Transformation system for prototrophic industrial yeasts using the *AUR1* gene as a dominant selection marker

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Abstract We show a new transformation system for prototrophic yeast strains including those of *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *K. marxianus*, and *Candida glabrata*. This system is composed of an antibiotic, aureobasidin A (AbA), and its resistance gene *AUR1*-C as a selection marker. Southern analysis of genomic DNAs of the transformants indicated that the copy number of the plasmid increased from one to more than four, depending on the concentration of AbA used for selection of the transformants. The *AUR1*-C gene was also effective as a selection marker for gene disruption, and was able to disrupt both copies of the gene on homologous chromosomes of diploid cells by a single round of transformation. This system has a broad application in the transformation and gene disruption of prototrophic strains of a variety of yeast species.

Key words: *AUR1*-C; Transformation system; Aureobasidin A; Drug resistance; Gene disruption; Industrial yeast

1. Introduction

Selection of transformants using a drug resistance gene as a marker has been commonly used for recombinant DNA technology. In the genetic manipulation of various yeasts including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida* spp., transformation experiments are mainly performed by transforming auxotrophic strains, which are genetically defined, with the corresponding wild-type gene as a marker and by selecting prototrophic transformed cells in a minimal medium. Therefore, prototrophic strains of industrial yeasts are not amenable to genetic manipulation using commonly applied auxotrophic markers. In addition, genetic breeding and expression of heterologous genes in industrial yeasts, most of which are prototrophic, are extremely restricted because of a lack of a genetic transformation system [1]. A dominant selective marker, such as resistance against a drug, is thought to be useful for recombinant DNA technology for industrial yeast strains because these cells are diploid or aneuploid, lack a sexual cycle, and are prototrophic. Several dominant selection markers in *Saccharomyces* have been established, including the G418-resistant gene of Tn*903* [2], a PDR4 gene imparting cerulenin resistance [3], and a *SMR1* gene conferring resistance to sulfometuron methyl [4]. Each of these markers, however, has limitations, in terms of suitable hosts and transformation efficiency.

Aureobasidin A (AbA), an antifungal antibiotic produced by *Aureobasidium pullulans*, is toxic to the budding yeast *S. cerevisiae* [5–7] and the fission yeast *S. pombe* [8]. We have isolated a dominant resistance gene to AbA, designated *AUR1*, from *S. cerevisiae* [9]. Here we constructed a new dominant selection marker *AUR1*-C from the *AUR1*^{R} gene and demonstrate that the marker is effective for transformations not only of *S. cerevisiae* but also of other yeast species.

2. Materials and methods

2.1. Yeast strains, media, and chemicals

We used prototrophic diploid strains of *S. cerevisiae*, sake yeast Kyokai no. 701 (our stock strain), shochu yeast Kyokai no. 2 (our stock strain), baker's dry yeast (Oriental yeast Co., Ltd.) and brewer's yeast (our stock strain) as well as a haploid strain DKD-5D (*MATa his3 leu2-3,112 trp1*) [10]. *Candida glabrata* TIMM1062 and TIMM1064 (Research Center for Medical Mycology, Teikyo University, Hachioji, Japan), *Kluyveromyces lactis* IFO1267, *Kluyveromyces marxianus* IFO1735, and *S. pombe* JY745 (h$^-$ *ade6*-M210 *leu1 ura4*-D18; kindly provided by C. Shimoda) were also used. Yeast cells were grown aerobically in YPD (1% yeast extract, 2% Bactopeptone, 2% glucose) at 30°C. Synthetic minimal medium (SD; 2% glucose, 0.7% yeast nitrogen base without amino acids, and appropriate amino acid supplements) was also used. The antibiotic AbA was prepared and used as described [5].

2.2. Plasmid construction

pTCR3 [9] contains the *AUR1*^{R}-1 mutant gene in a *Hin*dIII site of pWH5 [11]. The *AUR1*-C gene, with a two amino acid residue replacement from the wild-type *AUR1*$^+$ gene, was produced from *AUR1*^{R}-1 by site-directed mutagenesis using PCR. Briefly, a 3.5 kbp *Hin*dIII DNA fragment obtained by partial digestion of pTCR3 was subcloned into a *Hin*dIII site of pUC119 to construct pUaur1R. Oligonucleotide 5′-AATATGTATACTACATGTTTTTCAAATTCC-3′, in which the *Bst*1107I site is underlined, was used for PCR to make the replacement of residue 240, the second mutation site (described later), of *AUR1*^{R}-1. PCR amplification was done using the oligonucleotide and M13 primer M4 (Takara Shuzo Co., Ltd.) as primers, and pUaur1R as a template. The amplified DNA fragment was doubly digested with *Sal*I and *Bst*1107I, and the fragment obtained was used for the replacement of the corresponding fragment of pUaur1R, producing a plasmid pUAUR1-C (Fig. 1). The linearization of pUAUR1-C by digestion with *Stu*I enhanced integration into the chromosomal *AUR1*$^+$ locus of *S. cerevisiae*.

Other plasmids (pWHAUR1-C, pYCAUR1-C, and pΔleu2) used in this study were constructed as follows. The 3.5 kbp *Hin*dIII fragment from pUAUR1-C was inserted into a *Hin*dIII site of pWH5 to produce pWHAUR1-C. A DNA fragment containing *AUR1*-C obtained by PCR using pUAUR1-C as a template was inserted into a blunted *Eco*RI-*Bam*HI site of pYEUra3 (Clontech Lab., Inc.). The resultant plasmid was treated with *Xho*I and *Nae*I to delete a 0.6 kbp fragment, producing a plasmid pYCAUR1-C (Fig. 1). A *Eco*O65I-*Bst*XI DNA fragment of the *LEU2* coding region was blunt-ended and inserted into a *Sma*I site of pUAUR1-C, producing a plasmid pΔleu2 (Fig. 4) for disruption of the *LEU2* gene.

2.3. Yeast transformation

Transformation of all yeast strains was performed according to the lithium acetate method [12] with some modifications. Briefly, cells were grown in YPD medium to the logarithmic phase (1–2 at OD_{660}) at 30°C with shaking. The cells were harvested, washed once with water, suspended in solution A (100 mM lithium acetate, 1 mM

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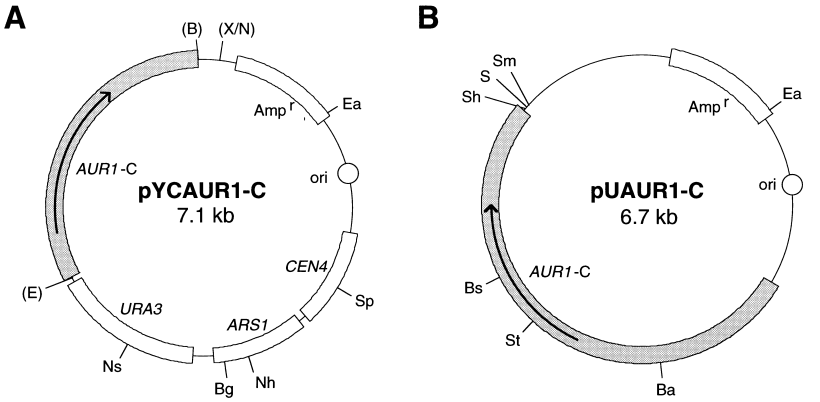

Fig. 1. Structure of the plasmids used for transformation experiments. A: The single copy plasmid pYCAUR1-C. B: The integrating plasmid pUAUR1-C. The direction of transcription of the *AUR1*-C gene is indicated by arrows. Restriction site abbreviations: B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; Bs, *Bst*1107I; E, *Eco*RI; Ea, *Eam*1105I; N, *Nae*I; Nh, *Nhe*I; Ns, *Nsp*V; S, *Sal*I; Sm, *Sma*I; Sp, *Spe*I; Sh, *Sph*I; St, *Stu*I; X, *Xho*I.

EDTA, 10 mM Tris-HCl pH 7.5) to a final OD_{660} of 150, and incubated at 30°C for 1 h. Then, 5 μg of plasmid DNA together with 150 μg of heat-denatured salmon testis DNA was added to the cell suspension (100 μl). The suspension was gently mixed with 850 μl of solution B (40% polyethylene glycol 4000 in solution A), and incubated at 30°C for 30 min. The suspension was kept at 42°C for 15 min and left at room temperature for 10 min. The cells were centrifuged, resuspended in 5 ml of YPD medium and incubated at 30°C for 6 h to overnight with shaking. Aliquots of the cell suspension were spread on YPD plates containing AbA or on SD plates. The one-step transformation method was also used [13].

2.4. DNA analysis

Chromosomal DNA of yeasts was isolated according to Philippsen et al. [14]. Southern hybridization was performed according to Sambrook et al. [15]. Hybridization probes were labeled with [^{32}P]dCTP by a random primer method.

3. Results and discussion

3.1. Construction of the AUR1-C marker gene

The *AUR1*^{R} dominant gene isolated from the AbA-resistant mutant of *S. cerevisiae* confers increased resistance to the host cells [9]. To examine whether a combination of AbA with *AUR1*^{R} is useful as a dominant selection system for transformation of *S. cerevisiae*, the multicopy plasmid pTCR3 containing *AUR1*^{R}-1 and *LEU2* as selection markers was introduced into *S. cerevisiae* haploid strain DKD-5D. Transformants were selected directly on YPD plates containing 0.5 μg/ml AbA or on SD plates supplemented with required nutrients except leucine. The number of AbA-resistant colonies was only a few percent of that of Leu$^+$ colonies (data not shown), showing that the transformation system using the *AUR1*^{R}-1 gene was inefficient in comparison to the system using the auxotrophic marker.

The AbA resistance gene of *S. pombe*, a homolog of the *S. cerevisiae AUR1*R gene, gave transformed colonies of *S. pombe* by selection on plates containing AbA at a frequency as high as auxotrophic marker selection (our unpublished data). In the resistant gene of *S. pombe*, glycine at amino acid position 240 was changed to cysteine [8], whereas in the resistance gene of *S. cerevisiae* phenylalanine was changed to tyrosine at amino acid position 158 [9]. These facts suggest that amino acid position 240 of the *aur1* gene product of *S. pombe* is important for the expressing AbA-resistant phenotype, and therefore we reasoned that an alteration of this position in the *AUR1*^{R}-1 gene product of *S. cerevisiae* would also result in an increase in transformation efficiency in *S. cerevisiae*. We constructed the mutant gene *AUR1*-C, in which alanine was replaced with cysteine at position 240 in *AUR1*^{R}-1 by in vitro mutagenesis, and the multicopy plasmid pWHAUR1-C containing *AUR1*-C. Then, *S. cerevisiae* strain DKD-5D was transformed with pWHAUR1-C and transformants were selected by AbA resistance or by leucine prototrophy (Leu$^+$). Transformants showing AbA resistance appeared at a similar frequency to Leu$^+$ colonies (Table 1), indicating that *AUR1*-C as a selection marker for transformation of *S. cerevisiae* is as effective as the auxotrophic marker.

3.2. Application of the AUR1-C transformation system to industrial strains of S. cerevisiae

A dominant selection marker is highly useful for transformation of prototrophic industrial yeasts. The antibiotic AbA was toxic to such yeasts at a dose toxic to the auxotrophic laboratory strain DKD-5D (Table 2). We constructed a single copy plasmid pYCAUR1-C which contains *AUR1*-C and URA3 as markers, and ARS1 and CEN4 for extrachromosomal maintenance of the plasmid (Fig. 1). When we performed transformation of industrial strains of *S. cerevisiae* (Table 3), pYCAUR1-C gave high transformation efficiencies upon direct AbA resistance selection, whereas no colony was formed without the plasmid. These results indicate the usefulness of the *AUR1*-C gene as a marker for transformation of industrial strains of *S. cerevisiae*.

3.3. Copy number of the AUR1-C marker in the transformants

To determine the copy number of the *AUR1*-C gene present in the AbA-resistant transformants, we used the integrating

Table 1
Comparison of transformation efficiencies of haploid strain DKD-5D of *S. cerevisiae* between AUR1-C and auxotrophic marker[a]

	Concentration of AbA (μg/ml)[b]		SD−leu[c]
	1.0	5.0	
Transformants/μg plasmid	2524	2044	2500

[a]Strain DKD-5D was transformed with pWHAUR1-C and spread on each plate to select transformants.
[b]The selective plates are YPD plates containing AbA at the concentration indicated.
[c]SD plates supplemented with the amino acids required except leucine.

Table 2
Antifungal activity of AbA against various yeast strains

Strain		(Ploidy)	MIC[a] (μg/ml)
S. cerevisiae	ATCC9763	(diploid)	0.2–0.4
	DKD-5D	(haploid)	0.4
	Sake yeast Kyokai no.701	(diploid)	0.1–0.2
	Shochu yeast Kyokai no. 2	(diploid)	0.1
	Brewer's yeast	(polyploid)	0.1
	Baker's yeast	(diploid)	0.2–0.4
S. pombe	JY745	(haploid)	0.1
C. glabrata	TIMM1064	(haploid)	0.7
	TIMM1062	(haploid)	0.7
K. lactis	IFO1267	(haploid)	0.05
K. marxianus	IFO1735	(haploid)	0.2

[a]Minimal inhibitory concentration (MIC) was measured as described before [5].

plasmid pUAUR1-C (Fig. 1), which is constructed to be maintained for integration into the chromosomal DNA. Since an uncut, circular form of the plasmid was unable to transform yeast strains, the plasmid was linearized by cutting at a unique *Stu*I site in the *AUR1*-C coding region to enhance integration into the chromosomal *AUR1*$^+$ locus by homologous recombination [16]. The linear plasmid was integrated into chromosomal DNA of sake yeast *S. cerevisiae* Kyokai no. 701 by transformation in which transformants were selected on YPD plates containing AbA at 0.5 or 2.0 μg/ml. The transformation efficiency (3×10^3 transformants/μg plasmid) obtained with YPD plates with 0.5 μg/ml AbA was about one-tenth of that of the replicating plasmid pYCAUR1-C and the colonies on the plates containing AbA varied in size. All transformants obtained, however, grew equally in AbA-free YPD medium. Genomic DNAs isolated from independent transformed clones derived from large- and small-size colonies were digested with *Hpa*I, and analyzed by Southern hybridization using the *AUR1*$^+$ gene as a probe. The result showed that the DNAs of all transformants had a 7.5 kbp band derived from the chromosomal *AUR1*$^+$ gene and one additional band (Fig. 2A). The extra band of all transformants (lanes 6–9) from the smaller colonies was a DNA fragment which was expected from integration with a single copy of the plasmid in the endogenous *AUR1*$^+$ locus (Fig. 2C). On the other hand, the size of bands of three (lanes 2–4) out of four transformants from the large-size colonies coincided with that of a DNA fragment having integration of two copies of the plasmid (Fig. 2C). This was verified by hybridization with pUC119 as a probe (Fig. 2B).

As the concentration of AbA increased, the colony number of transformants decreased (Table 3). When transformants were selected on the plate containing a high concentration of AbA, 2 μg/ml (Fig. 3), the copy number of the plasmid per cell apparently increased. In one of four transformants from large-size colonies, only the 20.7 kbp band was observed, indicating that the transformant had the pUAUR1-C plasmid integrated into the *AUR1*$^+$ loci on homologous chromosomes. Other clones have two copies of the plasmid at one locus on one of the homologous chromosomes. These results indicate that a single copy of the *AUR1*-C gene per cell is able to confer AbA resistance to sake yeast Kyokai no. 701 and probably to other strains of *S. cerevisiae*, and that selection on a medium containing higher concentrations of AbA increases the copy number of the *AUR1*-C gene integrated. This result is consistent with previous reports that the phenotype of resistance against antibiotics shows a dosage effect, and that transformation efficiency by the gene correlates well with copy number in the yeast cell [2,3,17].

3.4. Gene disruption using the AUR1-C gene as a selection marker

We examined whether *AUR1*-C is effective as a marker for selection of gene disruptants in industrial strains of yeasts. To disrupt a *LEU2* gene of the sake yeast by *AUR1*-C, a DNA fragment containing only the middle part of the *LEU2* gene was inserted at a *Sma*I site of pUAUR1-C, yielding the plasmid pΔleu2 (Fig. 4A). pΔleu2 was linearized by cutting a unique *Afl*II site and introduced into *S. cerevisiae* Kyokai no. 701. Four of 106 transformants selected by AbA resistance examined showed the Leu$^-$ phenotype. To confirm the disruption of the *LEU2* gene, Southern hybridization analysis was performed using the *Eco*O65I-*Bst*XI fragment of *LEU2* as a probe. Transformants showing Leu$^+$ had a 1.1 kbp band that originated from the intact *LEU2* gene and an additional band differing in size (Fig. 4B). The size of the extra band was related to the colony size of the transformants, that is, large-sized colonies had larger extra bands than small-sized colonies. The 8.2 kbp band was consistent with the expected size of a DNA fragment resulting from the integration of a single

Table 3
Transformation efficiencies with pYCAUR1-C containing the *AUR1*-C gene of the industrial strains of *S. cerevisiae*

Strain	Transformants/μg plasmid		
	Concentration of AbA (μg/ml)[a]		
	0.5	1.0	2.0
Sake yeast Kyokai no. 701	4.19×10^4	2.96×10^4	4.87×10^3
Shochu yeast Kyokai no. 2	2.10×10^4	8.36×10^3	1.59×10^3
Baker's dry yeast	2.49×10^3	868	236
Brewer's yeast	1.95×10^3	1.25×10^3	350

[a]The selective plates are YPD containing AbA at the concentration indicated.

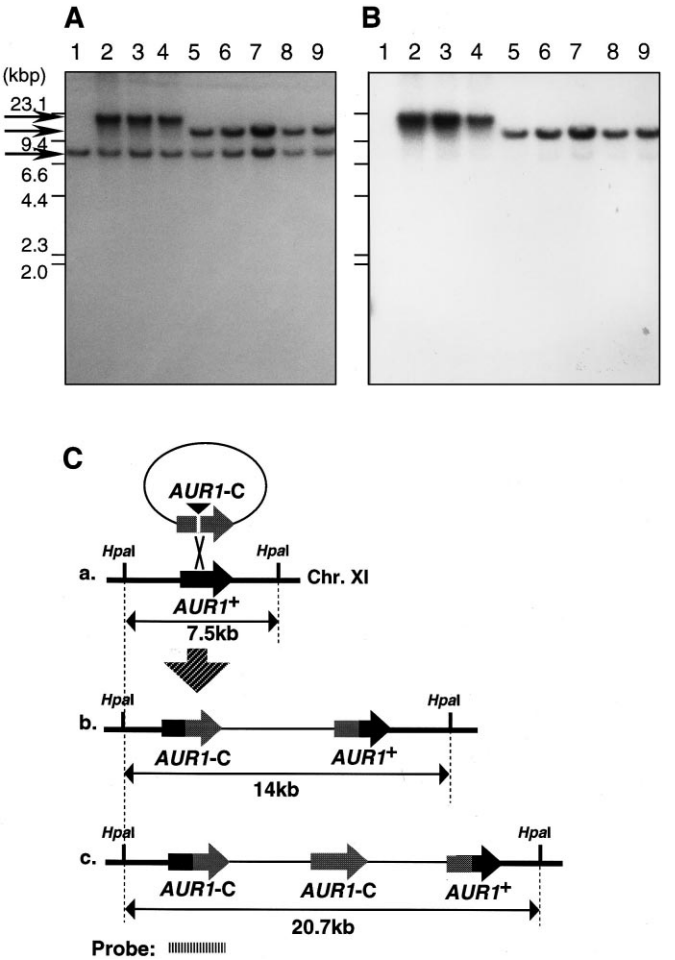

Fig. 2. Southern blot analyses of sake yeast Kyokai no. 701 of *S. cerevisiae* transformed with the linearized pUAUR1-C plasmid. Genomic DNAs were digested with *Hpa*I which does not cut pUAUR1-C, and Southern blot analysis was carried out with the *AUR1*$^+$ gene (A) and pUC119 (B) as probes. Lane 1 is the untransformed parent strain, Kyokai no. 701. Lanes 2–5 are four independent transformants derived from large-size colonies on YPD plates containing 0.5 μg/ml AbA. Lanes 6–9 are four independent transformants from smaller colonies on the same plates. Molecular size standards are shown to the left. The arrows indicate 7.5, 14, and 20.7 kbp bands. C: Schematic representation of integration of pUAUR1-C linearized at the *Stu*I site (arrowhead). The predicted arrangements are shown, in which one (b) or two (c) copies of the plasmid DNA are integrated at the *AUR1*$^+$ locus on the chromosome by homologous recombination. The thin line indicates the DNA fragment originating from pUC119. The thick line represents chromosomal DNA of *S. cerevisiae*.

copy of pΔleu2 in the *LEU2* locus, while the larger bands had a DNA size expected from integration of two or three copies of the plasmid. Four Leu$^-$ transformants tested had the 8.2 kbp band but not the 1.1 kbp band, suggesting that both the *LEU2* on homologous chromosomes in diploid cells were disrupted by insertion of the plasmid. These results showed that *AUR1*-C is useful as a marker for not only selection of gene disruptants of haploid cells but also the simultaneous disruption of two homologous genes in diploid cells by a single transformation. This disruption experiment also supports that the presence of a single copy of the *AUR1*-C marker in a diploid cell is sufficient to confer AbA resistance to sensitive cells.

3.5. Transformation of other species of yeasts

We examined whether *AUR1*-C can transform species of yeasts other than *S. cerevisiae*. Our initial trial to transform the fission yeast *S. pombe* with *S. cerevisiae AUR1*-C was unsuccessful (data not shown) although some genes of *S. cerevisiae* are known to function in *S. pombe*. Therefore, we chose yeast species related to *S. cerevisiae* phylogenetically [18], such

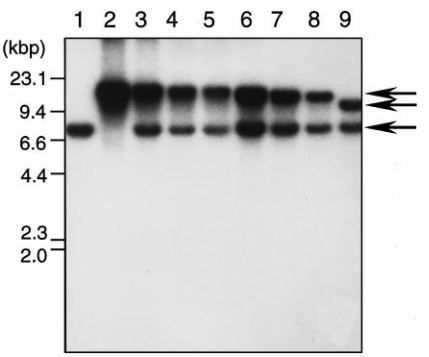

Fig. 3. Southern analysis of the transformants selected on plates containing 2 μg/ml AbA. Genomic DNAs were cleaved with *Hpa*I, and Southern blot analysis was carried out with the *AUR1*$^+$ gene as a probe. The untransformed parent strain (lane 1) and transformants from large (lanes 2–5) and small (lanes 6–9) colonies on YPD plates containing 2 μg/ml AbA were subjected to Southern hybridization. Molecular size standards are shown to the left. The arrows indicate 7.5, 14, and 20.7 kbp bands.

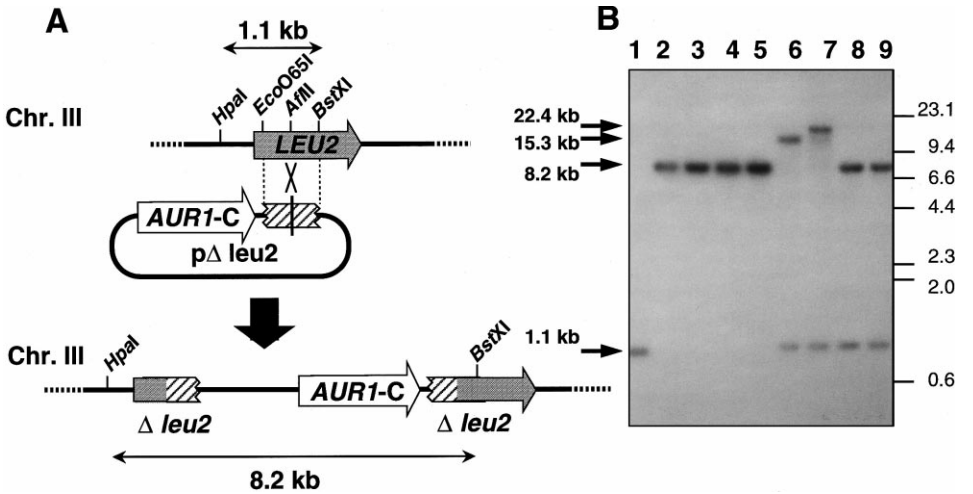

Fig. 4. Gene disruption using the *AUR1*-C gene as a selection marker. A: Schematic representation of disruption of the *LEU2* gene by integration of pΔleu2 linearized at an *Afl*II site. The sizes of the fragments calculated from the restriction map are shown. B: Southern analysis of sake yeast Kyokai no. 701 introduced with the linearized pΔleu2 DNA. Genomic DNAs were extracted from the AbA-resistant transformants and untransformed cells, and doubly digested with *Hpa*I and *Bst*XI which are absent in pΔleu2. The Southern blot was hybridized with an *Eco*O65I-*Bst*XI fragment of LEU2. Lane 1 is the untransformed parent strain. Lanes 2–5 are four independent AbA-resistant transformants showing the Leu^- phenotype. Among transformants showing the Leu^+ phenotype, those of large colonies (lanes 6 and 7) and of small colonies (lanes 8 and 9) on selective plates containing 0.5 μg/ml AbA were analyzed.

as *C. glabrata*, *K. marxianus* and *K. lactis*, as host candidates. The integrating plasmid pUAUR1-C was linearized with *Sal*I and was introduced into the above yeasts by a one-step transformation method [13]. AbA-resistant clones from strains of *C. glabrata*, *K. lactis* and *K. marxianus* were selected on YPD plates containing AbA at 0.7 μg/ml, 0.25 μg/ml and 0.5 μg/ml, respectively. In these strains, no colony formed on the plates without the plasmid. The transformation efficiencies of *C. glabrata* TIMM1062, *C. glabrata* TIMM1064, *K. marxianus*, and *K. lactis* were 1.7×10^3, 82, 1.4×10^2, and 11 transformants per μg of plasmid, respectively. The resistant clones were tested for the presence of pUAUR1-C in genomic DNA by Southern hybridization. The genomic DNAs were digested with *Sph*I, blotted, and hybridized with a labeled pUC119 (Fig. 5). All AbA-resistant clones of *C. glabrata*, *K. lactis* and *K. marxianus* tested had bands hybridizing with pUC119, indicating that these clones were transformed with pUAUR1-C. Some transformants of *C. glabrata* (lane 1), *K. lactis* (lanes 7, 8, and 9), and *K. marxianus* (lanes 11 and 12) had only one band which differed from the linearized pUAUR1-C (6.7 kbp) in size, and the size of each of these bands varied. This result indicates that each of these transformants has pUAUR1-C integrated at different, non-homologous sites in the chromosome. Three transformants of *K. lactis* showing the same band size, however, appear to have the plasmid integrated at the same site of the chromosome. Other transformants of *C. glabrata* (lanes 2, 4 and 5) and *K. marxianus* (lanes 13 and 14) had two hybridizing bands, one of which was the same size as linearized pUAUR1-C. This indicates that a few copies of the plasmid were integrated tandemly into different chromosomal sites in each transformant. We concluded that the AbA resistance gene *AUR1*-C functions in and is useful as a marker for selection of transformants of *C. glabrata*, *K. lactis* and *K. marxianus* as well as *S. cerevisiae*.

The transformation system composed of the *AUR1*-C gene and the antibiotic AbA is useful for the genetic manipulation including transformation and gene disruption of *S. cerevisiae* and its phylogenetically related yeasts, regardless of the ploidy of host cells. Furthermore, this system provides a means of genetically manipulating and producing heterologous protein in prototrophic, industrial yeasts. We have isolated homologs of the *AUR1* gene from *S. pombe*, *C. albicans*, *Aspergillus nidulans*, and *A. fumigatus* whose mutation provided resistance to AbA (in preparation). Such mutated genes would also serve as dominant selection markers in transformation experiments involving these and other species of yeast.

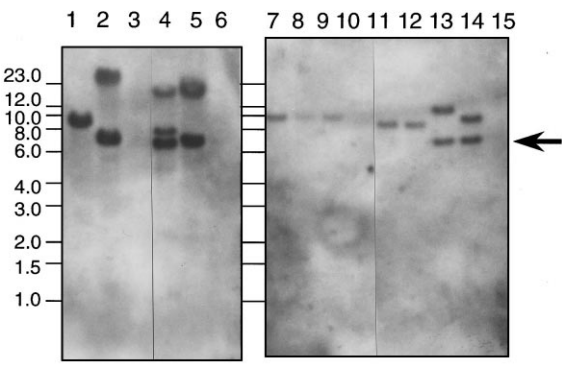

Fig. 5. Southern analysis of *C. glabrata*, *K. lactis* and *K. marxianus* transformed with the linearized pUAUR1-C plasmid bearing the *AUR1*-C gene. Genomic DNAs were cleaved with *Sph*I which cuts at a unique site of pUAUR1-C and probed with pUC119. Lanes 1 and 2 are transformants of *C. glabrata* TIMM1064. Lanes 4 and 5 are of *C. glabrata* TIMM1062. Lanes 7–9 are of *K. lactis* IFO1267. Lanes 11–14 are of *K. marxianus* IFO1735. Lanes 3, 6, 10, and 15 are the untransformed parent strains of *C. glabrata* TIMM1064, *C. glabrata* TIMM1062, *K. lactis* IFO1267, and *K. marxianus* IFO1735, respectively. The arrow indicates the size (6.7 kbp) of the linearized pUAUR1-C.

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