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Construction of a self-cloning sake yeast that overexpresses alcohol acetyltransferase gene by a two-step gene replacement protocol

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Abstract The commercial application of genetically modified industrial microorganisms has been problematic due to public concerns. We constructed a "self-cloning" sake yeast strain that overexpresses the *ATF1* gene encoding alcohol acetyltransferase, to improve the flavor profile of Japanese sake. A constitutive yeast overexpression promoter, *TDH3p*, derived from the glyceraldehyde-3-phosphate dehydrogenase gene from sake yeast was fused to *ATF1*; and the 5′ upstream non-coding sequence of *ATF1* was further fused to *TDH3p-ATF1*. The fragment was placed on a binary vector, pGG119, containing a drug-resistance marker for transformation and a counter-selection marker for excision of unwanted DNA. The plasmid was integrated into the *ATF1* locus of a sake yeast strain. This integration constructed tandem repeats of *ATF1* and *TDH3p-ATF1* sequences, between which the plasmid was inserted. Loss of the plasmid, which occurs through homologous recombination between either the *TDH3p* downstream *ATF1* repeats or the *TDH3p* upstream repeat sequences, was selected by growing transformants on counter-selective medium. Recombination between the downstream repeats led to reversion to a wild type strain, but that between the upstream repeats resulted in a strain that possessed *TDH3p-ATF1* without the extraneous DNA sequences. The self-cloning *TDH3p-ATF1* yeast strain produced a higher amount of isoamyl acetate. This is the first expression-controlled self-cloning industrial yeast.

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Introduction

Since the first commercial application of genetically modified (GM) plants, the planting and harvesting of GM plants has increased but consumers have not freely accepted GM organisms (GMO; Stewart et al. 2000; Falk et al. 2002; Harlander 2002; Hino 2002; Moseley 2002; Schilter and Constable 2002). In contrast to GM plants, GM microorganisms useful for the production of fermented foods and alcoholic drinks are not commercially available (for reviews, see Dequin 2001; Akada 2002; Pretorius and Bauer 2002). In 1990 and 1994, prior to the commercial application of GM plants, two GM yeast strains were approved by the British Government for commercial application (Aldhous 1990; Hammond 1995; Smith 1998). However, these GM yeast strains have never been used commercially. For both GM plants and microorganisms, the public remains concerned about the possible toxic and allergic effects of the products derived from the foreign genes in the GMOs when taken up into the human body (Lack 2002). In most cases, useful genes are introduced together with plasmid vectors that contain the drug-resistance genes necessary for gene manipulation in *Escherichia coli* and other microorganisms or plants. The risk posed by the gene products has been evaluated extensively and the drug-resistance marker genes are unlikely to cause problems if digested in the human stomach and gut (Jonas et al. 2001). However, the possibility of gene transfer to other organisms, such as from GM plants to weeds or from GM microorganisms to pathogenic microorganisms, cannot be neglected (Lorenz and Wackernagel 1994; Adam et al. 1999; Gasson 2000; Stewart et al. 2000; Dale et al. 2002; Gaugitsch 2002; Rieger et al. 2002). If drug-resistance genes and bacterial DNA derived from species other than the host organism were not present in the host organism, these potential problems would be eliminated.

In order to remove unwanted DNA sequences from GM yeast, we developed a method to excise unwanted DNA sequences from recombinant industrial yeast strains (Akada et al. 1999). The traditional method for the

elimination of unwanted DNA is a counter-selection system using the *URA3* marker, a *ura3*$^{-}$ yeast strain, and a drug, 5-fluoroorotic acid (5-FOA; Boeke et al. 1984, 1987). Yeast *URA3*$^{+}$ strains cannot grow on 5-FOA plates but *ura3*$^{-}$ strains can and therefore the loss of the *URA3* marker from transformants allows cells grow on 5-FOA plates. By combining homologous recombination with the counter-selection system, introduced plasmid sequences present on chromosomes can be eliminated completely (Rothstein 1991; Sikorski and Boeke 1991). One disadvantage of the *URA3*/5-FOA system is the requirement of a *ura3*$^{-}$ mutation in the host. Industrial yeast strains are usually polyploid and prototrophic. Therefore, counter-selection systems commonly used for laboratory yeast strains cannot be applied to industrial yeast strains. In contrast, our dominant counter-selection system is useful in any kind of yeast strain (Akada et al. 1999, 2002a; Kawahata et al. 1999).

The first example of the use of the dominant counter-selection system in industrial yeast was a *FAS2* replacement (Akada et al. 1999). Based on a two-step gene replacement protocol (Rothstein 1991; Sikorski and Boeke 1991), a *FAS2* mutant gene was replaced with a wild-type counterpart in a Japanese sake yeast strain in order to improve the flavor of sake. In this case, a self-cloning yeast that contained only yeast DNA but none of the other DNA sequences used during gene manipulations was constructed. Genetically identical mutant yeast strains, which were selected by drug-resistance screening (Ichikawa et al. 1991), have been used commercially for a long time in sake brewing, due to the value of the flavor profile caused by the *FAS2* mutation (Akada et al. 2001). The GM *FAS2* mutant yeast, which contains a single base change identical to that in the commercially distributed sake yeast, was approved as a self-cloning strain by the Japanese Ministry of Health, Labor, and Welfare in April 2001. This is the first GM microorganism approved in Japan. The yeast strain, which does not need to be designated as GM, is freely available to the public. As shown in this case, self-cloning is practical and safe and is applicable to the commercial food and drink market without the concerns caused by the presence of drug-resistance genes and bacterial DNA sequences.

If self-cloning is the most direct way to the commercial application of GM microorganisms, the development of methods to improve microorganisms with their own genes is necessary. In order to confer valuable properties to sake, we utilized the yeast *ATF1* gene encoding alcohol acetyltransferase in this study. The overexpression of *ATF1* in sake and wine yeast is known to affect the properties of the beverages by increasing specific flavor compounds, especially isoamyl acetate (Fujii et al. 1994; Fukuda et al. 1998; Lilly et al. 2000; Mason and Dufour 2000). These sake and wine yeast strains were constructed by transformation with plasmids containing *ATF1* and extraneous DNA. Due to the present situation for GM and self-cloning microorganisms, we planned to construct a self-cloning industrial yeast that overexpressed the *ATF1* gene by inserting a constitutive overexpression promoter from the yeast *TDH3* gene encoding glyceraldehyde-3-phosphate dehydrogenase (Schena at al. 1991; Velculescu et al. 1997) upstream of the 5′ end of *ATF1*. The resulting self-cloning yeast contained no extraneous DNA sequences derived from foreign species. The self-cloning strain produced a higher amount of isoamyl acetate, a valuable flavor component in sake.

Materials and methods

Strains and media

Japanese sake yeast Kyokai No. 7 strain was obtained from the Brewing Society of Japan. All strains in this study were derived from Kyokai No. 7. Plasmid integrants were RAK1807, RAK1808, and RAK1809. Counter-selected strains RAK1857, RAK1859, and RAK1861, containing *TDH3p-ATF1* but no extraneous DNA sequences, were derived from RAK1809. *E. coli* DH5α was used for plasmid constructions. The yeast strains were grown at 28°C in YPD medium (2% glucose, 2% Polypepton, 1% yeast extract, with 2% agar when necessary). Synthetic dextrose medium (SD) contained 0.17% yeast nitrogen base without amino acids and without ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose. YPGal medium contained 2% galactose instead of glucose in YPD. Cerulenin and cycloheximide were purchased from Sigma. Cerulenin was dissolved in ethanol and cycloheximide was dissolved in water and added to the media after autoclaving.

Plasmid constructions

Yeast chromosomal DNA was isolated by the protoplast method using Zymolyase 100T (Seikagaku Kogyo, Tokyo), followed by phenol/chloroform extraction and ethanol precipitation (Philippsen et al. 1991) and was used as a template for all PCR amplifications in this study. Primers (Table 1) were dissolved in sterile water at a concentration of 0.1 μg/μl. PCR was performed in a 25-μl reaction mixture, using KOD Dash DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's instructions. For amplification of the *TDH3* promoter, a 0.7-kb DNA fragment was amplified using TDH3-1 and TDH3-2 primers. The PCR was initiated at 94°C for 1 min, followed by 30 cycles of 94°C for 20 s, 55°C for 2 s, and 74°C for 30 s. The DNA fragment was digested with *Sal*I and *Xho*I and inserted into the *Sal*I-*Xho*I sites of pRS305 (Sikorski and Hieter

Table 1 Primers. Restriction sites are in italics

Primer	Sequence (5′→3′)
TDH3-1	TCAG*GTCGAC*TTTATCATTATCAATACTGCCATTTC
TDH3-2	GGG*CTCGAGCCATGG*TTGTTTGTTTATGTGTGTTTATTCG
ATF1-NCO	GGG*CCATGG*ATGAAATCGATGAGAAAAATCAGG
ATF1-APA	TTT*GGGCCC*CTAAGGGCCTAAAAGGAGAGC
ATF1-UPXBA	CCAATCATTAGCCGTTCC*TCTAGA*
ATF1-UCSAL	GGG*GTCGAC*AGCTGATAAATTGATGGTATT

1989) to form p305TDH3p. A 1.6-kb *ATF1* fragment was amplified with ATF1-NCO and ATF1-APA primers using the same PCR program. The fragment was digested with *Nco*I, which is located at the 3′ terminus of *ATF1*; and the resulting 1.3-kb fragment was inserted into the *Nco*I site of p305TDH3p to form p305TDH3pATF1-5. The *ATF1* gene was located downstream of *TDH3* promoter. For amplification of the 5′ upstream region of *ATF1*, a 2.0-kb fragment was amplified with ATF1-UPXBA and ATF1-UCSAL primers by the following PCR program: 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 56°C for 2 s, and 74°C for 2 min. The 2.0-kb fragment was digested with *Xba*I and *Sal*I and inserted into the *Xba*I-*Sal*I sites of p305TDH3pATF1-5 to form p305ATTDH3pATF1. A 3.8-kb *Xba*I-*Xho*I fragment isolated from p305ATTDH3pATF1 was inserted into the *Xba*I-*Sal*I sites of pGG119 vector (Akada et al. 2002a) to form pGG119TDH3pATF1 (Fig. 1).

Yeast manipulation

Yeast transformation was performed according to the simple lithium acetate/polyethylene glycol method (Chen et al. 1992). pGG119TDH3pATF1 was digested with *Sph*I and used to transform Kyokai No. 7. The transformation mixture was spread on SD plates containing 1.0 μg cerulenin/ml. The cerulenin-resistant colonies were streaked on SD plates containing 0.5 μg cycloheximide/ml as a double-check, because of the dual resistance caused by the *PGKp-YAP1* marker (Akada et al. 2002b). For counter-selection, transformants were grown at 28°C for 1 day in liquid YPGal medium and then cells were spread on YPGal plates at approximately 10^3–10^4 cells/plate. Colonies that grew on YPGal plates after 36–48 h incubation at 28°C were picked.

Southern blot analysis

Chromosomal DNA was digested with *Afl*II, run on an agarose gel, and transferred to a Nylon membrane. A 1.2-kb *Nco*I fragment containing *ATF1* from p305TDH3pATF1-5 and *Bam*HI-digested pBluescript KS^+ were used as probes. Probe preparation and hybridization were performed as described by Akada et al. (1999), using the DIG system (Roche Diagnostics, Germany) with digoxigenin.

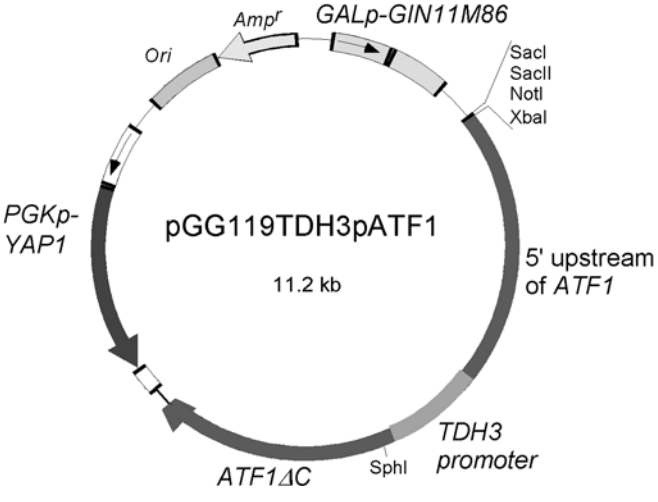

Fig. 1 Structure of the pGG119TDH3pATF1 plasmid. The pGG119 vector contained the *PGKp-YAP1* drug-resistance transformation marker and *GALp-GIN11M86* counter-selection marker. *TDH3p* (glyceraldehyde-3-phosphate dehydrogenase 3 gene promoter) was fused to *ATF1ΔC* (alcohol acetyltransferase 1 lacking its C-terminus) and the 5′ upstream non-coding sequence of *ATF1* was placed upstream of the *TDH3* promoter. The single *Sph*I site within *ATF1* was used for restriction enzyme digestion prior to integrative transformation

Sake brewing test

Sake brewing was performed at 10°C using a total of 500 g rice and 675 ml water. Fermentation was monitored by the loss of weight caused by CO_2 production. The general components of sake were analyzed by a standard method established by the National Tax Administration Agency (Brewing Society of Japan 1993). The concentrations of ethyl caproate and isoamyl acetate were measured as described by Akada et al. (1999).

Results

Construction of *TDH3p-ATF1* plasmid and self-cloning strategy

For the self-cloning of *TDH3-ATF1*, we constructed pGG119TDH3pATF1 (Fig. 1). The pGG119 vector contained a drug-resistance marker, *PGKp-YAP1*, for transformant selection, and a counter-selection marker, *GALp-GIN11M86*, for the selection of plasmid loss (Akada et al. 2002a). The vector was inserted with a 3.8-kb fragment containing the 5′ upstream region of *ATF1* (*5′-up*) and *TDH3p-ATF1ΔC*, which contains a deletion of the C-terminal sequence of *ATF1* for confirmation of correct integration by PCR.

The self-cloning strategy of *TDH3p-ATF1* is shown in Fig. 2A. The pGG119TDH3pATF1 plasmid was linearized by cutting within the *ATF1* open reading frame and was integrated into the *ATF1* locus of Kyokai No. 7. From the integrant, strains that lost the plasmid sequences were identified by counter-selection using *GALp-GIN11M86* as indicated by growth on galactose plates. Loss of the plasmid occurred through homologous recombination either between the 5′ upstream sequences of *ATF1* or between the *TDH3p* downstream sequences of *ATF1*. Recombination between the 5′ upstream regions resulted in a *TDH3p-ATF1* strain, but that between the downstream *ATF1* resulted in reversion to a wild-type strain.

Construction of a self-cloning *TDH3p-ATF1* yeast

pGG119TDH3pATF1 was digested with *Sph*I (Fig. 1) and used to transform the Kyokai No. 7 strain. Several colonies that grew on cerulenin plates (approx. 100 colonies/μg) were picked onto cycloheximide plates for the double selection (Akada et al. 2002b; Fig. 2B). Three strains (RAK1807, RAK1808, RAK1809) were selected as the correct plasmid integrants after Southern blot analysis using the *ATF1* probe, which showed 23-kb bands in addition to 12-kb bands, because the Kyokai No. 7 strain is diploid (Figs. 2, 3A)

From the RAK1809 integrant, counter-selected strains were selected by growth on YPGal plates. Colonies that grew on the galactose plates were streaked onto cerulenin

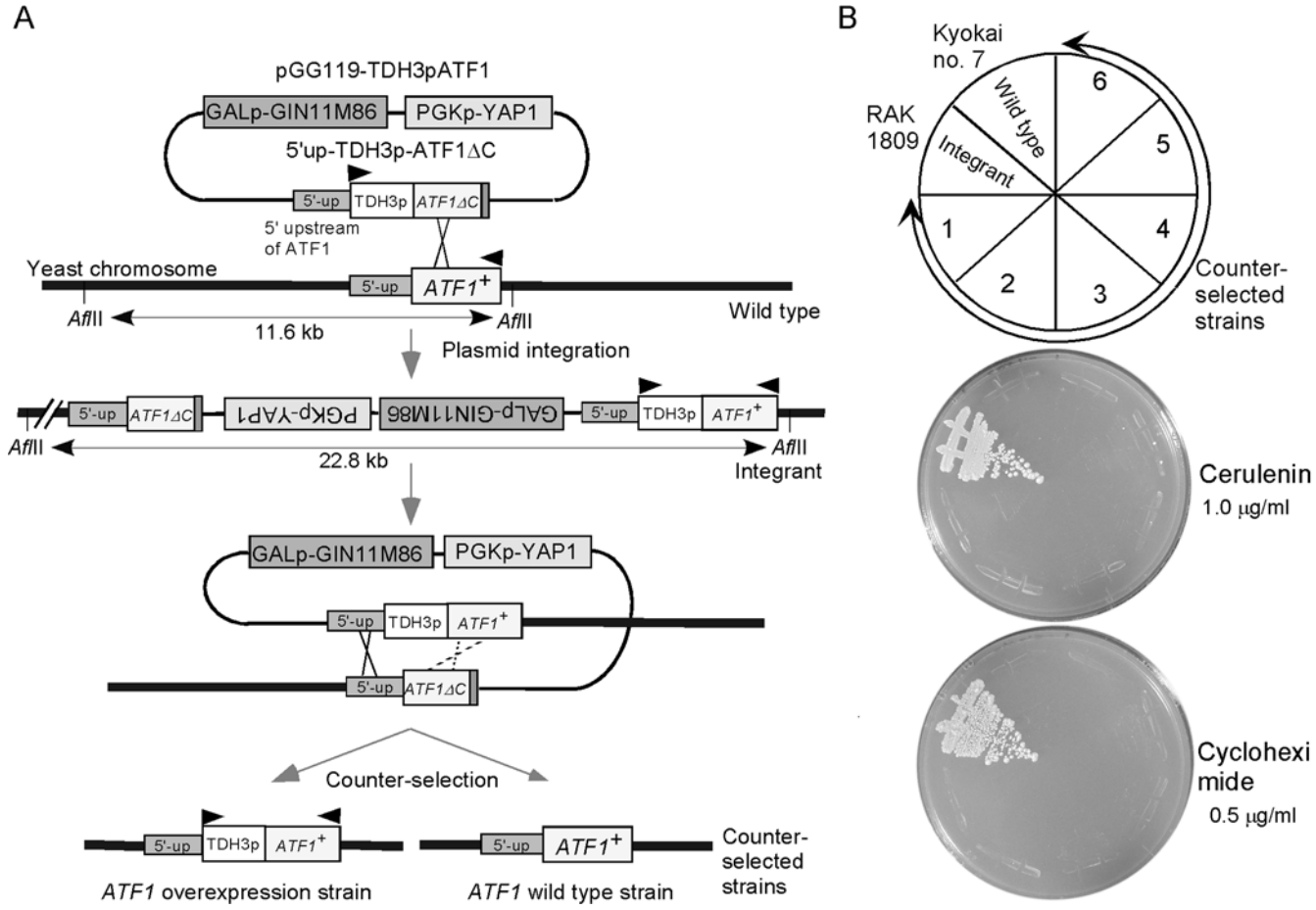

Fig. 2A, B Self-cloning strategy for promoter insertion by a two-step protocol. **A** pGG119TDH3pATF1 was digested with *Sph*I and inserted into the *ATF1* locus of a sake yeast strain. Plasmid integration resulted in the insertion of plasmid DNA sequences between the tandem repeats of *ATF1*. Homologous recombination between the *TDH3p*-downstream *ATF1* sequences resulted in a wild-type *ATF1* gene, but that between the 5′ upstream sequences resulted in *TDH3p-ATF1* without any extraneous DNA sequences. *Arrows* indicate the sites of primers used for PCR analysis (Fig. 3). *ATF1ΔC* does not contain the primer site and thus only the correct integrants can produce a 2.3-kb *TDH3p-ATF1* fragment. **B** Wild type, integrant, and counter-selected strains were streaked on cerulenin and cycloheximide plates. The integrant, but not the counter-selected strains (*sectors 1–6*) grew on the drug-resistance plates, indicating the loss of the transformation marker by counter-selection

and cycloheximide plates (Fig. 2B). All selected strains lost resistance to cerulenin and cycloheximide, indicating that these strains lost the *PGKp-YAP1* marker of the plasmid. These strains were either *TDH3p-ATF1* or *ATF1*, as shown in Fig. 2A. To select *TDH3p-ATF1* strains, PCR was performed with TDH3-1 and ATF1-APA primers and chromosomal DNA isolated from counter-selected strains. This PCR could not produce a product from pGG119TDH3pATF1 due to the absence of the annealing site of ATF1-APA. However, a 2.3-kb fragment was produced from integrants because correct integration generated *TDH3p-ATF1*, which contained the annealing sequence of the ATF1-APA primer. Among four counter-selected strains, three strains, RAK1857, RAK1858, and RAK1861, produced a 2.3-kb band, indicating that these were *TDH3p-ATF1* strains (Fig. 3B). The *ATF1* probe showed only the 12-kb band in hybridizations with these three strains. The 23-kb band was not present in the three integrants. The 0.7-kb difference between the inserted *TDH3* promoter and wild-type *ATF1* was not obvious in this Southern blot analysis. Loss of the extraneous plasmid DNA sequences in these strains was confirmed by Southern blot analysis using a pBluescript probe to detect the *E. coli* vector backbone of pGG119 (Fig. 3A). The pBluescript KS^+ probe hybridized to the 23-kb band of the integrants but showed no hybridization to the chromosomes of counter-selected strains or the Kyokai No. 7 strain, indicating that there were no bacterial vector sequences in these strains. From these results, we concluded that the self-cloning *TDH3p-ATF1* strain construction was successful.

Sake profiles

Laboratory-scale sake fermentation was performed with wild-type Kyokai No. 7, integrant RAK1809, and self-cloning RAK1857 strains. The fermentation profiles are shown in Table 2. The three strains showed similar fermentation profiles, except for acetic acid and isoamyl

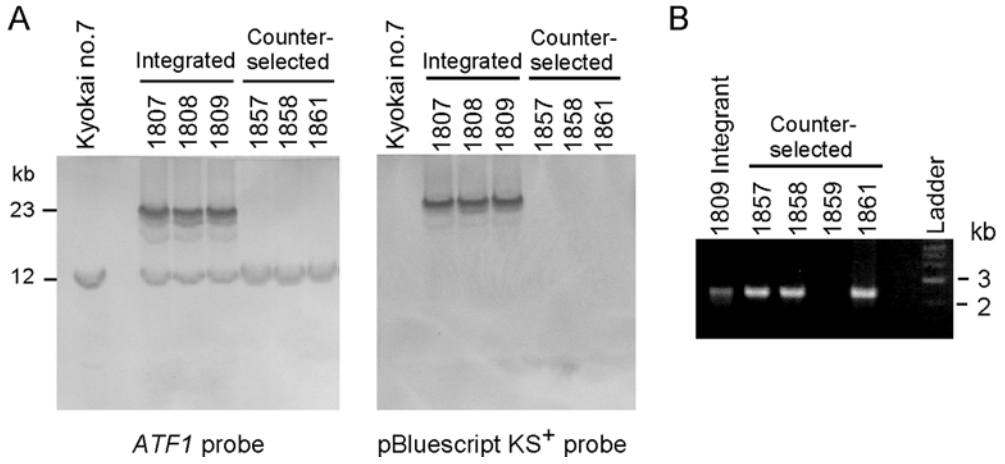

Fig. 3A, B Southern blot analysis and PCR detection of *TDH3p-ATF1*. **A** Chromosomal DNAs were isolated from Kyokai No. 7 strain, plasmid integrants, and counter-selected strains, digested with *Afl*II, and subjected to agarose gel electrophoresis. Integrants showed a 23-kb band in addition to a 12-kb band when hybridized with the *ATF1* probe, but counter-selected strains (*1857*, *1858*, *1861*) derived from the RAK1809 integrant showed only the 12-kb band. The bacterial vector DNA probe (*pBluescript KS*$^+$) hybridized to the 23-kb band but not to the chromosomal DNAs from Kyokai No. 7 or the counter-selected strains. **B** PCR amplification with primers TDH3-1 and ATF1-APA using template DNAs from the RAK1809 integrant and counter-selected strains produced a 2.3-kb band in strains RAK1857, RAK1858, and RAK1861 but not in RAK1859, indicating that the three counter-selected strains contained *TDH3p-ATF1*

acetate. The integrant and self-cloning strains produced three and five times more isoamyl acetate than the wild-type strain, respectively, and less acetic acid, indicating a higher alcohol acetyltransferase activity.

Discussion

We constructed a sake yeast strain that overexpresses the *ATF1* gene by placing a yeast constitutive overexpression promoter, *TDH3p*, upstream of the 5′ end of *ATF1*. The vector sequences used for gene manipulations were removed completely. The resulting strain contained only a 0.7-kb *TDH3* promoter sequence derived from the same Kyokai No. 7 strain upstream of *ATF1*. The strain construction process can be called “self-cloning”, which means cloning of its own DNA (Walgate 1979). The self-cloning *TDH3p-ATF1* strain produced a higher amount of isoamyl acetate due to the overexpression of the *ATF1* gene, as shown previously in winery, distillery, and sake yeast strains, in which *ATF1* was overexpressed by transforming shuttle plasmids (Fujii et al. 1994; Lilly et al. 2000). The originality of this study is the self-cloning process for constructing commercially applicable GM yeast strains that overexpress useful genes.

Table 2 Sake profiles

Profile	Kyokai No. 7 Wild type	RAK1809 Integrant	RAK1857 Self-cloning
Incubation (days)	27	27	28
Sake meter	+9.3	+9.0	+8.8
Acidity (ml)	1.9	1.8	1.8
Amino acid (ml)	1.0	1.1	1.1
Acetic acid (g/l)	0.24	0.18	0.19
Succinic acid (g/l)	0.07	0.09	0.09
Alcohol (%)	15.5	15.5	15.5
Ethyl caproate (ppm)	1.1	1.2	1.2
Isoamyl acetate (ppm)	4.9	15.4	24.2

The concept of self-cloning is important for the commercial use of GM microorganisms. Since the revision of the guidelines for safety assessments of GM organisms issued by the Japanese Ministry of Health, Labor, and Welfare, which became effective from April 2001 (Hino 2002), this self-cloning type of GM organism was eliminated from food safety assessments. Any GM microorganisms that are approved as self-cloning are not treated as GMO in Japan (Akada 2002). Actually, self-cloning is reasonable for the production of GM foods, because there is no risk of problematic gene transfer from GM foods to other microorganisms living in the human or animal body. Therefore, self-cloning gene manipulation is now practical for the commercial application of GM organisms.

A two-step gene replacement protocol was usually used for gene replacement between mutant and wild-type alleles in laboratory yeast strains by using a counter-selection system such as the *URA3*/*ura3*/5-FOA and *LYS2*/*lys2*/aminoadipate systems (Boeke et al. 1984; Sikorski and Boeke 1991). For industrial yeast strains, such gene replacement methods requiring recessive mutant yeast are not applicable. Using the dominant counter-selection system, *FAS2* point-mutant genes were successfully replaced with the wild-type *FAS2* in sake yeast to provide a valuable flavor profile in sake (Akada et al. 1999; Aritomi et al. 2004). In addition to the introduction of these point-mutation alleles, to the best of our knowledge, this report provides the first example of promoter insertion using the two-step protocol. The self-cloning procedure and the resulting *TDH3p-ATF1* strain, which confers an

improved flavor profile in sake, will be applicable to the Japanese sake market and also to the world GM market.

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