Adaptation of the Osmotolerant Yeast *Zygosaccharomyces rouxii* to an Osmotic Environment Through Copy Number Amplification of *FLO11D*

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ABSTRACT Copy number variations (CNVs) contribute to the adaptation process in two possible ways. First, they may have a direct role, in which a certain number of copies often provide a selective advantage. Second, CNVs can also indirectly contribute to adaptation because a higher copy number increases the so-called “mutational target size.” In this study, we show that the copy number amplification of *FLO11D* in the osmotolerant yeast *Zygosaccharomyces rouxii* promotes its further adaptation to a flor-formative environment, such as osmostress static culture conditions. We demonstrate that a gene, which was identified as *FLO11D*, is responsible for flor formation and that its expression is induced by osmostress under glucose-free conditions, which confer unique characteristics to *Z. rouxii*, such as osmostress-dependent flor formation. This organism possesses zero to three copies of *FLO11D*, and it appears likely that the *FLO11D* copy number increased in a branch of the *Z. rouxii* tree. The cellular hydrophobicity correlates with the *FLO11D* copy number, and the strain with a higher copy number of *FLO11D* exhibits a fitness advantage compared to a reference strain under osmostress static culture conditions. Our data indicate that the *FLO* gene-related system in *Z. rouxii* has evolved remarkably to adapt to osmostress environments.

AN organism adapts to adverse environments to survive and produce progeny. Adaptive evolution is the process through which a population becomes better suited to its environment via mutation, selection, and random drift (Bürger 2000; Cressman 2003; Ewens 2004; Nowak and Sigmund 2004). Types of mutations include nucleotide changes (Steiner *et al.* 2007; Barrick and Lenski 2009), transposition events (Wilke and Adams 1992; Aminetzach *et al.* 2005), and copy number variations (CNVs), including gene amplifications (duplication) and deletions (Brown *et al.* 1998; Perry *et al.* 2007; Gresham *et al.* 2010). CNVs can have a phenotypic impact and can consequently alter the fitness of an allele through the following mechanisms: (i) changing the coding sequence of a gene (Yamanaka *et al.* 2009; Schlattl *et al.* 2011), (ii) creating paralogs that can

diverge from each other and take on new or specialized functions (neofunctionalization or subfunctionalization, respectively) (Ohno 1970; Innan and Kondrashov 2010), and (iii) altering the expression level of a gene (gene dosage effect) (Stranger *et al.* 2007; Yamanaka *et al.* 2009; Iskow *et al.* 2012). In humans, most CNVs overlapping genes are under purifying (negative) selection (Conrad *et al.* 2010), but a handful of CNVs are thought to be under positive selection (Cooper *et al.* 2007; Hurles *et al.* 2010; Iskow *et al.* 2012) such as *AMY1* (Perry *et al.* 2007). In yeast, there are several examples of how the (increased) copy number can provide a direct selective advantage (Gresham *et al.* 2008; Voordeckers *et al.* 2012).

In nature, budding yeast exhibits a number of adaptive responses, such as filamentation, invasive growth, flocculation, and biofilm formation, to overcome a deleterious environment (Fidalgo *et al.* 2006). The *FLO* (flocculation) gene family plays a central role in the mediation of these adaptive responses. For example, *Saccharomyces cerevisiae* has five *FLO* genes: *FLO1*, *FLO5*, *FLO9*, *FLO10*, and *FLO11* (Teunissen and Ateensma 1995). *FLO1*, *FLO5*, *FLO9*, and *FLO10* confer cell–cell adhesion (flocculation). Smukalla

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et al. (2008) showed that flocculation protects *FLO1*-expressing cells from multiple stresses, including antimicrobial and ethanol stress. In contrast, *FLO11* is responsible for filamentation, invasive growth, and biofilm formation, such as a buoyant biofilm, which is also called flor (Guo *et al.* 2000; Zara *et al.* 2005; Fidalgo *et al.* 2006). The regulation of *FLO11* is remarkably complex. At least three well-known signaling cascades regulate *FLO11* expression in response to environment changes: the Ras-cAMP pathway, the MAP kinase-dependent filamentous growth pathway, and the glucose repression pathway (Madhani and Fink 1997; Rupp *et al.* 1999; Gagiano *et al.* 2002; Kuchin *et al.* 2002; Vyas *et al.* 2003; Schwartz and Madhani 2004; Verstrepen and Klis 2006). Accordingly, *FLO11*-dependent flor formation is considered as an adaptation to nutrient-limited and anoxic conditions because flor formation enables yeast to continue its growth through the utilization of oxygen and ethanol, which are produced by the yeast itself. In addition to these pathways, *FLO11* is also regulated by the pH response pathway, noncoding RNAs, and the chromatin state (Halme *et al.* 2004; Barrales *et al.* 2008, 2012; Bumgarner *et al.* 2009; Octavio *et al.* 2009). A recent study showed that the noncoding RNAs contribute to a variegated transcription of *FLO11* through the modulation of transcription factor recruitment (Bumgarner *et al.* 2012).

The *FLO* genes share a common structure, including a C-terminal glycosylphosphatidylinositol (GPI)-anchor addition site, an N-terminal carbohydrate- or peptide-binding domain or domain of unknown function, and a central domain containing multiple serine and threonine-rich repeats (Verstrepen and Klis 2006). The latter domain is also referred to as a domain with intragenic tandem repeats. The variation in the intragenic repeat number and/or the distribution of the different repetitive units provides a functional diversity of the cell-surface antigens that allows rapid adaptation to the environment (Verstrepen *et al.* 2005; Fidalgo *et al.* 2006, 2008; Zara *et al.* 2009). However, the relationship between adaptation and the copy number of the *FLO* gene itself, but not the number of the intragenic repeat units, has not been investigated.

It is known that not only *S. cerevisiae*, but also the osmotolerant yeast *Zygosaccharomyces rouxii*, form flor (Tomita and Yamamoto 1997). *Z. rouxii* has played a central role in the production of traditional fermented foods (e.g., soy sauce and soybean paste) in Japan; however, it is also known to cause yeast spoilage in the food industry. In soy sauce manufacturing, for example, *Z. rouxii* produces alcohol and aromatic flavors, such as 4-hydroxy-2(or 5)-ethyl-5 (or 2)-methyl-3(2H)-furanone. In addition, the flor-forming strain produces off-flavors, such as isovaleric acid, in soy sauce. Interestingly, it is known that flor is formed in a NaCl-dependent manner in *Z. rouxii* (Tomita and Yamamoto 1997). We predicted that the NaCl-dependent ability to form flor by osmotolerant yeast is a reflection of a physiological role of flor formation under high-osmolarity conditions.

In this study, we demonstrate the molecular mechanism that underlies the ability of NaCl-dependent flor formation in *Z. rouxii*. Through a series of experiments, we found that

FLO11D, which is responsible for flor formation in *Z. rouxii*, is a good example of a gene that exhibits copy number amplification that was positively selected. We also show that the copy number amplification of *FLO11D* enables *Z. rouxii* to be further adapted to a flor-formative environment, such as osmostress static culture conditions, through direct competition experiments.

Materials and Methods

Strains, media, and genetic methods

The yeast strains used in this study are listed in Table 1. *Z. rouxii* Z3 and Z1 were isolated from the flor of yeasts grown on the surface of soy sauce mash at a soy sauce factory (Yamasa Corporation, Chiba, Choshi, Japan) and the laboratory, respectively, in 2009. *Z. rouxii* 3K was isolated from soy sauce mash as a non-flor forming strain at the soy sauce factory. *Z. rouxii* 1501 was isolated from soy sauce as a flor-forming strain in 1972. *S. cerevisiae* 133d *flo11Δ* was generously provided by J. I. Ibeas (Fidalgo *et al.* 2006), and BY4743 was purchased from Funakoshi Co. (Tokyo). The *FLO11D* gene disruption was performed using PCR-mediated gene replacement, and the gene deletion was confirmed using PCR and Southern blot analyses. pCUG, pCUZ, and pCUN were used as PCR templates for gene disruption, and each of these possesses G418-, Zeocin-, and Nourseothricin-resistant genes surrounded by two loxP sequences. The primers that were used in this study are listed in [Supporting Information, Table S1](#). The transformations of *Z. rouxii* and *S. cerevisiae* were performed by electroporation and lithium acetate procedure, respectively (Gietz *et al.* 1995; Watanabe *et al.* 2010). The bacterial transformations, bacterial DNA manipulation, and plasmid constructions were performed using standard methods (Sambrook and Russell 2001).

Standard YPD medium (1% yeast extract, 2% Bacto peptone, and 2% glucose) containing 200 mg/liter G418, 200 mg/liter Zeocin, and/or 5 mg/liter Nourseothricin and synthetic complete dextrose medium lacking uracil (SCD *ura*⁻) were used for the transformant selection. YPD containing 1.8 M NaCl was used as the flor-forming media for *Z. rouxii*. Synthetic complete galactose medium lacking uracil (SCG *ura*⁻) was used as the flor assay media for *S. cerevisiae*. YPD plates supplemented with 0.35% agar and YPD plates supplemented with 0.40% agar and 1.8 M NaCl were used to investigate mat formation. All of the yeasts were incubated at 28°. For the incubation of *ura3Δ* mutants in *Z. rouxii*, 0.17% uracil was used to supplement the medium due to their significant requirement for uracil.

Assays for flor formation, mat formation, and determination of cellular hydrophobicity

The flor formation in *Z. rouxii* was assayed by inoculating precultured cells in glass tubes containing 10 ml of YPD or YPD supplemented with 1.8 M NaCl. The mat formation was assayed by inoculating cells from a fresh colony onto YPD

Table 1 Strains used in this study

Strains	Genotype	Source
<i>Z. rouxii</i> 3K		Soy sauce
1501		Soy sauce
ATCC46251		Unknown
ATCC2623		Black-grape must
Z1		Soy sauce
Z3		Soy sauce/Watanabe <i>et al.</i> (2010)
NBRC0505		Shoyu yeast, Kikkoman
NBRC0506		Shoyu yeast, Yamasa
NBRC0845		Mash of tamari-soya
NBRC1876		Soy bean paste
NBRC1877		Soy bean paste
NBRC1877 <i>ura3Δ</i>	<i>ura3Δ::loxP/ura3Δ::loxP</i>	This study
Z3 <i>flo11aΔ</i>	<i>flo11aΔ::loxP-KanMX-loxP</i>	This study
Z3 <i>flo11bΔ</i>	<i>flo11bΔ::loxP-KanMX-loxP</i>	This study
Z3 <i>flo11cΔ</i>	<i>flo11cΔ::loxP-ZeoMX-loxP</i>	This study
Z3 <i>flo11abΔ</i>	<i>flo11abΔ::loxP-KanMX-loxP</i>	This study
Z3 <i>flo11abcΔ</i>	<i>flo11abΔ::loxP-KanMX-loxP flo11cΔ::loxP-ZeoMX-loxP</i>	This study
Z3 <i>ura3Δ</i>	<i>ura3Δ::loxP/ura3Δ::loxP</i>	This study
Z3 <i>flo11dΔ1</i>	<i>ura3Δ::loxP/ura3Δ::loxP flo11dΔ::loxP-KanMX-loxP</i>	This study
Z3 <i>flo11dΔ2</i>	<i>ura3Δ::loxP/ura3Δ::loxP flo11dΔ::loxP-KanMX-loxP</i>	This study
	<i>flo11dΔ::loxP-ZeoMX-loxP</i>	
Z3 <i>flo11dΔ3</i>	<i>ura3Δ::loxP/ura3Δ::loxP flo11dΔ::loxP-KanMX-loxP</i>	This study
	<i>flo11dΔ::loxP-ZeoMX-loxP flo11dΔ::loxP-NatMX-loxP</i>	
ATCC46251 <i>flo11dΔ1</i>	<i>flo11dΔ::loxP-KanMX-loxP</i>	This study
ATCC46251 <i>flo11dΔ2</i>	<i>flo11dΔ::loxP-KanMX-loxP flo11dΔ::loxP-ZeoMX-loxP</i>	This study
ATCC46251 <i>flo11dΔ3</i>	<i>flo11dΔ::loxP-KanMX-loxP flo11dΔ::loxP-ZeoMX-loxP</i>	This study
	<i>flo11dΔ::loxP-NatMX-loxP</i>	
NBRC0845 <i>flo11dΔ1</i>	<i>flo11dΔ::loxP-KanMX-loxP</i>	This study
NBRC0845 <i>flo11dΔ2</i>	<i>flo11dΔ::loxP-KanMX-loxP flo11dΔ::loxP-ZeoMX-loxP</i>	This study
1501 <i>flo11dΔ1</i>	<i>flo11dΔ::loxP-KanMX-loxP</i>	This study
<i>S. cerevisiae</i> BY4743	<i>MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0 /MET15</i>	Funakoshi Co.
	<i>ura3Δ0/ ura3Δ0</i>	
133d <i>flo11Δ</i>	<i>MATa ura3-52 flo11Δ</i>	J. I. Ibeas/Fidalgo <i>et al.</i> (2006)

soft agar plates containing 1.8 M NaCl. The flor formation in *S. cerevisiae* was assayed by inoculating cells from a fresh colony into glass tubes containing 10 ml of SCG *ura⁻* medium. The cells were grown at 28° for 3–7 days under static conditions and photographed. The exact incubation periods are written in the legend for Figures 1–6. The assays for the determination of the cellular hydrophobicity were performed as previously described (Barrales *et al.* 2008) with little modification. Briefly, the cells were grown in SCD containing 1.8 M NaCl, and then a portion of the cells was transferred to fresh medium. The cells were grown to an OD₆₀₀ of ~1.0, and then 1.2 ml of the culture was overlaid with 600 μl of octane and vortexed for 3 min. The OD₆₀₀ of the aqueous layer was recorded, and the relative difference compared with the initial OD₆₀₀ was used to determine the percentage of cellular hydrophobicity. The detailed colony morphology was observed using a digital microscope VHX-1000 equipped with VH-Z250R/Z250W (Keyence, Osaka, Japan).

***ura3* mutant construction in *Z. rouxii* NBRC1877 and Z3**

We attempted to identify the *URA3* gene coded on the P subgenome in *Z. rouxii* because of the requirement for the auxotrophic mutant in the allopolyploid of *Z. rouxii*. The

genomes from ATCC2623 and ATCC46251 were digested with *Bgl*II, *Spe*I, and *Sac*I, and the fragments were analyzed by Southern blot using the coding region of *URA3* from ATCC2623 as a probe. The specific signal from *URA3* coded on the P subgenome was detected at ~2.2 kb in the *Bgl*II-digested ATCC46251 genome. The band was recovered and cloned into the same site of pZEAK, which was constructed from pZEU (Pribylova *et al.* 2007b) by removing the *URA3* marker genes and disrupting the *Bam*HI recognition site derived from pSRI. *S. cerevisiae* BY4743 (a uracil auxotrophic mutant) was transformed with the resulting plasmid and selected on an SCD *ura⁻* plate. The sequence analysis of the plasmid extracted from the transformants revealed the *URA3* gene coded on the P subgenome (AB665419).

Z. rouxii NBRC1877 and the Z3 *ura3* mutant were obtained through a two-step deletion of the *ura3* gene (XM002499041, AB665419). The primers that were used in the gene disruption are indicated in Table S1. The removal of the G418 and Zeocin resistance markers from the genomes were performed as previously described (Pribylova *et al.* 2007b) with little modification. The cells were transformed with pZCRE, and the transformants were grown selectively on SCD *ura⁻* plates for 2 days and then nonselectively in liquid YPD supplemented with 0.17% uracil (YPDU)

for 24 hr. The cultures were diluted and plated on YPDU to obtain single colonies. Two days later, the colonies were replated on YPDU, YPDU + G418, YPDU + G418 + Zeocin, and SCD ura⁻ plates, and those clones with both G418- and Zeo-sensitive and Ura⁻ phenotypes were selected. The removal of the G418 and Zeocin resistance markers from the genome was confirmed using PCR analysis.

Plasmid construction

To identify the flor-forming gene through functional cloning, Z3 genomic libraries were prepared. The Z3 genomic DNA was partially digested with *Sau3AI*, and the resulting fragments were separated by agarose gel electrophoresis. The 6- to 9-kb DNA fragments were recovered using a PCR and Gel Purification Kit (Promega, Fitchburg, WI) and cloned into the *Bam*HI site of pZEUneo, which was constructed from pZEU (Pribylova *et al.* 2007b) by disrupting the *Bam*HI recognition site derived from pSRI. *Escherichia coli* DH5 α cells were transformed with the resulting plasmids, and the transformants were selected by Luria broth medium containing carbenicillin. The resulting Z3 genomic library was extracted from these bacteria and used for functional cloning. The *FLO11D* gene was amplified by PCR from the *Z. rouxii* Z3 genomic DNA using the primers pFLO11D_F and pFLO11D_R to append the *Eco*RI and *Sal*I digestion sites onto either end of the PCR product. The PCR product was digested with the respective restriction enzymes and ligated into *Eco*RI- and *Sal*I-digested pZEU (Pribylova *et al.* 2007a) to generate pFLO11D. To drive *FLO11D* under the *GAL1* promoter, pYFLO11D were constructed. Briefly, the coding sequence of *FLO11D* was amplified by PCR from the *Z. rouxii* Z3 genomic DNA using the primers pYFLO11D_F and pYFLO11D_R. The resulting DNA fragments and pYES2 (Life Technologies, Carlsbad, CA) digested with *Bam*HI and *Hind*III were introduced into *S. cerevisiae* BY4743 and then plated onto a SCD ura⁻ plate. After the resulting plasmids were introduced into *E. coli* and were amplified, BY4743 and 133d *flo11* Δ were transformed by the plasmid. The PCR analyses were initiated using KOD plus neo (Toyobo, Osaka, Japan) according to the manufacturer's instructions.

DNA sequencing, alignment, and analysis

All of the DNA sequences were obtained through outsourcing (Fasmac, Atugi, Japan). All of the BLASTP and PSI-BLAST searches (Altschul *et al.* 1997) applied an expected value cutoff of 10^{-5} at the hemiascomycete yeast genome database (Sherman *et al.* 2009; <http://www.genolevures.org/>) and through the National Center for Biotechnology Information. The protein sequences were aligned using ClustalX (Larkin *et al.* 2007). The initial formatting of the alignments was conducted in GeneDoc (<http://www.psc.edu/biomed/genedoc>). For the phylogenetic inferences, we used the neighbor-joining method (Saitou and Nei 1987) with 1000 bootstrap interactions. The tree was visualized in NJplot (Perrière and Gouy 1996). The predicted N-terminal signal peptides were detected with the SignalP

3.0 algorithm (Emanuelsson *et al.* 2007). The C-terminal GPI anchor attachment signals were detected using the fungal big-II prediction algorithm (Eisenhaber *et al.* 2004).

Southern and Northern blot analyses

For the Southern blot analysis, the genomic DNA of yeast cells was extracted using standard protocols (Sambrook and Russell 2001) and digested with the appropriate restriction enzyme. The digested DNA was separated by 1% agarose gel electrophoresis and transferred onto a Hybond-N⁺ membrane (GE Healthcare, Buckinghamshire, UK) by upward capillary transfer.

For the Northern blot analysis, the total RNA was prepared with Isogen (NIPPON gene, Tokyo) according to the manufacturer's instructions with little modification. Briefly, the total RNA was extracted after the cells were homogenized in a Micro Smash MS-100 bead beater (TOMY, Tokyo) at $3000 \times g$ for 300 sec with adequate amounts of glass beads (0.1 mm in diameter) and Isogen solution. A 5- μ g aliquot of the total RNA was run on a gel and blotted.

The Flo11 domain-coding region, which is associated with the Flo11p N-terminal region in the Pfam database (Finn *et al.* 2010), was used to probe the membranes. These experiments were performed using a PCR digoxigenin probe synthesis kit (Roche, Basel, Switzerland) and detection starter kit II (Roche) according to the manufacturer's instructions.

Chromosomal DNA isolation and pulsed-field gel electrophoresis

The chromosomal DNA in plug was prepared according to a previous study with little modifications (Pribylova *et al.* 2007a). Instead of Lyticase, Zymolyase 100T (SEIKAGAKU Corporation, Tokyo) was used, and the cell treatment was performed overnight at 30°. The *Not*I-digested plug was prepared according to a previous study (Lim *et al.* 1994). The 1% agarose gels were prepared from pulsed-field certified agarose (Bio-Rad, Hercules, CA) and run on a CHEF apparatus (Bio-Rad) in $0.5 \times$ TBE buffer at 10°. The running conditions for chromosomal DNA separation were as follows: switch time, 350–400 sec; run time, 240 hr; angle, 120°; and voltage, 3 V/cm. The running conditions for *Not*I-digested chromosomal DNA separation were as follows: switch time, 60–120 sec; run time, 24 hr; angle, 120°; and voltage, 6 V/cm.

The DNA banding patterns were analyzed with Quantity One Basic (Bio-Rad) and manually. The cluster analysis of the band patterns was performed using SPSS version 11.5 with a word algorithm.

Fitness measurements

Yeast cells (wild-type, single, double, and triple *FLO11D* gene mutants) were independently grown in YPDU, and then a portion of the cells was transferred to fresh medium. The cells were grown to an OD₆₀₀ of 1.0, and then 100 μ l from each culture was mixed with its reference strain: wild type was mixed with single mutant, single mutant was mixed with double mutant, and double mutant was mixed with triple mutant. The mixture was inoculated into YPDU

Figure 1 Spontaneous flor on the surface of soy sauce mash and in manufactured soy sauce. (A) Spontaneous flor on the soy sauce mash in a concrete tank. The white pixels on the mash are the flor. (B) Spontaneous flor on manufactured soy sauce in glass bottles. (C) Appearance of the flor formation process. *Z. rouxii* Z3 was inoculated into soy sauce mash or soy sauce and incubated at 28° for 3 days. Bar, 30 µm.

containing 1.8 M NaCl and incubated at 28° with or without shaking. After 7 days of incubation, the culture was vortexed for 3 min, and 100 µl of the culture was harvested. The ratio of the two competitors was quantified at the initial and final time points as follows: The culture was diluted and plated on YPDU medium to obtain a single colony. Three days later, the colonies were counted to calculate the colony formation units, and at least 208 colonies were randomly selected and transferred to YPDU or YPDU containing G418, Zeocin, or Nourseothricin to calculate the composition ratio of wild-type, single, double, and triple *FLO11D* gene mutants. The selective advantage, s , of each strain was calculated as $s = [\ln(S_f/R_f) - \ln(S_i/R_i)]/T$, where S and R are the numbers of sample and reference strains, respectively; the subscripts refer to the final and the initial populations, and T is the number of generations during the competition. The fitness of the reference strain was designated 1, and the fitness of the sample strain was $1 + s$.

Results

Identification of the gene conferring floatability to *Z. rouxii*

In soy sauce manufacturing, flor is naturally formed on the surface of soy sauce mash and soy sauce products by the flor-forming *Z. rouxii* (Figure 1, A and B). This strain reduces the

quality of soy sauce products by producing off-flavors. We first observed the flor formation process to investigate how a flor would be developed on the surface of soy sauce mash and soy sauce. After the cells elongated and formed chains on the surface of the substrate, the cells began to elongate along the z -axis (Figure 1C, arrows), and this elongation was followed by the acquisition of a fluffy morphology (Figure 1C). It is known that the *Z. rouxii* F51 strain forms flor in NaCl-containing medium, but *Z. rouxii* never forms flor under NaCl-free conditions (Tomita and Yamamoto 1997). We replicated the experiment using a different genetic background in liquid static culture and on soft agar plates or normal agar plates to compare the morphologic changes. Flor yeasts display morphological development, such as flor, fluffy colony morphology, and mat, in NaCl-containing medium (Figure 2). These results are in agreement with the previous report, with the exception of the weak flor formation observed in the absence of NaCl. In contrast, non-flor-forming yeasts never developed flor, regardless of whether the medium contained NaCl (Figure 2A). These results suggest that the morphological development in *Z. rouxii* is strongly induced by NaCl.

It is known that the *FLO11* gene is responsible for flor formation in *S. cerevisiae*. A recent study showed that the N-terminal domain of Flo11p, which is referred to as the Flo11 domain, self-interacts and explains the cell-cell interaction

Figure 2 NaCl-dependent morphological changes. (A) Flocculation in YPD or YPD containing 1.8 M NaCl. A fresh colony was inoculated into YPD or YPD containing 1.8 M NaCl and incubated at 28° for 5 days under static culture conditions. (B) Mat formation on YPD soft agar plates or YPD soft agar plates containing 1.8 M NaCl. A fresh colony was inoculated onto YPD plates supplemented with 0.35% agar or 0.4% agar containing 1.8 M NaCl and incubated at 28° for 7 days. (C) Assay for colony fluffiness. *Z. rouxii* Z3 was plated on YPD or YPD containing 1.8 M NaCl and incubated at 28° for 3 or 4 days, respectively. Bar, 0.3 mm.

capacity of *FLO11*-expressing cells (Goossens and Willaert 2012). The Flo11 domain is conserved in the *S. cerevisiae* Flo11 protein and in the respective proteins from some related species within Saccharomycotina but was not found in any other fungal lineage (Linder and Gustafsson 2008). We searched for the *FLO11* homolog in the *Z. rouxii* genome database (Sherman *et al.* 2009) to identify the gene conferring floatability to *Z. rouxii*. BLASTP searches were used with an expected value cutoff of 10^{-5} using the *S. cerevisiae* Flo11 domain as the query. As a result, three candidates were found, including ZYR00F02728g, ZYR00F02750g, and ZYR00A13662g, which were designated *FLO11A*, *FLO11B*, and *FLO11C*, respectively (Figure S1A). These candidates have the Flo11 domain in the N terminus, Ser/Thr-rich repeats in the middle domain, and the GPI-attachment signal in the C terminus, indicating that not only the N-terminal region but also the overall structure of these candidates is similar to that of *S. cerevisiae* Flo11p. To investigate the role of *FLO11A*, *FLO11B*, and *FLO11C*, we constructed single, double, and triple gene mutants; however, all of the mutants

formed flor and mat normally (Figure S1, C and D). The Southern blot analysis using the Flo11 domain coding region as a probe indicated that these strains had *FLO11A*, *FLO11B*, and *FLO11C*, as well as additional and multiple *FLO11*-like genes (Figure S1B). The signals from the additional *FLO11*-like genes would be derived from the P sub-genome because it is presumed that the *Z. rouxii* in soy sauce mash is primarily of an allopolyploid and/or diploid mosaic lineage (Tanaka *et al.* 2012; Watanabe *et al.* 2013) with one parent similar to *Z. rouxii* CBS732 and one NCYC3042 parent, which is informally called *Z. pseudorouxii* (James *et al.* 2005; Solieri *et al.* 2006, 2007, 2013; Gordon and Wolfe 2008).

We then attempted conventional functional cloning strategies by transforming the non-flor yeast NBRC1877 *ura3Δ* with Z3 genomic libraries that contain 20,000 independent clones (average insert size: 6 kbp) to select the floating clones. Approximately 200 colonies that formed on the SCD *ura⁻* plate were inoculated into 10 ml of SCD *ura⁻* containing 1.8 M NaCl, and the yeasts growing on the

Figure 3 Identification of *FLO11D* gene. (A) Flor formation in the transformants obtained from functional cloning. A fresh colony was inoculated to 10 ml of SCD *ura*⁻ containing 1.8 M NaCl and incubated at 28° for 4 days. (B) A DNA restriction map of the *FLO11D* gene using *ScaI* endonuclease (top) and the primary structure of Flo11p (bottom). (C) Detection of *FLO11D* by Southern blot and PCR analyses. *ScaI*-digested genomic DNA was analyzed by Southern blot analysis using the Flo11 domain-coding region of the *FLO11D* gene as a probe (top). *FLO11D* was amplified by PCR using *FLO11D*-specific primers (bottom). The *URA3* gene was used as a positive control in both analyses. (D) Sequential flor formation assay in YPD containing 1.8 M NaCl. Z3 was inoculated into 20 ml of YPD containing 1.8 M NaCl and incubated at 28° for 3 days under static culture conditions. (E) Detection of *FLO11D* transcripts by Northern blot analysis. The total RNA was extracted from the yeast cells, and the *FLO11D* expression was measured by Northern blot analysis using the Flo11 domain-coding region of the *FLO11D* gene as a probe.

surface of the medium were recovered and purified. Consequently, we recovered a total of four colonies that form weak flor from the ~ 20,000 colonies that were screened (Figure 3A). The sequence analysis of the plasmid extracted from the abovementioned four colonies identified *FLO*-like genes (Figure 3B). All of these genes were homologous to each other, except for a difference in the number of repeats in the middle domain and a few base substitutions in the promoter region (Figure S2). The BLASTP analysis of the N-terminal region of this gene revealed a Flo11 domain. To compare the sequence of the Flo11 domain, we searched and aligned homologous sequences. Similar to the result reported by Linder and Gustafsson (2008), the homology search did not detect a Flo11 domain in any other fungal lineages except Saccharomycotina. The phylogenetic analysis revealed that the Flo11 domain obtained above is similar to that of Flo11ap and Flo11bp, but some differences existed (Figure S3). These results suggest that the gene is novel. Thus, we designated it *FLO11D*. The *FLO11D*-specific signals detected by Southern blot and PCR analyses were obtained

only from the flor-forming strain (Figure 3C), indicating that *FLO11D* may be responsible for flor formation. If *FLO11D* truly played a role in flor formation in *Z. rouxii*, its expression would be detected during the flor-forming phase. We prepared total RNA sequentially from sedimenting cells and floating cells to investigate the *FLO11D* gene expression (Figure 3D). *FLO11D* transcripts were detected in floating cells after 2 and 3 days of culture by Northern blot analysis (Figure 3E). In contrast, *FLO11A*, *FLO11B*, and *FLO11C* gene expression was not observed by Northern blot analysis, but the expression of these genes was slightly detected by RT-PCR analysis regardless of the culture phase (data not shown). These results suggest that only *FLO11D* is upregulated at the flor-forming phase. Finally, to demonstrate the role of the *FLO11D* gene in the morphological change, we constructed a *FLO11D* deletion mutant in Z3. As shown in Figure 4, Z3 possesses three copies of *FLO11D*, and the complete deletion of *FLO11D* in Z3 abolished flor, mat, and fluffy colony morphology development. The plasmids containing *FLO11D* restored the ability to form flor, mat, and fluffy

Figure 4 *FLO11D* is required for NaCl-dependent morphological changes. (A) PCR amplification of the *FLO11D* locus. (B) Flor formation in wild-type, single, double, and triple *FLO11D* gene mutants with an empty vector or a *FLO11D*-containing vector (pFLO11D). A fresh colony was inoculated in 10 ml of YPD containing 1.8 M NaCl and incubated at 28° for 5 days. (C) Mat formation in wild-type, single, double, and triple *FLO11D* gene mutants with an empty vector or pFLO11D. A fresh colony was inoculated onto YPD plates supplemented with 0.4% agar and 1.8 M NaCl and incubated at 28° for 7 days. (D) Microscopic analysis of C. Bar, 0.5 mm. (E) Assay for colony fluffiness. *Z. rouxii* Z3 or *flo11* $\Delta 3$ strains were plated on YPD containing 1.8 M NaCl and incubated at 28° for 4 days. Bar, 0.3 mm. (F) Flor formation assay in *S. cerevisiae* BY4743 and 133d *flo11* Δ with an empty vector or a *FLO11D*-containing vector (pYFLO11D). A fresh colony was inoculated in 10 ml of SCD ura⁻ and SCG ura⁻ and incubated at 28° for 4 days.

colony morphology, indicating that this gene is required for the morphological development (Figure 4, B–E). To verify that *FLO11D* is directly responsible for flor formation, *FLO11D* was heterologously expressed under the *GAL1* promoter in *S. cerevisiae* BY4743 and 133d *flo11* Δ . As a result, *S. cerevisiae* harboring pYFLO11D formed weak flor under galactose- but not glucose-containing conditions (Figure 4F). These results suggest that *FLO11D* is directly responsible for flor formation.

Osmotic stress-induced *FLO11D* expression under glucose-free conditions

Z. rouxii forms flor in a NaCl-dependent manner, and, to the best of our knowledge, this ability is a *Z. rouxii*-specific characteristic. We hypothesized that this unique feature was due to NaCl-dependent *FLO11D* expression. To test this hypothesis, Z3 was incubated with shaking in synthetic complete dextrose medium (SCD) and was then transferred to various conditions (SCD, SCD containing NaCl, SC, or SC containing NaCl). We prepared total RNA from these cultures

after 30, 60, and 90 min and then measured *FLO11D* expression by Northern blot analysis. *FLO11D*-specific signals were detected only in SC containing 1.8 M NaCl (Figure 5A), suggesting that *FLO11D* is repressed by glucose and induced by NaCl under glucose-free conditions. To examine other carbon sources in the repression of *FLO11D*, *FLO11D* expression was measured by Northern blot analysis in SC containing 1.8 M NaCl and other carbon sources, such as galactose, ethanol, and glycerol. As a result, these carbon sources did not repress the *FLO11D* expression under our experimental conditions (data not shown).

To demonstrate whether *FLO11D* expression is specifically induced by NaCl, we observed flor formation in YPD medium containing various chemicals (NaCl, MgCl₂, KCl, MgSO₄, Na₂SO₄, NaNO₃, and sorbitol). *Z. rouxii* Z3 formed flor in medium containing 1.8 M NaCl, KCl, NaNO₃, and sorbitol after a 7-day incubation (Figure 5B) and in the medium containing 0.9 M MgCl₂, 1.8 M MgSO₄, and Na₂SO₄ after a 16-day incubation (Figure S4). The result of the Northern blot analysis clearly demonstrated that

Figure 5 *FLO11D* expression and flor formation under osmotic stress conditions. (A) The regulation of *FLO11D* expression. The cells were grown to an OD₆₀₀ of 1.0 in SCD, washed, and transferred to SCD, SCD containing 1.8 M NaCl, SC, or SC containing 1.8 M NaCl. After 30, 60, and 90 min, the total RNA was extracted and analyzed by Northern blot analysis using the Flo11 domain-coding region of the *FLO11D* gene as a probe. (B) Flor formation of Z3 in YPD medium or YPD medium containing 1.8 M of various chemicals (NaCl, KCl, NaNO₃, and sorbitol). A fresh colony was inoculated into the above-mentioned medium and incubated at 28° for 7 days. (C) *FLO11D* gene expression in response to various osmotic stress conditions. The cells were grown to an OD₆₀₀ of 1.0 in SCD, washed, and transferred to SC, SC containing 1.8 M NaCl, SC containing 1.8 M KCl, SC containing 1.8 M NaNO₃, or SC containing 1.8 M sorbitol. After 60 min, the total RNA was extracted and analyzed by Northern blot analysis using the Flo11 domain-coding region of the *FLO11D* gene as a probe.

FLO11D was induced not only by NaCl but also by KCl, NaNO₃, and sorbitol under glucose-free conditions (Figure 5C). These results indicate that *FLO11D* is most likely induced by osmotic stress under glucose-free conditions. Consequently, flor formation can be observed in the late phase of culture, when the depletion of glucose occurs and *Z. rouxii* cells are subjected to osmotic stress.

FLO11D copy number variations

To confirm the requirement of *FLO11D* for flor formation in other genetic background strains, we constructed *FLO11D* gene mutants in the ATCC46251, NBRC0845, and 1501 strains. This analysis also showed that *FLO11D* played a role in flor formation, and there were *FLO11D* copy number variations (i.e., Z3, ATCC46251, NBRC0845, and 1501 possessed three, three, two, and one copies of *FLO11D*, respectively) (Figure 6, A–C).

Using a combination of pulsed-field gel electrophoresis (PFGE) and Southern blot analyses, we investigated how many *FLO11D* allele amplification events occurred in *Z. rouxii*. With respect to the NBRC1877, 1501, NBRC0845, ATCC46251, Z3, and Z1 strains, zero, one, one, three, two, and three copies of *FLO11D*, respectively, were detected by chromosomal PFGE analysis, whereas zero, one, two, three, three, and three copies, respectively, were detected by *NotI*-digested chromosomal PFGE analysis (Figure 6D). The latter analysis is in agreement with the *FLO11D* gene disruption experiment, indicating that the NBRC1877, 1501, NBRC0845, ATCC2623, Z3, and Z1 strains possess zero, one, two, three, three, and three copies of the *FLO11D* allele, respectively. These results also indicate that the first

and second *FLO11D* alleles are located on either the same or different chromosomes, which have the same mobility in NBRC0845 and Z3. Interestingly, the mobility of the third *FLO11D* allele of Z3 was different from that of ATCC46251 and Z1, indicating that either the acquisition of the third *FLO11D* allele occurred independently two times or a sequence alteration in the third *FLO11D* allele occurred in Z3. We next compared the *NotI*-digested karyotype profiles of various *Z. rouxii* strains by cluster analysis, and the *FLO11D* amplification events were mapped on the tree (Figure 6E). The loss or gain of *FLO11D* allele events can be explained by the dendrogram, which suggested that the *FLO11D* allele originated from a single origin and that sequence alteration in the third *FLO11D* allele occurred in Z3 after its acquisition.

Adaptation to osmotic stress environments by copy number amplification of *FLO11D*

In *S. cerevisiae*, it has been reported that the length and type of repetitive units in the Flo11p coding repeats domain affect the Flo11p-associated functions, including cellular hydrophobicity (Fidalgo *et al.* 2006; Fidalgo *et al.* 2008; Zara *et al.* 2009); however, the relationship between its functions and the copy number of the *FLO* gene itself has not been elucidated. To investigate this relationship, we measured the cellular hydrophobicity of *FLO11D* mutants in Z3. As a result, the cellular hydrophobicity was correlated with the *FLO11D* copy number (Figure 7A).

We hypothesized that the copy number amplification of *FLO11D* promoted adaptation to osmotic stress environments

Figure 6 Copy number variations of *FLO11D* in *Z. rouxii*. (A) PCR amplification of the *FLO11D* locus. (B) Flor formation in wild-type, single, double, and triple *FLO11D* gene mutants. A fresh colony was inoculated in 10 ml of YPD containing 1.8 M NaCl and incubated at 28° for 5 days. (C) Mat formation in wild-type, single, double, and triple *FLO11D* gene mutants. A fresh colony was inoculated onto YPD plates supplemented with 0.4% agar and 1.8 M NaCl and incubated at 28° for 7 days. (D) Karyotype profiles of *Z. rouxii* strains. The untreated chromosome (top) and *NotI*-digested chromosome (bottom) pattern was analyzed by Southern blot using the Flo11 domain-coding region of the *FLO11D* gene as a probe. (E) Summary of the loss and gain of *FLO11D* allele events. The tree shows the cluster based on the *NotI*-digested chromosome length polymorphism in D. Each event was manually mapped on the tree.

because of their osmo-ergic properties and the correlation between the *FLO11D* copy number and cellular hydrophobicity. To test this hypothesis, we incubated an equivalent mixture of wild-type and single *FLO11D* mutant, single and double *FLO11D* mutants, and double and triple *FLO11D* mutants and sequentially traced the population that remained in the culture. In a comparison between static culture and shaking culture conditions in YPD medium containing 1.8 M NaCl, the strain with more copy numbers of *FLO11D* showed a considerable fitness advantage compared to the reference strain under static culture conditions: the wild-type strain was dominant over the single mutant, the single *FLO11D* mutant was dominant over the double *FLO11D* mutant, and the double *FLO11D* mutant was dominant over the triple *FLO11D* mutant (Figure 7B). These results suggest that the *FLO11D* copy number amplification promotes further adaptation to a flor-formative environment, such as osmostress static culture conditions.

Discussion

Previous phenotypic studies have suggested that the flor formation of *Z. rouxii* occurs in a NaCl-dependent manner

(Tomita and Yamamoto 1997). However, at the outset of this study, the molecular mechanism that underlies NaCl-dependent flor formation was unclear. In this study, we identified *FLO11D* as a gene responsible for buoyant biofilm formation in *Z. rouxii* (Figure 4), and the *FLO11D* gene expression is induced by osmostress under glucose-free conditions (Figure 5). Their osmo-ergic properties must permit the unique characteristics in *Z. rouxii*, such as osmostress-dependent buoyant biofilm formation.

Regulation of *FLO11D*

The osmostress-dependent expression of *FLO11D* is very unique. In *S. cerevisiae*, *FLO11* is activated by nitrogen and carbon depletion via the MAP kinase-dependent filamentous growth pathway and the glucose repression pathway (Madhani and Fink 1997; Rupp *et al.* 1999; Gagiano *et al.* 2002; Kuchin *et al.* 2002; Vyas *et al.* 2003; Schwartz and Madhani 2004). Therefore, the formation of flor can be considered as an adaptive mechanism to nutrient starvation (Zara *et al.* 2011) because it ensures access to oxygen and therefore permits continued growth on nonfermentable ethanol. The glucose repression pathway between *S. cerevisiae* and *Z. rouxii* could be common because not only *FLO11* expression but

Figure 7 Biological roles of *FLO11D* copy number variation. (A) Cellular hydrophobicity. The cell cultures were overlaid with octane and mixed. The OD₆₀₀ of the aqueous layer was recorded, and the relative difference compared with the initial OD₆₀₀ was used to determine the percentage of cellular hydrophobicity. The error bars represent the standard deviation between three independent experiments. The results were analyzed by one-way ANOVA with Bonferroni post test. (B) Effects of changes in the *FLO11D* copy number on the yeast fitness under osmotic stress conditions. An equal number of reference and sample strains (combinations of wild-type and single *FLO11D* gene mutants, single and double *FLO11D* gene mutants, and double and triple *FLO11D* gene mutants) were inoculated into 10 ml of YPD containing 1.8 M NaCl and incubated at 28°C with or without shaking. The ratio of the two competitors was quantified at the initial and final time points (7-day incubation) based on its drug resistance. The fitness of the reference strain was designated as 1 and the fitness of the sample strain as 1 + *s*. The selective advantage, *s*, of each strain was calculated by the formula described in *Materials and Methods*. The error bars represent the standard deviation between three independent experiments. The results were analyzed by *t*-test.

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also *FLO11D* expression was repressed by glucose, suggesting that the flor formation is important to the adaption to nutrient starvation in *Z. rouxii*. Interestingly, a previous study showed that hyperosmotic stress inhibits the development of the fluffy colony morphology in *S. cerevisiae* (Furukawa *et al.* 2009), which are the opposite results from ours (Figure 2C). This fact raises the question of why the *FLO11D*-dependent morphological change is induced by osmotic stress in *Z. rouxii*. We hypothesize three possible reasons: (1) the *FLO11D* gene is under the *HOG1* MAP kinase pathway, (2) there is signaling cross talk between the filamentous growth MAP kinase pathway and the *HOG1* MAP kinase pathway because the two pathways share common components (Ste11p, Ste50p, Ste20p, and Cdc42p) in their signal transduction (Chen and Thorner 2007), or (3) there is an unknown pathway. To assess the possibility of hypothesis 1, we constructed the *hog1Δ* strain under the Z3 background and compared the expression level of *FLO11D* between Z3 and *hog1Δ*. As a result, *FLO11D* gene expression was observed under osmotic stress conditions in both Z3 and *hog1Δ* (data not shown). These results disprove hypothesis 1. We therefore infer that hypothesis 2 or 3 may contribute to the osmotic stress *FLO11D* expression. However, we cannot exclude the possibility that *FLO11D* activation in *hog1Δ* under osmotic stress conditions is an artificial consequence because hyperosmotic shock induces inappropriate activation of the MAP kinase-dependent filamentous growth pathway in *S. cerevisiae* *hog1Δ* (Davenport *et al.* 1999). Clearly, a more detailed analysis is needed to elucidate the pathway required for NaCl-dependent *FLO11D* expression in *Z. rouxii*. We speculate that the osmo-ergic *FLO11D* expression in *Z. rouxii* would be important for ecological niche segregation among yeast. For *Z. rouxii*, the adaptation to nutrient starvation through the formation of flor in a non-osmotic stress environment would not be effective for the expansion of *Z. rouxii*'s natural habitat because its competitor, *S. cerevisiae*, has advantages in growth rate in this type of environment. Conversely, the adaptation to nutrient starvation by forming flor observed in *Z. rouxii* under osmotic stress conditions would

ensure its increased survival and thereby permit its occupancy of this type of niche.

Implications of *FLO11D* copy number amplification

We showed that there are *FLO11D* copy number variations in a *Z. rouxii* population and that the cellular hydrophobicity correlates with the *FLO11D* copy number (Figure 6 and Figure 7A). We also showed that the strain with more *FLO11D* copy numbers has a fitness advantage in a flor-formative environment, such as osmotic stress static culture conditions (Figure 7B), even though one copy of *FLO11D* is sufficient to form flor (Figure 4B and Figure 6B). These results suggest that the copy number of *FLO11D* is under positive selection under osmotic stress static culture conditions. A salt-containing food fermentation process, such as soy sauce and soy bean paste, is similar to the osmotic stress static culture conditions; therefore, these traditional food production processes can act as a positive selection pressure for the *FLO11D* copy number of *Z. rouxii*. This raises the question of why soy sauce mash is not occupied by flor-forming yeast with a high copy number of *FLO11D*. In fact, we demonstrated that the *FLO11D* copy number was variable in a *Z. rouxii* population isolated from salt-containing conditions (Figure 6). One possibility is that counterselection pressure exists in these artificial environments. During soy sauce fermentation, the flor is usually discarded by the manufacturer to maintain the organoleptic quality of the products, and a non-flor-forming strain of *Z. rouxii* is often added to soy sauce mash to stabilize the alcohol fermentation. The addition of yeast to the mash as a starter may lead to a cross between flor-forming and non-flor-forming yeast, which have variable *FLO11D* copy numbers, followed by sporulation.

In general, gene amplification (duplication) can have an important role in evolution through changes in the sequence, the acquisition of new or specialized functions (neofunctionalization or subfunctionalization), and increases in the expression level (gene dosage effect). For example, a high copy number of the human salivary amylase gene (*AMY1*), the yeast copper-binding metallothionein gene (*CUP1*), and the yeast

high-affinity sulfate transporter (*SUL1*) acts as a gene dosage, and the yeast α -glucosidase gene *MALS* family exhibits aspects of three classical models, such as gene dosage, neo-, and sub-functionalization (Perry *et al.* 2007; Gresham *et al.* 2008; Warringer *et al.* 2011; Voordeckers *et al.* 2012). This study clearly indicates that the copy number amplification of *FLO11D* also contributes to adaptation in a gene-dosage-dependent manner. This study therefore may have profound implications that support part of the classical models of gene duplication built with past knowledge.

In this study, we have demonstrated that the osmo-ergic *FLO11D* gene is responsible for the NaCl-dependent morphological changes in *Z. rouxii*. Through a gene amplification event and natural selection during its evolution, *Z. rouxii* acquired an additional copy of the *FLO11D* gene, enabling its further adaptation to flor-formative environments, such as osmostress static culture conditions.

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Supporting Information

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Adaptation of the Osmotolerant Yeast *Zygosaccharomyces rouxii* to an Osmotic Environment Through Copy Number Amplification of *FLO11D*

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Figure S1 *FLO11*-like gene-related phenotypes in the deletion mutants. (A) A DNA restriction map of the *FLO11A*, *B* and *C* genes using the *HincII* endonuclease. (B) Southern blot analysis of the *FLO11A*, *B* and *C* genes. (C) Flor formation in the *FLO11*-like gene deletion strains. (D) Mat formation in the *FLO11*-like gene deletion strains.

GATCATTCAATAGGTCACGTG

1501 GCTTCCCTAGTGCCTCGTCTGTTACAAGTAGTGGCGGTTCTCTGGTAGTACCTCTGCACCAACCGGCAGTGCTTTCAGTAGTTCTACCGTGAACAGCAACGCTTCCCCTAGTGCTTCTCTGTTACAAGCAGTGGTGCTTCATCTGGT
501 A S P S A S S V T S S G G S S G S T S A P T G S A F S S S T V N S N A S P S A S S V T S S G A S S G
1651 GCTTCTTCTACAACAAGCAGCGGTGCTTCATCTGGTAGTACCTCTGCGCCAACCGGCAGTGCTTTCAGTAGTTCTACCGTGAACAGCAACGCTTCCCCTAGTGCTTCTGTTACAAGCAGCGGTGGTTCTCTGGTCTTCTTCTACA
551 A S S T T S S G A S S G S T S A P T G S A F S S S T V N S N A S P S A S S V T S S G G S S G A S S T
1801 CCAAGCAGTGGTGCTTCATCTGGTGCTTCTTCTACAACAAGCAGCGGTGCTTCATCTGGTAGTACCTCTGCACCAACCGGCAGTGCTTTCAGTAGTTCTACCGTGAACAGCAACGCTTCCCCTAGTGCTTCTGTTACAAGTAGTGGC
601 P S S G A S S G A S S T T S S G A S S T K T N A V S S G S S P A P T R S T S A S S T T S S A S S S V T S S G
1951 GGTTCCTCTGGTAGTACCTCTGCACCAACCGGCAGTGCTTTCAGTAGTTCTACCGTGAACAGCAACGCTTCCCCTAGTGCTTCTTCTGTTACAAGCAGCGGTGGTTCTCTGGTGCTTCTTCTGTTACAAGCAGCGGTGGTTCTCTGGT
651 G S S G S T S A P T G S A F S S S T V N S N A S P S A S S V T S S G G S S G A S S V T S S G G S S G
2101 GCTTCTTCTACAACAAGCAGTGGCACTTCTCTGGTGCTTCTTCTACAAAACCAATGCAGTTTCTCTGGTGCTTCCCCTGCTCCAACCTAGAGTACCTCCGCTAGCTCTACTACCAGCAGCGCTTCTTCCAGTTCTCCCGCACCAGCT
701 A S S T T S S G T S S G A S S T K T N A V S S G S S P A P T R S T S A S S T T S S A S S S S P A P A
2251 AGTAGTGGTTTCAGTAATTCTGGTGTAACAGCATCATTTCCTATGGTCTTCTCTCGGCACCAATAGTAGTGTTCTCTCAGTCATTTTCTGCTACAAGTAGCGCGCTCCCTCCGGTCTCTCTGCATCAACCAGCAATGCTTTTAAAGT
751 S S G F S N S G V T T S I I S Y G P S S A P N S S V S F S H F A T S S G A P S G S P A S T S N V F K S
2401 TCCATTATAACCAATGGGCTTCTCTGGTGCTTCTGCTGCTCCAAGCAGTGCTCCTTCTGCTAGTTCTCTACGCTAACCAACGACGTCTCTTCTGGTTCCAGTAACAATGGTCTAGCAGTTTCGACTGCTGTACCAAGGATCCCAAGC
801 S I I T N G A S S G P S A A P S S A P S A S S S T L T N D V S S G S S N N G P S S S T A V P R I P S
2551 GATGACCTTCTGGTACAACAGTAGTGCTAATCTGCTACTTCCGGTCTGATGAATCTACTACCGGACCAAGCACTTCGACTGGTAAACCTTCTGACACAACCAAGTGGTACTGGATCTGCTACCTCTAATTACAACGAGTCAACTACT
851 D A P S S G T T S A N S A T S G S D E S T T G P S T S T G K P S D T S G T G S A T S N Y N E S T T
2701 GGTCCAAGCGCTTCAACTGGTGCTCCTTCTGGCACAACCACTGGTACTATCACTAGCAGCTTCCCTTTCAAGCGGTAGTTGGTGCTGAGTTCACTTTCGAAGTCTCAAGCAGCATCATCCAGGCACCACTATCCAGGCAGCAGCGTC
901 G P S A S T G A P S G T T S G T I T S T S L S S G S G V A S S S F E V S S S I I P G T T I P G S T V
2851 CCAGCAGCAGTACCAGGAATCTATCCCAGCAAGCAGCGTCCCAACGAGCAGCGCCCGAGCAACAGTTCAGGCACCACTATCCATCAAGCACTGTACCAGGCACCTATTATCCAGGCACCTACCATCTCAAGTAGTACCATC
951 P I P A R T V P G T T I P A S T V P T S S A P A N T V S G T T I L P S S T V P G T I I P G T T I S S T S I
3001 TCAGGAAGCATAATCCCAGGCACAATAATCCCAGGTAGCACAATCCAGAAAGCACTGTCCAGGTACTACTATACCAGCAAGTACCATTCTGCTAGCGTTATCCCAGGCACCACTATCCCAGGCAGCACCCTCCAGCAGCACAGTA
1001 S G S I I P G T I I P G S T I P E S T V P G T T I P A S T I P A S V I P G T T I P G S T V P A R T V
3151 CCAGGAATCTACTATCCCAGCAGCAGCGTCCCAACGAGCAGCGCCCGAGCAACAGTTCAGGCACCACTATCCATCAAGTAGTACCATCTCAGGAGCATAATC
1051 P G T T I P A S T V P T S S A P A N T V S G T T I L P S T V P G T T I P G T T I S S T I S G S I I
3301 CCAGGCACAATAATCCCAGGTAGCACAATCCAGAAAGCACTGTCCAGGTACTACTATACCAGCAAGTACCATTCTGCTAGCGTTATCCCAGAAAGCACCATTCCAGCTACAACAATCCAGTAGCGTCATCCCAGGTAGCACTTTC
1101 P G T I I P G S T I P E S T V P G T T I P A S T I P A S V I P E S T I P A T T I P S S V I P G S T F
3451 TCAGATATTACTATTCAGCTGAAACCGTTTTTCAGCAGCGTAGCTACTCCATCCACCGTTACCTCTCCAGCCAGCAGCAAGTATCAATAAATGGCGGTAATAGCGAACAGAACCAAGTCCAACTATCGTCACCCT
1151 S D I T I P A E T V F S S V A T P S T V T S P A T A A S A S I N N N G G N S E Q N P Q V Q T I V T T
3601 ATTCAACCAGGCAGCGGTAGCAACGGCAGTAACGGTCAACCACCTCACAAATTCAAACCATCACCAACCGCTCAGCCAACCGGTGGTAGTAACGGTAACAACAGCAATAATGGTAACAACAACAACAGT
1201 I Q P G S S G S N G S N G Q P P S Q I Q T I T T T A Q P T G G S N G N N S N N G N N N N D N N N N S
3751 CCAGTCTCTGTTCCGCTCTATCCAAACTGTACACACGTTCAAACCCAACTGTCACTGAGACAGTTACCAACAACCTGCGTATGTCCAACAGCTGCGGTGTCAGGTCTTCTTCCCTCTAATTCAGGTATTTTCAACAACACTCTA
1251 P V S V S V S I Q T V T H V Q T Q T V T E T V T N N C V C P T A A A A G P S S P S N S G Y F N N T L
3901 CCTGTAAGAGAGAACTTCAGTGGTTAAACCCGTCAGTAGAAACACTTCTGCTCAGCCCAACAATTGCACCCCAACCCCTATCTCAGGGCGTAGCTGGAAGAATGATGGGCCAATTAACGGAGTTTAAATGGGTCTATTGACACTGTTG
1301 P V K R E T S V V K P V S R N T S A Q P T I A P Q T L S Q G V A G R M M G P I N G V L M G L L T L L
4051 TCTCTCGTTTTCTGAATGTAAACATAATACCATTCTTCTAACTGTTGAATAGTTTCGTACAATGTGTATCAAATTTAGCTGATCATTATTATTCGACCTTCATAATTATGAAGCCATTGAAATAATACCTTACTACATAAAACATTGTA
1351 S L V F *
4201 TAATGTATTTCTTTATTTTACAGTAGGACTGTTTCAATAAGGTTGAGTGAATGAATTTGCCCGTTGCAAAATTCATATGCGGGTAACACTTTTCGTTAGTGTCTCGAGTATCGGAACCTTCTATTGTATTATAGTCTCGGTAGTTTCT
4351 CAGATGATTTCTCGGATTCTCGAAATGTACAATAGCTGCGTAAACATTCTAGAAAGTGTTTGTAGGTATTGTCGTGTTCTAGAAACGATCGTTTGTGCATACGATATAGGCTAATTAGGAAATAGTAAGAGGAAGAACCAGGCATTTC
4501 CAATGTGATATTTCTGACGCTTGAATACCTGAGAGCTGAAGAAATGTGAAGAGACTTGTGGACAGTAATTTTGCATATCACTTGGTCCAGCATTACAGAGGATCTCAAAAAAGTTTGTCTATGCTATCCATGGACTAGGAATCTA
4651 TGCAATATTACTTAATTTATTCACCGCTTACCGAATCTTACGCTTCAACAATAAACTATATCGTATGTTGCTGAGCTGATCGCAAAACAATTGAAGGAACAGGGGCTAAACAGATGCCAATAATGAGGTCCAGTAGTGTAGCCTG
4801 AAACATCAATACCAATATGATC

Figure S2 Synthetic DNA and deduced amino acid sequence of the *FLO11D* gene assembled from 4 plasmid sequences with genome walking results. The p1-0 plasmid contains sequences from -2,597 to 4,405 with little mutation; sequences from 1,518 to 1,619 were deleted, from 2,074 to 2,109 were deleted, and 3,110 to 3,394 were deleted (AB769971). The p1-1 plasmid contains sequences from -152 to 4,734 bp with little mutation; C-115 was substituted with G, T-108 was deleted, G-107 was substituted with C, and sequences from 1,635 to 2,048 were deleted (AB769972). The p1-4 plasmid contains sequences from -749 to 4,826 bp with little mutation; C-115 was substituted with G, and T-108 was deleted (AB769973). The p4-1 plasmid contains sequences from -908 to 4,606 with little mutation; C-115 was substituted with G, and T-108 was deleted (AB769974).

Figure S3 Conservation of the Flo11 domain. (A) Multiple sequence alignment of Flo11 domains. Sequence were collected with PSI-BLAST (for GenBank) with an expect value threshold of 10^{-5} using the Flo11 domain of *S. cerevisiae* Flo11p as a query. (B) Unrooted phylogram of Flo11 domains. *S. pombe* was used as outgroup. Bootstrap values (>0.60) based on 1000 replicates are indicated at the nodes. Bar, 0.1 substitutions per site.

Figure S4 Flor formation of Z3 in YPD medium or YPD medium containing 0.45 M, 0.9 M, and 1.8 M of various chemicals (MgCl_2 , MgSO_4 , or Na_2SO_4). A fresh colony was inoculated into the medium and incubated for 5 or 16 days at 28°C.

Table S1 Primers used in this study

Primer name	Sequence	Corresponding fig.
FLO11A_F	5'-CTTGTGCAGGTCGAAGCTTT-3'	Fig. S2 for probe synthesis
FLO11A_R	5'-CCATTGCTATAGGTATATGTCAATTG-3'	Fig. S2 for probe synthesis
FLO11B_F	5'-ACGCTTGTCCAGTTCGAAAC-3'	Fig. S2 for probe synthesis
FLO11B_R	5'-ACCGTTGCTATATTCGTAGACCA-3'	Fig. S2 for probe synthesis
FLO11C_F	5'-TACAAATATGTGCCCAGGTTTAAAC-3'	Fig. S2 for probe synthesis
FLO11C_R	5'-TAGTGGGAAGTCCGCATTG-3'	Fig. S2 for probe synthesis
TsubURA3Δ_F	5'-GAAGGTGAAAAATTATCGACGATGAGATTAGTTCAACACTCTTTCGTGT GATAAGGTTCTCAGAAAACAGTCGTCTCCTTCGTACGCTGCAGGTCG-3'	Fig. 3 for <i>ura3Δ</i> construction
TsubURA3Δ_R	5'-ATTCACCATGTGCTAACGATCCTTTAGAGGACAATTCTGCAAGCATTAGA AGACCACGAGGTTCTGTGTAGTCTCTTGCACTAGTGGATCTGATATCACC-3'	Fig. 3 for <i>ura3Δ</i> construction
PsubURA3Δ_F	5'-ATTTAAAGATCCGACCTCTCTTCCAATGCATAGAAGAGTTGATTAACTT TTTCCAAGAAGACGCATCGAAAGTGAAAAATTGACAGCTATGAGATCAGC TTCGTACGCTGCAGGTCG -3'	Fig. 3 for <i>ura3Δ</i> construction
PsubURA3D_R	5'-CCTTTCTGTAACGTTACCTTCAACACTTGGATCACGACCTTTGGCGAATA AACCTCTGCCGACGATAATTATGTCTGATCCCGTGGATACTACGTCA CACTAGTGGATCTGATATCACC -3'	Fig. 3 for <i>ura3Δ</i> construction
URA3_F	5'-ATGAGCCATCTCACTTATCAAG -3'	Fig. 3 for PCR and probe
URA3_R	5'-TTATTTATTGCATCTCTTTAAATACGC -3'	Fig. 3 for PCR and probe
FLO11D_F	5'-GACATTCACGACGCCTGTTA-3'	Fig. 3 for PCR and probe

FLO11D_R	5'-ATCATTACCGTAAGTATATTCCAATTG-3'	Fig. 3 for PCR and probe
FLO11D_F	5'-CATCTAATATAAATTGATAATCAATTCGCAAGCTTGTCCAAAAGAAGT TCCCTAGCTTATTCTTGTTCCATACCAACATAAAAAGAAAGCAACGAAGG <u>AATTCGTACGCTGCAGGTCG</u> -3'	Fig. 4 for <i>flo11d</i> Δ construction
FLO11DΔ_R	5'-GAACTAGTAGTAGCTAGCACACCTGGCAAACCGTTACTGTAGGTATAT GTTAATTGGAAGGCTGGTAGCCAGATGTCGTCACCATTTTGAATACCATA <u>AGCACTAGTGGATCTGATATCACC</u> -3'	Fig. 4 for <i>flo11d</i> Δ construction
FLO11DΔcomf_F	5'-GTCCGAAACTTTCAGTCCTG-3'	Fig. 4 for PCR analysis
FLO11DΔcomf_R	5'-CTAGATTTGGACAACCGGCA-3'	Fig. 4 for PCR analysis
pFLO11D_F	5'-TTTTGAATTCTGCCTATCTGGTCAGTCACA -3'	Fig. 4 for pFLO11D
pYFLO11D_F	5'-GCTGTAATACGACTCACTATAGGGAATATTATGAAATCTTCTCGTAACGTT-3'	Fig. 4 for pYFLO11D
pYFLO11D_R	5'-CTAATTACATGATGCGGCCCTCTAGATGCATCAGAAAACGAGAGACAACAG-3'	Fig. 4 for pYFLO11D
pFLO11D_R	5'-TTTTGTCGACGATCAGCTCAGCAACATACG -3'	Fig. 4 for pFLO11D
ACT1_F	5'-ACATCAAGGTATCATGGTTGG-3'	Fig. 5 for probe synthesis
ACT1_R	5'-TTTCAGCAGTAGTGGAGAAAG-3'	Fig. 5 for probe synthesis

1 Underline indicates the annealing sequence to the plasmid.