes; Lentinula edodes; long terminal repeat (LTR); transformation

marY1 is a gypsy-type long terminal repeat (LTR) retroelement from the ectomycorrhizal homobasidiomycete Tricholoma matsutake.1) We previously documented that (i) a 5'-LTR region of marY1 is expressed in the budding yeast Saccharomyces cerevisiae, and (ii) nucleotide sequences that compose the 426-bp LTR are present in various higher fungi.2,3) In fact, the structure of LTR of marY1 resembles to some extent that of retrotransposons from S. cerevisiae, such as the copia-type Ty1 and the gypsy-type Ty3.1) The LTR of Ty1 and Ty3 are dispersed throughout the genome of the budding yeast as the solo elements designated δ and σ, respectively.4) Similarly, our unpublished data showed that the LTR of marY1, which is designated σ_{marY1} after σ of Ty3, is dispersed throughout the genome of T. matsutake.

In this study, we questioned whether σ_{marY1} is useful in recombinant systems of homobasidiomycetes. To answer this question, we analyzed the recombination efficiency of σ_{marY1} using a genetic transformation system based on pLC1-hph.5) pLC1-hph is a vector designed for transformation of the homobasidiomycete Lentinula edodes, which expresses hygromycin phosphotransferase (hph) through the ras gene promoter (Pras) and confers hygromycin resistant (Hyg') on recombinants.5) We constructed a pLC1-hph derivative carrying σ_{marY1}, introduced it into L. edodes A-567-Pm17 (Akiami Shukin, Yamanashi, Japan), and compared their transformation efficiency and the copy number of the integrated DNA in the genome with those of the control vector.

Prior to the construction of such a vector, an attempt was made to replace the BamHI site (GGATCC) located at bp 308 of σ_{marY1} with the EcoRI site (GAATTC) so that the unique BamHI site of pLC1, wherein hph is cloned, could be retained in the resulting construct (Fig. 1). To make such σ_{marY1}, the 308-bp sequence upstream of the BamHI site of σ_{marY1} and the 118-bp downstream sequence were separately prepared by PCR with a set of primers [5’σ_{marY1}-XbaI/5’σ_{marY1}-EcoRI], and [3’σ_{marY1}-EcoRI/3’σ_{marY1}-EcoRV-SalI], respectively, and the plasmid pHHM149 carrying σ_{marY1} as a template

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Nucleotide sequence of σ_{marY1} (= LTR of marY1) has been deposited in the DDBJ data base under the accession number AB028236.
Abbreviations: Amp', ampicillin resistance; hph, hygromycin phosphotransferase gene; Hyg', hygromycin resistance; LTR, long terminal repeat; PCR, polymerase chain reaction; Pras, ras gene promoter; TpriA, priA gene terminator
(Table 1). The resulting fragments were cloned into appropriate cloning sites of pBluescript SK+ and examined for the nucleotide sequences with the ABI prism 377 autosequencer (Applied Biosystems, Foster City, CA). The inserts that correspond to the upstream 308-bp fragment carrying the correct sequence and the downstream 118-bp one were ligated together using the XbaI, EcoRI, and SalI sites of pBluescript SK+. The resulting \( \sigma_{marY1}^* \) was excised from pBluescript SK+ with XbaI and EcoRV, and cloned into the appropriate restriction sites of pLC1-hph to produce pLC1-hph-\( \sigma_{marY1}^* \)-1 (Fig. 1). Please note that \( \text{Pras} \) is present within the 466-bp sequence in front of the BamHI site of pLC1. Therefore, deletion of further upstream sequences, such as deletion of the 1.7-kb \( \text{HindIII} \) fragment that contains the restriction sites used to ligate \( \sigma_{marY1}^* \), does not affect the expression (Fig. 1). Such a deletion vector, i.e., pLC1-hph-dH, was used as a control in the following analysis.

Genetic transformation of \( L. \text{edodes} \) strain A-567-Pm17 was essentially carried out based on a standard published procedure with the modified enzyme solution (10 mg/ml cellulase ONOZUKA R-10 [Yakult, Tokyo], 10 mg/ml zymolyase 100T [Seikagaku Inc., Tokyo], 1 mg/ml chitinase RS [Sigma, St Louis, MO], and 1 mg/ml \( \beta \)-glucuronidase [Sigma] for protoplast formation). \( L. \text{edodes} \) strain A-567-Pm17 (1 \( \times \) 10\(^7\) protoplasts) was transformed with plasmid DNA (25 \( \mu \)g) using polyethylene glycol (PEG), and plated on MS agar containing hygromycin B to a 10-fold higher concentration of the antibiotic than that described in a standard protocol (200 \( \mu \)g/ml; Wako Pure Chemicals, Osaka, Japan). Thus far, pLC1-hph-\( \sigma_{marY1}^* \)-1 conferred 0.8–1.3 Hyg\( ^{\prime} \) transforms per 1 \( \mu \)g DNA, the transformation efficiency equivalent to that of pLC1-hph-dH. However, transforms carrying pLC1-hph-\( \sigma_{marY1}^* \)-1 generally grew much better than those carrying pLC1-hph-dH on the selection agar plate (Fig. 2).

Southern hybridization analysis was done on genomic digests of the transforms. The \( \text{BamHI-EcoRV-XbaI} \) digests were hybridized with the \( [\alpha-\text{32P}]\text{dCTP}-\text{labeled} \) 0.70-kb EcoRI fragment of pLC1-hph-\( \sigma_{marY1}^* \)-1 containing \( \text{Pras} \) (Fig. 1). When 1.0 \( \mu \)g digests were tested, hybridization signals were detected in the sample of \( L. \text{edodes} \) A-567-Pm17 carrying pLC1-hph-\( \sigma_{marY1}^* \)-1 as a sharp 1.2-kb band (Fig. 3A, B), which corresponds to the

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<tr>
<th><strong>Table 1. Primers</strong></th>
<th><strong>Sequence (( \text{TTN} \pm \text{C} )), and Description</strong></th>
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<tr>
<td>( 3\sigma_{marY1}-\text{EcoRI} )</td>
<td>5'-CGGAATTTCCTAGGTTCCTTTCTTGGATTTCCCTTGTC-3' (58), designed based on a sequence right behind the unique ( \text{BamHI} ) site of ( \sigma_{marY1} ), the EcoRI site and a few extra bases (CG) attached to the 5'-end</td>
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<td>( 3\sigma_{marY1}-\text{EcoRV-Sall} )</td>
<td>5'-GGCTGAGGATATCTATAAGGCAACTGAGCTCTTAG-3' (60), designed based on a complementary sequence at the 3'-end of ( \sigma_{marY1} ), the Sall-EcoRV site and a few extra bases (GC) attached to the 5'-end</td>
</tr>
<tr>
<td>( 5\sigma_{marY1}-\text{EcoRI} )</td>
<td>5'-CGGAATTCTCCCTTGCTGATGCCCAGTATATGT-3' (60), designed based on a complementary sequence in front of the unique ( \text{BamHI} ) site of ( \sigma_{marY1} ), the EcoRI site and a few extra bases (GC) attached to the 5'-end</td>
</tr>
<tr>
<td>( 5\sigma_{marY1}-\text{XbaI} )</td>
<td>5'-CGCTTAGATGTAAGGGGACTAGGGGGGTGC-3' (63), designed based on a sequence of the 5'-end of ( \sigma_{marY1} ), the XbaI site and a few extra bases (GC) attached to the 5'-end</td>
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**Fig. 2.** Expression of the Hyg' Trait in \( L. \text{edodes} \) A-567-Pm17 Carrying pLC1-hph-\( \sigma_{marY1}^* \)-1 and pLC1-hph-dH.

Transformants of \( L. \text{edodes} \) A-567-Pm17 were grown on MS agar with hygromycin B (200 \( \mu \)g/ml, 1–3). \( L. \text{edodes} \) A-567-Pm17 carrying pLC1-hph-\( \sigma_{marY1}^* \)-1; 4–5, \( L. \text{edodes} \) A-567-Pm17 carrying pLC1-hph-dH; 6, \( L. \text{edodes} \) A-567-Pm17 without a plasmid.

\( \text{BamHI-EcoRV} \) fragment of \( \text{Pras} \) from the vector DNA (Fig. 1). In contrast, such signals were barely detected in the constructs carrying pLC1-hph-dH (Fig. 3A, B). In the sample of \( L. \text{edodes} \) A-567-Pm17 without a plasmid, a signal that corresponds to a native single copy \( \text{Pras} \) lighted up when 6 \( \mu \)g or 60 \( \mu \)g DNA was hybridized, though the signal still remained barely detectable in the 6- \( \mu \)g digests (Fig. 3A, B).

Similarly, the digests were hybridized with the [\( \alpha-\text{32P}]\text{dCTP}-\text{labeled} \) 1.07-kb \( \text{BamHI} \) fragment containing hph and the [\( \alpha-\text{32P}]\text{dCTP}-\text{labeled} \) 0.43-kb XbaI-EcoRV fragment of \( \sigma_{marY1}^* \) (Fig. 1, 3A, 3C). While the hph probe hybridized with the 1.1-kb fragment from transformants with pLC1-hph-\( \sigma_{marY1}^* \)-1 and pLC1-hph-dH, the \( \sigma_{marY1}^* \) probe only hybridized with the 0.43-kb fragment from the former transformants, indicating that \( \text{Pras-hph-Tpr} \) of pLC1-hph-\( \sigma_{marY1}^* \)-1 integrated along with \( \sigma_{marY1}^* \) (Fig. 3C).

An image analysis revealed that the hybridization signal of \( \text{Pras} \) was about 95-times stronger in the samples with pLC1-hph-\( \sigma_{marY1}^* \)-1 than that with a single copy \( \text{Pras} \), which was about 16 times stronger with
correlates to the intensity of the hybridization signals, indicating that the copy number of Pras-hph-TpriA integrated in the L. edodes genome is reflected in the levels of the expression of the phenotypes in the transformants.

Although it did not improve transformation efficiency, a σ_marY1-mediated system markedly increased the copy number of the vector DNA integrated in the genome. Please note that two types of vectors, pLC1-hph-σ_marY1 and pLC1-hph-dH with nearly equal sizes, the former of 6.3 kb, and the latter 5.7 kb, were applied to protoplast suspensions in a standard protocol. The procedures are not expected to affect the efficiency of introduction of vectors into the targeted cells, unlike systems using viral infections or Agrobacterium-mediated transformation.7,8) If, however, multicopy DNA integration by σ_marY1 were due to the increased copy number of specific integration sites, such as repeated sequences homologous to those of σ_marY1 in the targeted genome, the σ_marY1-mediated system could improve the transformation efficiency due to increased probability of recombination. A plausible hypothesis for the increased copy number of the vector integrated without improving transformation efficiency is that the vector proliferated in the presence of σ_marY1 in the recombinants of L. edodes during the protoplast regeneration process.

Mechanisms of genetic recombination through σ_marY1 are yet to be clarified. The muticopy DNA integration system achieved by σ_marY1 may, however, be useful in the production of a large quantity of proteins encoded in the constructs through a gene dosage effect. The system may influence both basic and applied research in basidiomycetes since many fungi belonging to the basidiomycetes are important in agricultural production, biochemical industries, and environmental conservation. Some produce edible or pharmaceutical mushrooms, and others catalyze biomass conversion, or promote arboreal habitat through symbiotic relationships.9,10) It may be interesting to apply such a genetic tool to the development of systems that allow the mass-production of specific proteins useful in various facets of such processes in basidiomycetes.

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**References**

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