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Functional Correlation between the Nuclear Localization of Fht1p and Its Flocculation and Heat Tolerance Activities in Budding Yeast *Saccharomyces cerevisiae*

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Key Words

Fht1p/Gts1p · Green fluorescent protein (S65T/S147P) · Subcellular localization · Flocculation · *Saccharomyces cerevisiae*

Abstract

Fht1p is involved in the flocculation and heat tolerance machinery of budding yeast *Saccharomyces cerevisiae*. Despite knowledge of its involvement in those phenotypes, a precise mechanism has yet to be discovered. To this end, we monitored the relationship between subcellular localization of Fht1p and its flocculation or heat tolerance function using newly developed expression vectors with a recombinant green fluorescent protein (GFP; S65T/S147P) of *Aequorea victoria* added at both the N- and C-terminus of Fht1p. The main fluorescent signal of the GFP tagged with either a wild-type Fht1p or mutants which preserve their flocculation function was detected in the nucleus, whereas signals of functionless mutants were dispersed to the cytoplasm.

Introduction

Flocculation of the budding yeast *Saccharomyces cerevisiae* is a mannose-sensitive, asexual, cell- to-cell interaction which is carried by the noncovalent Ca^{2+}-dependent interaction of a lectin-like protein and an outer-chain mannan side branch on the cellular surface [20, 21]. Genes controlling flocculation display two kinds of action, i.e. the suppression of flocculation and its activation. Examples of the former are shown in the recessive mutations of *sfl1* [3], *sfl2/tup1* [4, 24] and *cyc8* [22], and examples of the latter are dominant *FLO1, FLO5, FLO8* and *FLO11* [9, 12, 19, 23].

Fht1p, a protein which was originally called Gts1p [2, 13], is also involved in flocculation machinery via flocculation formation and heat tolerance when overexpressed [2]. Unlike other flocculation activators, except for Flo8p [9], Fht1p has no obvious sequence similarity with a lectin-like cell surface protein Flo1p [1]. Fht1p is predicted to localize in the nucleus due to its characteristic zinc finger and polyglutamine stretch [5], but no evidence for its subcellular localization has been provided so far. To investigate the functional relationship between the subcellular localization of Fht1p and the Fht1p-dependent flocculation phenotype, we constructed green fluorescent protein (GFP)-tagging vectors which express the engineered

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protein GFP (S65T/S147P) [14], which emits fluorescence about four times brighter than that of GFP (S65T) at 37°C [7, 8], and monitored microscopically the subcellular localization of the Fht1p-GFP (S65T/S147P) fusion protein (GFP-Fht1p) in *S. cerevisiae*. The overexpressed GFP-Fht1p confers the flocculation and heat tolerance phenotype to cells as a wild-type protein dose. GFP-Fht1p was localized mainly in the nucleus, whereas functionless mutant proteins are dispersed to the cytoplasm.

Materials and Methods

Strains and Media

Escherichia coli strain ER2267 [F′ *proAB lacI*^{q} *Δ(lacZ)M15 zzf::mini-Tn10 (Kan*^{r}*)/e14-λ-endA1 supE44 thi-1 relA1 spoT1 Δ(mcrC-mrr)114::IS10 Δ(argF-lac)U169 recA1*] was provided by New England Biolabs. *S. cerevisiae* strain YPH500 *(MATϑ ura3-52 leu2-1 his3-200 trp1-63 ade2-101 lys2-801)* was used throughout this experiment [17]. 2YT (1.6% Bacto-peptone, 1% yeast extract and 0.5% NaCl, with or without 1.5% agar) was used for *E. coli* cell propagation. Yeast cells were grown in YPD (2% Bacto-peptone, 1% yeast extract and 2% glucose, with or without 1.5% agar), SD (0.67% Bacto-yeast nitrogen base without amino acids, 20–400 μg/ml amino acid mixture and adenine sulfate and uracil, and 2% glucose, with or without 1.5% agar) or SG (same as SD except that 2% galactose was added instead of glucose).

Plasmid Construction

pGGN1 containing the *GAL1–10* promoter followed by the GFP open reading frame (ORF) with the Ser^{65} to Thr mutation, GFP (S65T), without a termination codon [10], was digested with *Eco*RI, blunted with T4 DNA polymerase, digested with *Sph*I and cloned into the *Hinc*II-*Sph*I site of pUC119 with a disrupted *Hin*dIII site (pGAL-GFPN119dHd). pGAL-GFPN119dHd was digested with *Pst*I, blunted and self-ligated to generate pGAL-GFPN119dPst. pGAL-GFPN119dPst was digested with *Hin*dIII, blunted and self-ligated to generate pGGN119. The *Nco*I-*Hpa*I fragment of pGGN119 was substituted with the same fragment from pQB2, having the additional Ser^{147} to Pro mutation GFP (S65T/S147P) to emit four times or more intense fluorescent light at 37°C compared to that of GFP (S65T) [7, 8], to generate pGGNe119. The *Sph*I-*Eco*RI fragments of pGGNe119 were used to construct pGN series of GFP N-terminal tagging vectors.

The *Sph*I-*Hin*dIII (blunted) fragment of *GAL1–10* promoter from pGAL-GFPN119dHd was cloned into the *Sph*I-*Hinc*II site of pUC118 (pGALs118), and the *Sph*I-*Eco*RI fragment of pGALs118 was used to replace the same promoter regions of the original pGM series [6]. These new pGM series (259 bp smaller than those of original ones) were used to generate pGC series. The blunted *Kpn*I-*Kpn*I fragment of pQB2 was cloned into the blunted *Sac*I-*Sal*I site of pUC119 (pGFPCe119). The *Eco*RI-*Sal*I fragment of pGFPCe119 was used to construct pGC series of GFP C-terminal tagging vectors. The characteristics of the constructed vectors are shown in table 1.

The *FHT1* ORF was amplified by standard PCR method with primers GLN [5′-TCATCTAGATGAGGTTTAGGAGTTCTTCC-C-3′ (containing the *Xba*I site)] and LC [5′-ATTGAATTCAAT-TGTGTGTAGAAATAACCTTG-3′ (containing the *Eco*RI site)], digested with *Bam*HI and *Eco*RI and cloned into pGCU20 [6] (pGCU20-FHT1BE). The *Eco*RI (blunt-ended)-*Kpn*I fragment of *FHT1* from pGAD424-FHT1, which contains the whole *FHT1* ORF within its *Eco*RI-*Sal*I sites, beginning with 5′-GAATTCATGAGG and ending with AATTAAGTCGAC-3′, was cloned into the *Xba*I (blunt-ended)-*Kpn*I site of pGCU20-FHT1BE to obtain pGCL20-FHT1. pGNL20-FHT1 was constructed by subcloning the *Eco*RI-*Sal*I fragment of pGAD424-FHT1 into the same sites of pGNL20. Deletion mutants of GFP-Fht1p were constructed by proper restriction endonuclease digestion of pGNL20-FHT1.

Table 1. Characteristics of the vectors

Marker	Name	Size, kb	GFP[1]	myc tag	Restriction sites[2]
HIS3	pGNH20	6.1	N	yes	6
	pGCH20	6.0	C	no	6
LEU2	pGNL20	6.7	N	yes	6
	pGCL20	6.7	C	no	6
TRP1	pGNT20	5.9	N	yes	5
	pGCT20	5.9	C	no	5
URA3	pGNU20	6.2	N	yes	6
	pGCU20	6.1	C	no	6

[1] GFP was connected at the N-terminus (N) or C-terminus (C) of the cloned gene products.
[2] Unique restriction sites in multiple cloning sites in each vector are indicated.

Microscopic Observation of the GFP-Tagged Proteins

After 9–12 h of incubation in SG medium at 28°C, cells carrying GFP fusion vectors were washed once with distilled water, resuspended in phosphate-buffered saline with or without 1 mg/ml of Hoechst 33342 for 15 min and viewed with a No. 9 or No. 13 filter for GFP fluorescence observation or Nomarski optics for differential interference contrast microscopy on a Zeiss Axiophoto microscope (Carl Zeiss Ltd., Tokyo, Japan). Cells were photographed with a ×100 objective and microscopy pictures were processed using Adobe Photoshop (Adobe Systems Inc., San Jose, Calif., USA).

Results

The GFP-Fht1p expression vectors are represented in figure 1. The *Sph*I-*Eco*RI fragment of the pGML20 myc-tagging yeast shuttle vector [6] was replaced with the *Sph*I-*Eco*RI fragment of pGGNe119 encoding the GFP (S65T/S147P) gene (see Materials and Methods) to generate the N-terminal tagging vector (fig. 1A). The *Eco*RI-*Sal*I fragment of the myc-tagging sequence of pGMU20 [6] was replaced with the GFP (S65T/S147P) *Eco*RI-*Sal*I frag-

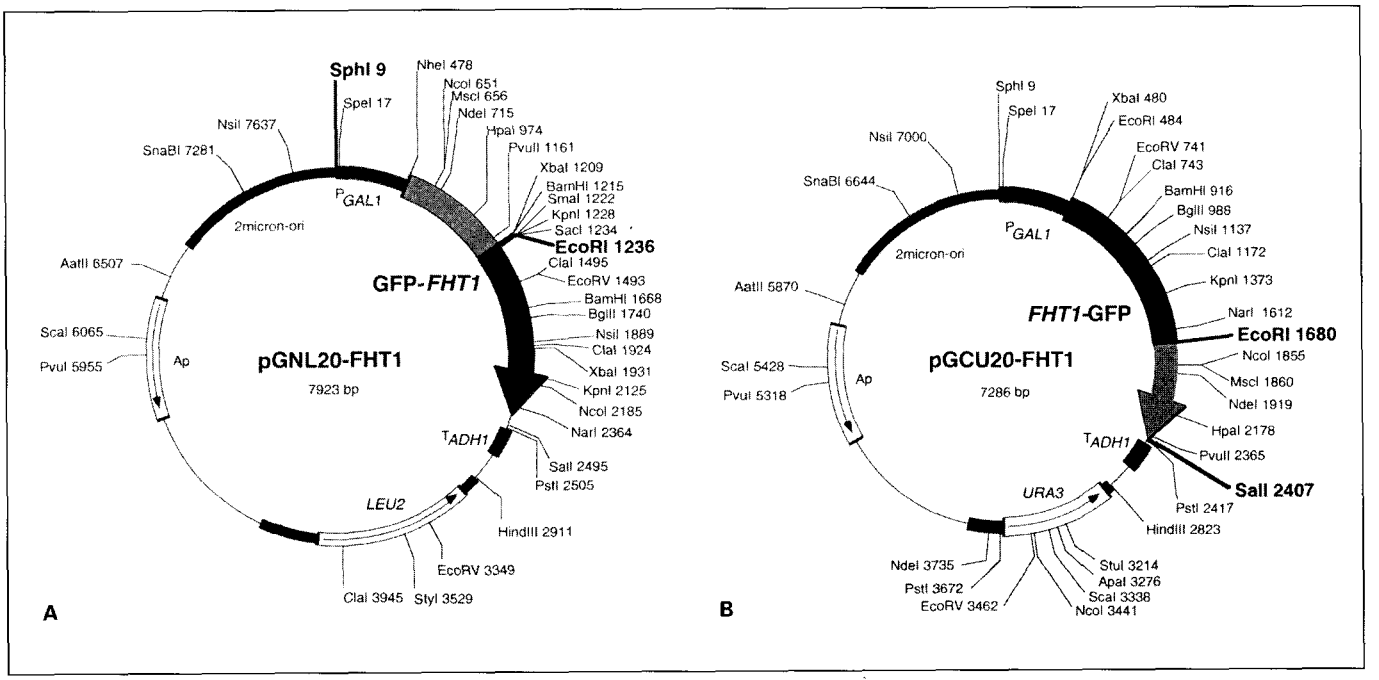

Fig. 1. Schematic representation of the GFP-Fht1p expression vectors. Genetically engineered GFP protein, GFP (S65T/S147P), was fused to the N-terminus (**A**) and the C-terminus (**B**) of Fht1p. The GFP (S65T/S147P) gene cassette for both vectors is indicated by *Sph*I-*Eco*RI and *Eco*RI-*Sal*I in bold (see Materials and Methods for details).

ment of pGFPCe119 (see Materials and Methods) to generate the GFP (S65T/S147P) C-terminal tagging vector (fig. 1B). The characteristics of each vector constructed for this experiment are shown in table 1.

To investigate the relationship between flocculation function and subcellular localization of Fht1p, we generated GFP-Fht1p expression vectors (fig. 1) and observed this microscopically (fig. 2). Since the main signal of GFP-Fht1p was colocalized with Hoechst 33342 emission (data not shown), Fht1p could be a nuclear protein as predicted by PSORT analysis [5]. We then made various deletion mutants of GFP-Fht1p to verify whether nuclear localization of GFP-Fht1p is necessary for the flocculation phenotype or not (fig. 2). The wild-type and deleted mutants which have flocculation activities showed their intense fluorescence in the nucleus, but flocculation-negative mutants were dispersed to the cytoplasm (fig. 2). The C-terminal Gln-Ala repeats (331–360 amino acids, indicated by shaded boxes, fig. 2) turned out to be indispensable for those functions. The flocculation-positive GFP-Fht1p also conferred a heat tolerance phenotype against 55°C for 10 min of treatment (data not shown). These results indicate that localization to the nucleus is necessary for the flocculation and heat tolerance function of Fht1p.

Discussion

In this report, we described the clear correlation between nuclear localization and the flocculation function of Fht1p with the help of a newly developed GFP-tagging vector system. The *FHT1* gene was originally cloned with the oligonucleotide probe of highly conserved -GGCACA- repeats, which potentially codes for amino acid repeats of Gly and Thr, found in some biological clock genes such as PERIOD of fruit fly *Drosophila melanogaster* [16] and thus called *GTS1,* which means poly Gly-Thr or Gly-Ser (GT/S) repeats [13]. However, structural analysis of this protein has indicated that Gts1p/Fht1p codes for the poly Gln-Ala repeats instead of GT/S repeats (fig. 2) in its C-terminus [2, 6]. Fht1p functions to confer flocculation and heat tolerance (i.e. FHT) phenotypes to cells when overexpressed [2, 25], thus we strongly propose to call this gene *FHT1* instead of *GTS1.*

Recently, the involvement of the flocculation gene *SFL1* in pseudohyphal development of *S. cerevisiae* was shown [15], and this morphological change is highly related to hyphal growth of pathogenic fungi *Candida albicans* [11, 15]. Interestingly, we identified a physical interaction between Fht1p and Sfl1p by two-hybrid assay (data not

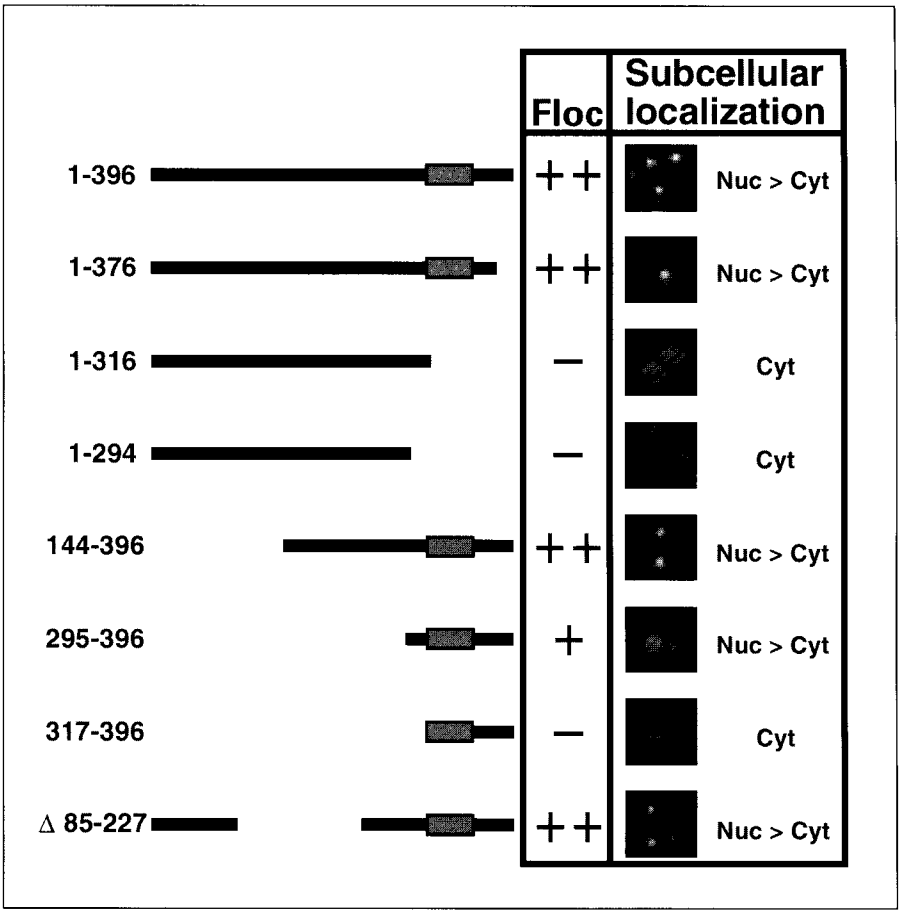

Fig. 2. Relationship between subcellular localization and flocculation function of GFP-Fht1p and its deletion mutants. Amino acid sequences of wild-type Fht1p (396 amino acids) and mutants are indicated on the left. The poly-QA repeats (331–360 amino acids) are indicated as shaded boxes. The flocculation activity comparable to wild-type Fht1p is indicated as ++, weak but perceivable flocculation activity is indicated as + and no flocculation is indicated as –. Intense fluorescence of GFP-Fht1p in the nucleus is indicated as Nuc > Cyt and dispersed fluorescence in the cytoplasm is indicated as Cyt.

shown). Sfl1p is also a nuclearprotein and acts as a transcription suppressor for several genes [15, 18]. It is intriguing to know how these two proteins interact with each other physically and genetically and also interesting to know whether Fht1p is involved in pseudohyphal development or not. Our ongoing studies will be published elsewhere.

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