Evidence that the *Saccharomyces cerevisiae CIF1* (*GGS1/TPS1*) gene modulates heat shock response positively

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Abstract **The *CIF1* gene (also called *GGS1/TPS1*) encodes a protein of the trehalose synthase complex that affects trehalose accumulation and general glucose sensing by *Saccharomyces cerevisiae* cells. There is considerable debate as to whether *CIF1*-dependent trehalose accumulation is a determinant in heat shock-acquired thermotolerance. Thermosensitivity of *cif1* mutants could alternatively, or also, be related to gene expression-signalling defects in such strains. Because many signal-dependent factors are involved in stress protection and repair in yeast, we have compared the expression of various stress response and heat shock genes in 'isogenic' *CIF1* and *cif1* strains growing exponentially in galactose medium. Transcription of *CTT1, CIF1, HSP26, HSP82, HSP104, SSA4* and *UBI4* was notably lower in the *cif1* mutant following heat shock. Moreover, a single copy of chromosomally integrated *HSP104-lacZ* fusion gave up to 5.5-fold more heat shock induction in the *CIF1* strain compared to the *cif1* mutant. We conclude that reduced heat shock-acquired thermotolerance in *cif1*-deletion mutants growing exponentially on galactose is more likely to result from a general reduction in expression of stress response and heat shock genes, than simply or solely through deficiency of trehalose accumulation. The possible role of *CIF1* in modulating stress response is discussed.**

Key words: Stress response; Heat shock; *CIF1* (*GGS1/TPS1*); Trehalose; Thermotolerance; Yeast (*Saccharomyces cerevisiae*)

1. Introduction

The *CIF1* gene of *Saccharomyces cerevisiae*, which is also known as *BYP1, FDP, GGS1, GLC6, TPS1* and *TSS1*, encodes a 56 kDa protein [1,2]. This protein is a subunit of the trehalose synthase complex and mutants deficient in *CIF1* function are unable to accumulate the disaccharide trehalose [1–5]. Additionally, *cif1* mutants are unable to grow on glucose, fructose, mannose or sucrose, but they are able to utilise galactose, maltose and non-fermentable carbon sources [3,6,7]. Inability of *cif1* mutants to adapt to rapidly fermentable carbon sources is associated with a lack of glucose-induced regulatory effects including induction of cAMP synthesis, induction of glycolytic enzymes, inactivation of gluconeogenic enzymes, phosphatidylinositol turnover, activation of cation transport and stimulation of H^+-ATPase [3,7]. These observations have led to the concept that *CIF1* has a general glucose sensing function that involves interaction with the Ras-adenylate cyclase pathway [6,7].

The physiological function of trehalose in yeast is not fully understood. It may be involved in regulation of glycolysis and sugar-phosphate/free phosphate balance [7,8]. Furthermore, there is some evidence that the disaccharide is involved in protection of cells against stress. Trehalose accumulates in cells exposed to non-optimal physiological conditions such as growth on respiratory carbon sources, nutrient starvation, or exposure to heat shock or noxious chemicals [9–13]. Studies with yeast growing by respiratory metabolism imply that trehalose is significant as an intrinsic stress protectant [4,14,15]. Results with fermenting cells that have been induced for thermotolerance by prior heat shock are, however, equivocal. Whilst some workers assert that trehalose concentration correlates closely with appearance and persistence of thermotolerance [4,16], others claim that trehalose accumulated under such circumstances is insufficient, at least by itself, to explain acquired thermotolerance [15,17–19]. Thus, even though trehalose protects cell-free proteins and lipids against damage by freeze-thaw, high temperature shift and desiccation [20], its role in protecting rapidly fermenting heat-shocked cells in vivo is unclear.

Many of the studies aimed at determining the role of trehalose in stress tolerance of yeast have employed *CIF1* (trehalose-proficient) or *cif1* (trehalose-deficient) strains that are otherwise identical in genetic background. However, because of the pleiotropic effects of *cif1* mutation, it cannot be assumed that trehalose accumulation deficiency is specifically responsible for reduced acquired thermotolerance. Thus, it is important to test whether expression of genes encoding various known heat inducible proteins is similar in *CIF1* and *cif1* 'isogenic' strains. We have therefore analysed expression of various stress response and heat shock genes in isogenic *CIF1* and *cif1* strains in an attempt to separate trehalose from other stress tolerance factors.

2. Materials and methods

2.1. Yeast strains, growth and heat shock conditions

S. cerevisiae strains W303-1A (*a ade2 his3 ura3 leu2 trp1 CIF1*) and WDC-3A (isogenic except for *cif1::HIS3*) [2] were used for studies of stress gene transcription. Strains W303-1A-16, W303-1A-25, WDC-3A-20 and WDC-3A-28 were used to assay *HSP104*-regulated β-galactosidase expression. These strains carry single copies of an in-frame *HSP104-lacZ* gene fusion integrated at *ura3*. To construct the strains, an approximately 800 kb *Kpn*I-*Eco*RI fragment of *HSP104*, which encodes the entire promoter region of the gene plus the first 74 amino acids, was isolated from pYS104 [21]. The fragment was ligated into *Kpn*I-*Eco*RI digested polylinker of the *URA3* integrative *lacZ*-fusion vector YIP358R [22]. This construct was cleaved with *Stu*I within the *URA3* gene and then transformed into W303-1A and WDC-3A with selection for growth on medium without uracil. DNA from transformants was subjected to Southern hybridisation analyses using *HSP104* and *URA3* specific DNAs to determine location and copy number of integrated *HSP104-lacZ*.

Strains were grown for approximately 8 h to mid-exponential respiro-

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fermentative phase at 25°C in GalYP or GYP broth as described previously [14,23]. Heat shock involved transfer of cultures from 25°C to 39°C for 15 min prior to extraction of total RNA, or for 1 h prior to assaying β-galactosidase.

2.2. Extraction of total RNA and mRNA analyses

Total RNA was extracted [24] from control or heat shocked (39°C) yeast, separated by electrophoresis in 1.2% w/v agarose gels with 2.2 M formaldehyde and transferred to nylon membranes [25]. Identical concentrations (10 μg) of total RNAs were loaded for probing separately with either *ACT1* or a specific heat inducible gene, to allow for normalisation of mRNA concentrations to *ACT1* transcript. Specific mRNA transcripts were probed using open reading frames of *ACT1*, *CIF1*, *CTT1*, *HSP26*, *HSP82/90*, *HSP104*, *SSA1*, *SSA4* or *UBI4* genes subcloned into pUC-based vectors. DNA for hybridisation was labelled with [α-^{32}P]dCTP using a Prime-it RmT labelling kit (Stratagene) following the manufacturer's protocol. Hybridisation at 42°C and washing conditions were as described [26]. X-ray film (DuPont Reflection™) was exposed to post-hybridised membranes at room temperature for times ranging from 6 to 96 h. Exposed films were scanned using an UltraScan XL densitometer (Pharmacia LKB), and results processed using ImageMaster (version 1.0) software. Analyses of densitometric scans indicated that films had not been saturated during exposure. Sizes of transcripts were obtained by reference to ribosomal RNA bands in ethidium bromide stained gels. Results presented are typical of two cultures tested in duplicate.

2.3. Assay of β-galactosidase activity

50 ml of either heat shocked or non-heat shocked, control cultures were placed on ice for 5 min prior to harvest of cells by centrifugation at 3000 × *g* and 4°C. Pellets were washed twice in ice cold 25 mM sodium phosphate buffer, pH 6.5 and cells finally resuspended in 1 ml of the same buffer. Cells were homogenised by addition of 1 g glass beads and vortex mixing for 6 × 30 s, with 30 s intervals on ice. Homogenates were centrifuged in Eppendorf tubes at 10 000 × *g* and 4°C for 5 min and cell extract supernatants decanted. β-Galactosidase activity was assayed as follows: 2 ml of 25 mM sodium phosphate buffer, pH 6.5 containing 1.6 mg *o*-nitrophenyl β-D-galactopyranoside was preincubated at 30°C and the reaction started by addition of 0.2 ml cell extract. Reaction mixtures were incubated for up to 15 min and reactions stopped at intervals by addition of 1 ml of 1 M sodium carbonate. Absorbances were read at 420 nm against a blank without added cell extract. Protein assays were carried out on cell extracts [27] and β-galactosidase activities calculated as 420 nm absorbance units/min per mg protein. Results shown are means with standard deviations of triplicated experiments.

3. Results and discussion

Differences in *CIF1* and *cif1* isogenic yeast strains are broader than a mere proficiency or deficiency in trehalose accumulation ([3,6–8]; unpublished data of this laboratory). If *CIF1* is involved in key regulatory processes, it might affect the heat shock induction of stress response genes. In order to establish whether this is so, we measured transcription of various stress response and heat shock genes by Northern analyses and normalised these to *ACT1* (actin) mRNA levels measured in the

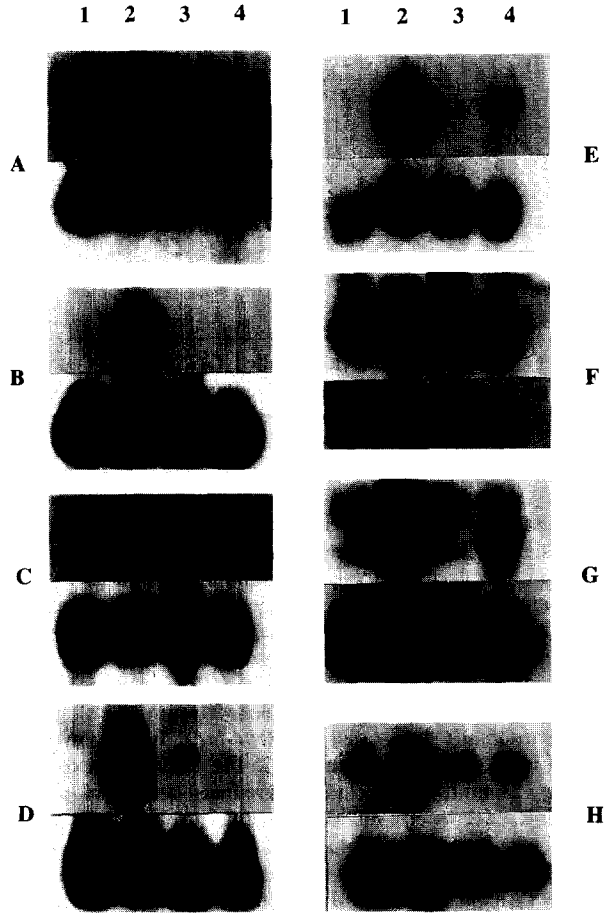

Fig. 1. Transcription of heat inducible genes before and after heat shock in *S. cerevisiae* strains with functional or deleted *CIF1* activity. Strains W303-1A (*CIF1*) and WDC-3A (*cif1::HIS3*) were grown to mid-exponential phase in GalYP medium at 25°C and subjected to heat shock for 15 min at 39°C. Total RNA was probed separately for *ACT1* or other specific mRNA transcripts. Lanes: 1, W303-1A no heat shock; 2, W303-1A heat shocked; 3, WDC-3A no heat shock; 4, WDC-3A heat shocked. The lower panels in each alphabetical set of photographs show *ACT1* transcript present in the same amount of total RNA extract also probed for heat inducible genes. A, *CIF1*; B, *CTT1*; C, *HSP26*; D, *HSP82/90*; E, *HSP104*; F, *SSA1*; G, *SSA4*; H, *UBI4*.

Table 1
Transcription of different heat shock and stress response genes in *S. cerevisiae* strains with functional or deleted *CIF1* activity

Strain and conditions	*CIF1*[a]	*CTT*	*HSP26*	*HSP82/90*	*HSP104*	*SSA1*	*SSA4*	*UBI4*
W303-1A *CIF1* at 25°C	0.08	ND	ND	ND	ND	1.08	0.14	0.12
W303-1A *CIF1* at 39°C for 1 h	0.46	0.56	0.58	1.79	1.07	2.32	2.77	0.56
WDC-3A *cif1::HIS3* at 25°C	0.03	ND	ND	ND	ND	3.26	0.16	0.12
WDC-3A *cif1::HIS3* at 39°C for 1 h	0.11	ND	0.09	ND	0.14	4.06	1.40	0.29

Levels of heat inducible gene transcripts were normalised to *ACT1* mRNA in the same extracts (Fig. 1). ND indicates transcripts not detected.
[a]*CIF1* mRNA in WDC-3A was detected as a 1.1 kb truncated transcript encoded by the insertionally deleted *cif1::HIS3* locus [2]. *SSA4* transcript is represented by the lower, approx. 2.0 kb, bands in the upper panel of Fig. 1G. The larger, approx. 2.7 kb, and less concentrated transcripts seen in the same panel, represent non-specific binding. These were omitted as background in densitometric scanning.

same RNA preparations. There was no detectable transcription of *CTT1, HSP26, HSP82/90, HSP104*, and only low level transcription of *CIF1, SSA4* and *UBI4* in either *CIF1* or *cif1::HIS3* cells growing exponentially in galactose medium at 25°C (Fig. 1, Table 1). When strains were heat shocked at 39°C for 15 min. W303-1A *CIF1* showed generally higher levels of transcription of *CIF1, HSP26, HSP104, SSA4* and *UBI4* than the mutant WDC-3A *cif1::HIS3* (Fig. 1, Table 1). Moreover, the *CIF1* strain showed significant induction of *CTT1* and *HSP82/90* transcription following heat shock, but the *cif1::HIS3* mutant still failed to transcribe these genes at detectable levels (Fig. 1, Table 1). Thus, there appears to be a general dependence on *CIF1* function for efficient expression of heat shock and stress tolerance genes following heat shock treatment. An exception to this amongst the genes we tested was *SSA1*, which was significantly transcribed in non-heat shocked cells growing exponentially on galactose, and significantly induced following heat shock, regardless of *CIF1* functionality (Fig. 1, Table 1). Appearance of significant levels of *SSA1* transcript in non-heat shocked cells is not surprising since *SSA1* is known to be expressed at high levels relative to other heat shock genes under non-stressed conditions [28]. The significance our finding that it is present at greater levels in the *cif1::HIS3* strain is unclear, but it could relate to generally defective carbon metabolism and altered physiological status of such mutants even when growing on galactose ([6,7]; unpublished data of this laboratory). *SSA1* is a member of a subfamily of 70 kDa proteins implicated in protein translocation across endoplasmic reticulum and mitochondrial membranes, regulation of the overall stress response in the cell, degradation of misfolded proteins, and possibly as a chaperone for nascent polypeptides as they are synthesized at the ribosomal complex [28,33]. We speculate that the defective metabolism of *cif1* mutants results in a greater need for function of genes such as *SSA1*.

To test whether generally reduced basal and heat shock-induced transcription of stress response and heat shock genes in the *cif1::HIS3* mutant is manifest as lowered level of translation, we utilised an *HSP104-lacZ* promoter fusion. This was integrated as a single copy into the *URA3* loci of the *CIF1* and *cif1* strains, which were then grown at 25°C in galactose medium and assayed for β-galactosidase before and after heat shock at 39°C. Heat shock induction ratios were 2.9–5.5-fold greater in the W303-1A *CIF1* derivatives than in the WDC-3A *cif1* mutants (Table 2). W303-1A derivatives grown in glucose medium gave β-galactosidase induction ratios of 7.8 (isolate number 16) and 9.70 (isolate number 25) (data not shown). Notably, β-galactosidase activities in control, non-heat shocked W303-1A derivatives grown in glucose were about 50% of those measured in galactose grown cells, and activities obtained after heat shock were similar following growth in GYP or GalYP (results not shown). The greater induction ratio in glucose medium was therefore associated with reduced basal expression of *HSP104-lacZ* in GYP relative to GalYP. Other workers have found greater levels of hsp104 protein in non-heat shocked cells growing on galactose or respiratory carbon sources [29]. Thus we consider that the *HSP104-lacZ* fusion is an accurate model system.

It has been reported recently that *HSP26* expression is reduced in *ggs1/tps1* (*cif1*) deletion mutants, which corroborates our findings [30]. The implication is that reduced acquired thermotolerance in heat-shocked *cif1* mutants is likely to result from generally lowered stress response and expression of key protection/repair factors such as hsp104 [29] and catalase T [32] rather than simply or solely through trehalose deficiency. We therefore propose that whilst thermotolerance of respiratory phase yeast may be significantly influenced by trehalose concentration [14,15], fermentative phase yeast exposed to mild heat shock treatment acquire thermotolerance through broader functions of *CIF1* rather than solely through its trehalose synthetic activity.

How *CIF1* influences general stress response is unclear. Activation of genes by stress conditions involves the presence of specific sequences within their promoter regions. These include HSE (heat shock elements [31]) and STRE (stress response elements [32]). Analysis of *CIF1, CTT1, HSP26, HSP82, HSP104, SSA1, SSA4* and *UBI4* indicates that they all carry HSE and, with the exception of *SSA1*, STRE sequences in their promoters (EMBL sequence data base). STRE appears to be required for Ras-adenylate cyclase-dependent transcriptional regulation of stress genes [32]. Cyclic AMP levels are relatively high in cells growing exponentially on fermentable sugars and transcription of genes with STREs is down-regulated [6]. Thus, under conditions of unstressed, fermentative growth, there is only very low transcription of *CIF1* and other STRE-influenced genes (Fig. 1, Table 2; unpublished data of this laboratory). Absence of STRE sequences in the *SSA1* promoter region explains why this gene can be transcribed at relatively high levels when cells are growing unstressed on glucose or galactose (Fig. 1, Table 1 [28]). We propose that when cells are exposed to stress or non-optimal growth conditions, *CIF1* positively modulates expression of itself and other heat inducible genes. It could do this, for example, by functioning in overriding negative cAMP-dependent transcriptional regulation. Alternatively, or as well, the role of positive-acting transcriptional regulators that bind HSEs and STREs might be somewhat *CIF1*-dependent.

The molecular basis of *CIF1* function remains unknown, but it is evident that it has extremely wide ranging effects on cellular

Table 2
Heat shock-induced expression of β-galactosidase encoded by a single integrated *HSP104-lacZ* fusion in *S. cerevisiae* strains with functional or deleted *CIF1* activity

Strain genotype	W303-1A-16 *CIF1*	W303-1A-25 *CIF1*	WDC-3A-20 *cif1::HIS3*	WDC-3A-28 *cif1::HIS3*
Control, non-heat shocked cells at 25°C	0.13 (0.05)	0.11 (0.05)	0.14 (0.06)	0.20 (0.08)
Heat shocked cells at 39°C for 1 h	0.35 (0.06)	0.33 (0.03)	0.13 (0.06)	0.11 (0.01)
Induction ratio	2.69	3.00	0.93	0.55

Numbers are means of specific activities (420 nm absorbance units/min per mg protein) derived from three separate experiments. Figures in brackets are standard deviations. Induction ratios were calculated by dividing the specific activity measured after heat shock by specific activity measured in control, non-heat shocked cells.

metabolism, not only in terms of general glucose sensing [6,7], but apparently in general stress response too. The complex phenotype of *cif1* mutants lends itself to speculation that *CIF1* protein may be a broadly acting modulator of regulatory/ signalling processess in yeast cells, rather than being a specific glucose-sensing factor.

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