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FLO11 is essential for flor formation caused by the C-terminal deletion of *NRG1* in *Saccharomyces cerevisiae*

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Abstract

The flor strains of *Saccharomyces cerevisiae* form a flor on the surface of wine after alcoholic fermentation. High hydrophobicity of the cell surface is suggested to be important for flor formation by the flor wine yeasts. However, the molecular mechanism of flor formation is not clear. We found that expression of C-terminal deleted *NRG1* lacking its two C_2H_2 zinc finger motifs (*NRG1*$^{1–470}$) on the multicopy plasmid conferred the ability to form a flor to a non-flor laboratory strain. The cell surface hydrophobicity of *NRG1*$^{1–470}$ was higher than of the non-flor strain. Disruption of the Nrg1p-repressed gene *FLO11*, which encodes a cell surface glycoprotein that functions as a flocculin or an adhesin, abolished flor formation. Moreover, expression of *FLO11* on a multicopy plasmid could also cause flor formation. These results indicate that *FLO11* is essential for flor formation by *NRG1*$^{1–470}$. In addition, the results suggest that the C-terminal truncated form of Nrg1p exerts a dominant negative effect on *FLO11* repression, resulting in *FLO11* expression and, thus, flor formation.

Keywords: *FLO11*; *NRG1*; *Saccharomyces cerevisiae*; Flor formation

1. Introduction

Some strains of *Saccharomyces cerevisiae* form a biofilm call a 'flor' on the surface of wine after ethanolic fermentation. During this time, further growth of the cells depends on aerobic assimilation of ethanol because of the depletion of grape sugar [1]. In the production of Sherry-type wines, biological wine ageing occurs in the so-called 'Solera' system (different sets of aged oak casks) under a flor of yeasts (up to 1 cm thickness) growing on the surface of the wine, which contains about 15% (v/v) ethanol. Growth of yeasts on the surfaces results in variable changes to the characteristics of the wines due to the oxidative metabolism of the flor yeasts. For example, the flor yeasts produce important quantities of acetaldehyde as the result of ethanol oxidation by alcohol dehydrogenase ADHII [2]. In general, the biology of flor formation can be viewed as an adaptive mechanism that maintains access to oxygen and thus permits yeast growth on a non-fermentable carbon source in an aerobic environment [1].

Previous studies have established that the flor formation is induced in a medium containing ethanol as a carbon source but not in a medium containing glucose [3]. In contrast to other microbial biofilms, the flor is thought to consist of a layer of buoyant cells without a suspending extracellular matrix [1] because a polysaccharide or protein matrix has not been found in the flor. It has been suggested that the buoyancy of the cells is due to an elevated and/or altered lipid content [3–6].

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In general, the molecular mechanism of flor formation is unclear, although *HSP12*, which encodes a heat-shock protein, is known to be essential for flor formation [1].

In this report, we found that expression of the C-terminally truncated form of *NRG1*, designated *NRG1*$^{1-470}$, on a multicopy plasmid increases the hydrophobicity of the cell surface conferring flor formation to a non-flor laboratory strain. Disruption of the Nrg1p-repressed gene *FLO11* abolished flor formation caused by *NRG1*$^{1-470}$. Finally, expression *FLO11* on the multicopy plasmid also caused flor formation. These results strongly suggest that Flo11p is a key factor in flor formation.

2. Materials and methods

2.1. Strains, media and plasmids

The *S. cerevisiae* strains used in this work are listed in Table 1. The *Escherichia coli* strain JM109 [7] was used as a host for propagation and manipulation of plasmid DNA. The Δ*flo11::kanMX4* disruptant strain BY4741 [8] constructed by EUROSCARF was purchased from Invitrogen and designated MI1. The Δ*flo11::kanMX4* strain of DBY746 [9], MI2, was constructed as follows. A 2.9-kbp fragment containing Δ*flo11::kanMX4* allele was amplified by PCR from MI1 chromosomal DNA as a template and using oligonucleotides primers 5′-ACATGCATGCGAGGACATTGCCCAACCCTA-3′ (forward; corresponding to nucleotides (nt) −569 to −550 of *FLO11* relative to the first base of the ATG start codon of *FLO11*) and 5′-CTCGCATGCTGTGCCAAGGCAATATCAGG-3′ (reverse; corresponding to nt +5170 to +5151 of *FLO11*). The PCR product was used for transformation of DBY746 to obtain MI2. The minimal (SD) medium [10] for *S. cerevisiae* and LB medium [7] for *E. coli* were prepared as described previously. Flor medium consists of synthetic minimal medium [10] containing 3% (v/v) ethanol as the sole carbon source in place of glucose and adjusted to pH 3.5 with hydrochloric acid [4]. The plasmid pFTF4, which causes flor formation, was obtained from the flor-forming transformant designated FTF4. Plasmid pNRG1$^{1-470}$, which carries *NRG1* lacking the C-terminal region, including its two C_2H_2 zinc finger motifs, on a multicopy vector was constructed as follows. A 1.9-kbp fragment of *NRG1* (nt −1063 to +861) was amplified by PCR using the pFTF4 plasmid DNA as a template and the oligonucleotides primers 5′-ACAAAAGCTTGCTACCCGTCGTATGTATGG-3′ (forward; corresponding nt −1063 to −1044 of *NRG1* and with a *Hin*dIII linker) and 5′-GAGGGCATGCAAGATTCCGAATACCGCAAG-3′ (reverse; corresponding to +861 to +842 of *NRG1* and with a *Sph*I linker). The PCR product was digested with *Hin*dIII and *Sph*I and inserted into the *Hin*dIII–*Sph*I gap of YEp13 [11] to obtain the plasmid pNRG1$^{1-470}$. The plasmid pNRG1, which contains full length *NRG1* in YEp13, was constructed as follows. A 2.0-kbp fragment of *NRG1* was amplified by PCR using chromosomal DNA of *S. cerevisiae* strain DBY746 as a template and oligonucleotides primers 5′-AGACGGATCCAGGGACCTAGAACGCCAAGC-3′ (forward; corresponding to nt −988 to −969 of *NRG1* and with a *Bam*HI linker) and 5′-CAGGAAGCTTGGAGGTAGTCACAGTCTCGT-3′ (reverse; corresponding to nt +1034 to +1015 of *NRG1* and with a *Hin*dIII linker). The PCR product was digested with *Bam*HI and *Hin*dIII and inserted into the *Bam*HI–*Hin*dIII gap of YEp13 to obtain the plasmid pNRG1. The plasmid pHEM13, which carries *HEM13* on YEp13 was constructed as follows. A 4.5-kbp fragment of *HEM13* was amplified by PCR using pFTF4 as a template and the oligonucleotides primers 5′-CTCGGATCCTTCAAGGTTCTTTAGCAGACC-3′ (forward) and 5′-ATGCGGATCCGAGCCACTATCGACTACGCG-3′ (reverse). The PCR product was digested with *Bam*HI and cloned into the *Bam*HI gap of YEp13 to obtain the plasmid pHEM13. The plasmid pFLO11, which carries *FLO11* on the multicopy vector was constructed as follows. A 5.7-kbp fragment of *FLO11* was amplified by PCR using chromosomal DNA of the *S. cerevisiae* strain MI1 as a template and the oligonucleotides primers 5′-ACATGAATTCGAGGACATTGCCCAACCCTA-3′ (forward; corresponding to nt −569 to −550 of *FLO11* and containing an *Eco*RI linker) and 5′-CTCGCATGCTGTGCCAAGGCAATATCAGG-3′ (reverse; corresponding to nt +5170 to +5151 of *FLO11* and containing a *Sph*I linker). The PCR product was digested with *Eco*RI and *Sph*I and inserted into the *Eco*RI–*Sph*I gap of YEplac181 [12] to obtain the plasmid pFLO11.

2.2. Determination of hydrophobicity of yeast cell surfaces

The hydrophobicity of yeast cell surfaces was determined as described previously by measuring the distribution ratio of yeast cells in a two phase system

Table 1
S. cerevisiae strains used

Strain	Genotype	Source/reference
DBY746	*MATα leu2-3,112, his3Δ1, ura3-52, trpl-289*	[9]
MI1	*MATα, leu2Δ0 ,his3Δ1, ura3Δ0 ,met15Δ0, YIR019c :: kanMX4*	EUROSCAF
MI2	*MATα leu2-3,112 his3Δ1, ura3-52, trpl-289, YIR019c :: kanMX4*	This study

consisting of a buffer solution and an organic solvent [4]. Briefly, harvested cells were washed three times with water and suspended in four ml McIlvaine buffer, pH 3.5, adjusting the cell population to an optical density at 660 nm (OD_{660}) of approximately 0.5 in a test tube with a stopper. Four ml of this suspension were transferred to a test tube (15 × 150 mm) with a stopper. An equivalent volume of hexane was gently layered over the buffer layer. This test tube was vigorously vortexed for 5 min being careful to avoid emulsification. The OD_{660} of the initial and the residual buffer layers were measured, and the degree of hydrophobicity degree of the yeast cell surfaces (HD) was calculated from the equation:

$$HD(\%) = 100(I - R)/I,$$

where I and R are the OD_{660} of the initial and the residual layers, respectively.

2.3. Genetic and biochemical methods

Saccharomyces cerevisiae and *E. coli* were transformed as described by Ito et al. [13] and Sambrook et al. [7], respectively. Yeast chromosomal and plasmid DNAs were prepared as described previously [14]. Bacterial plasmid DNA was isolated by the alkaline lysis method [7]. Subcloned DNA fragments for sequencing were obtained from the plasmid pUC18 [7]. Nucleotide sequences were determined by the dye terminator cycle sequencing method with an CEQ2000XL DNA Analysis System (Beckman Coulter) using the DTS Quick Start Master Mix (Beckman Coulter) according to the manufacturer's instructions.

3. Results and discussion

3.1. Construction of flor S. cerevisiae strains from a non-flor strain

To elucidate the mechanism of flor formation in yeast, we first searched for genes that can cause flor formation by overexpression using the plasmid vector of multicopy in a non-flor *S. cerevisiae* laboratory strain. The non-flor strain DBY746 was transformed with a genomic DNA library from the non-flor *S. cerevisiae* strain AB320 [15] in the multicopy vector YEp13. Approximately 1.0×10^4 transformants selected for leucine prototrophy were mixed and cultivated in flor medium for 10 days at 30 °C under static conditions. The cells from the flor that formed on the surface of the medium were isolated and grown on SD solid medium without leucine. The colonies that formed on the medium were inoculated into the flor medium independently and examined for their abilities to form a flor. As a consequence, three flor transformants were isolated, FTF4, FTF12 and FTF65 (Fig. 1). Interestingly, these transformants differed in the time required for flor formation and in the thickness of the flor (data not shown), suggesting that these transformants harbor different plasmid DNAs. Because FTF4 formed the most abundant flor, it was selected for further analysis.

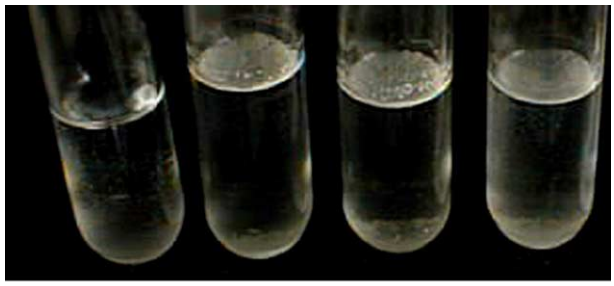

Fig. 1. Flor formation by flor transformants FTF4, FTF12 and FTF65. Cells of each strain were cultivated overnight at 30 °C in 1 ml of SD medium without leucine and with vigorous shaking. These cells were collected by centrifugation, washed once with sterile distilled water, and resuspended in 5 ml flor medium in 16 × 165 mm test tubes. The tubes were photographed after seven days of static incubation at 30 °C.

3.2. $NRG1^{1–470}$, which lacks its two C_2H_2 zinc finger motifs, confers flor formation

To identify the genes required for flor formation, the plasmid DNA, designated pFTF4, was recovered from the flor transformant FTF4. By sequencing parts of the insert, we found that the plasmid harbors a 4.8-kbp insert including part of the *NRG1* and *HEM13* genes. The *NRG1* gene encodes a transcriptional repressor for glucose repression of *STA1* [16], *DOG2* [17], *SUC2* and *GAL* genes [18]. Nrg1p contains two C_2H_2 zinc finger motifs near the C-terminus and specifically binds to two regions in the upstream activation sequence of *STA1* [16]. In contrast, the *HEM13* gene encodes an enzyme, coproporphyrinogen oxidase, which catalyzes the sixth step in the heme biosynthetic pathway [19]. The *NRG1* in pFTF4 lacks the region following nucleotide position +471 relative to the first base of the ATG start codon, resulting in the deletion of the two C_2H_2 zinc finger motifs. Therefore, *NRG1* in pFTF4 was designated $NRG1^{1–470}$.

To clarify which gene causes flor formation, we constructed $pNRG1^{1–470}$ and pHEM13 which contain the $NRG1^{1–470}$ and *HEM13* in YEp13, respectively, and introduced them into the non-flor strain DBY746. We next examined whether the resultant transformants form flor in the flor medium. The cells harboring pFTF4 or $pNRG1^{1–470}$ formed a flor after cultivation for seven days (Fig. 2). However, the cells harboring pHEM13 or YEp13 did not form a flor, indicating that $NRG1^{1–470}$ is responsible for the flor formation.

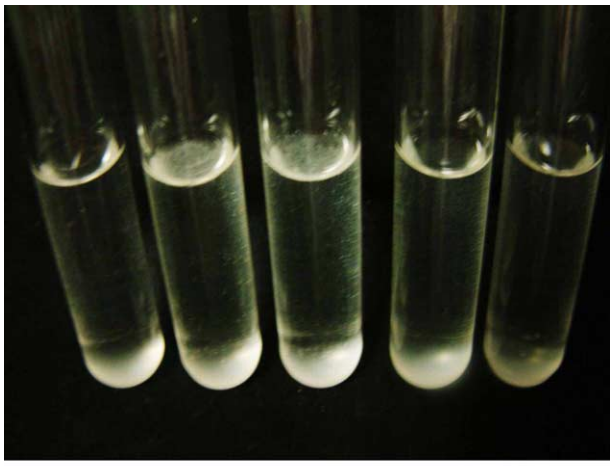

Fig. 2. *NRG1*$^{1–470}$ lacking the two C_2H_2 zinc finger motifs causes flor formation. The cells harboring pFTF4, pNRG1$^{1–470}$, pNRG1, pHEM13 and YEp13 were cultivated overnight and inoculated into flor medium as described in the legend to Fig. 1. The tubes containing each culture were photographed after seven days of static incubation at 30 °C.

The Nrg1p expressed from pNRG1$^{1–470}$ lacks zinc finger motifs. To examine whether the deletion of the zinc finger motifs is essential for the flor formation, we constructed pNRG1, which contains the entire *NRG1* in YEp13. We introduced it into DBY746 and examined whether the cells harboring pNRG1 form a flor. Interestingly, the cells harboring pNRG1 did not form a flor (Fig. 2), indicating that the deletion of the C-terminal domain of Nrg1p is essential for flor formation.

Based on these results, we speculate that there are two mechanisms by which *NRG1*$^{1–470}$ causes flor formation. One is the titration of transcriptional factors that are involved in the repression of flor formation and that bind to *NRG1* promoter region by virtue of the many target sequences for these transcriptional factors in the *NRG1*$^{1–470}$ promoter regions. However, the cells harboring pHEM13, which also contains full-length promoter region of *NRG1*, did not form a flor (Fig. 2), suggesting that the titration of transcriptional factors by the *NRG1* promoter is not involved in the flor formation. The other possible mechanism by which *NRG1*$^{1–470}$ causes flor formation is that the Nrg1p expressed from *NRG1*$^{1–470}$ exerts a dominant negative effect on functional Nrg1p expressed from endogenous *NRG1*, which normally functions as a repressor of flor formation.

3.3. *NRG1*$^{1–470}$ on a multicopy plasmid increases cell surface hydrophobicity

It has been reported that, compared with non-flor yeasts, flor wine yeasts possess a strong affinity for hydrophobic solvents and a high cell surface hydrophobicity [4]. We speculated that the cells harboring pNRG1$^{1–470}$, which causes the flor formation, have these same characteristics as flor wine yeasts. We examined this possibility by assessing the affinities of the cells harboring pNRG1$^{1–470}$ or YEp13 to olive oil droplets as observed by light microscopy. Many cells harboring pNRG1$^{1–470}$ adhered to oil droplets, while the cells harboring YEp13 rarely adhered (date not shown). These results indicate that pNRG1$^{1–470}$ confers a high affinity to the olive oil droplets on the cells.

We further examined the hydrophobicities of the cells harboring pNRG1, pHEM13, pNRG1$^{1–470}$ and YEp13 by comparing the distribution ratios of the cells between buffered aqueous and organic solvent phases. The HD value of the cells harboring pNRG1$^{1–470}$ was much higher than those of the cells harboring pNRG1, pHEM13 or YEp13, which do not cause flor formation (Fig. 3). The similar phenomena have been shown in the wine yeast flor by Iimura [4]. These results indicate that the expression of *NRG1*$^{1–470}$ on a multicopy plasmid confers high hydrophobicity to the cell surface and suggests that the flor transformant constructed by introducing pNRG1$^{1–470}$ into the non-flor laboratory strain has the same hydrophobic characteristics as flor wine yeasts. Therefore, functional analysis of *NRG1*$^{1–470}$ seems to have clarified the molecular mechanism of flor formation in flor wine yeasts.

3.4. *FLO11* is essential for flor formation caused by *NRG1*$^{1–470}$

In addition to glucose repression of *STA2*, *DOG2*, *SUC2* and *GAL* genes, Nrg1p was recently found to be involved in the repression of *FLO11* [20]. *FLO11* encodes a cell surface flocculin [21] and is required for invasive growth in haploid cells as well as pseudohyphal differentiation in diploid cells [22,23] and adherence to

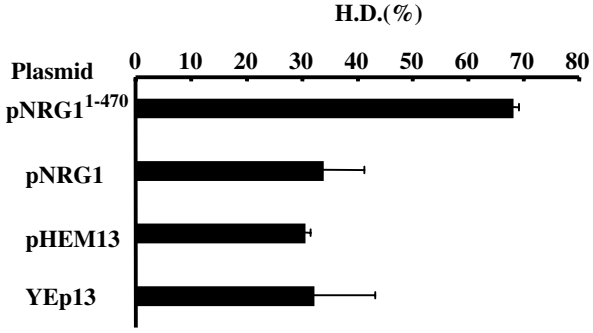

Fig. 3. *NRG1*$^{1–470}$ on a multicopy plasmid increases cell surface hydrophobicity. Measurement of cell surface hydrophobicity. The cells harboring pNRG1$^{1–470}$, pNRG1, pHEM13, and YEp13 were cultivated on flor medium for 10 days, and the hydrophobicities of cells were measured as described in Section 2. HD (%) indicates the hydrophobic degree of the cell surface. The results are averages of at least three independent determinations, and error bars represent standard deviations.

plastic surfaces [20]. Because cell–cell interactions appear to be important for flor formation, we suspected that *FLO11* is essential for flor formation caused by *NRG1*$^{1–470}$.

To test this possibility, we introduced pNRG1$^{1–470}$ or YEp13 into the wild-type and Δ*flo11* disruptant strains of DBY746, and examined their ability to form a flor (Fig. 4(a)). The wild-type cells harboring pNRG1$^{1–470}$ formed a thick flor after seven days of cultivation. Interestingly, the Δ*flo11* disruptant cells harboring pNRG1$^{1–470}$ did not form the flor at all. These results indicate that *FLO11* is essential for flor formation caused by *NRG1*$^{1–470}$.

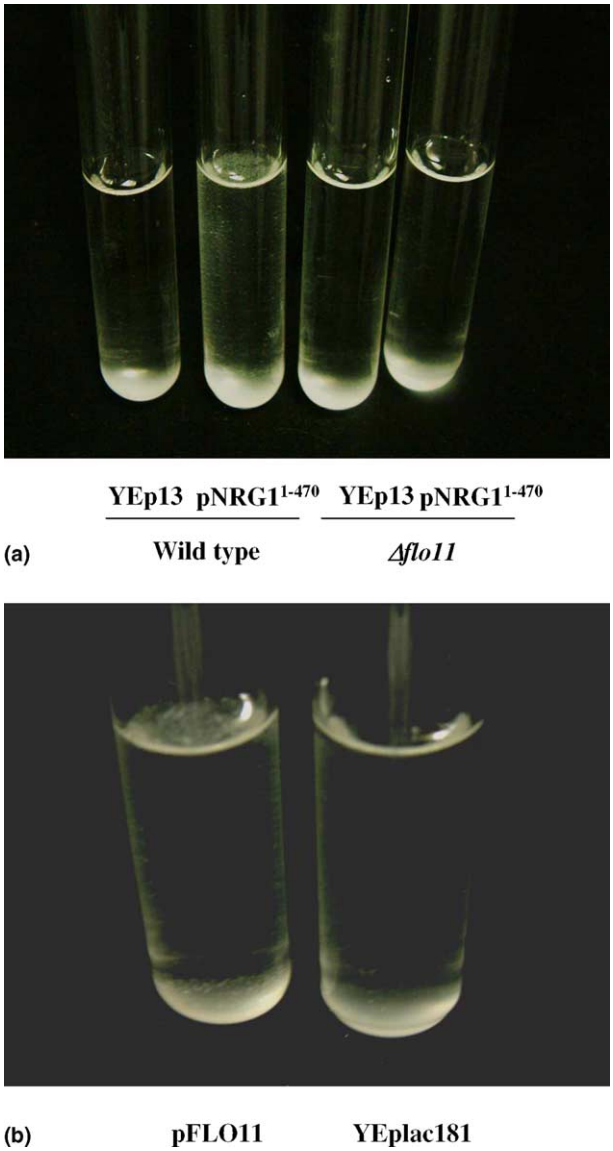

Fig. 4. *FLO11* is essential for flor formation caused by *NRG1*$^{1–470}$. (a) Cells of wild-type (*FLO11*) and Δ*flo11* disruptant strains harboring pNRG1$^{1–470}$ or YEp13 were cultivated overnight, inoculated into flor medium as described in the legend to Fig. 1. (b) The cells harboring pFLO11, YEplac181 were cultivated overnight and inoculated into the flor medium as described in the legend to Fig. 1. In (a) and (b), the tubes containing each culture were photographed after seven days of static incubation at 30 °C.

To examine whether overexpression of *FLO11* is sufficient for flor formation, we introduced pFLO11, which is a multicopy plasmid containing *FLO11*, into the non-flor laboratory strain DBY746. The cells harboring pFLO11 formed a flor after seven days cultivation, while the cells harboring a multicopy vector YEplac181 did not (Fig. 4(b)). This indicates that because the expression of *FLO11* is repressed the overexpression of this gene is sufficient for flor formation in the laboratory strain.

Based on the findings of this study, we propose a molecular mechanism for flor formation by flor wine yeasts. During the primary fermentation when grape sugar is anaerobically converted to ethanol, the *FLO11* transcription of flor strains may be repressed by the glucose repressor Nrg1p and Nrg2p (which is a C_2H_2 zinc finger protein homologous to Nrg1p) [17]. Based on our results, we can say that Nrg2p appear not to be active or at least is antagonized by the overexpression of *NRG1*$^{1–470}$. Therefore, flor formation does not occur during alcoholic fermentation. However, when the glucose repression of the *FLO11* transcription is abolished because of the depletion of grape sugar after alcoholic fermentation, the flor yeasts begin to form flor by a *FLO11*-mediated mechanism and begin to produce acetaldehyde as the result of ethanol oxidation. Further analysis of the functional role of Flo11p in flor formation will help clarify the molecular mechanism of flor formation.

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