Investigation into the mechanism of action and biological role of *Saccharomyces cerevisiae* mannoproteins which reduce visible haziness in white wine

by

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DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Shauna L Brown

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THESIS SUMMARY

Heat induced protein haze is a common problem in white wine. Grape derived pathogenesis related proteins slowly denature and aggregate during wine storage and this gives rise to light dispersing haze. Protein haze formation is currently prevented by removing proteins using bentonite, an aluminium silicate clay, but this method has drawbacks. A potential alternative or complementary method is the use of haze protective factors (HPF), specific mannoproteins from *Saccharomyces cerevisiae* that visually reduce protein haze.

Hpf1p was originally isolated from Muscat Gordo Blanco wine and Hpf2p from a synthetic grape juice ferment. Based on partial amino acid sequences, putative structural genes, *HPF1* and *HPF2*, for these proteins were identified. *HPF1* has a homologue, *HPF1'*, (71% similarity) in *S. cerevisiae*. Sequence analysis suggests that Hpf1p, Hpf1'p and Hpf2p are localised to the cell wall or plasma membrane.

This study aimed to determine the biological function of the *HPF* genes in *S. cerevisiae*. *HPF* overexpression and deletion strains were constructed and analysed for cell wall related phenotypes. Under a number of conditions, including cold temperature and ethanol stress, the *hpf1Δ hpf1'Δ* strain was more tolerant than the wild type strain. However, mating efficiency of the *hpf1Δ hpf1'Δ* strain was significantly less than the wild type strain and this was found to be correlated with the persistence of a septum between the mating partners. The decreased mating efficiency was also mating type specific, only occurring in *MAT*α cells.

This study also aimed to establish conclusively that the *HPF* genes do indeed encode proteins with haze protective properties. Haze protective activity of the material from ferment supernatants was assessed. Material from the *HPF* deletion strains exhibited significantly less haze protective activity than the wild type. Moreover, material derived from *HPF1* and *HPF1'* overexpressors was more active than material from the wild type. A 6xHis-tagged Hpf2p was expressed and purified using immobilised metal affinity chromatography. This Hpf2p had significant haze protective activity. Modification of *N*-glycans of 6xHis-Hpf2p by Endoglycosidase H decreased its haze protective activity.

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ABBREVIATIONS

6xHis	six consecutive histidine amino acids
AWRI	Australian Wine Research Institute
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
cDNA	complementary deoxyribonucleic acid
CDGJM	chemically defined grape juice medium
Da	Dalton
DIC	direct interference contrast
DNA	deoxyribonucleic acid
Endo H	Endoglycosidase H
ER	endoplasmic reticulum
FARA	flexible approach to random analysis
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GluNAc	*N*-acetylglucosamine
GPI	glycosylphosphatidylinositol
HPF	haze protective factor
IMAC	immobilised metal affinity chromatography
kb	kilobase
kDa	kilo Dalton
mA	milli-Amps
Man	mannose
MAP	mitogen activated protein
MEN	mitotic exit network
M-Pol	mannan polymerase
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
NBT	nitro blue tetrazolium
Ni-NTA	nickel-nitrilotriacetic acid

ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
PI(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
PNGase F	peptide-*N*-(acetyl-β-glucosaminyl) asparagine amidase
PR	pathogenesis related
PRE	pheromone response element
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SC	synthetic complete
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS-T	tris buffered saline-Tween 20
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)aminoethane
V	Volts
YPD	yeast extract/peptone/dextrose medium or Yeast Protein Database

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Heat induced protein haze is a common problem for makers of white wine (Høj *et al.*, 2000). It is caused by pathogenesis-related proteins of *Vitis vinifera*, namely thaumatin-like proteins and chitinases, which slowly denature and aggregate, resulting in light dispersing particles. Currently, haze formation is prevented in commercial wines by removing the proteins by absorption onto bentonite, but this method has drawbacks. The use of so called haze protective factors, almost certainly represented by at least two classes of mannoproteins from the yeast *Saccharomyces cerevisiae*, is potentially a novel and feasible alternative method for reducing or even preventing haze formation in white wines. The biological role of these mannoproteins in *S. cerevisiae* is unknown. The research described in this thesis aims not only to firmly establish the role of the two classes of mannoproteins as haze protective factors but also to elucidate the biological function of these gene products.

1.2 HAZE

1.2.1 Types of Haze

Wine quality is determined by many factors including palate characteristics, aroma characteristics, colour and physical condition. For white wine, clarity is an essential aspect in determining quality. A microbial haze can form from microorganisms that are present at bottling (Van de Water, 1985). Haze can also be caused by the crystallisation of salts, including potassium bitartrate (Dunsford and Boulton, 1981; Rodriguez-Clemente *et al.*, 1990) and calcium tartrate (Clark *et al.*, 1988), by polysaccharides (Vernhet *et al.*, 1996) and by polyphenolic compounds (Siebert, 1999; Siebert *et al.*, 1996). Protein haze formation is always a potential problem that can occur after bottling (Figure 1.1). Slow denaturation of wine proteins may result in aggregation of the proteins into light dispersing particles (Waters *et al.*, 1993). Heating accelerates this process, as does the reaction of proteins with polyphenolic compounds (Siebert *et al.*,

Figure 1.1 Protein haze formation in white wine. The glass on the left shows wine that has not been bentonite fined and protein haze has formed after heating. The wine on the right has been bentonite fined and protein haze formation has been prevented.

1996). Currently, winemakers use various methods to prevent protein haze formation, but each of these has its drawbacks, as described in Section 1.3.

1.2.2 Heat Unstable Proteins

Early studies on wine proteins suggested that proteins with low isoelectric point (pI) and low relative molecular mass (M_r) were most important in haze formation (Bayly and Berg, 1967; Moretti and Berg, 1965). More recent work has generally supported these early investigations. Hsu and Heatherbell, (1987a and b) and Waters *et al.*, (1991 and 1992) showed that protein fractions of M_r 12600 and 20000-30000 with a low pI, between 4.1-5.8, are heat unstable and thus make a significant contribution to instability of wine.

Further investigation found that the proteins that cause wine instability were relatively stable in the short to medium term and that, under conventional winemaking conditions, these proteins are resistant to proteolysis (Waters *et al.*, 1992). Waters *et al.*, (1996), identified these ‘nuisance’ proteins as pathogenesis-related (PR) *Vitis vinifera* proteins present in both the pulp and skin of grapes.

Two of the major grape PR proteins isolated from wine were identified by amino acid and cDNA sequencing as a thaumatin-like protein (Tattersall *et al.*, 1997; Waters *et al.*, 1996) and a chitinase (Robinson *et al.*, 1997; Waters *et al.*, 1998; Waters *et al.*, 1996). Thaumatin-like proteins and chitinases were found to constitute the major soluble protein component of grapes from all *V. vinifera* varieties studied so far (Pocock *et al.*, 2000; Tattersall *et al.*, 1997). These proteins are synthesised during normal fruit development (Tattersall *et al.*, 1997). The level of these two groups of PR proteins increases at the onset of vèraison (berry softening) and continues until the berries reach maturity, therefore, the riper the berries used to make the wine, the higher the haze-forming potential (Pocock *et al.*, 2000; Robinson *et al.*, 1997; Tattersall *et al.*, 1997).

1.3 REMOVAL OF HEAT UNSTABLE PROTEINS

1.3.1 The use of bentonite as the protein fining agent

Bentonite addition is the most widely used procedure to achieve protein stability in wine (Høj *et al*., 2000). Bentonite is a hydrated aluminium silicate clay that exists in small plates (Rankine, 1987). Sodium bentonite, with its monovalent ions, is preferred in the wine industry over calcium bentonite since calcium bentonite has a poorer adsorption capacity attributed to less extensive swelling (Blade and Boulton, 1988). Upon addition to wine, exchange of sodium or calcium cations and positively charged proteins can occur (Blade and Boulton, 1988).

The level of heat unstable protein in wine may range from 50-100 mg/L, but occasionally reaches up to several hundred mg/L (Høj *et al*., 2000). Dawes *et al*., (1994), showed that a Gewürztraminer wine with 240 mg/L total soluble protein content was heat stable after fining with 1300 mg/L bentonite that reduced the protein level to 31 mg/L. In another four wines with initial concentration of protein ranging from 19 to 44 mg/L, stability was achieved when the protein concentration was reduced to ~5 mg/L (Hsu and Heatherbell, 1987a).

The use of bentonite to remove protein from wine has disadvantages. Protein stabilisation of wine by bentonite has been postulated to result in a loss of aroma and flavour (Leske *et al*., 1995; Miller *et al*., 1985; Simpson, 1986). This is because bentonite is not specific in its interactions with wine constituents, thus compounds other than protein, such as fermentation esters and alcohols, can be removed as a result of bentonite fining (Miller *et al*., 1985; Somers and Ziemelis, 1973). Due to bentonite's extensive swelling and inferior settling capabilities, between 3% and 10% of the wine volume can be lost as bentonite lees (Tattersall *et al*., 2001). Most of the wine can be recovered from the lees by rotary-drum filtration, but the quality may be significantly lowered because oxidation can occur during the process (Rankine, 1987). In general, a loss of 3% volume is considered a reasonably accurate estimate by major wine producers (D. McWilliam, T. James, personal communication). The estimated cost of bentonite fining to the wine industry worldwide is US $300-500 million per annum (Høj *et al*., 2000).

1.3.2 Alternatives to the use of bentonite for protein fining

Ultrafiltration has been considered as an alternative to bentonite fining. This method has been found to be effective in removing soluble protein from grape juice and wine, but even with the use of membranes with a molecular weight cut off as low as 10 kDa, small amounts of heat unstable proteins remain in the filtered wine (Flores *et al*., 1990; Hsu *et al*., 1987), which generally results in heat instability. Ultrafiltration has also been reported to result in some loss of aroma compounds (Miller *et al*., 1985; Simpson, 1986). High establishment and running costs have also made ultrafiltration unattractive to the wine industry as a means to remove heat unstable protein.

Proteolytic enzymes have been used for more than 40 years to prevent protein chill haze in beer (Sharpe, 1982) and their use has been suggested as an alternative to bentonite fining in wine. Lagace and Bisson (1990) showed that proteolytic enzymes from various yeast strains were active in wines at 37^{o}C and Pocock *et al*., (2003), demonstrated that high temperature for a short time period (90^{o}C for one minute) or moderate temperature (45^{o}C for one day) coupled with proteolytic enzyme addition reduced the protein concentration of treated wine considerably. However, under winemaking conditions, at 15^{o}C, proteolytic enzymes were not able to hydrolyse wine proteins responsible for haze (Waters *et al*., 1995; Waters *et al*., 1992).

1.4 HAZE PROTECTIVE FACTOR

A polysaccharide-rich fraction isolated from Muscat Gordo Blanco wine was shown to reduce the visible turbidity formed upon heating of wine protein fractions (Waters *et al*., 1991). The use of this so called haze protective factor (HPF) may be an alternative or, at the very least a complement to the currently used methods to prevent haze formation due to heat unstable proteins.

The fraction with haze protective activity was isolated from Muscat Gordo Blanco wine by affinity, anion and cation exchange chromatography (Waters *et al*., 1993). This mannoprotein fraction was of high molecular weight and was able to protect against haze caused by wine proteins or bovine serum albumin (BSA) when either was heated in white wine (Waters *et al*., 1993). Carbohydrate and amino acid analysis has indicated that the mannoprotein is derived from the yeast cell wall (Waters *et al*., 1994a). Haze protective activity was also exhibited by a crude mannoprotein fraction isolated from yeast cell walls (Dupin *et al*., 2000a). Investigation of methods to extract Hpf from yeast and immunological studies of the location of Hpf demonstrated that Hpf was located in the cell wall (Dupin *et al*., 2000a).

A haze protective mannoprotein was subsequently isolated and purified from 600 litres of Carignan Noir wine in milligram amounts (Waters *et al*., 1994a) and is referred to as Hpf1p. Hpf1p is apparently analogous to the HPF isolated from Muscat Gordo Blanco wine described above (Waters *et al*., 1994a). The amino acid sequence of the protein portion of Hpf1p was determined and, from this, a putative structural gene in *S. cerevisiae,* YOL155c (*HPF1*), has been identified, (Waters *et al*., unpublished) (Appendix 1). This gene has a homologue, YIL169c, which will be referred to as *HPF1'* (Appendix 1).

A second haze protective mannoprotein (Hpf2p) was isolated by ethanol precipitation from a chemically defined grape juice fermented by the wine making strain of *S. cerevisiae*, Maurivin™ PDM. A putative structural gene has been identified for Hpf2p, YDR055w, from the amino acid sequence (Stockdale, 2000) (Appendix 1). Methylation analysis of the carbohydrate component of Hpf2p showed the presence of (1→2), (1→3) and (1→2, 1→6) mannosyl residues suggesting that *N*- and *O*-glycosylation are present. PNGase F digestion of Hpf2p resulted in a decrease in molecular weight, confirming the presence of *N*-linked mannosylation. Removal of *N*-linked glycans reduced, but did not eliminate, the haze protective activity of Hpf2p (Stockdale, 2000).

The haze protective effect of yeast mannoproteins was independently confirmed by Ledoux *et al*., (1992), when they showed that white wine aged on yeast lees had reduced haze potential and lower bentonite requirements than white wine aged without lees contact. The wine contained the same amount of total protein as white wine that had not been aged on lees. A mannoprotein fraction isolated from yeast cell walls was shown to protect against haze (Ledoux *et al*., 1992). Moine-Ledoux and Dubourdieu, (1999), showed that the mannoprotein responsible was a fragment of invertase from *S. cerevisiae*. The susceptibility of wine to haze formation and hence bentonite fining requirements can be reduced by the addition of this fragment. Other glycoproteins have been shown to exhibit haze protective activity including whole yeast invertase (McKinnon, 1996; Moine-Ledoux and Dubourdieu, 1999), a wine arabinogalactan protein (Waters *et al*., 1994b), gum Arabic and an apple arabinogalactan protein (Pellerin *et al*., 1994).

The exact mechanism by which mannoproteins afford haze protection is unclear. Waters *et al*., (1993), determined that the presence of mannoproteins in the wine decreased the particle size of the haze rather than prevented aggregation of the wine proteins. It was shown that with the addition of an unpurified yeast mannoprotein fraction, the particle size of the haze formed upon heating was reduced from 30 μm to 5 μm, making the haze barely detectable to the naked eye (Waters *et al*., 1993). McKinnon, (1996), showed that invertase from *S. cerevisiae* was present in the wine after heating and removal of the haze. It was suggested that, since the majority of invertase was in the supernatant, haze protective factors act by competing with other wine proteins for wine components required for the formation of large insoluble aggregations of denatured protein (McKinnon, 1996).

1.5 THE SECRETORY PATHWAY IN YEAST

Many yeast mannoproteins are localised in the cell wall or plasma membrane (Costanzo *et al*., 2000). Following translation on the ribosomes, they are directed through the secretory pathway. The secretory pathway deals with a large number of proteins, for example, approximately 2000 of the 6000 Open Reading Frames (ORFs) in the *S. cerevisiae* genome are predicted to encode membrane proteins (Cherry *et al*., 1997). Proteins destined for the cell surface are directed through the endoplasmic reticulum (ER) and Golgi complex which, together with the vesicles that transport proteins between these two organelles, comprise the secretory pathway (Sutterlin *et al*., 1997). On the way to their final destination, proteins are sorted, modified and assembled into complexes in the ER and Golgi.

1.5.1 The Yeast Cell Wall

Previously, the yeast cell wall was considered a static, rigid exoskeleton whose sole purpose was to give structure to the organism. More recently, it has been realised that the cell wall is a dynamic organelle that changes depending upon the physiological status of the cell (Popolo and Vai, 1999; Stratford, 1994). The prime functions of the cell wall are osmotic and physical protection of the plasma membrane and cytoplasm, but it also represents a selective permeability barrier and support for immobilised enzymes. The cell wall functions in cell-cell recognition and adhesion especially during mating and flocculation (Stratford, 1994).

The cell wall constitutes 15-25% of the dry weight of the cell (MacWilliam, 1970). It contains β1,3-glucan (~50% (w/w)), mannan (~35%), β1,6-glucan (~5%), chitin (~1-2%) which is mostly found in bud scars (Cid *et al.*, 1995), and proteins (~10%). The protein content of 10% (w/w) includes the protein moieties of all the mannoproteins for cellular functions such as adhesion and cell recognition (Fleet, 1991). The glucan and chitin give form and strength to the cell wall and the mannoproteins are suggested to act as a barrier to retain periplasmic proteins (Kapteyn *et al.*, 1999) and protect the cell against damaging enzymes from the exterior (Lipke and Ovalle, 1998).

1.5.2 Sorting of proteins through the secretory pathway

Sorting begins with the co-translational recognition of a signal sequence at the amino-terminus of the protein. These signal sequences can be extremely degenerate, but the hydrophobic character of the amino acids for direction into the ER is conserved. The signal sequence determines whether a protein will translocate co- or post-translationally (Brown and McDonald, 1999). The more hydrophobic a signal sequence is, the more chance there is of the protein being translocated co-translationally. Several important processes occur in the ER including signal sequence cleavage, *N*- and *O*-glycosylation, protein folding and addition of glycosylphosphatidylinositol (GPI) anchors (Brown and McDonald, 1999).

Cleavable amino-terminal signal sequences are precisely removed by signal peptidase. Signal peptidase activity is essential since deletion of genes encoding components of the complex such as *SEC11* or *SPC3* results in lethality (Bohni *et al.*, 1988; Fang *et al.*, 1997).

The *HPF* genes, *HPF1* and *HPF2*, have predicted amino-terminal hydrophobic signal sequences (Figure 1.2) that direct the protein's translocation across the ER (Caro *et al.*, 1997). According to the computer algorithm of Caro *et al.*, (1997), *HPF1'* is not predicted to have an N-terminal secretion signal sequence. This is most likely due to the limitations of the program used to identify N-terminal signal sequences, which is only 75-80% accurate (von Heijne, 1986).

1.5.3 Glycosylation in the endoplasmic reticulum

Mannoproteins contain *N*- and *O*-linked glycans. As with all eukaryotes, the glycan structures are progressively elaborated in the secretory pathway. *N*-linked oligosaccharides have *N*-acetylglucosamine linked to asparagine (Asn) and *O*-linked sugars have a mannosyl residue from a dolichol-phosphate donor linked to serine (Ser) or threonine (Thr) (Haselbeck and Tanner, 1983). The *N*-linked sugars in yeast have a core structure with up to 200 mannose molecules attached on the outer chain, known as 'mannan.' Mannan consists of a long α-1,6-linked backbone of approximately 50 residues to which short branches of 3-4 residues are attached. The first two mannose residues of these branches are α-1,2-linked and the final residue is α-1,3-linked. Some branches are modified with the addition of a phosphomannose residue (Dean, 1999). *O*-linked oligosaccharides are shorter with only four or five mannose molecules.

N-linking initiates when a 14-sugar residue, $Glc_3Man_9GlcNAc_2$ is transferred, *en bloc*, from the lipid carrier dolichyl phosphate to a selected Asn within the sequence Asn-X-Ser/Thr (where X is any amino acid except proline) of the nascent polypeptide. This process occurs in the ER and is catalysed by the oligosaccharyltransferase complex (Knauer and Lehle, 1999). After transfer and prior to transport to the Golgi, three glucose molecules are cleaved and a specific mannose is removed resulting in $Man_8GlcNAc_2$.

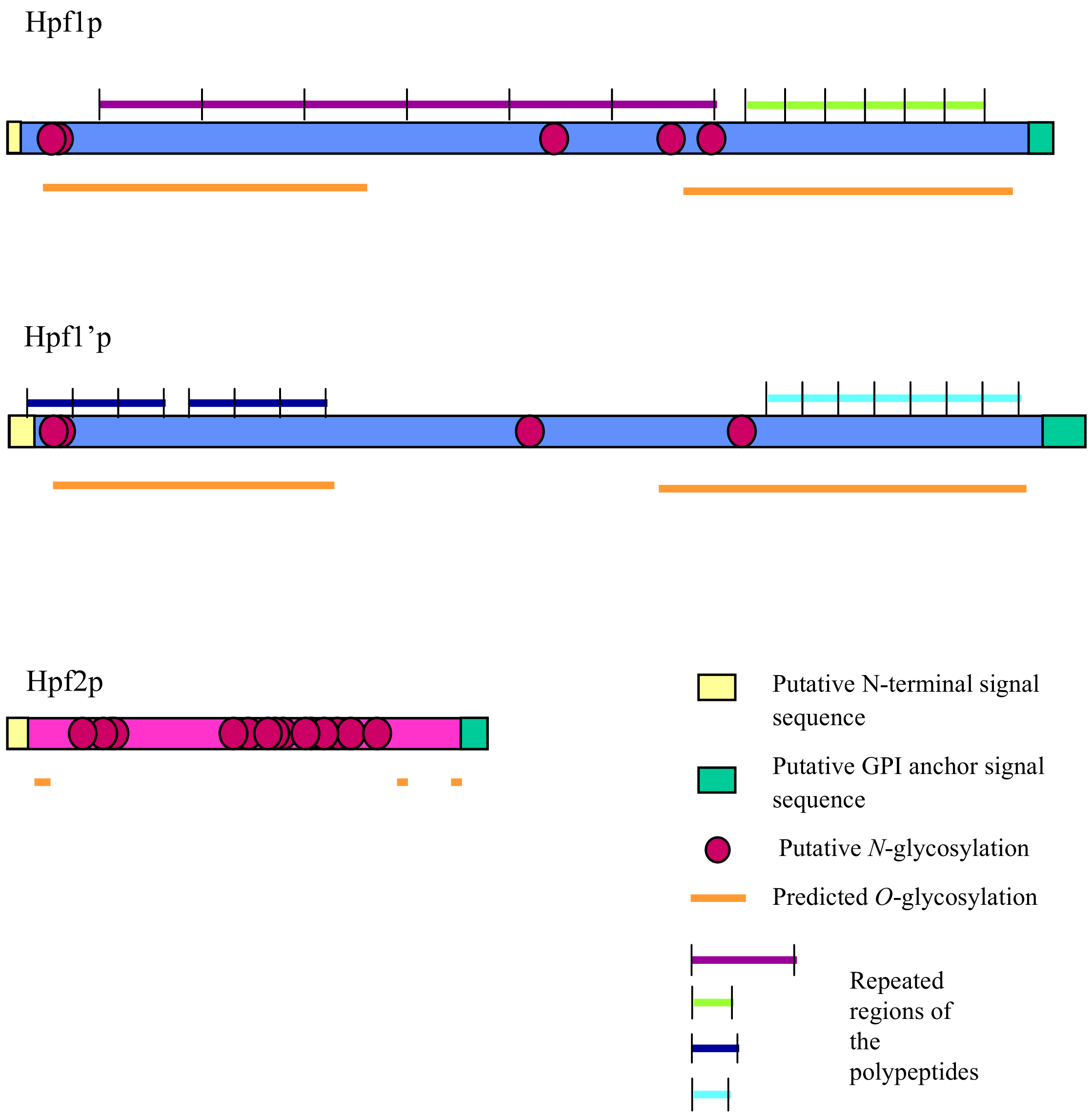


Figure 1.2 The predicted motifs and repeated regions of the Hpf proteins.

O-mannosylation begins in the ER with dolichyl phosphate-bound mannose serving as the mannose donor in the initial transfer reaction catalysed by mannosyltransferases (Pmt1p/Pmt2p). An α-D-mannosyl linkage is formed when this mannose is attached to serine or threonine residues of secretory proteins (Strahl-Bolsinger *et al.*, 1999). The donor of subsequent mannosyl residues is GDP-mannose (guanosine diphosphate-mannose). A minimum level of *O*-glycosylation has been found to be necessary for yeast cells to grow normally. Double or triple *pmt* mutants have been found to be either lethal, only able to grow when osmotically stabilised, or to be sensitive to cell wall destabilising agents (Gentzsch and Tanner, 1996). *O*-linked mannosylation is often required for stabilisation or correct localisation of proteins and may influence protein function.

1.5.4 Glycosylphosphatidylinositol (GPI) anchors

The addition of GPI anchors to proteins with the appropriate signal sequence is essential for direction to the cell wall or plasma membrane, and cells with defective GPI anchoring mechanisms have defects in cell wall synthesis. GPI proteins have a carboxy-terminal signal sequence which, like the amino-terminal signal sequence, is extremely degenerate, but does have some characteristic features (Figure 1.3). The residue to which the GPI anchor is attached, the ω site, and the ω+2 residue are always small, usually alanine or glycine (Gerber *et al.*, 1992; Hamada *et al.*, 1998; Nuoffer *et al.*, 1993). Eight to 12 amino acids downstream of the ω site, at or very near to the C-terminus, are 15 to 20 hydrophobic amino acids (Englund, 1993; McConville and Ferguson, 1993; Takeda and Kinoshita, 1995). The amino residues upstream of the ω site (ω-minus region) determine the final location of a GPI anchored protein (Hamada *et al.*, 1998; Hamada *et al.*, 1999). Valine, isoleucine or leucine at ω-4 or ω-5 and tyrosine or asparagine at ω-2 are signals for cell wall localisation, and a dibasic residue motif in the ω-minus region is also required for plasma membrane localisation (Hamada *et al.*, 1999). In contradiction to this, De Sampaio *et al.*, (1999), suggests that GPI anchors have a necessary but not sufficient role in cell wall targeting of proteins. Thus, hybrid proteins containing an α-galactosidase marker at the N-terminus and the C-terminus of the plasma membrane proteins Gas1p or Yap3p are not retained in the plasma membrane as would be expected if the dibasic motif was sufficient for this retention.

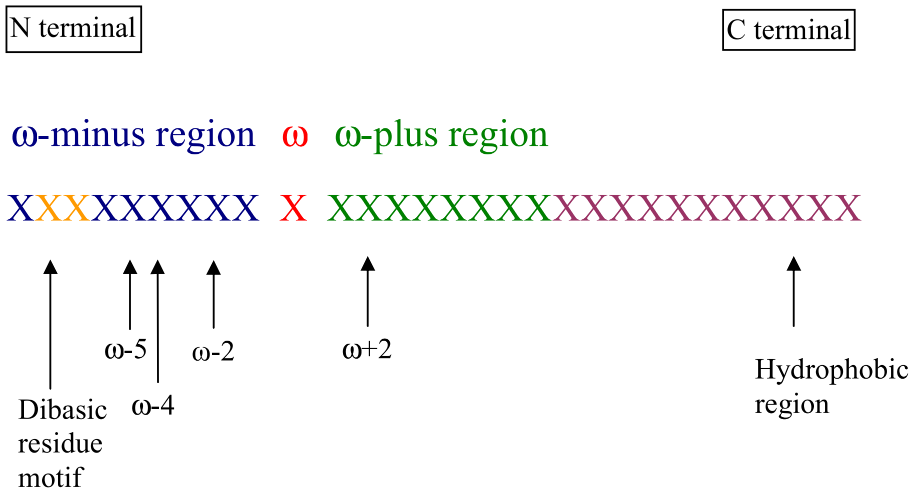


Figure 1.3 GPI anchor signal sequences in yeast as proposed by Hamada *et al*., (1999). It is proposed that the GPI anchor is attached to the protein at the ω site. The amino acids in the ω-minus region determine the final location of the protein (Hamada *et al*., 1999).

This implies that a more distant additional sequence is necessary (De Sampaio *et al*., 1999). Different sequence contexts in this domain apparently result in a continuum of residencies and thus the idea of a purely cell wall or plasma membrane GPI protein is unlikely (Huang *et al*., 2003).

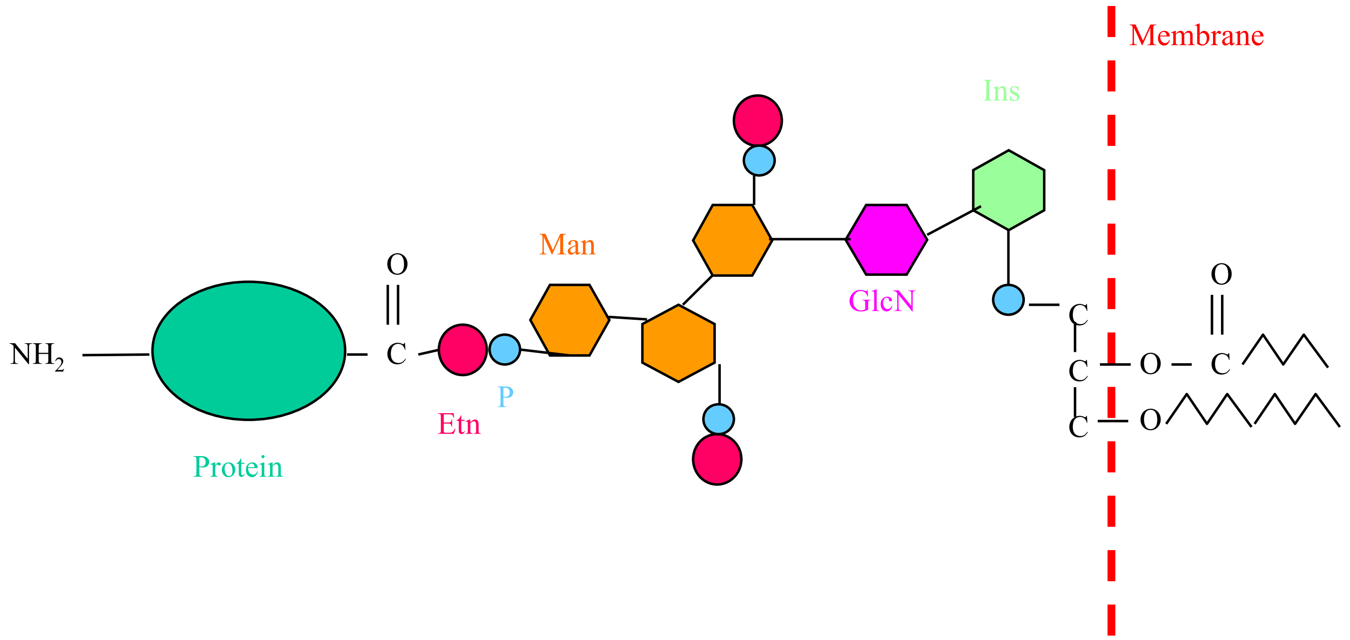
During GPI anchor attachment, the C-terminal sequence is cleaved from the peptide at the ω site and replaced with a pre-assembled GPI anchor precursor (McConville and Ferguson, 1993; Takeda and Kinoshita, 1995) by a GPI protein transamidase complex (Hamburger *et al*., 1995) in the luminal face of the ER (Hamada *et al*., 1998). Synthesis of the GPI anchor precursor occurs stepwise in the ER (Takeda and Kinoshita, 1995).

1.5.5 Further glycosylation in the Golgi apparatus

Following correct attachment of the GPI anchor to the protein in the ER (Figure 1.4), proteins are again sorted and transported to their correct destination by vesicular trafficking. The cell surface proteins are transported to the Golgi complex but their pathway through the Golgi remains unclear. During the process some glycosyl side-chain addition and modification does occur. All enzymes involved in elongation of *O*-linked chains have been either shown or predicted to be located in the Golgi (Strahl-Bolsinger *et al*., 1999). Ktr1p, Ktr3p and Kre2p/Mnt1p have been shown to be involved in the transfer of the second α-1,2-linked mannosyl residue (Figure 1.5). Other members of the Ktr, Mnn and Mnt mannosyltransferase families have been shown to participate in *O*-mannosyl synthesis (Lussier *et al*., 1999; Strahl-Bolsinger *et al*., 1999).

N-linked mannan cores are then extensively ‘decorated’ by the addition of mannan outer chains to the core. The first mannose connected to the inner core, which is attached to the Asn residue, with an α-1,6-linkage in the Golgi apparatus by the Och1p mannosyltransferase (Figure 1.6). This mannose is attached to all *N*-linked glycans but only a select group is extended further with α-1,6-linked mannosyl residues by the mannan polymerases, M-Pol I and M-Pol II, to form the mannan backbone. Those which will be elaborated further have an α-1,2-linked mannose residue added and α-1,3-linked residues will be added further. Branches on the mannan backbone are

Figure 1.4 The structure of a Glycosylphosphatidylinositol (GPI) anchored protein. Etn = ethanolamine, P = phosphate, Man = mannose, GlcN = glucosamine, Ins = inositol. This figure was adapted from Takeda and Kinoshita (1995).


Membrane
Ins
Man
GlcN
NH$_2$
O
C
Etn
P
Protein
O
C
C
C
O
C
O

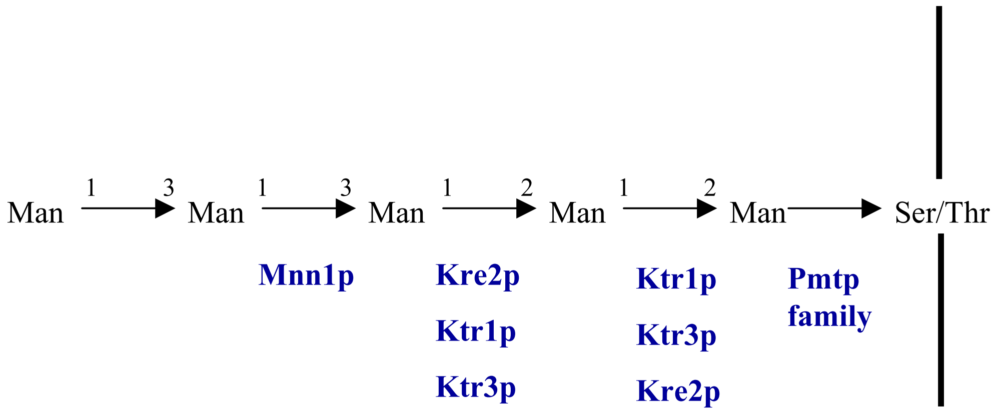


Figure 1.5 *O*-glycosylation in yeast. Generally, *O*-glycosylation consists of five mannose residues linked to either a serine or threonine amino acid in the protein backbone. The arrows indicate α-1,3 or α-1,2 linkages between Man (mannose) residues. This figure was taken from Lussier *et al*., (1999).

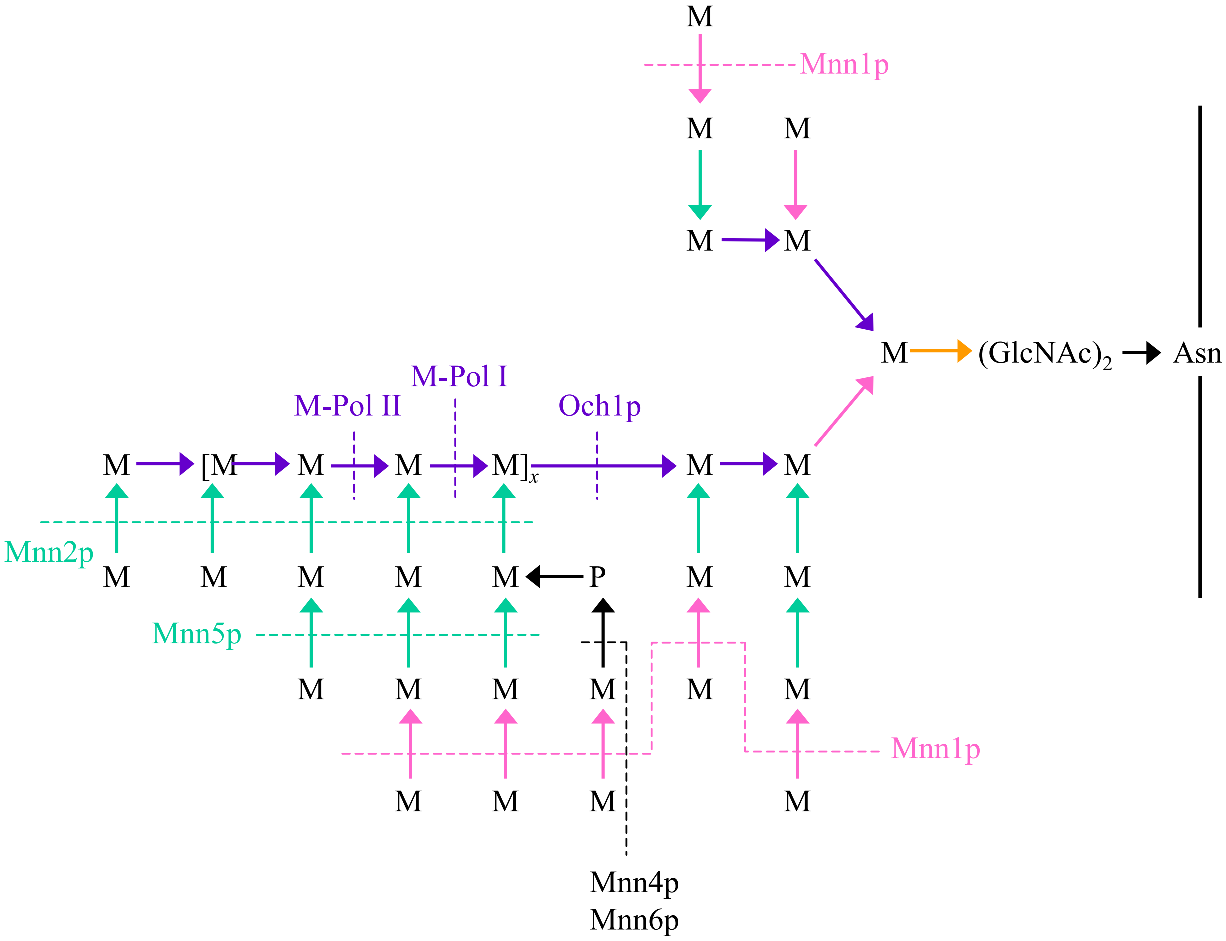


Figure 1.6 In yeast, *N*-glycosylation cores are extensively 'decorated' by the addition of mannan outer chains. *N*-glycosyl structures are attached to the Asn residue in the sequence Asn-X-Ser/Thr (where X is any amino acid except proline). Green arrows indicate α-1,2 linkages, orange arrows indicate β-1,4 linkages, pink arrows indicate α-1,3 linkages and blue arrows indicate α-1,6 linkages. M-Pol I consists of Mnn9p and Van1p. It acts when *x* is approximately ten. M-Pol II consists of Mnn9p, Anp1p, Mnn10p, Mnn11p, Hoc1p. It acts when *x* is approximately 50. *x* equals ten on average. GluNAc = *N*-acetylglucosamine; M = mannose; P = phosphate. Adapted from Lussier *et al.*, (1999) and Munro *et al.*, (2001).

extended by the α-1,2-mannosyltransferases Mnn2p and Mnn5p. Terminal α-1,3-linked mannose is added by Mnn1p and Mnn6p adds mannosylphosphate, under the regulation of Mnn4p.

Mannoproteins are sorted in the *trans*-Golgi and those destined for the cell surface are separated, packaged into vesicles and targeted to the plasma membrane. GPI proteins that are linked to the plasma membrane have an intact GPI anchor but those in the cell wall have their GPI anchor cleaved at the plasma membrane prior to incorporation into the cell wall by covalent linkages to β-1,6-glucan (Kollár *et al*., 1997; Lu *et al*., 1995; Lu *et al*., 1994; Müller *et al*., 1996) and in turn to β-1,3-glucan.

1.6 GPI ANCHORED CELL WALL/PLASMA MEMBRANE PROTEINS IN *SACCHAROMYCES CEREVISIAE*

The cell wall of *S. cerevisiae* is known to contain more than sixty different mannoproteins (Caro *et al*., 1997; Cherry *et al*., 1997; Klis *et al*., 2002). Each is considered to play a role in building, maintenance or modification of the cell wall during the cell cycle or changing environmental conditions (Cabib *et al*., 1997; Smits *et al*., 1999).

The mannoproteins have been classified into three groups based upon their mode of attachment to the cell wall (Mrsa *et al*., 1999a). The first group of mannoproteins are non-covalently attached to structural polysaccharides of the cell wall. All of these types of proteins identified have been demonstrated to have hydrolytic activity or are homologues to such enzymes, for example Bgl2p, an endo-β-1,3-glucanase (Mrsa *et al*., 1993).

Proteins covalently attached to the cell wall that can be extracted by 30 mM sodium hydroxide form a second group of mannoproteins. The actual alkali labile link by which these proteins are attached to the cell wall remains unclear. These proteins have recently been identified as a family consisting of proteins with seven to ten internal repeats of 18-19 amino acids (*PIR*) (Mrsa and Tanner, 1999; Toh-e *et al*., 1993). These proteins do not have GPI anchors but are highly *O*-glycosylated. It has been suggested that the *O*-

glycosylation may link the Pir proteins to an as yet unidentified cell wall component (Mrsa *et al.*, 1999a; Mrsa *et al.*, 1997; Mrsa and Tanner, 1999).

The third group are those that are covalently linked to the polysaccharide components of the cell wall and are extractable by glucanases. α-agglutinin was the first protein identified from this group and it contains a putative GPI-anchoring sequence (Wojciechowicz *et al.*, 1993). The study of its passage through the secretory pathway showed that this protein travels the entire pathway in the GPI-anchored form, before it is localised in the plasma membrane (Wojciechowicz *et al.*, 1993). The GPI-anchor is then released and α-agglutinin is translocated to the cell wall (Wojciechowicz *et al.*, 1993). Other proteins from this group include Gas1p, Yap3p and Tir1p.

Other glucanase extractable cell wall proteins are mannoproteins that share a great deal of structural similarity: they all possess putative GPI-anchoring signal sequences, are rich in hydroxy amino acids (serine and threonine) and are highly glycosylated by both *N*- and *O*-glycosylation (Cherry *et al.*, 1997; Mrsa *et al.*, 1999a). If the structure of Hpf mannoproteins is considered, they would be classified as belonging to this group of glucanase-extractable mannoproteins. Experiments on methods to extract Hpf mannoproteins have shown, however, that they can be extracted simply with boiling or by EDTA containing buffers: glucanases are not required but they do accelerate the extraction process (Dupin *et al.*, 2000a).

The physiological role of most yeast mannoproteins is unclear, except for those expressed during particular events such as agglutination or flocculation. Strains with mutations in individual genes have been found to exhibit a weakening of the cell wall and have increased sensitivity to cell wall perturbing agents, such as Calcofluor white, Congo red and zymolyase (Moukadiri *et al.*, 1997; Van Der Vaart *et al.*, 1995). Against this, a strain with multiple mutations (*ccw12Δ ccw13Δ cwp1Δ tip1Δ cwp2Δ*) did not show any significant growth problem and did not require osmotic stabilisation (Mrsa *et al.*, 1999b). Cell wall proteins may also be involved in cell wall signalling pathways such as the pheromone response, cell integrity or high osmolarity glycerol signal transduction pathways (Molina *et al.*, 1999).

1.7 CONCLUSIONS AND AIMS OF THE PROJECT

Protein haze in wine is formed when heat unstable proteins, the pathogenesis related proteins from grapes, slowly denature and aggregate, resulting in light-dispersing particles. Current methods to prevent haze, such as the use of bentonite as a fining agent, have drawbacks. It is suggested that an alternative or at the very least a complement to bentonite fining may be the use of HPFs.

The experiments described in this thesis seek to establish whether the genes inferred from partial amino acid sequences of isolated Hpf1p and Hpf2p preparations indeed can be concluded to encode proteins with haze protective characteristics. A further aim of the study is to use strains deleted for or overexpressing the *HPF* genes to elucidate the biological function of these Hpf mannoproteins in *Saccharomyces cerevisiae*, through analyses of the phenotypic consequences.

In Chapter 2, the experimental materials and methods utilised in this project are explained including the construction of the deletion and overexpression strains, the phenotypic analysis assays and purification of the tagged Hpfs. Phenotypic analyses of the Hpfs are described in Chapter 3. This includes determination of the possible involvement of the Hpfs in cell wall signalling, cell wall structure or stress responses. An investigation into the role of these mannoproteins in yeast mating is described in Chapter 4. Chapter 5 describes the use of *hpfΔ* deletion and *HPF* overexpression strains to demonstrate that *HPF* genes do encode haze protective mannoproteins, and the use of 6xHis-tagged Hpf to ensure that this is so. In Chapter 6, a preliminary investigation into which components of the mannoprotein are responsible for haze protective activity is presented through the use of yeast glycosylation mutants overexpressing a 6xHis-tagged Hpf, and Endoglycosidase H to remove *N*-linked glycans from 6xHis-Hpf2p. The work is summarised and possible further research discussed in Chapter 7.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MOLECULAR BIOLOGY TECHNIQUES

2.1.1 Enzymatic manipulations of DNA

Restriction enzymes were sourced from Roche Biochemicals and were used according to the manufacturer's instructions. Briefly, this involved diluting 10x restriction enzyme buffer stock (supplied with the enzyme) (Ausubel, 2001) to a final concentration of 1x in the DNA sample to be digested. The enzyme was added and the digest was incubated at 37^{o}C for between one and 16 hours (Ausubel, 2001).

Plasmid DNA was isolated as described in Section 2.1.3 and following digestion, dephosphorylated using Shrimp Alkaline Phosphatase (SAP) (Roche Biochemicals). This involved diluting 10x SAP buffer (supplied with the enzyme) to 1x in the DNA sample. The SAP enzyme (20 U/µg DNA) was added to a final volume of no more than 20 µl. The sample was incubated at 37^{o}C for 10 minutes and the enzyme was inactivated by incubation at 65^{o}C for 15 minutes (Ausubel, 2001).

Plasmid and insert DNA (1:3) were ligated using T4 DNA ligase (Roche Biochemicals). A 10x ligase buffer stock was diluted to 1x in a mixture of the DNA samples and water. The enzyme (20 U/µg DNA) was added to a final volume of 10 µl. The ligation mix was incubated at 16^{o}C overnight (Ausubel, 2001).

2.1.2 Competent *E. coli*

Competent DH5α *E. coli* cells were prepared following the method of Inoue *et al*., (1990), or competent JM109 *E. coli* cells were sourced from Promega. Competent *E. coli* were used for propagation of plasmids. Transformation of *E. coli* was performed following the manufacturer's instructions or by the calcium chloride method of Inoue *et al*., (1990).

2.1.3 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* by the alkaline lysis method of Sambrook and Russell, (2001).

2.1.4 Preparation of chromosomal DNA from yeast

Yeast chromosomal DNA was isolated following the method of Ausubel, (2001), using breaking buffer (Appendix 2) and glass beads to break open the cells, except cells were shaken using a Mini-Beadbeater 8 (Biospec Products), instead of vortexing.

2.1.5 Preparation of *S. cerevisiae* for transformation and introduction of DNA into *S. cerevisiae*

S. cerevisiae was transformed by the lithium acetate/polyethylene glycol method described by Ausubel, (2001).

2.2 YEAST MATING, SPORULATION AND MICRODISSECTION

Yeast strains of opposite mating type were mixed on a YPD plate (Appendix 2) and incubated overnight at 30°C to allow mating. A loopful of the mixed cells were resuspended in sterile water and plated on to a fresh YPD plate to obtain single colonies. The colonies were replica plated to potassium acetate medium (Appendix 2) to allow sporulation whilst incubating at 30°C over 4-5 days. The sporulated cells were transferred to a fresh YPD plate and asci were microdissected using an Olympus CHT microscope that was modified for microdissection.

2.3 GENERATION OF DELETION STRAINS USING SHORT FLANKING HOMOLOGY POLYMERASE CHAIN REACTION (SFH-PCR)

Each of the putative *HPF* genes was disrupted using Short Flanking Homology PCR (SFH-PCR) (Wach *et al.*, 1997). The marker gene for *HPF1* was *URA3* from *Kluyveromyces lactis* (Längle-Rouault and Jacobs, 1995) and for *HPF1'* and *HPF2* was

HIS5 from *Schizosaccharomyces pombe* (Wach *et al*., 1997). These were used to complement the *S. cerevisiae ura3* and *his3* mutations (Wach *et al*., 1997). This method involved amplifying the marker gene by PCR using primers that have approximately 20 bp homology to the marker gene and 80 bp homology to the *HPF* gene. Chromosomal DNA was isolated from *K. lactis* as described in Section 2.1.4, in order to amplify the *URA3* gene. Plasmid DNA (p3xHA-HIS5) was isolated from *E. coli* as described in Section 2.1.3. The plasmid p3xHA-HIS5 was kindly donated by Peter Walsh and Trevor Lithgow, La Trobe University. The *K. lactis URA3* gene was amplified from the genome using the primers SLB3 and SLB4. The *S. pombe HIS5* gene was amplified from p3xHA-HIS5 using SLB2 and SLB5 or SLB6 and SLB7. The primers used are listed in Appendix 3. All primers used in this study were sourced from Life Technologies.

Typical PCR mixtures (50 μl) contained $(NH_4)_2SO_4$ (20 mM), Tris-HCl (pH 8.8) (75 mM), Tween 20 (0.01%), dNTPs (200 μM of each), primers (0.5 μM of each), $MgCl_2$ (1.5 mM), Taq polymerase (Advanced Biotechnologies) (1.25 U) and template DNA (10 ng). The reactions were performed in an MJ Research PTC-100 programmable thermal cycler programmed as follows: Denaturation of DNA at 94^{o}C for 5 minutes followed by 30 cycles of 30 second denaturation of DNA at 94^{o}C, 30 seconds primer annealing at 60^{o}C and 2.5 minutes DNA extension at 72^{o}C. The final extension period was 10 minutes and tubes were then cooled to 4^{o}C. The PCR products were digested with restriction enzymes, run on a 1% (w/v) agarose gel (Scientifix) and the gel stained with ethidium bromide to visualise the DNA, to ensure correct amplification.

S288c diploid yeast (Winston *et al*., 1995) was transformed with the PCR products, as described in Section 2.1.5 and those in which homologous recombination had taken place were selected for on appropriate synthetic complete dropout plates.

Disruption of the *HPF* genes was confirmed by PCR using primers as follows: For *HPF1*, SLB11 and SLB12, for *HPF1'*, SLB13 and SLB14 and for *HPF2*, SLB15 and SLB16 (Appendix 3). The PCR products were digested with appropriate restriction enzymes to confirm that the expected region was amplified.

2.4 AMPLIFICATION OF HPF GENES AND CLONING INTO P415GAL1

The *HPF1* gene was amplified from the genome of a haploid strain of S288c with the primers SLB11 and SLB12. *HPF1'* was amplified with primers SLB17 and SLB18 and *HPF2* with primers SLB19 and SLB20 (Appendix 3). The forward primers incorporated *Xba*I restriction enzyme sites and reverse primers, *Sal*I sites. PCR mixes (50 µl) contained 1x strength Platinum Pfx amplification buffer (supplied with Pfx DNA polymerase), dNTPs (300 µM of each), primers (0.3 µM of each), $MgSO_4$ (1 mM), Platinum Pfx DNA polymerase (Life Technologies) (1.0 U) and template DNA (10 ng). Platinum Pfx possesses proofreading 3' to 5' exonuclease activity and provides high fidelity amplification. An antibody is bound to Platinum Pfx polymerase in an inactive form at ambient temperature allowing for automatic 'hot start.' The thermal cycler was programmed as follows: Denaturation of DNA at 94^{o}C for 2 minutes followed by 30 cycles of denaturation of DNA at 94^{o}C for 15 seconds, annealing of primers at 50^{o}C for 30 seconds and extension of DNA at 68^{o}C for 2.5 minutes.

To confirm that the PCR had amplified the correct region containing the *HPF* genes, 1 µl of the reaction was digested with restriction enzymes and run on a 1.0% agarose gel whereafter fragments were visualised and their sizes determined with reference to standards. The remainder of the PCR product was purified using the QIAquick PCR purification kit (QIAGEN) and the DNA was eluted with 10 mM Tris.Cl (pH 8.5). The DNA was digested with *Sal*I and then with *Xba*I as described in Section 2.1.1. This digested insert DNA was run on a 0.8% agarose gel and the fragment was gel purified using a silica milk procedure supplied in the UltraClean™ kit (MoBio).

The plasmid (p415GAL1 ATCC #87330, (Mumberg *et al.*, 1994), Appendix 5) was prepared from *E. coli* by the method described in Section 2.1.3. The plasmid was purified using the QIAquick PCR purification kit (QIAGEN) and then digested successively with *Sal*I and *Xba*I. The restriction enzymes were denatured by heating the digestion mixture to 65^{o}C for 15 minutes. The digested vector was dephosphorylated as described in Section 2.1.1. This digested, dephosphorylated plasmid DNA was run on a 0.8% agarose gel and the fragment was gel purified using a silica milk procedure supplied in the UltraClean™ kit (MoBio).

The plasmid and insert DNA were mixed at a 1:3 ratio and ligated using T4 DNA ligase (Roche) at 12°C for 16 hours. The ligation product was transformed into competent *E. coli* and transformants were selected on ampicillin. Plasmid DNA was prepared from the transformants by the method described in Section 2.1.3 and this DNA was digested with restriction enzymes to identify correct clones. The resultant plasmids were named p415GAL1-HPF1, p415GAL1-HPF1' and p415GAL1-HPF2 (Appendix 5).

To ensure that no amplification artefacts had been introduced by the Platinum Pfx polymerase, the plasmid DNA was sequenced at the Australian Genome Research Facility (Brisbane, Australia) using ABI BigDye Terminator chemistry. The primers used for sequencing are listed in Appendix 3. S288c haploid yeast were transformed with purified plasmid DNA by the method described in Section 2.1.5.

2.5 AMPLIFICATION OF HPF GENES FOR 6XHIS TAGGING AND CLONING INTO PYES2/GS

The *HPF1* gene was amplified from the p415GAL1-HPF1 plasmid with the primers SLB57 and SLB58. *HPF1'* and *HPF2* were amplified from their respective p415GAL1-HPF plasmids and the primers used for *HPF1'* were SLB62 and SLB63 and for *HPF2* were SLB64 and SLB65. The forward primer incorporated a *Pvu*II restriction enzyme site, the N-terminal signal sequence of the *HPF* genes, 6xHis tag sequence and a 5' section of the gene. The reverse primer incorporated a 3' section of the gene and an *Xba*I restriction enzyme site (Appendix 3). A scheme outlining the introduction of the 6xHis tag into the *HPF* gene sequence is shown in Figure 2.1. PCRs were prepared as described in Section 2.4. The PCR products were cleaned as described in Section 2.4 and were digested with *Pvu*II and *Xba*I. The fragment was gel purified using the UltraClean™ kit (MoBio) following the manufacturer's instructions.

The plasmid (pYES2/GS, Invitrogen) initially carried *HPF1*, followed by a V5 epitope and a 6xHis tag. The vector was prepared from *E. coli* by the method described in Section 2.1.3. The plasmid was cleaned using the QIAquick PCR purification kit (QIAGEN) and digested with *Pvu*II and *Xba*I, simultaneously. The enzymes were inactivated by heating to 65°C for 10 minutes and the vector was then dephosphorylated

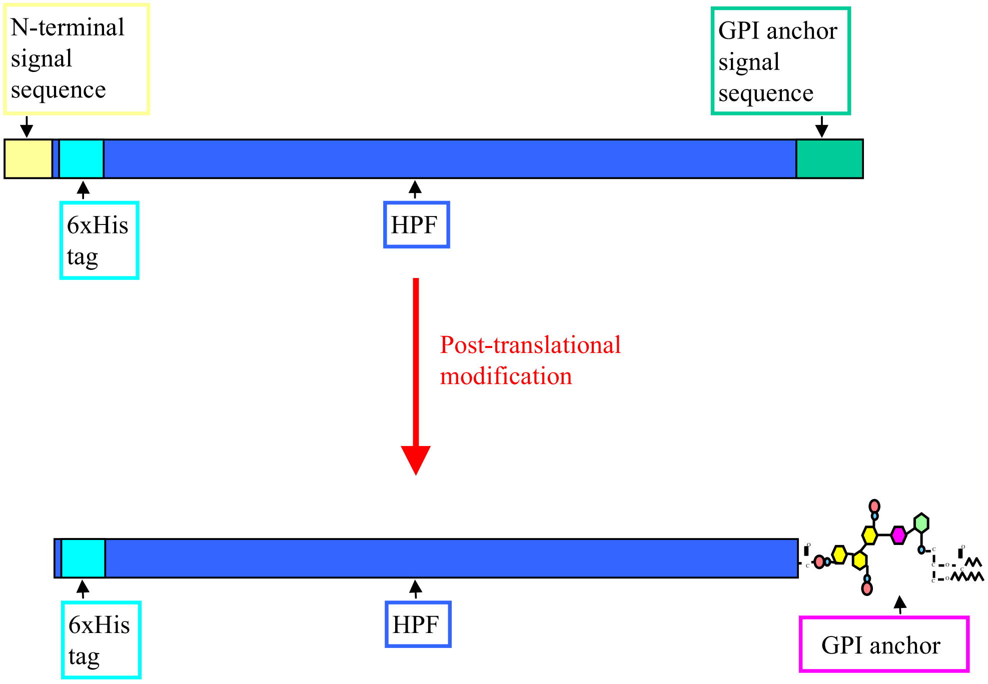


Figure 2.1 Schematic to illustrate the introduction of the 6xHis tag at the N-terminus of the Hpf mannoprotein, following the signal sequence. When the signal sequence is cleaved during post-translational modification, the 6xHis tag will be close to the N-terminus of the mature protein. The GPI anchor signal sequence is also cleaved and a GPI anchor will be attached.

as described in Section 2.1.1. The digested, dephosphorylated DNA was run on a 1.0% agarose gel and the vector fragment was cut out and purified using the UltraClean™ kit (MoBio).

Plasmid and insert DNA (1:3) were ligated and transformed into *E. coli* as described in Section 2.1.2. Correct clones were identified by restriction enzyme analysis. The resultant plasmids were named p6xHis-HPF1, p6xHis-HPF1' and p6xHis-HPF2 (Appendix 5). To ensure that the 5' region was aligned with the gene, the plasmids were sent to the Australian Genome Research Facility (Brisbane, Australia) and the 5' region was sequenced. The sequence of the region was that expected. Purified plasmid DNA was transformed into S288c haploid yeast by the method described in Section 2.1.5.

2.6 FERMENTATION IN CHEMICALLY DEFINED GRAPE JUICE MEDIUM

Fermentation was done in Chemically Defined Grape Juice Medium (CDGJM) (adapted from Henschke and Jiranek, (1993)) (Appendix 2) with appropriate carbon sources as described below. The medium was adjusted to pH 3.2 and filtered through a 0.2 µm filter (Pall Gelman Laboratory). The medium composition was modified to allow growth of the laboratory strain S288c by the addition of a synthetic complete amino acid mix (Appendix 2). Ferments were performed in triplicate in Schott bottles with fermentation airlocks.

The medium in which all yeast fermentations were conducted was CDGJM supplemented with appropriate carbon sources. The starter cultures were inoculated from precultures that had been in stationary phase for at least one week. Starter culture medium for the deletion ferments was 50% CDGJM, diluted in sterile water. For the overexpression ferments, CDGJM with 20 g/L raffinose was diluted 50% with MilliQ water for the starter cultures. These starter cultures were grown aerobically until the cell density reached $1x10^7$ cells/mL and then they were used to inoculate the ferments. The medium for the deletion ferments was CDGJM with 200 g/L glucose and for the overexpression ferments was CDGJM with 20 g/L galactose. Ferments were inoculated at $1x10^6$ cells/mL and were monitored by measuring the OD_{600}. The ferments were incubated at 30^oC with shaking at 100 rpm. When the yeast had reached stationary phase, the ferments were allowed to continue for 64 hours (deletion strains) or 96 hours (overexpression strains) and then were stopped by pelleting the yeast by centrifugation.

2.7 ETHANOL PRECIPITATION FROM FERMENTATION SUPERNATANTS

Mannoproteins and other materials were precipitated from clarified fermentation supernatants (typically one litre) by addition of ethanol. Four volumes of acidified (60 mM hydrochloric acid) 95% (v/v) ethanol were added to the supernatant. This was stored at least overnight at –20^{o}C. The precipitated material was removed by centrifugation in a Beckman J2-21M/E centrifuge at 9500rpm at -20^{o}C for 15 minutes in a Beckman JA10.5 rotor. The pelleted material was washed with cold 80% (v/v) ethanol followed by centrifugation and redissolved in MilliQ water. After freezing at –80^{o}C, the sample was freeze-dried on a Dynavac Engineering FD3 freeze-dryer with Edwards RV5 Rotary Vane pump. The dry material was resuspended in 3 mL MilliQ water and desalted into water using Econo-Pac 10DG columns (BioRad). The 4 mL eluate was frozen and freeze-dried.

2.8 MANNOSE ASSAY

The freeze-dried material was weighed out and resuspended at 2 mg dry weight/ml. This was used for the mannose assay previously described Dupin *et al*., (2000b). Briefly, the samples were hydrolysed in 1.5 M sulphuric acid for 90 minutes at 100^{o}C. Using the COBAS-FARA, the samples were neutralised with sodium hydroxide and buffered with triethanolamine buffer containing NADP, ATP and magnesium sulphate from a D-glucose/D-fructose spectrophotometric kit (Roche Biochemicals). Glucose concentration was determined by measuring the difference in OD_{340} before and 20 minutes after the addition of hexokinase and glucose-6-phosphate dehydrogenase. Fructose concentration was determined after the addition of phosphoglucose isomerase, however this was always negligible as fructose degrades during hydrolysis. Mannose concentration was determined 90 minutes after the addition of phosphomannose isomerase (Sigma).

2.9 HEAT TEST FOR PROTEIN HAZE POTENTIAL

2.9.1 Preparation of wine for the assay

The wine used for this assay was a 2001 Semillon from McLaren Vale, South Australia, kindly donated by Southcorp Wines. The wine had not been bentonite fined and was thus heat unstable. Sixty litres of wine was allowed to cold settle at 4°C until yeast was collected at the bottom of the containers. Twenty litres of the wine was racked into a 20 litre container containing 0.2 g/L SO_2 in the form of potassium metabisulphite to reduce oxidation. The wine was sterile filtered (0.2 µm) into sterile one litre Schott bottles using VacuCap 60 bottle top filter devices (Pall Gelman Laboratory). All wine was stored at 4°C in darkness until required.

2.9.2 Preparation of samples for the 'heat test' assay

The heat test assay was based on the method of Pocock and Rankine, (1973), with a modification of the micromethod described by Waters *et al.*, (1991), and Stockdale, (2000). Usually, based on the results of the mannose assay (Section 2.8), material to be tested for haze protective activity was resuspended in MilliQ water so that the concentration of mannose was normalised. A stock solution of 10 mg mannose equivalent/mL was prepared and this was diluted into wine for the assay. In other cases, the material for the heat test was normalised based on the volume of supernatant from which it was extracted. Four replicates of each sample were prepared in PCR strip tubes (Eppendorf), heated to 80°C for six hours and then cooled to 4°C for at least 12 hours in an MJ Research PTC-100 programmable thermal cycler. Samples were allowed to come to room temperature and were transferred to a 96 well flat bottom plate (Greiner). The optical density at 490 nm was measured using either a UV max or BioRad plate reader. The minimum haze (0%) was set by the OD_{490} of the unheated wine samples with only water added. It was assumed that the maximum haze (100%) was thrown by the heated wine with only water added.

2.10 STATISTICAL ANALYSIS

All statistical analysis was performed using the Jmp computer program (Version 3.2.2, SAS Institute).

2.11 OVEREXPRESSION OF 6XHIS-HPF IN *S. CEREVISIAE*

Yeast were inoculated into 5ml of synthetic complete medium without uracil (Appendix 2) and grown until the yeast were in stationary growth phase. From this, the yeast were diluted down appropriately into synthetic complete medium without uracil containing 2% glucose and incubated overnight until the cells were in mid-exponential growth phase. The yeast were washed once with water to remove any glucose and diluted to $1x10^6$ cells/mL in synthetic complete medium containing 2% (w/v) galactose and 1% (w/v) raffinose as the carbon source to induce expression of Hpf. These cultures were incubated at 30°C for 24 hours. The cultures were centrifuged and the cells and supernatant were stored at -20°C until needed.

2.12 PURIFICATION OF 6XHIS-HPF USING IMMOBILISED METAL AFFINITY CHROMATOGRAPHY

The supernatant prepared as described in Section 2.11 was defrosted overnight at 4°C. A ¼ volume of a 5x stock of wash buffer (Appendix 2) was added to the supernatant. The pH was adjusted to 8.0 with 1.0 M hydrochloric acid. Alternatively, mannoproteins were precipitated from the supernatant by ethanol, desalted and resuspended in 1x wash buffer. Composition of the buffer used was as described by the manufacturer (Appendix 2).

The Nickel-Nitrilotriacetic acid (Ni-NTA) immobilised metal affinity chromatography (IMAC) resin (QIAGEN) was resuspended and packed into an Econo-Pac column (BioRad). The storage buffer was removed from the resin by flowing though the column and the column was equilibrated in wash buffer (five column volumes). The buffered supernatant was then applied to the column and the unbound fraction was collected. The resin was then washed with wash buffer (five column volumes) and the sample was eluted with wash buffer fortified with imidazole (250 mM). The fractions were desalted using an Econo-Pac 10DG column (BioRad) and freeze dried individually.

2.13 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Yeast cells were resuspended in an equal volume of 2x SDS-PAGE sample buffer (Appendix 2), mixed briefly and boiled for 5 minutes. Glass beads were added, the cells were vortexed at high speed for 30 seconds followed by boiling again for 5 minutes. The samples were centrifuged at 15000 rpm in a micro-centrifuge for 5 minutes at room temperature and the supernatant was transferred to a clean tube. Samples were briefly vortexed, boiled at 100°C for five minutes and allowed to cool to room temperature before loading on the gel. SDS-Polyacrylamide gel electrophoresis was performed as described by Ausubel, (2001). Tris-glycine polyacrylamide gels (4-20% gradient) were sourced from Gradipore (Australia). Gels were run in SDS-glycine running buffer (Appendix 2) at 100 V (constant voltage), 60 mA, until the dye front had run off the end of the gel. Gels were either Periodate-Schiff and Coomassie blue stained or prepared for transfer on to nitrocellulose for Western blotting.

2.14 PERIODATE-SCHIFF'S STAIN FOR DETECTION OF CARBOHYDRATE ON SDS-PAGE GELS

Gels were stained with Schiff's reagent to detect carbohydrate as described by Carlsson, (1993). This involved fixing the gel in fixative solution (Appendix 2) for at least one hour. The gel was incubated in freshly prepared periodate solution (Appendix 2) for one hour. Metabisulphite solution (Appendix 2) was freshly prepared and the gel was transferred to this solution and incubated until it turned yellow (usually 5 to 10 minutes). The solution was replaced with fresh metabisulphite solution and incubated for a further 5 to 10 minutes until the gel just cleared. The gel was incubated in Schiff's reagent (Sigma) until dark pink bands appeared but for no greater than two hours. Excess stain was removed by washing in fixative solution.

2.15 COOMASSIE BLUE STAINING FOR DETECTION OF PROTEINS ON SDS-PAGE GELS

Following Periodate-Schiff's stain, the gels were incubated in Coomassie blue stain (Appendix 2) for approximately one hour. The stain was discarded and the gel was destained in fixative solution (Appendix 2). The destain was changed several times until the background gel colour was reduced sufficiently to detect bands.

2.16 TRANSFER OF PROTEINS TO NITROCELLULOSE

Wet transfer of proteins on to nitrocellulose (BA83, Schleicher and Schuell) was done using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell following the manufacturer's instructions. Immediately after the SDS-PAGE gel had finished running, the gel was equilibrated in cold transfer buffer (Appendix 2) for 15 minutes. The fibre pads (Bio-Rad), filter paper (3MM, Whatman) and nitrocellulose were also soaked in transfer buffer (Appendix 2) for 15 minutes. The transfer cassette was assembled as described by the manufacturer. Transfer was conducted at 100 V (constant voltage) for one hour in transfer buffer.

2.17 WESTERN BLOT ANALYSIS

After transfer, the membrane was covered with Ponceau S stain (Appendix 2) for five minutes and rinsed with MilliQ water until the background was removed to ensure that the proteins had indeed transferred. The remaining Ponceau S was washed from the membrane with TBS-T (Appendix 2). The membrane was blocked in blocking buffer (Appendix 2) either overnight at 4^{o}C or for two hours at room temperature, with gentle rocking. This was followed by a rinse and four 10 minute washes in TBS-T. The blot was probed with the primary antibody, mouse anti-6xHis (Sigma), diluted 1:3000 in blocking buffer for at least one hour at room temperature. The membrane was briefly rinsed and washed for 10 minutes in TBS-T four times. The secondary antibody, goat anti-mouse conjugated to alkaline phosphatase (Promega) was diluted 1:7500 in blocking buffer and the membrane was incubated for at least one hour at room temperature. The membrane was rinsed and washed twice for 10 minutes with TBS-T and then washed in sarkosyl wash reagent (Appendix 2) for 15 minutes. Finally, the membrane was washed twice for five minutes in TBS-T prior to rinsing in MilliQ water. To develop the blot, it was incubated in the presence of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Promega) in alkaline phosphate buffer (Appendix 2) until bands appeared. The membrane was gently blotted with filter paper and allowed to dry.

2.18 PHENOTYPIC ANALYSES OF *HPF* OVEREXPRESSION AND DELETION MUTANTS

2.18.1 Growth at various temperatures

2.18.1.1 Plate assay

The *HPF* deletion and overexpression strains were assayed for their ability to grow at various temperatures. Serial 10-fold dilutions of exponential cultures were plated (5 µl of each dilution) on YPD plates (Appendix 2) and incubated at 4, 7, 11, 16, 24, 27, 30, 37, 40, 44°C until appropriate growth was observed (Hampsey, 1997).

2.18.1.2 Liquid assay

Following the method described by Stolz *et al.*, (1998), growth of a yeast with a cold resistant phenotype was measured. Yeast strains were grown overnight to mid-exponential phase at 30°C. Cell number was determined the next morning and cells were diluted into fresh YPD (100 mL) at 2×10^5 cells/mL and incubated at 10, 12 and 14°C. The OD_{600} was followed for 200 hours.

2.18.2 Growth whilst under an osmotic stress

Tolerance of the wild type and *hpfΔ* strains to osmotic stress was assayed on YPD plates containing 1.0, 1.25, 1.5, 2.0, 2.5, 3.0 and 5.0 M sorbitol (Hampsey, 1997). Ten-fold serial dilutions of exponential cultures in YPD were plated (5 µl of each dilution) on YPD plates containing sorbitol and incubated at 30°C for up to six days.

2.18.3 Ethanol tolerance

Ethanol tolerance of the deletion strains was tested by plating 5 µl of 10-fold serial dilutions of exponential cultures on YPD plates containing various concentrations of ethanol (6, 8, 10, 12, 14% (v/v)) (Hampsey, 1997). Plates were incubated at 30°C for up to six days.

2.18.4 Oxidative stress

Oxidative stress tolerance was tested by the method described by Stephen *et al.*, (1995). This involved growing the cells to exponential phase and making serial 10-fold dilutions and spotting 5 μl of each dilution on to YPD plates containing 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mM hydrogen peroxide. Plates were incubated at 30°C for up to two days.

2.18.5 Sensitivity towards Calcofluor white and Congo red

The ability of the *hpfΔ* and *HPF* overexpression strains to grow in the presence of Calcofluor white and Congo red was assayed by growing cells to exponential phase, preparing serial dilutions and spotting 5 μl of each dilution on YPD plates containing Calcofluor white or Congo red, following the method described by Van Der Vaart *et al.*, (1995). Calcofluor white and Congo red were sourced from Sigma-Aldrich Chemicals.

2.18.6 Zymolyase sensitivity

The sensitivity of the wild type and *hpfΔ* strains to zymolyase was assayed by the method of Van Der Vaart *et al.*, (1995). Cultures were grown to mid-exponential phase and diluted down to OD_{600} of 0.3 in 10 mM Tris-HCl (pH 7.0). Zymolyase 20T (50 μg/mL) was added and the decrease in OD_{660} was monitored.

2.18.7 Killer phenotype

Sensitivity of *hpfΔ* strains to killer toxins was tested by the method of Burke *et al.*, (2000). Briefly, tester strains (*hpfΔ* strains) were grown to mid-exponential phase and spread on to low pH blue YPD plates (Appendix 2). Killer yeast were then dotted on to the plates (7.5 μl of stationary culture). The killer yeast tested in this assay, selected for their known killing ability (Young and Yagiu, 1978), are shown in Table 2.1. The plates were incubated at 27°C for 48 hours. Differences in the response to killer yeast were scored by measurement of the zone of growth inhibition.

Table 2.1 Killer yeast used to test sensitivity of *hpfΔ* strains to killer toxins

Killer yeast strain	**Source**
Kluyveromyces lactis var. *drosophilarum*	CBS 2896
Kluyveromyces marxianus	NCYC 587
Pichia anomala	NCYC 434
Pichia membranifaciens	CBS 638
Pichia membranifaciens	CBS 107
Pichia subpelliculosa	NCYC 16
Williopsis saturnus var. *mrakii*	CBS 1707

2.18.8 Electron microscopy

Cells in mid-exponential growth phase were washed twice with MilliQ water, resuspended in fixative (Appendix 2) and stored at 4°C, overnight. The cells were pelleted and washed twice in washing buffer (Appendix 2) for 10 minutes. The cells were then stained with 2% (w/v) osmium tetroxide in PBS, with mixing for one hour. Dehydration involved washing the cells three times for 15 minutes in each of the following ethanol concentrations: 70% (v/v) ethanol, 90% (v/v) ethanol, 95% (v/v) ethanol and 100% ethanol. Cells were then incubated in 100% ethanol for one hour. Each of the incubations were done while slowly rotating the tubes. The samples were resuspended in a 1:1 mixture of epoxy resin and 100% ethanol and mixed overnight. The cells were resuspended in 100% resin (Procure–Araldite embedding kit) and the resin was changed after eight hours and again at 24 hours. After another four hours, the cells were embedded in fresh resin and this was incubated at 70°C for at least 12 hours to allow the resin to polymerise. Thin sections (80 nm) were prepared, mounted on mesh grids and stained with Reynolds' lead citrate (Wright, 2000). Samples were observed using a Philips CM100 Transmission Electron Microscope with automated stage, including multiple specimen and tilt rotate holder and SIS Megaview II Image Analysis software.

2.18.9 Cell integrity - MAP kinase mediated signal transduction pathway involvement

2.18.9.1 Detection of alkaline phosphatase

An assay for the release of alkaline phosphatase from lysed cells was performed. The *hpfΔ* strains were grown to mid-exponential phase and spotted on to YPD, YPD + 40 μg/mL BCIP and YPD + 40 μg/mL BCIP + 1 M sorbitol, in duplicate. One replicate was incubated at 24°C and the other at 37°C for two days (Molina *et al.*, 1999).

2.18.9.2 Caffeine sensitivity

Sensitivity to caffeine was assayed by plating mid-exponential cultures of the *hpfΔ* strains on YPD containing 3, 6, 9, 12 and 15 mM caffeine at 24°C for five days (Molina *et al.*, 1999).

2.18.10 Oleic acid utilisation

The ability of the *hpfΔ* strains to utilise oleic acid as a carbon source were analysed by the method of Entian *et al.*, (1999). Briefly, cells in mid-exponential growth phase were centrifuged and resuspended in oleic acid induction medium (Appendix 2) at $3x10^6$ cells/mL. Serial dilutions were made after seven hours and 5 μl of each dilution was plated on to selective oleic acid medium (Appendix 2). Plates were incubated at 30°C for six days before photographing.

2.18.11 Competitive growth assay

This assay was performed following the method described by Thatcher *et al.*, (1998), using four replicate cultures. Sub-culturing involved diluting the culture back 32-fold at the same time every day and this was repeated for 27 days.

2.18.12 α-factor growth arrest

The relative sensitivity of cells to α-factor induced growth arrest was measured with a 'halo' bioassay. Cells ($1x10^5$) of a mid-exponential culture were added to 7 mL of YPD top-agar and well mixed. The top agar was poured on to a YPD plate and allowed to set. Four micrograms of α-factor was spotted in the centre of the plate and the plate was incubated at 30°C for 48 hours and the diameter of the halo measured (Sprague, 1991).

2.18.13 Shmoo formation

Yeast cultures were grown overnight to mid-exponential phase. Cell number was determined the next morning by counting with a haemocytometer. α-factor was added to the cultures to a final concentration of 5 μg/ml and incubated at 30°C for two hours. After the appropriate time, the proportion of cells that had formed shmoos was determined (McCaffrey *et al*., 1987). (OD_{600} should be less than 0.3 because of Bar1p in *MAT***a** cells – S. E. Erdman).

2.18.14 Agglutination assay

Mid-exponential cultures of yeast were grown overnight. One millilitre of culture of each mating type was mixed, as well as mixes of each mating type with itself (as controls) in 18 mm round-bottom tubes. The tubes were shaken at 200 rpm for one hour at 30°C. Cells were pelleted at 735 g, for five minutes in a Hettich Universal 32R centrifuge. In order to resuspend any clumps, the cells were gently mixed and tubes were allowed to stand for 15 minutes to allow any agglutinated cells to settle. A fraction of the supernatant was removed, the OD_{600} measured, and this was compared to the controls (Erdman *et al*., 1998).

2.18.15 Mating efficiency

2.18.15.1 Liquid assay

Mating efficiency of the *hpfΔ* strains in liquid media was measured by following the method of Gehrung and Snyder, (1990). Briefly, yeast were grown to mid-exponential phase. Cells ($3x10^6$) of each mating type were mixed in 18 mm round-bottom tubes and allowed to stand, without shaking at 30°C for four hours. The tubes were vortexed vigorously and serial dilutions were spread plated on to selective medium. The mating efficiency of the wild type strains was taken to be 100% and the difference in efficiency was calculated from this value. (Cells should be sonicated as multiple zygotes can occur in mating aggregates, diluting and plating together, making the assay slightly less quantitative – S. E. Erdman).

2.18.15.2 Limited filter assay

Limited filter mating assays were performed essentially as described by Sprague, (1991). Briefly, cultures of *MAT***a** and *MAT*α cells to be mated were grown overnight to mid-exponential growth phase in YPD or selective medium (to retain plasmids). Equal quantities of cells from each parent were mixed and concentrated on a 0.45 μm nitrocellulose filter (Millipore) using a Swinnex 25 apparatus (Millipore). Using sterile forceps, the filter was placed on a pre-warmed YPD plate with the cell side up. The mating mixture was incubated for two to six hours at 30°C and the filter was then transferred into a 1.5 mL Eppendorf tube containing 1 mL of phosphate buffered saline (PBS) (Appendix 2). The tube was vigorously vortexed to remove the cells from the filter. An appropriate dilution for each time point was prepared and a sample of the mating mixture was plated on to selective medium to determine diploid formation. The plates were incubated at 30°C until colonies had formed.

2.18.16 Frequency of zygote formation using a cytoplasmic mixing assay

One mating partner was transformed with a constitutively expressed cytoplasmic green fluorescent protein (GFP) construct as described in Section 2.1.5. This plasmid, pGAL-GFP, was kindly provided by Cindy Tobery and Mark Rose, Princeton University. Yeast strains were grown overnight to mid-exponential phase in selective medium. Limited matings were prepared as described in Section 2.18.15.2. After mating, the mating mixtures were rinsed off the 0.45 μm filters with 1 ml of ice-cold PBS into a microfuge tube. The cells were pelleted by brief centrifugation and this washing procedure was repeated twice. The cells were fixed for no more than 15 minutes in 3.7% formaldehyde so to arrest mating without destroying GFP fluorescence. The mating mixture was washed three times in 1 ml of PBS. The cells were wet-mounted and examined by phase contrast microscopy and then with fluorescence microscopy using an FITC filter set on an Olympus BX51 microscope. The cells were photographed using a Bio-Rad MRC-1000UV Confocal Laser Scanning Microscope with a Nikon Diaphot 300 inverted microscope and two lasers, krypton-argon and UV-argon attached.

2.18.17 Differential Interference Contrast Microscopy of mating cells

Mating cells were viewed using a Zeiss Axiophot microscope with a JVC 3CCD C-Mount camera and AcQuis Bio (Syncroscopy) software (Version 3.01) attached.

2.18.18 Transmission Electron Microscopy of mating cells

Mating cells were prepared for transmission electron microscopy essentially as described by Gammie and Rose, (2002), and cells were viewed using the same transmission electron microscope procedure described in Section 2.18.8.

2.18.19 Indirect immunofluorescent localisation of Hpf1p and Hpf1'p

Cells were prepared for immunofluorescence using the method described by Guo *et al*., (2000). This involved growing the *MAT***a** strains carrying p6xHis-Hpf1 and p6xHis-Hpf1' (SB39 and SB57, respectively; Appendix 4) in synthetic complete medium without uracil, containing glucose, to mid-exponential. The cultures were washed in MilliQ and inoculated in synthetic complete medium without uracil, containing galactose and raffinose, in duplicate to induce expression for four hours. α-factor was added at 5 μg/mL to one replicate culture. After one hour, another 5 μg/mL α-factor was added and the culture was incubated for another hour. Cells were fixed with formaldehyde and washed in PBS. Cells were blocked in PBS + 1% (w/v) bovine serum albumin (BSA) at 4^{o}C overnight. The primary antibody used was mouse anti-6xHis (Sigma) (1:100) and the secondary antibody was anti-mouse IgG (Fab specific) fluorescein isothiocyanate (FITC) conjugate (Sigma) (1:128). The cells were wet-mounted and examined by phase contrast microscopy and then with fluorescence microscopy using an FITC filter set on an Olympus BX51 microscope at 100x magnification.

CHAPTER THREE

PHENOTYPIC ANALYSES OF *HPF* DELETION AND OVEREXPRESSION STRAINS

3.1 INTRODUCTION

The genome sequence of *S. cerevisiae* was published in 1996 and it was the first eukaryotic sequence completed (Goffeau *et al.*, 1996; Oliver, 1996). Of the approximately 6000 predicted genes, less than one third had known function, including the *HPF* genes. The experiments described in this chapter investigated possible phenotypes for mutants of the genes which encode the putative Hpf mannoproteins, the so-called *HPF* genes, YOL155c (*HPF1*) and YDR055w (*HPF2*), in order to determine their biological function in the yeast *S. cerevisiae*.

A second gene with significant homology to *HPF1*, *HPF1'*, was identified in the *S. cerevisiae* genome. At the protein level, Hpf1p and Hpf1'p exhibit 71% positional identity. Hpf1p and Hpf1'p also have similarity to two duplicate smaller proteins, Yhr214p and Yar066p, both of unknown function, at 74% identity to Hpf1p (over 203 of the 967 amino acids) and 77% identity to Hpf1'p (over 203 of the 995 amino acids). No other protein from human, mouse, rat, fruit fly, worm, fission yeast or *C. albicans* exhibits greater than 27% identity to either Hpf1p or Hpf1'p. Hpf1p and Hpf1'p have similarity to amino acid sequences in *S. bayanus* (Table 3.1) (Feldmann, 2000). YDR055w (also named *PST1* - protoplasts secreted), the gene encoding Hpf2p, has sequence similarity to Ecm33p (58% identity) (extracellular mutant), a sporulation specific protein, Sps2p, (31% identity) and Ycl048p (29% identity), all of unknown function. No related proteins have been detected in human, mouse, rat or fruit fly. Identity between Hpf2p and proteins in worm, fission yeast and *C. albicans* is no greater than 35%. Hpf2p exhibits similarity to amino acid sequences from the yeasts *S. bayanus*, *Zygosaccharomyces rouxii*, *Kluyveromyces lactis*, *Pichia sorbitophila* and *Candida tropicalis* (Table 3.1) (Feldmann, 2000). Table 3.2 shows the predicted length (expressed as amino acids in primary translation product), pI, molecular weight and codon bias for the Hpf protein sequences derived from the *Saccharomyces* genome sequence before any post-translational modification.

Table 3.1: Similarity of Hpf proteins to proteins from sequenced DNA of other yeasts (Feldmann, 2000).

S. cerevisiae **Hpf**	**Amino acids encoded by Hpf**	**Species**	**Number of amino acids in aligned region**	**% identity in alignment (amino acids)**
Hpf1p	967	*Saccharomyces bayanus*	108	41
Hpf1p	967	*Saccharomyces bayanus*	33	81
Hpf1p	967	*Saccharomyces bayanus*	86	48
Hpf1p	967	*Saccharomyces bayanus*	76	42
Hpf1p	967	*Saccharomyces bayanus*	213	80
Hpf1'p	995	*Saccharomyces bayanus*	108	41
Hpf1'p	995	*Saccharomyces bayanus*	34	70
Hpf2p	444	*Saccharomyces bayanus*	204	63
Hpf2p	444	*Zygosaccharomyces rouxii*	53	60
Hpf2p	444	*Zygosaccharomyces rouxii*	119	52
Hpf2p	444	*Kluyveromyces lactis*	180	49
Hpf2p	444	*Kluyveromyces lactis*	256	55
Hpf2p	444	*Kluyveromyces lactis*	236	52
Hpf2p	444	*Kluyveromyces lactis*	253	54
Hpf2p	444	*Pichia sorbitophila*	201	31
Hpf2p	444	*Pichia sorbitophila*	170	31
Hpf2p	444	*Candida tropicalis*	218	34

Table 3.2: Details of the sequence of the *S. cerevisiae* Hpf proteins (Cherry *et al*., 1997). These figures assume that the protein is not modified post-translationally. aa, amino acids; MW, Molecular weight.

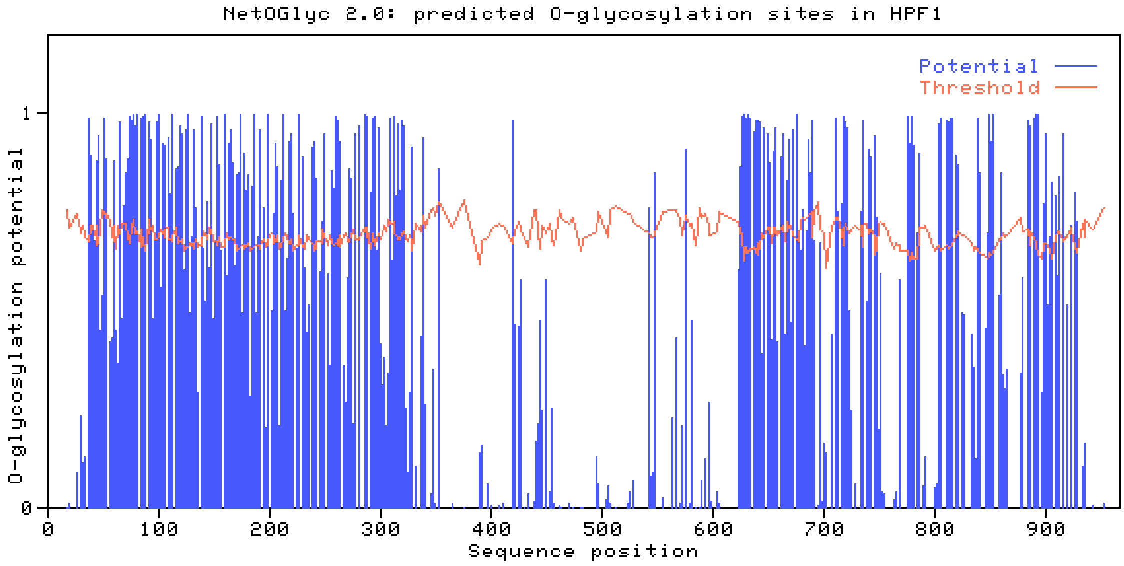
Feature	**Hpf1p**	**Hpf1'p**	**Hpf2p**
Length (aa)	967	995	444
Predicted pI	4.04	4.45	9.40
MW (Da)	94648	99681	45783
Codon bias	0.449	0.464	0.383

Since the Hpf proteins have a high serine and threonine content (Hpf1p, 42.4%; Hpf1'p, 40.7%; Hpf2p, 28.6% as a molar ratio), they are likely to be highly glycosylated (Stratford, 1994). As described in Section 1.5.3, the amino acid motif for *N*-glycosylation is Asn-X-Ser/Thr (where X is any amino acid except proline). Hpf1p, Hpf1'p and Hpf2p have five, four and fifteen potential *N*-glycosylation sites, respectively (Figure 1.2). The required motif for *O*-glycosylation remains unknown however, the glycosylation occurs when an α-D-mannosyl linkage is formed with serine or threonine residues of proteins (Strahl-Bolsinger *et al.*, 1999), as described in Section 1.5.3. The Hpf proteins are also predicted to be highly *O*-glycosylated using the programme NetOGlyc 2.0 (Figure 1.2 and Figure 3.1) (Hansen *et al.*, 1995; Hansen *et al.*, 1997; Hansen *et al.*, 1998) which has been found to correctly determine 51% of *O*-mannosylated sites and 85% of the non-*O*-mannosylated sites in fungal proteins. Previous work indicates that Hpf1p is *N*-glycosylated, as shown by the change in mobility after Endo H treatment (Waters *et al.*, 1994). Hpf1p is also *O*-glycosylated as shown by size-exclusion chromatography of the products after β-elimination (Waters *et al.*, 1994). Hpf2p is *N*- and possibly *O*-glycosylated, as shown by the change in mobility following PNGase F treatment and methylation linkage analysis of the polysaccharide (Stockdale, 2000).

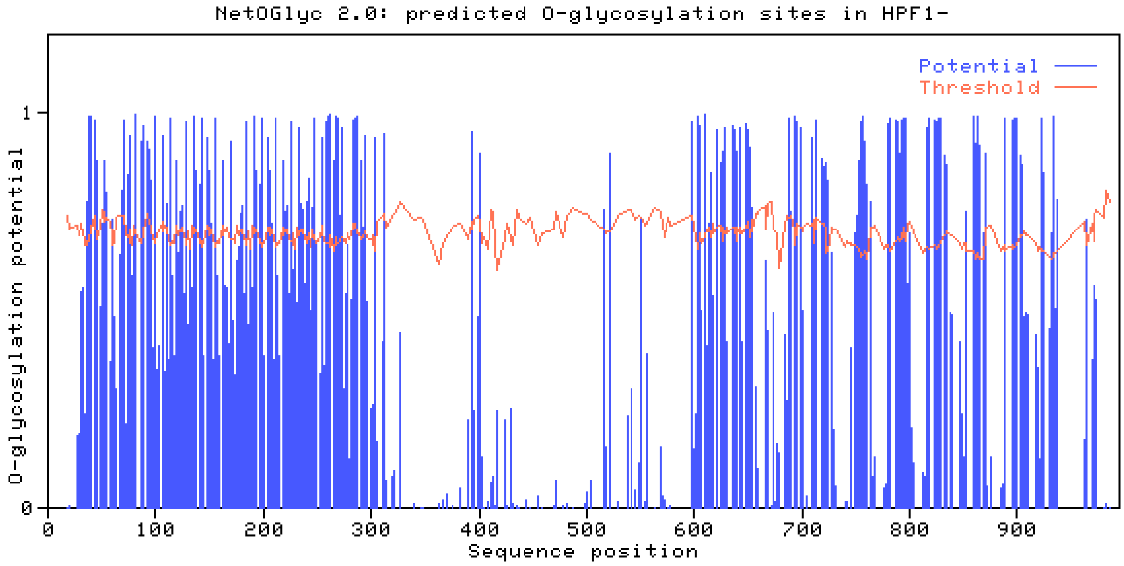
As described in Section 1.5.2, the Hpf proteins are likely to have N-terminal signal sequences and be GPI anchored proteins (Caro *et al.*, 1997). This suggests that the proteins are located at the cell wall or plasma membrane. Hpf1p has been putatively localised in the cell wall and Hpf2p in the plasma membrane (Caro *et al.*, 1997). Hpf1'p is not predicted to have a cell surface location using the GPI anchor prediction programme of von Heijne, (1986), used by Caro *et al.*, (1997). However, the programme has limitations and is only 75-80% accurate (von Heijne, 1986). Also, the significant predicted *N*- and *O*-glycosylation is further evidence that Hpf1'p is likely to be a cell surface protein. A polyclonal antibody was raised against purified Hpf1p and used in electron microscopic immunolocalisation of this protein (Dupin *et al.*, 2000a). These results indicated that Hpf1p was localised primarily to the innermost and outermost layers of the cell wall (Dupin *et al.*, 2000a).

Figure 3.1 Prediction of *O*-mannosylation of Hpf mannoproteins using NetOglyc 2.0. (a) Hpf1p, (b) Hpf1'p, (c) Hpf2p.

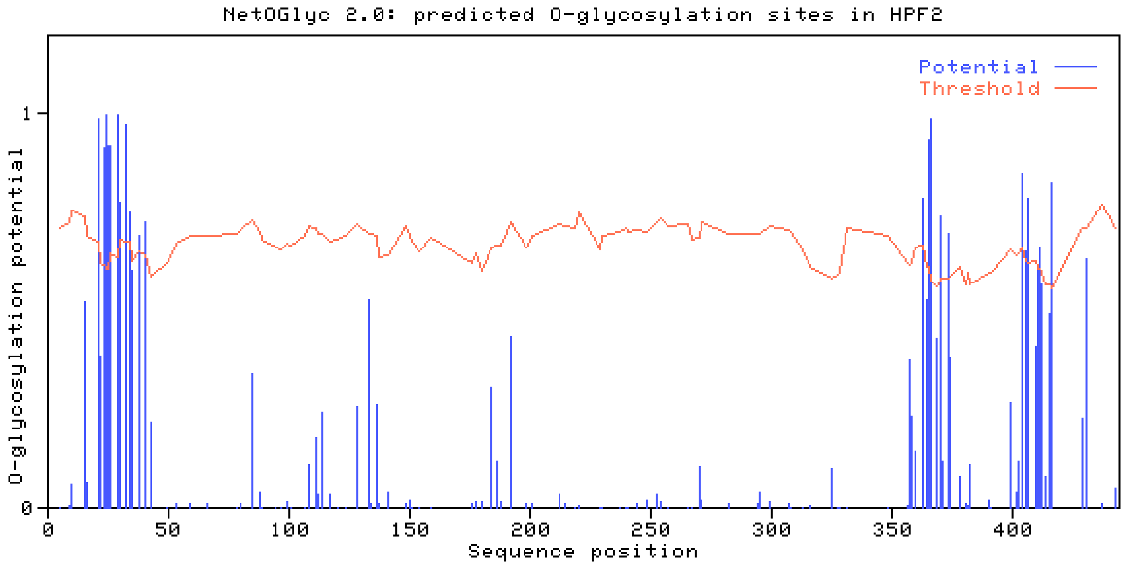
(a)


NetOGlyc 2.0: predicted O-glycosylation sites in HPF1
Potential
Threshold
O-glycosylation potential
1
0
0
100
200
300
400
500
600
700
800
900
Sequence position

(b)


NetOGlyc 2.0: predicted O-glycosylation sites in HPF1-
Potential
Threshold
O-glycosylation potential
1
0
0
100
200
300
400
500
600
700
800
900
Sequence position

(c)


NetOGlyc 2.0: predicted O-glycosylation sites in HPF2
Potential
Threshold
O-glycosylation potential
1
0
0
50
100
150
200
250
300
350
400
Sequence position

The probable cell surface localisation of these proteins may aid identification of their function in the cell. The yeast cell wall acts as an osmotic and physical structure to protect the cell from lysis (Fleet, 1991). Some cell wall proteins function in signalling changes in the outside environment, such as stresses and cell-cell recognition. These signals activate pathways that in turn modify the cell wall structure appropriately to protect or adapt to the changes (Molina *et al.*, 1999). Involvement of the *HPF* gene products in maintenance or alteration of the cell wall structure was analysed using *HPF* overexpression and *hpfΔ* deletion strains. To do this, the strains were grown in the presence of compounds that challenge the integrity of the cell wall structure and the signalling pathways that are required to maintain proper cell wall structure. These experiments are described and discussed below.

3.2 RESULTS

3.2.1 Construction of *HPF* overexpression strains and *hpfΔ* deletion strains

The putative *HPF* genes were amplified by PCR and cloned into the plasmid p415GAL1. Gene expression was under the control of the *GAL1* promotor and the *LEU2* gene provided for selection of the plasmid. After cloning, the *HPF* genes were sequenced to ensure that no mutations had occurred. The entire sequences of all three genes were found to be 100% identical to the sequence of the genes in the *Saccharomyces* Genome Database (Cherry *et al.*, 1997). The p415GAL1-HPF plasmids were transformed into a haploid S288c strain.

Deletion strains of each of the *HPF* genes were constructed using Short-Flanking Homology PCR (Wach *et al.*, 1997) which involved amplifying a marker gene (in this case *URA3* or *HIS5*) by PCR with primers that had homologous regions to the *HPF* genes at the 5’ end. The resultant PCR product was transformed into diploid S288c yeast and those yeast that had undergone homologous recombination were selected for on appropriate media. The diploid yeast, which were expected to be heterozygous at the *HPF* loci, were dissected to obtain *MAT***a** and *MAT*α *hpfΔ* haploid strains. The haploid strains were mated and dissected to construct haploid *hpf1Δ hpf1’Δ* and *hpf1Δ hpf1’Δ hpf2Δ* strains and homozygous diploid *hpf1Δ*, *hpf1’Δ* and *hpf2Δ* strains.

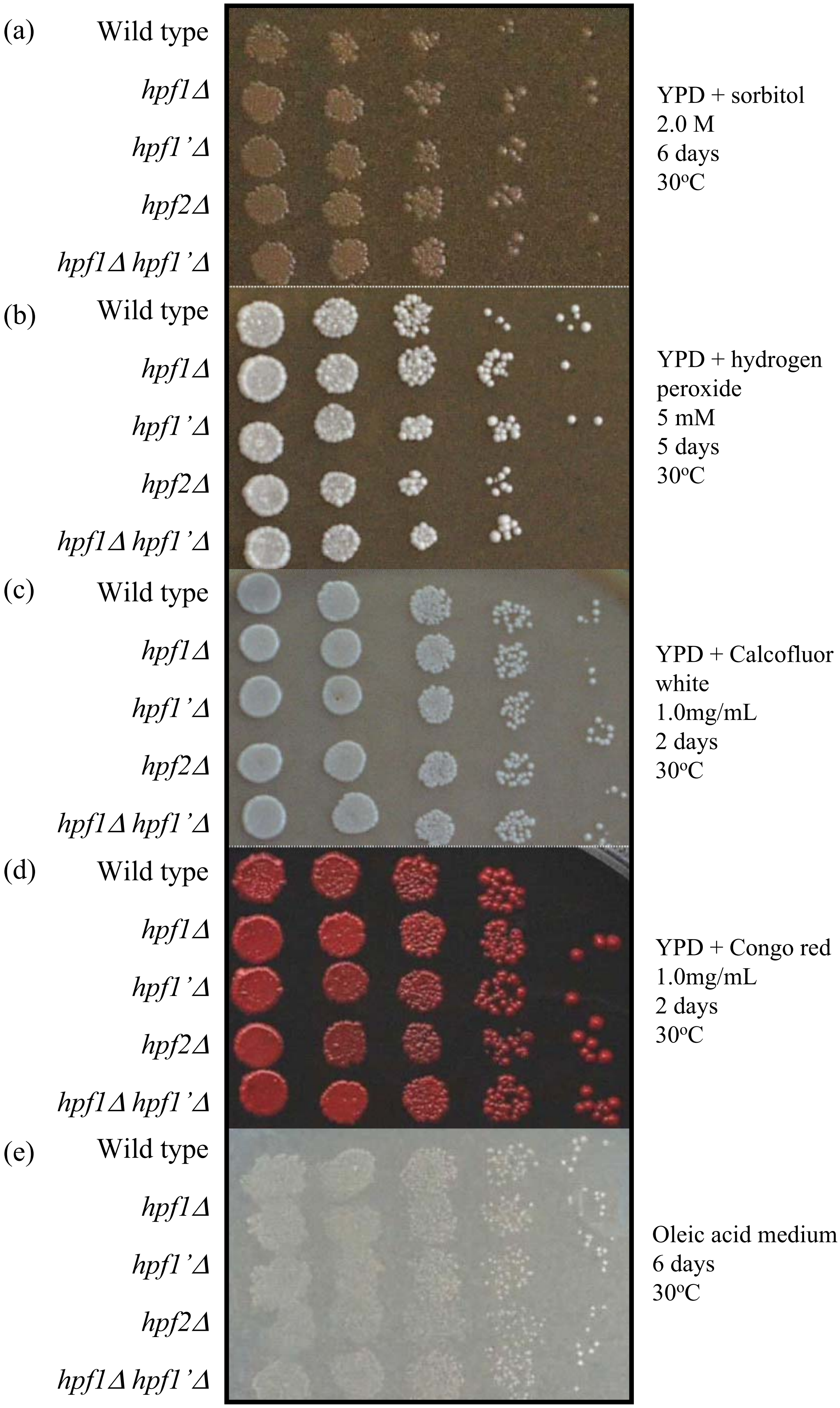
3.2.2 *HPF* deletion strains show no phenotypes linked to cell wall signalling responses

The yeast cell wall provides protection against changes in osmotic balance as rapid alterations in cell volume may cause the cell to lyse (Fleet, 1991). To respond to osmotic stress, yeast have osmosensors such as Sho1p and Sln1p-Ypd1p-Ssk1p (Maeda *et al*., 1995; Posas and Saito, 1997) on the cell surface that activate the high osmolarity glycerol (HOG) MAP kinase signal transduction pathway, which plays an important role in mediating the cellular response to the increased external osmolarity. The ability of the *hpfΔ* strains to grow under conditions of high osmotic stress was assayed by plating the yeast on to rich media with increasing sorbitol concentrations. No difference in the growth of the yeast strains under these conditions was found (Figure 3.2 (a)).

Oxidative stress is brought about by the generation of reactive oxygen species by normal aerobic metabolism, environmental radiation and certain chemicals. The sensor that induces a response to oxidative stress by hydrogen peroxide and diamide is the two-component protein Sln1p-Ssk1p (Singh, 2000) whereas Sho1p is responsible for signalling hydrogen peroxide-specific damage (Singh, 2000). Oxidative stress tolerance was tested by plating the yeast on to rich media containing hydrogen peroxide. No clear difference in the ability of the *hpfΔ* strains to grow under these conditions, relative to the wild type, was identified (Figure 3.2 (b)).

Alterations to the cell integrity MAP kinase signal transduction pathway may cause defects in cell wall assembly or maintenance. Inactivation of any of the components of the MAP kinase pathway results in cell lysis due to increased cell permeability and release of intracellular contents into the medium. This defect is limited to the cell wall, not the plasma membrane, thus it can be remedied by adding an osmotic stabiliser to the medium. The cell wall proteins Mid2p (Ketela *et al*., 1999) and Wsc1p (Lodder *et al*., 1999) are upstream sensors of the cell integrity pathway. If a defect in the cell integrity pathway occurs in the MAP kinase branch, then cell lysis only occurs at high temperatures or in the presence of low concentrations of caffeine, a purine analogue (Hampsey, 1997; Molina *et al*., 1999). Proteins involved in the cell integrity pathway can be identified by detecting the extracellular activity of the normally intracellular enzyme alkaline phosphatase from mutants or by determining the sensitivity of the

Figure 3.2 Growth conditions where no difference was seen between the wild type and *hpfΔ* mutants. The spots show serial 10 fold dilutions of an exponentially growing culture. These results were representative of those seen repeatedly.


(a)
Wild type
hpf1Δ
hpf1'Δ
hpf2Δ
hpf1Δ hpf1'Δ
YPD + sorbitol
2.0 M
6 days
30°C
(b)
Wild type
hpf1Δ
hpf1'Δ
hpf2Δ
hpf1Δ hpf1'Δ
YPD + hydrogen peroxide
5 mM
5 days
30°C
(c)
Wild type
hpf1Δ
hpf1'Δ
hpf2Δ
hpf1Δ hpf1'Δ
YPD + Calcofluor white
1.0mg/mL
2 days
30°C
(d)
Wild type
hpf1Δ
hpf1'Δ
hpf2Δ
hpf1Δ hpf1'Δ
YPD + Congo red
1.0mg/mL
2 days
30°C
(e)
Wild type
hpf1Δ
hpf1'Δ
hpf2Δ
hpf1Δ hpf1'Δ
Oleic acid medium
6 days
30°C

mutants to caffeine (Molina *et al*., 1999). No difference was seen in cell lysis between the wild type and *hpfΔ* mutant strains, either at 24 or 37°C and no difference in growth ability of wild type and *hpfΔ* mutant strains was seen on YPD-caffeine plates.

3.2.3 *hpf1Δ, hpf1'Δ* and *hpf2Δ* deletion mutants do not have a visible cell wall structural defect

The effect of deleting the *HPF* genes on cell wall structure was examined. Several studies have shown that some yeast cell wall deletion mutants, such as *cwp2Δ*, *ccw14Δ* or *mid2Δ*, are sensitive to Calcofluor white and Congo red (Ketela *et al*., 1999; Lussier *et al*., 1997; Moukadiri *et al*., 1997; Van Der Vaart *et al*., 1995). Calcofluor white and Congo red interfere with cell wall assembly. Calcofluor white is a fluorescent, anionic dye that preferentially binds to chitin, chitosan and cellulose, and to a lesser extent, β1,3-glucan (Pringle, 1991; Ram *et al*., 1994). Congo red is a dye that interacts with a variety of polysaccharides and exhibits a high affinity for yeast cell wall chitin (Roncero and Durán, 1985; Roncero *et al*., 1988). Calcofluor white and Congo red had no noticeable effect on the growth of the *hpfΔ* mutants (Figure 3.2 (c) and (d)).

Zymolyase is an enzyme preparation that digests glucan in the yeast cell wall. Sensitivity of yeast to zymolyase is used as a measure of changes in cell wall strength. Null mutants of several known cell wall proteins are sensitive to zymolyase, including *sed1Δ* (Shimoi *et al*., 1998), *ccw14Δ* (Moukadiri *et al*., 1997) and *cwp2Δ* (Van Der Vaart *et al*., 1995). No significant difference in zymolyase sensitivity was found between the wild type and *hpfΔ* cells (data not shown).

Killer action of yeast is the result of toxin secretion by specific killer strains. The toxin may be a protein or glycoprotein which is lethal to sensitive yeast (Golubev, 1998; Shimizu, 1993). Several yeast surface proteins are receptors for these killer toxins, and by deleting these genes the yeast become 'killer resistant.' For example, *KRE1* encodes the plasma membrane receptor for the yeast K1 viral toxin (Breinig *et al*., 2002). Also, the cell wall receptor for the HM-1 killer toxin that inhibits β1,3-glucan synthesis (Kasahara *et al*., 1994) is encoded by *RHK1* (Kimura *et al*., 1997). The sensitivity of the *hpfΔ* strains to a range of killer species was tested to detect structural changes in the cell wall (Table 2.1). No significant differences were seen between the wild type and *hpfΔ* mutants to killer sensitivity with the killer strains that were tested.

Transmission electron microscopy was used to closely look at the structure of the cell wall and plasma membrane to determine if a structural difference could be visualised. No significant difference was observed between the wild type and *hpf1Δ hpf1'Δ* mutant cells from two preparations in the structure of the cell surface including the thickness of the cell membrane or cell wall (Figure 3.3).

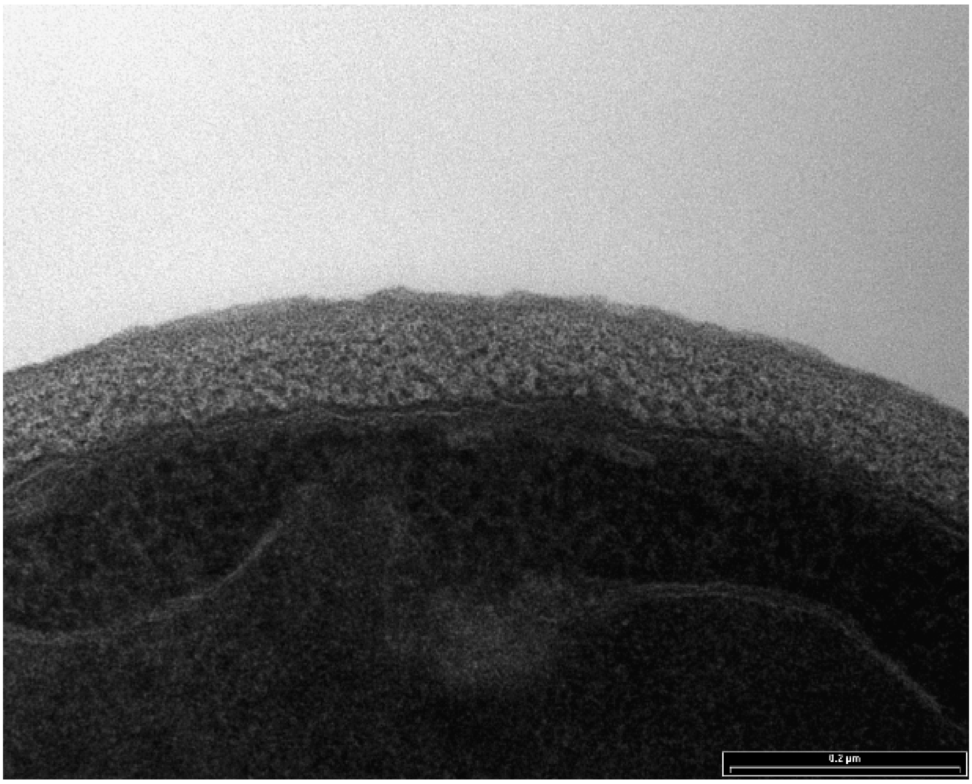
3.2.4 ***hpf1Δ hpf1'Δ*** **deletion mutants are not affected by the use of oleic acid as their sole carbon source**

Due to findings of Kal *et al*., (1999), that shows *HPF1* mRNA abundance increases 70-fold following a shift from glucose to oleate as the carbon source, the ability of the *hpfΔ* mutants to grow on oleic acid as the sole carbon source was tested. Fatty acids such as oleic acid are catabolised by fatty acid oxidation. Other genes induced by growth on oleate were involved in the biogenesis and function of peroxisomes, mitochondrial function, communication between peroxisomes, cytosol and mitochondria, and stress response pathways (Kal *et al*., 1999). Kal *et al*., (1999), also discovered that the oleate-induced increase in *HPF1* mRNA abundance is eliminated in a *pip2Δ oaf1Δ* mutant. Pip2p and Oaf1p encode transcription factors that are required for the induction of genes encoding peroxisomal proteins required for β-oxidation of fatty acids. These transcription factors regulate transcription of genes which have the oleate response element (ORE) present in their promotor region. *HPF1* does not have the consensus ORE in its 5' region (Karpichev and Small, 1998). It was found that growth of wild type and *hpfΔ* mutants were similar when grown on oleic acid as the carbon source (Figure 3.2 (e)).

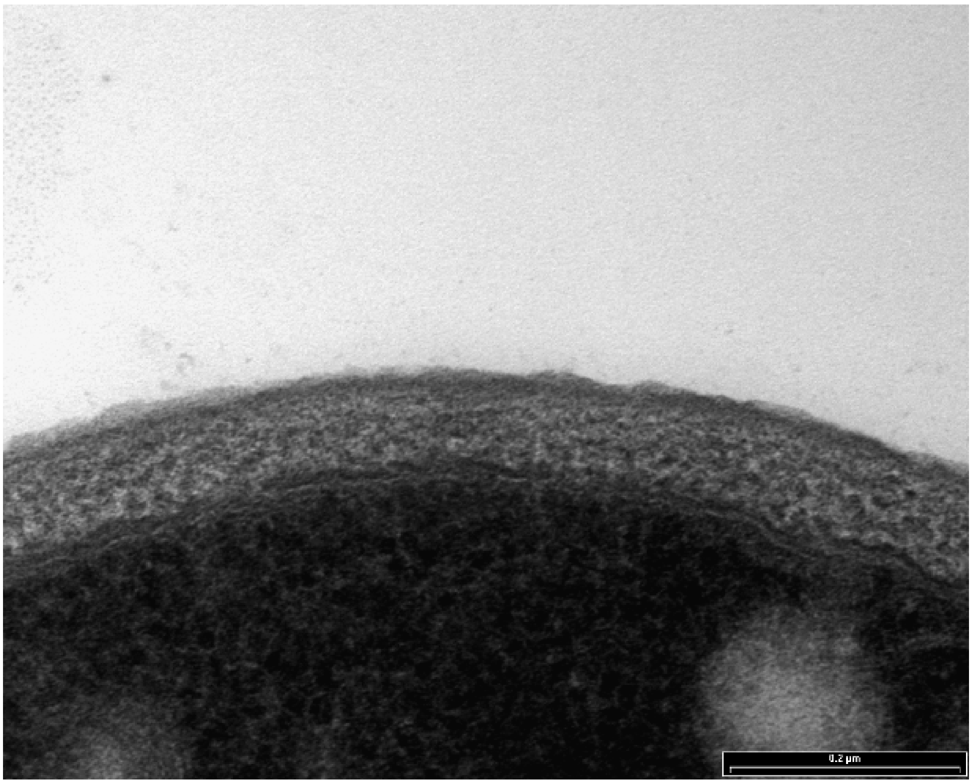
The unsaturated fatty acid composition of membranes of *S. cerevisiae* is almost exclusively palmitoleic (Δ^9Z-$C_{16:1}$) and oleic acids (Δ^9Z-$C_{18:1}$), with the majority being palmitoleic acid (You *et al*., 2003). Palmitoleic and oleic acids are essential for homeoviscous adaptation (to maintain constant membrane fluidity) in various

Figure 3.3 Transmission electron micrographs of cell wall and cell membrane from (a) wild type (S288c) and (b) *hpf1Δ hpf1'Δ* mutant cells. The structure of the cell wall and membrane shown in these micrographs is representative of that seen for at least 50 cells of each strain. The scale bar is 0.2 µm.

(a)



(b)



environments such as temperature, pH, osmotic pressure and the presence of salt or ethanol (Hazel and Williams, 1990; Sajbidor, 1997). This led us to investigate the growth of the *HPF* deletion strains under temperature and ethanol stress.

3.2.5 *hpf1Δ hpf1'Δ* deletion strains have improved growth at low temperature

The ability of the mutant strains to grow at various temperatures ranging from 4 to 40°C was tested because of a possible involvement of the genes in maintenance of membrane fluidity as described in Section 3.2.4. The strains in the S288c background constructed during this study were used as well as *hpf1Δ*, *hpf1'Δ* and *hpf1Δ hpf1'Δ* in the W303 background (kindly donated by Dr. Trevor Lithgow and Traude Beilharz, University of Melbourne). On solid growth medium, at regular yeast growth temperatures (24-30°C), and higher temperatures (30-44°C) all the mutant strains grew as well as the wild type strains, up to the temperature where no strain could grow (44°C). However, at low temperatures (4-24°C), the *hpf1'Δ* and *hpf1Δ hpf1'Δ* deletion mutants grew at a faster rate than the wild type showing that they are more tolerant to the reduced temperature (Figure 3.4). This difference was seen more clearly in the W303 background than the S288c background. To further define this difference in growth rate at low temperature, the strains were cultured in triplicate in liquid medium at 10, 12 and 14°C. This clearly showed that the *hpf1Δ hpf1'Δ* mutant had a faster growth rate at every low temperature tested. The *hpf1'Δ* strain also had a faster growth rate than the wild type, but not as fast as the double deletion mutant (Figure 3.5).

The *HPF* overexpression strains, in the S288c background, were tested for their growth at various temperatures on solid growth medium. At regular temperatures (24-30°C), all overexpressors grew as well as the wild type on both glucose and galactose media. At low temperatures, the *HPF1* overexpressor did not grow as well as the wild type on glucose or galactose. It is possible that 'leaky' expression of Hpf1p on glucose prevented growth as did overexpression on galactose.

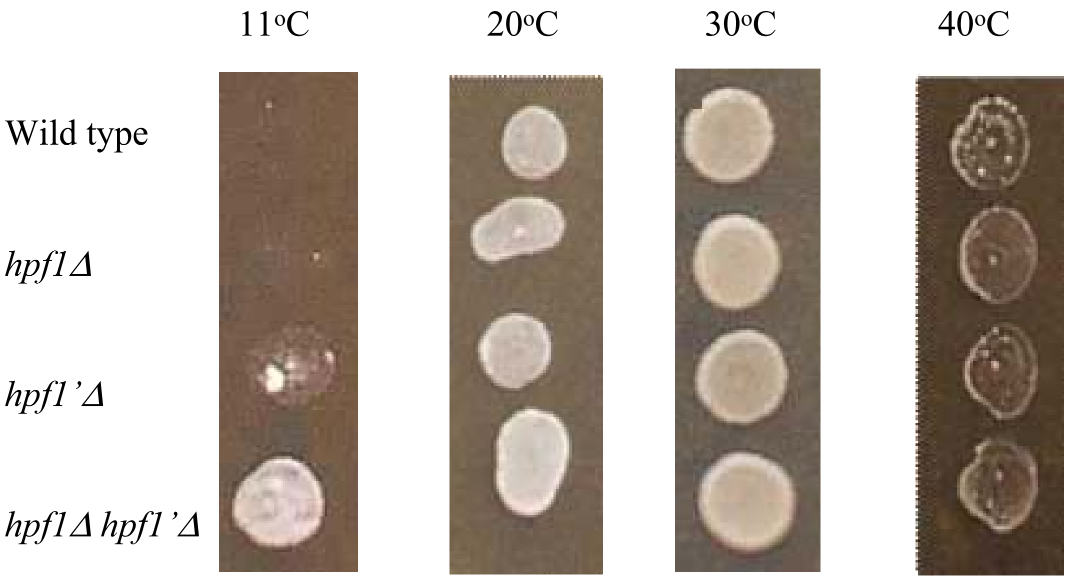
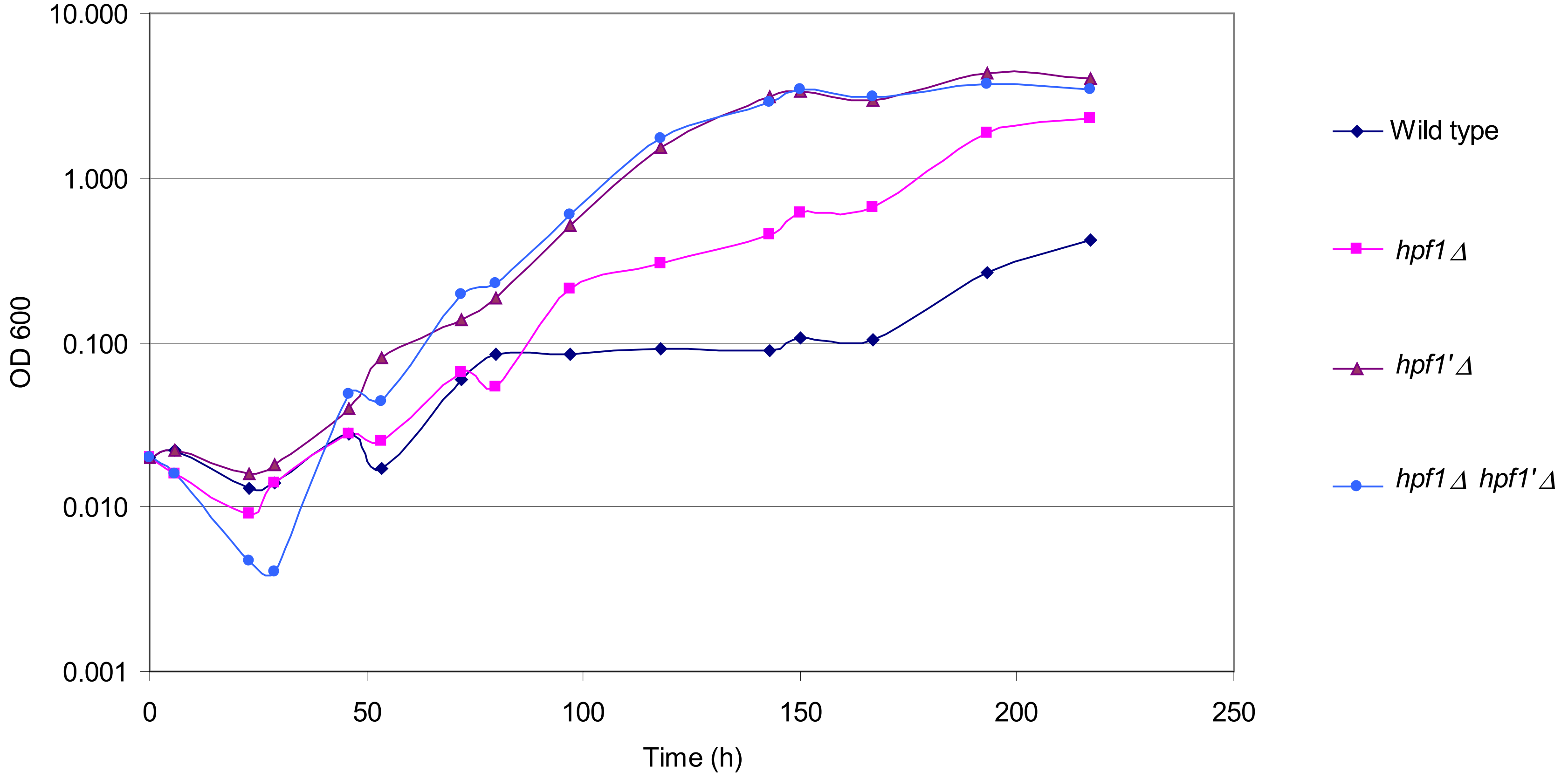


Figure 3.4 Difference in growth of *hpfΔ* strains in the W303 background on rich solid medium at 11, 20, 30 and 40^{o}C. The *hpf1'Δ* and *hpf1Δ hpf1'Δ* were able to grow and therefore more tolerant to low temperature.

Figure 3.5 The growth curve of wild type and *hpfΔ* mutants in the W303 background in YPD medium at 10ºC as determined by measurement of optical density at 600nm. Similar results were seen at 12 and 14ºC.


10.000
1.000
0.100
0.010
0.001
OD 600
0
50
100
150
200
250
Time (h)
Wild type
hpf1Δ
hpf1'Δ
hpf1Δ hpf1'Δ

3.2.6 ***hpf1Δ hpf1'Δ*** deletion strains have improved growth in the presence of ethanol

The growth of the *hpfΔ* strains was tested in the presence of various concentrations of ethanol because of the possible involvement in maintenance of membrane fluidity as described in Section 3.2.4. Although it is difficult to be sure of the final ethanol concentration in the medium because of the volatility of ethanol (Aguilera and Benitez, 1986), at addition concentrations of 12%-15% (v/v), the *hpf1Δ* and *hpf1Δ hpf1'Δ* mutants were more tolerant to ethanol than the wild type. This could be seen in both the S288c and W303 backgrounds (Figure 3.6). The ethanol tolerance of these strains was also tested in liquid media, over a short time period, but no difference was seen after 24 hours.

3.2.7 The ***hpf1Δ hpf1'Δ*** deletion mutant out-competes the wild type under laboratory growth conditions

A majority of yeast genes are not essential in laboratory conditions and fail to show a discernable phenotype, even when disrupted (Thatcher *et al*., 1998). It is possible that (i) these genes have important functions in environments not yet tested in the laboratory and would show a conditional phenotype if the right conditions were found. This possibility was tested as described in Sections 3.2.5 and 3.2.6. (ii) The non-essential genes make small but significant contributions to fitness, even under routine growth conditions, but these are unable to be detected by conventional methods (Thatcher *et al*., 1998).

In this work, an assay that measured the possible 'marginal benefit' of non-essential genes was used. Using the method of Thatcher *et al*., (1998), the wild type and *hpf1Δ hpf1'Δ* strains were co-inoculated into four replicate cultures. The cultures were diluted back daily for 27 days and on some days, a sample of the culture was taken and plated on YPD to determine total cell number. Cells were also plated on selective medium for the *hpfΔ* mutant in order to determine the proportion of cells in the culture that were wild type and mutant (Figure 3.7). The results in Figure 3.7 show that, over time, the proportion of the *hpf1Δ hpf1'Δ* mutant cells increased to 80% in the mixed culture and

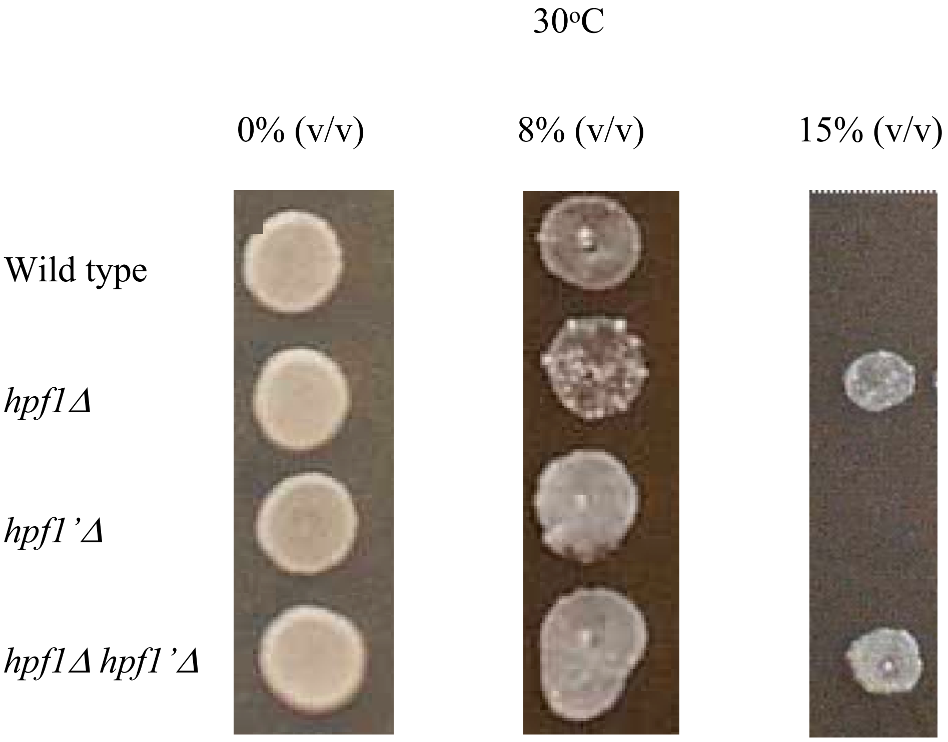
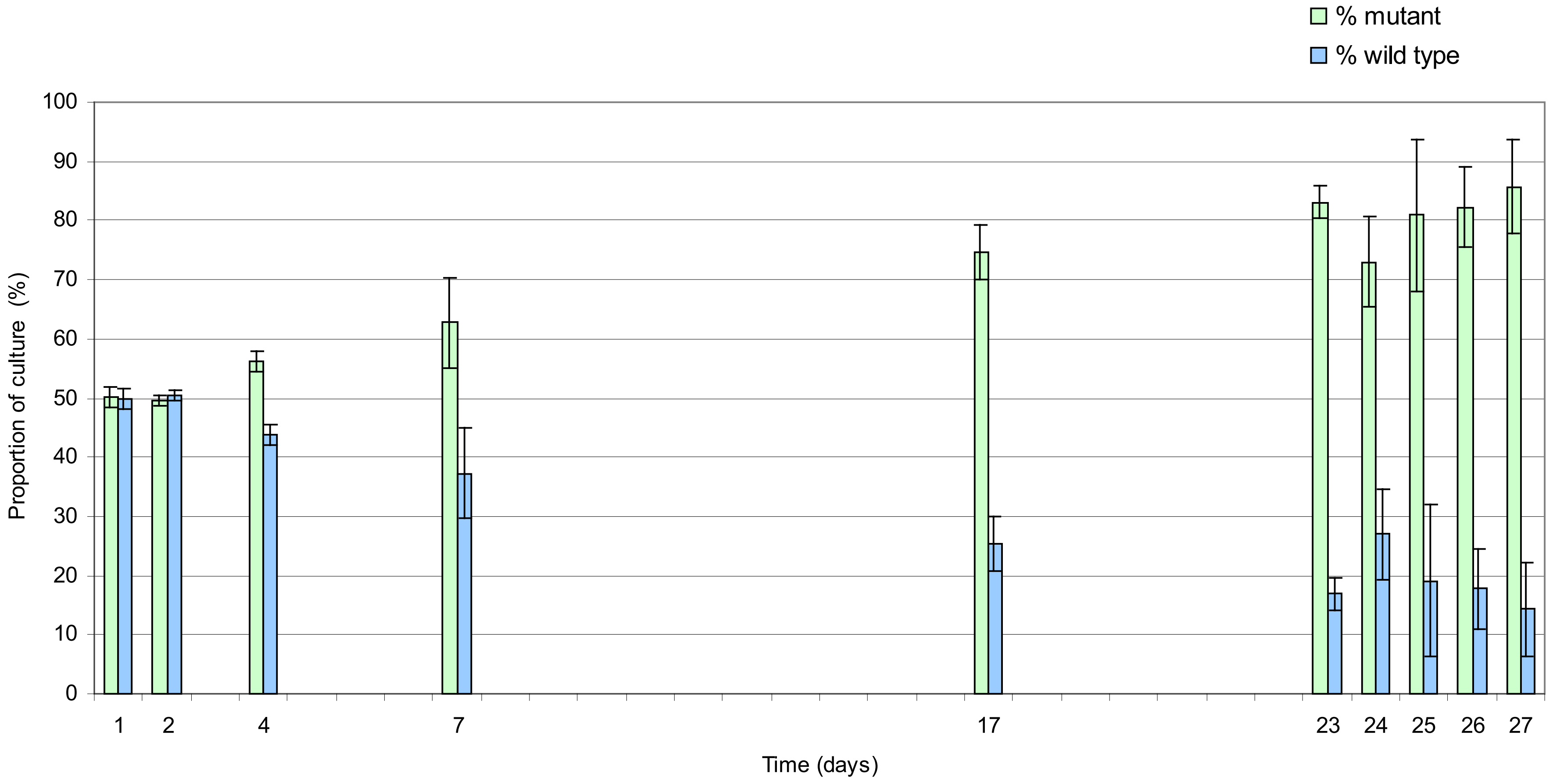


Figure 3.6 Difference in growth of *hpfΔ* strains in the W303 background on solid rich medium with various concentrations of ethanol at 30^{o}C grown for up to 14 days.

Figure 3.7 Competitive growth assay of wild type and *hpf1Δ hpf1'Δ* in YPD medium. Performed as described in Chapter 2 following the method of Thatcher *et al*., (1998). Results show the mean and standard deviation of four replicate cultures.


% mutant
% wild type
Proportion of culture (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
4
7
17
23
24
25
26
27
Time (days)

therefore these genes appear to be detrimental to growth under normal laboratory growth conditions.

3.3 DISCUSSION

In this chapter, the construction of the *HPF* deletion and overexpression strains was described. The experiments aimed to determine the phenotype of the *hpfΔ* mutants and *HPF* overexpression strains in order to uncover the biological function of the *HPF* genes in *S. cerevisiae*. The *HPF* gene products are predicted to be localised to the cell surface, as explained in Section 3.1, therefore cell wall related phenotypes were investigated for the *hpfΔ* mutants. The experiments described in Section 3.2.2 examined several cell wall signalling response phenotypes namely osmotic stress, oxidative stress and defects in cell wall maintenance. It was found that growth of *hpf1Δ*, *hpf1'Δ*, *hpf2Δ* and *hpf1Δ hpf1'Δ* mutants were not different from the wild type yeast under these conditions. Alterations to cell wall structure upon deletion of the *HPF* genes was also investigated using transmission electron microscopy and the cell wall perturbing agents Calcofluor white, Congo red and zymolyase. No difference in the structure of the cell surface or growth of the *hpf1Δ*, *hpf1'Δ* and *hpf2Δ* strains was seen under these conditions. This corresponded with the results of Lafuente and Gancedo, (1999), who showed that with respect to cell wall associated phenotypes, such as equinocandine, papulacandine and Calcofluor white sensitivity, no appreciable difference in phenotype between wild type and *HPF1* and *HPF1'* mutant strains was found. Chitin distribution and sonication resistance were also found to be normal (Lafuente and Gancedo, 1999). It is possible that the phenotype of the *hpf1Δ hpf1'Δ* strain may be partially complemented by Yhr214p and Yar066p, the homologues to Hpf1p and Hpf1'p.

The similarity between the wild type and *hpf2Δ* strain was somewhat unexpected considering *HPF2* transcription was previously shown to be induced under conditions of cell wall stress (Roberts *et al*., 2000). *HPF2* transcription was increased 34.51 fold when *PKC1*, the gene encoding protein kinase C, was overexpressed and 9.42 fold when *RHO1* was overexpressed (Roberts *et al*., 2000). The Slt2p/Mpk1p MAP kinase pathway, which is controlled by Pkc1p, is directly involved in signalling cell wall damage (de Nobel *et al*., 2000). Rho1p, a small GTPase, binds to and activates Pkc1p.

The signal is transmitted through the cascade to the MAPKKK Bck1p, the MAPKKs Mkk1p and Mkk2p and the MAPK Slt2p/Mpk1p. *HPF2* was found to be positively regulated by Slt2p/Mpk1p and this was mediated by the Rlm1p transcription factor (Jung and Levin, 1999). This cell integrity pathway is induced by several environmental stimuli including elevated temperature, hypo-osmotic shock, oxidative stress, treatment with Calcofluor white, caffeine and mating pheromone (Jung and Levin, 1999; Singh, 2000). These results indicate an involvement for Hpf2p as a final target in response to cell wall damage. Although *HPF2* transcription is induced by overexpression of components of the Slt2p/Mpk1p MAP kinase pathway, deletion of this gene does not seem to affect yeast growth even under conditions when this pathway should be upregulated. Many cell wall proteins have been shown to have overlapping functions, especially if the proteins have a structural role. For example, a strain with multiple deletions, *ccw12Δ ccw13Δ cwp1Δ tip1Δ icwp1Δ*, did not show any extensive growth problems (Mrsa *et al*., 1999). It is possible that other cell wall proteins were able to compensate for these deletions.

Non-essential genes to which no phenotype can be found may be important in an environment that has not yet been simulated in the laboratory or make small but significant contributions to fitness. The results in Section 3.2.7 show that the *hpf1Δ hpf1'Δ* mutant was able to grow at a faster rate than the wild type yeast in a competitive growth assay thus these genes appear to be detrimental to growth under laboratory conditions. The results of the competitive growth assay show that the proportion of mutant cells in a mixed culture increases over time, possibly due to a faster growth rate than the wild type. Together, these results suggested that these genes are not contributors to 'marginal fitness' in the conditions investigated (Thatcher *et al*., 1998). In fact, by deleting *HPF1* and *HPF1'*, the fitness of the yeast appeared to improve. The deletion of these genes showed that they do not contribute to the 'marginal fitness' proposal of Thatcher *et al*., (1998), therefore it is likely that *HPF1* and *HPF1'* are required in environments that have not yet been tested in the laboratory.

Although competition experiments are a sensitive way to measure differences in growth rate (Baganz *et al*., 1998; Baganz *et al*., 1997), the competitive growth assay does have limitations involved with the replacement of the *HPF* genes with the nutritional markers

HIS5 and *URA3* (Baganz *et al*., 1997). Depending upon the physiological conditions used for the assay, *HIS3* was shown to give the yeast a significant advantage (Baganz *et al*., 1997). The change in fitness due to the presence of nutritional markers in deletions strains may be attributed to differences in the kinetics and energetics of amino acid uptake for the auxotrophic parent strain in relation to those of amino acid synthesis in the mutant strain (Baganz *et al*., 1997). Even so, these results indicate that deleting *HPF1* and *HPF1'* has no detrimental effect on cell growth.

The possibility of *HPF1* being involved in oleic acid metabolism was investigated because Kal *et al*., (1999), showed that transcription of *HPF1* is up-regulated 70-fold when yeast are grown on oleic acid as the sole carbon source, compared to glucose. No difference in growth between the wild type and *hpfΔ* mutants on oleic acid medium was observed. Although deletion of the *HPF* genes did not alter growth on oleic acid, the link with oleic acid was investigated further. Some of the other genes that were significantly upregulated were directly involved in oleic acid metabolism, such as biogenesis and function of peroxisomes or mitochondrial function, and others were stress response proteins. *HPF1* does not contain an oleate response element which is required for induction by the β-oxidation related transcription factors Pip2p and Oaf1p. Oleic acid is an important unsaturated fatty acid of membranes in *S. cerevisiae* because it is required for maintenance of membrane fluidity (the measure of the rate of the lateral motion of molecules within the membrane). Membrane fluidity may be altered by variations in growth environments such as temperature or the presence of ethanol so the involvement of the Hpfs in temperature stress and ethanol stress was investigated. These growth conditions showed that the *hpf1Δ hpf1'Δ* mutants were more tolerant than the wild type yeast to cold temperature and to ethanol.

The molecular basis for cold tolerance in plants has been the focus of extensive research. Lowered temperatures result in decreased membrane fluidity and increased fatty acid unsaturation in many species (Harwood *et al*., 1994; Murata and Los, 1997; Nakagawa *et al*., 2002). Miquel *et al*., (1993), along with others, have shown a correlation between increased fatty acid unsaturation and cold resistance. A study of genetic response in yeast cells to low temperature showed that genes related to rRNA synthesis, ribosomal proteins and several stress responses are up-regulated (Sahara *et al.*, 2002).

The increased cold temperature and ethanol tolerance of the *hpf1Δ hpf1'Δ* strain is striking as temperature and ethanol tolerances have been correlated previously, but often the temperature response was to heat (Aguilera and Benitez, 1986). Published data shows that the response of cells to cold temperature and ethanol is usually inversely correlated in terms of membrane fluidity and fatty acid unsaturation. Cold temperature generally causes decreased membrane fluidity and increased fatty acid saturation in cells (Harwood *et al.*, 1994; Miquel *et al.*, 1993; Murata and Los, 1997) whereas ethanol and heat exposure causes increased membrane fluidity and decreased fatty acid saturation (Alexandre *et al.*, 1994; Piper, 1995; Sajbidor and Grego, 1992). *S. cerevisiae* has a single integral membrane Δ^9 fatty acid desaturase encoded by *OLE1*, which acts to modulate the degree of fatty acid unsaturation and therefore membrane fluidity (Murata and Los, 1997). This homeoviscous adaptation response can be relatively rapid (Jones and Greenfield, 1987; Piper, 1995). The stress response of yeast to sublethal ethanol and heat exposure are similar, but vary dramatically depending upon the strain and medium composition (Piper, 1995). This response includes the induction of heat shock proteins, and changes to membrane protein composition such as reduction of levels, but increased activity of plasma membrane H^+-ATPase (Piper, 1995). The adverse effects of ethanol are generally more severe at higher temperatures.

However, links between ethanol and cold tolerance have been seen previously. Peyou-Ndi *et al.*, (2000), cloned a Δ^{12} fatty acid desaturase from *Caenorhabditis elegans* and demonstrated its activity by heterologous expression in *S. cerevisiae*. This yeast was tolerant to both cold temperature and ethanol, as was the *hpf1Δ hpf1'Δ* mutant. The gene's expression resulted in accumulation of $\Delta^{9,12}$Z-$C_{16:2}$ and $\Delta^{9,12}$Z-$C_{18:2}$ (linoleic) acids and thus an increase in membrane fluidity. This altered fatty acid composition and increased membrane fluidity also led to increased resistance to oxidative stress. The *hpf1Δ hpf1'Δ* mutants did not grow differently in response to oxidative stress. The deletion of *HPF1* and *HPF1'* has some of the same growth phenotypes as overexpression of a Δ^{12} fatty acid desaturase indicating that these *HPF* genes may be involved with the pathway that enhances fatty acid saturation and therefore decreases membrane fluidity.

The increased ethanol tolerance described above was observed on agar plates. No difference in growth of the *hpf1Δ hpf1’Δ* strain, relative to the wild type, was seen following short-term exposure to ethanol in liquid medium. The reason for this discrepancy may be that cell wall and membrane changes are long-term adaptation processes (Alexandre *et al*., 2001). The expression profile of yeast genes in response to short-term ethanol stress has been previously investigated and it was found that environmental stress response genes including heat protection, antioxidant defence, ionic homeostasis and trehalose synthesis genes were upregulated (Alexandre *et al*., 2001). Transcription of genes involved in lipid metabolism or cell wall biosynthesis were not upregulated even though the cell wall and membrane are known targets of ethanol stress response (Alexandre *et al*., 2001). This may be because changes in membrane composition are a long-term response to ethanol stress.

The occurrence of a cold tolerant phenotype in a yeast null mutant, as was seen for *hpf1’Δ* and *hpf1Δ hpf1’Δ*, has been described by one other group only (Stolz *et al*., 1998a; Stolz *et al*., 1998b) and this may provide a hint towards characterising the function of Hpf1p and Hpf1’p. An *inp51Δ* mutant grew significantly faster at temperatures below 15^{o}C compared to the wild type. An *inp52Δ* strain also exhibited this phenotype, but to a lesser extent. *INP51* and *INP52* (inositol polyphosphate 5-phosphatase numbers 1 and 2) have similarity in their amino- and carboxyl-terminal regions to mammalian inositol polyphosphate 5-phosphatases. *INP51* displays intrinsic phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) 5-phosphatase activity which is critical to maintain proper cellular levels of $PI(4,5)P_2$. Deletion mutants of *INP51* have a 2-4 fold increase in $PI(4,5)P_2$ and inositol-1,4,5-trisphosphate, whereas cells overexpressing *INP51* have a 35% decrease in $PI(4,5)P_2$. $PI(4,5)P_2$ is normally localised to the plasma membrane and is required for normal cell morphology and membrane trafficking (Stefan *et al*., 2002). Upon deletion of the *INP5* genes, $PI(4,5)P_2$ inappropriately accumulated in intracellular compartments as well as the cell surface (Stefan *et al*., 2002). Measurement of the levels and distribution of inositol phosphates in the *hpfΔ* mutants may further explain the cold tolerant phenotype.

3.4 CONCLUSION

The results presented in this chapter have shown that the growth ability of the wild type and *hpfΔ* mutants is similar under osmotic stress, oxidative stress and in the presence of caffeine indicating that the *HPF* gene products do not contribute to the cell wall signalling responses assessed in this study. The *hpfΔ* mutants are not affected by compounds which alter the structure of the cell wall such as Calcofluor white, Congo red or zymolyase. When *HPF1* and *HPF1'* are deleted from yeast, the yeast become more tolerant to cold temperature and ethanol and the cells have a faster growth rate than the wild type. This suggests that these genes do not have a 'marginal benefit' contribution to the fitness of the yeast. In fact, when these genes are deleted the yeast is at an advantage relative to the wild type under certain growth conditions. These genes may be important in an, as yet, untested environment, or in conditions that yeast encounter in their natural habitat and remain to be defined. Alternatively, the genes may have a role in secondary development pathways, which is examined further in Chapter 4.

CHAPTER FOUR

BIOLOGICAL FUNCTION OF THE *HPF* GENE PRODUCTS IN YEAST

4.1 INTRODUCTION

The role of *HPF1* and *HPF1'* in the yeast mating pathway is examined in this chapter. To further investigate the biological function of the *HPF* genes, it was instructive to examine what is known about the genes and homologues in other organisms as a result of other investigations. *HPF1* exhibits homology to *STA2* that encodes glucan 1,4-α-glucosidase, a protein found in only one yeast strain; *S. cerevisiae* var. *diastaticus* (Costanzo *et al.*, 2000). Sta2p is an extracellular glucoamylase enzyme, 25% of which is secreted (Vivier *et al.*, 1999). Expression of glucoamylase takes place only in haploids, not in a/α diploids. This enzyme is important for degradation of starch (Balogh and Maráz, 1996). This may be an important clue to the function of *HPF1* in *S. cerevisiae* but the similarity is limited to the serine/threonine rich region and this may only imply that both are glycosylated proteins. Lo and Dranginis, (1996), discovered a novel flocculin, *MUC1*, a member of the *FLO* gene family that is related to the *STA* genes of yeast. A recent investigation of another *FLO* family gene of a bottom-fermenting lager yeast (Lg-*FLO1*) identified a new gene in a lager yeast that had lost its flocculation ability (*ILF1*-inactivated Lg-*FLO1*). *ILF1* was a fusion gene of the N-terminal of Lg-*FLO1* and a part of chromosome IX from *S. cerevisiae*, and the C-terminal domain was thought to contain a part of *HPF1'* (Sato *et al.*, 2002). Southern analysis indicated that the translocation of an approximately 20kb region of chromosome XI to the Lg-*FLO1* region results in the conversion of a flocculent cell to a non-flocculent one (Sato *et al.*, 2002). A flocculation phenotype has not been described for *hpfΔ* mutants and was not observed in this study. *HPF1* exhibits similarity to *AWA1*, ('awa' – the Japanese word for foam), which was responsible for the foaming ability of the sake yeast K7 (Shimoi *et al.*, 2002). The extent of the similarity was not described by Shimoi *et al.*, (2002). *AWA1* is not found in laboratory yeast.

As a contribution to the European Functional Analysis Network (EUROFAN), Lafuente and Gancedo, (1999), undertook a systematic analysis to determine the function of several previously uncharacterised yeast genes on chromosome XV, including YOL155c (*HPF1*). Due to the notable similarity between YOL155c and YIL169c (*HPF1'*), as described in Section 3.1, Lafuente and Gancedo, (1999), constructed a double disruption mutant (*yol155cΔ yil169cΔ*) and analysed its phenotype. No obvious growth, mating or sporulation phenotype was observed for deletion strains in either the CEN.PK2 or FY1679 (S288c) backgrounds (Lafuente and Gancedo, 1999). Expression of Yol155p was similar in several carbon sources in rich or minimal medium (Lafuente and Gancedo, 1999).

HPF1 has been identified as a multicopy suppressor of lethality in *mnn9Δ sed1Δ* or *mnn10Δ sed1Δ* double mutants (Horie and Isono, 2001). High copy suppression is often an indicator of the ability of a gene to act downstream or in a parallel pathway (Gammie *et al.*, 1998). Mnn9p and Mnn10p act in the *N*-glycosylation pathway (Dean, 1999; Jungmann *et al.*, 1999) and Sed1p is likely to be a structural cell surface glycoprotein (Shimoi *et al.*, 1998). This result suggests that Hpf1p acts in the same or a parallel pathway to Sed1p. A Yol155p-GFP fusion protein was localised to the cell wall during vegetative growth and its localisation was especially prominent in the region of the bud sites (Horie and Isono, 2001). This localisation may not be accurate as the Hpf1p-GFP construct is questionable. The GFP was located at the C-terminus of the immature Hpf1p, however, the GPI anchor signal sequence at the C-terminus of the protein is most likely cleaved in post-translational modification. This means that the GFP localised in this experiment may not necessarily be attached to Hpf1p or may not be representative of the final mature protein.

The prime function of the cell wall is to protect the cell from osmotic or physical lysis (Fleet, 1991). Apart from providing protection, however, the cell wall must be a dynamic structure which is constantly rearranged to accommodate cell modifications such as budding, filamentous growth and mating projection formation. As described in Section 1.5.1, the yeast cell wall also functions in cell-cell recognition and adhesion during mating. For cell fusion of mating partners, the cell wall degrades prior to plasma membrane fusion. As a result, mating is a potentially hazardous act for the yeast and must be tightly regulated.

The pheromone response pathway is induced when a peptide pheromone (**a**-factor or α-factor) interacts with the plasma membrane receptor of the opposite mating type (Ste2p or Ste3p) (Figure 4.1). This recognition activates a heterotrimeric G-protein (Gα (Gpa1p); Gβ (Ste4p)-Gγ (Ste18p)), which in turn initiates a mitogen activated protein (MAP) kinase cascade consisting of MAPKKK Ste11p, MAPKK Ste7p and MAPKs Fus3p and Kss1p. A scaffolding protein, Ste5p, organises the structure of these proteins (Elion, 2000). When the final MAPK in the cascade, Fus3p, is activated, the signal is divided into three response pathways, namely transcriptional activation of pheromone regulated genes by Ste12p (using Pheromone Response Elements (PRE)), post-transcriptional blocking of the cell cycle in G_1 and an independent but uncharacterised pathway of fusion activation (Brizzio *et al*., 1998; White and Rose, 2001). High levels of **a**- and α-factor are required for cell fusion to occur (Brizzio *et al*., 1996), however, even in the presence of excess pheromone, fusion will not necessarily proceed until the mating partners come into contact. This indicates that some aspects of mating are activated by contact with a mating partner (Brizzio *et al*., 1998; White and Rose, 2001).

The mating pheromones prepare the cells for fusion by inducing expression of cell fusion proteins, such as Fus1p and Fus2p (Gammie *et al*., 1998; Trueheart *et al*., 1987), Rvs161p (Brizzio *et al*., 1998; Gammie *et al*., 1998), Axl1p (Elia and Marsh, 1998), Fig1p, Fig2p and Fig4p (Erdman *et al*., 1998; Zhang *et al*., 2002). These proteins, together with new cell wall material, are deposited near the site of future cell wall contact. To fuse, the two mating cells must make contact, seal the junction area, degrade the cell wall between the mating partners and fuse the two plasma membranes. Attachment is mediated by the agglutinins followed by irreversible attachment of the cell walls. A seal to prevent lysis due to osmotic pressure is formed at the rim of cell contact. The cell wall begins to thin from the central point of cell-cell contact and proceeds gradually towards the periphery (Gammie *et al*., 1998). Inappropriate activation of cell wall degradation is likely to be perilous for cells and thus a mechanism to regulate cell fusion during mating must exist. The mechanisms by which cell wall removal is regulated and occurs and the machinery that controls plasma membrane

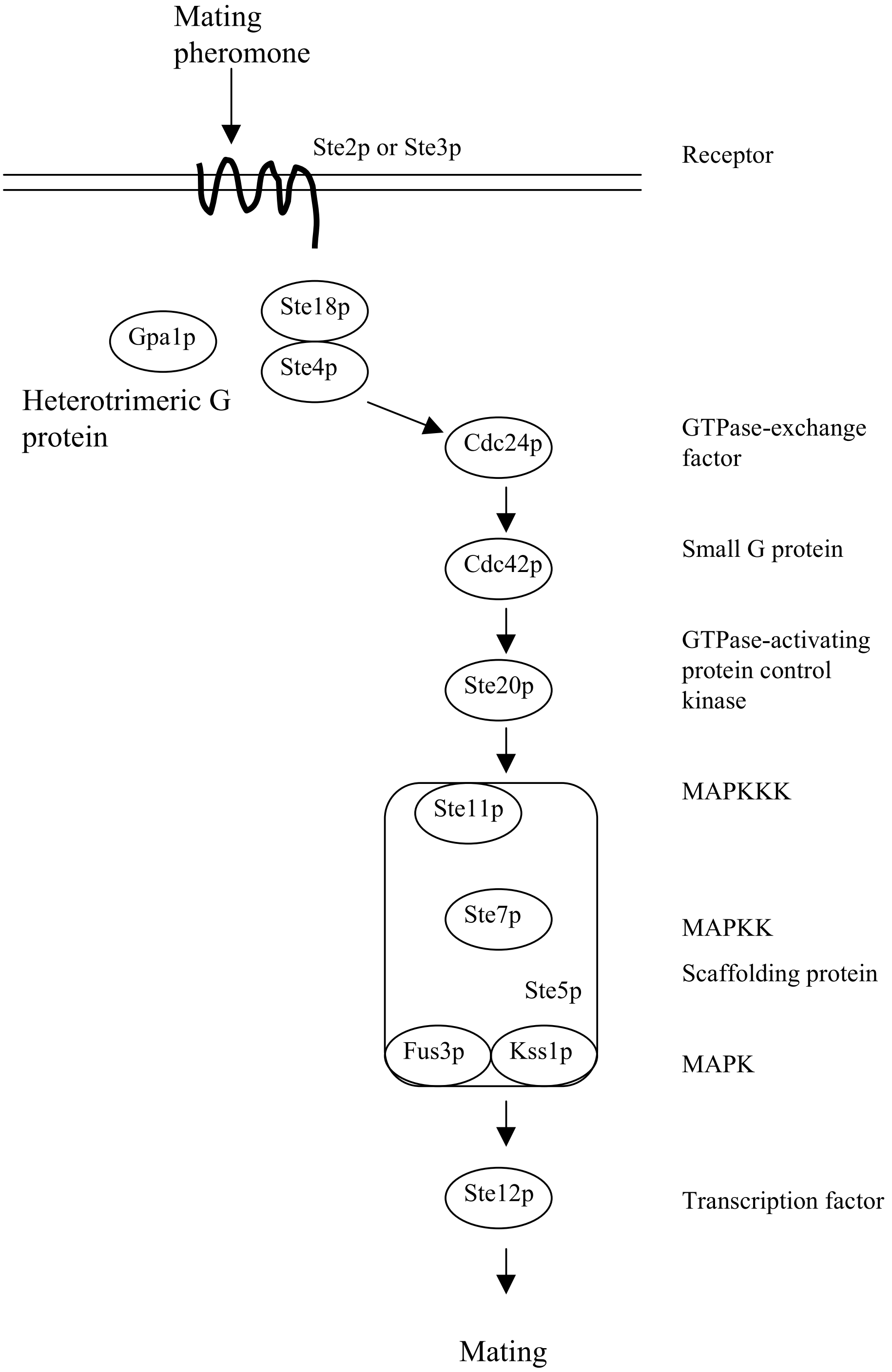


Figure 4.1 The pheromone response MAPK-mediated signal transduction pathway of *S. cerevisiae*.

fusion remain largely unknown. However, Prm1p was recently identified as a protein with a possible role in plasma membrane fusion. In *prm1Δ* matings, the cells begin zygote formation, degrade the cell wall but the plasma membranes fail to fuse (Heiman and Walter, 2000).

Cell fusion mutants can be distinguished by the presence of a septum (a dividing membrane or wall with semipermeable properties) between the mating partners (Gammie *et al*., 1998; Gammie and Rose, 2002). Zygote formation is blocked after the cells have attached but before the cell wall has degraded (Philips and Herskowitz, 1997), or after the cell wall has degraded but before the plasma membranes have fused (Heiman and Walter, 2000). Some mutants display a complete block, which can be seen as two cells with a septum between them and unfused nuclei either side of the septum. Other mutants have a partial defect that is displayed as a septum and a single nucleus (Gammie *et al*., 1998; Gammie and Rose, 2002).

As Hpf1p and Hpf1'p are predicted to be cell surface proteins, a role in mating is possible. More importantly, transcription of *HPF1* and *HPF1'* is upregulated in response to the mating pheromones (Ren *et al*., 2000; Roberts *et al*., 2000). This activation requires the Ste12p transcription factor. This transcriptional response to α-factor was shown to be slow suggesting that the 5' regions of *HPF1* and *HPF1'* are bound by Ste12p only after exposure to pheromone as opposed to many other Ste12p regulated genes, such as Fig2p and Fus1p, that are bound both before and after exposure (Ren *et al*., 2000). Chromatin immunoprecipitation demonstrated that *HPF1* and *HPF1'* are bound by Ste12p only after exposure to pheromone is the case (Ren *et al*., 2000). The experiments described in this chapter aim to determine if Hpf1p and Hpf1'p have a function in the mating response, and if so, what that role is.

4.2 RESULTS

4.2.1 Pheromone response elements upstream of *HPF1* and *HPF1'*

Transcription of a number of pheromone induced genes involved in the mating response is controlled by the presence of a regulatory element called the pheromone response element (PRE) (Van Arsdell *et al.*, 1987). The consensus sequence, TGAAACA, is a potential binding site for Ste12p, the transcription factor regulating pheromone induced transcription, and is generally found upstream of pheromone induced protein coding sequences. The results of Ren *et al.*, (2000), and Roberts *et al.*, (2000), showed that transcription of *HPF1* and *HPF1'* is upregulated in response to the mating pheromones. For this reason, the presence of PRE sequences in the upstream regions of *HPF1* and *HPF1'* was investigated. Several sequences similar to the PRE consensus sequence were found (Table 4.1). As suggested by Erdman *et al.*, (1998), variations from the PRE consensus are likely to be important for Ste12p dependant regulation of some genes.

4.2.2 Mating

4.2.2.1 Mating efficiency

Mating efficiency was assayed by both the liquid mating assay and the limited filter mating assay. The liquid mating assay tests mating under conditions of reduced cell densities (Gehrung and Snyder, 1990). The limited filter mating assay places cells in close proximity at a defined cell density to maximise zygote formation (Gammie and Rose, 2002; Sprague, 1991). In liquid medium, the efficiency of a bilateral *hpf1Δ* x *hpf1Δ* mating was slightly but significantly less ($P<0.05$) than a wild type x wild type mating by 6.73%. The *hpf1'Δ* x *hpf1'Δ* cross had 70.65% efficiency which was significantly less ($P<0.05$) and the double deletion bilateral cross was 28.87% efficient ($P<0.05$) (Table 4.2). The results from limited filter mating assays gave a similar trend, where *hpf1Δ* and *hpf1'Δ* single deletion mutant bilateral matings reduced mating efficiency to approximately 70%, which was significantly less ($P<0.05$) than the wild type mating. For *hpf1Δ hpf1'Δ* x *hpf1Δ hpf1'Δ* crosses mating efficiency reduced significantly ($P<0.05$) to 11.74% (Table 4.2).

This mating defect persists over time. Figure 4.2 shows that the mating efficiency of *hpf1Δ hpf1'Δ* x *hpf1Δ hpf1'Δ* crosses remains at less than 20% compared to wild type x wild type matings, when measured by the limited filter mating assay, even as the number of cells mating increases.

Table 4.1: Possible pheromone response element (PRE) sequences in the upstream regions of *HPF1* and *HPF1'* with five or more alignments to the consensus sequence (as shown in blue).

HPF1:

DNA Strand	Sequence	Position from ATG
Consensus	**TGAAACA**	
Sense	**TCAAA**G**A**	-7
Anti-sense	GT**AAACA**	-293
Anti-sense	**T**T**AAACA**	-412
Sense	G**GAAA**T**A**	-523

HPF1':

DNA Strand	Sequence	Position from ATG
Consensus	**TGAAACA**	
Sense	**TGAAA**G**A**	-7
Sense	A**GAAACA**	-56
Anti-sense	C**GAAA**T**A**	-66
Anti-sense	A**GAAA**G**A**	-402
Anti-sense	A**GAAA**T**A**	-550

Table 4.2: Mating efficiency, as a percentage, compared to wild type x wild type, measured by the liquid mating assay and the limited filter mating assay as described in the Chapter 2. *Significantly different from wild type x wild type (P<0.05, Student’s t-test).

Mating partners *MAT***a** x *MAT*α	Liquid mating assay	Limited filter mating assay
Wild type x wild type	100.00	100.00
Wild type x *hpf1Δ*	100.48	101.58
Wild type x *hpf1’Δ*	78.27*	80.51*
Wild type x *hpf1Δ hpf1’Δ*	28.05*	11.44*
hpf1Δ x wild type	103.20	69.11*
hpf1’Δ x wild type	101.49	73.53*
hpf1Δ hpf1’Δ x wild type	107.59*	62.59*
hpf1Δ x *hpf1Δ*	93.27*	70.45*
hpf1’Δ x *hpf1’Δ*	70.65*	71.90*
hpf1Δ hpf1’Δ x *hpf1Δ hpf1’Δ*	28.87*	11.74*

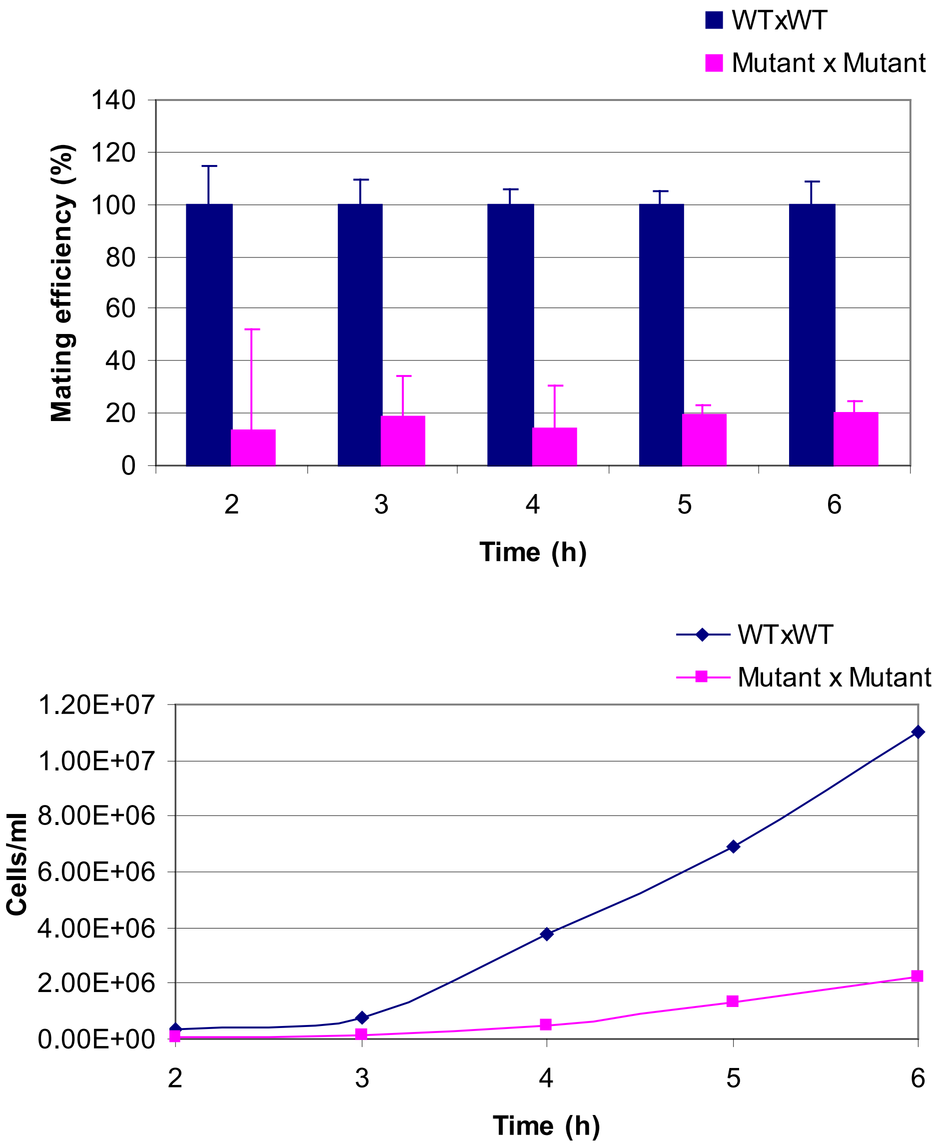


Figure 4.2 Mating efficiency of the *hpf1Δ hpf1'Δ* mutant compared to the wild type strain in bilateral matings, measured by the limited mating filter assay, over time. WT = wild type x wild type; Mutant = *hpf1Δ hpf1'Δ* x *hpf1Δ hpf1'Δ*.

4.2.2.2 The mating defect of *hpfΔ* mutants is mating type specific

The mating strains were also tested against wild type yeast in unilateral matings in liquid medium and in limited filter assays (Table 4.2). Unilateral *hpf1Δ* matings had similar efficiency to wild type matings. When wild type *MAT*α cells were mated with *MAT***a** *hpf1'Δ* or *hpf1Δ hpf1'Δ* strains, the mating efficiency was comparable to a wild type x wild type cross. However, when wild type *MAT***a** strains were mated with *MAT*α *hpf1'Δ* or *hpf1Δ hpf1'Δ* strains, the reduction in mating efficiency was similar to the bilateral matings. This suggests a possible function of the two Hpf proteins in *MAT*α, but not *MAT***a** cells during mating. Macroscopic evidence of the mating defect was also seen in the form of small colonies. In wild type x wild type matings, around 10% of colonies were small and in *hpf1Δ hpf1'Δ* bilateral matings and *MAT***a** wild type x *MAT*α *hpf1Δ hpf1'Δ* matings, around 50% of the colonies were small.

Fusion of yeast cells to form zygotes during mating involves several steps: the pheromone response, prezygotic signalling, osmotic sensing, polarisation of growth, cell wall digestion and removal and plasma membrane fusion (White and Rose, 2001). It was determined whether deletion of *HPF1* or *HPF1'* affected any of these processes.

4.2.3 α-factor growth arrest

The first step in yeast mating is the growth arrest response of the yeast cells to pheromone. To determine if this was responsible for the mating defect of *hpf1Δ hpf1'Δ* cells, cell-cycle arrest was assayed using the 'halo test.' The halo test examines the sensitivity of cells to pheromone of the opposite mating type. When cells of opposite mating type are mixed, they respond to the opposite mating type pheromone by temporarily arresting in G_1. Exposure to higher concentrations of pheromone causes the yeast to undergo a permanent growth arrest, and in a plate assay, this is seen as a zone of inhibition. No difference in growth arrest response was seen between the wild type and *hpfΔ* strains in response to α-factor in halo tests (Figure 4.3). As the mating defect occurred in *MAT*α *hpfΔ* cells, it would be appropriate to examine sensitivity to **a**-factor. However, the results described in Section 4.2.6 suggest that the cause of the mating

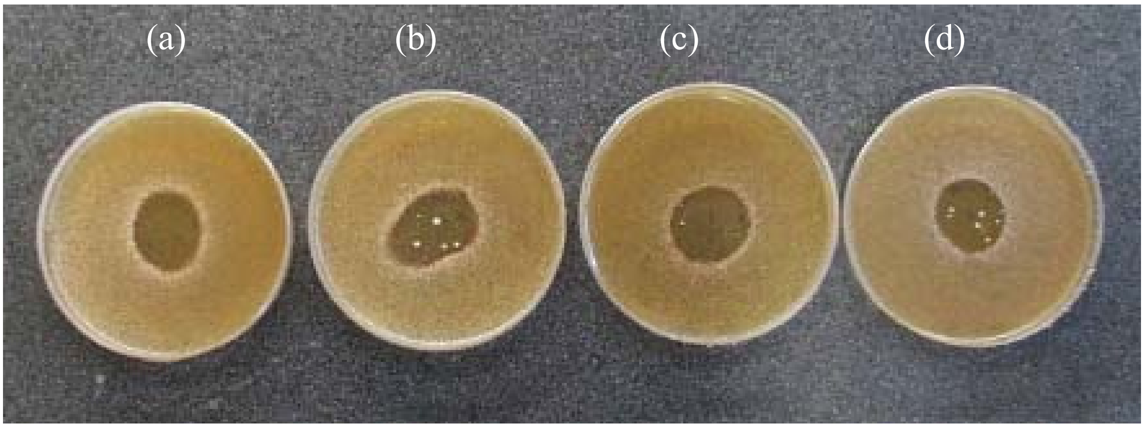


Figure 4.3 α-factor sensitivity assays ('halo' tests) using the method described in Chapter 2. (a) S288c wild type; (b) S288c *hpf1Δ*; (c) S288c *hpf1'Δ*; (d) S288c *hpf1Δ hpf1'Δ*.

defect is not due to pheromone sensitivity and therefore growth arrest was not examined any further.

4.2.4 Agglutination assays

Agglutination assays were performed to establish if the *HPF* gene products are involved in agglutination during mating, allowing the mating partners to interact. When *MAT***a** wild type was mixed with *MAT***a** *hpf1Δ hpf1'Δ* and *MAT*α wild type with *MAT*α *hpf1Δ hpf1'Δ*, the OD_{600} of the cell mixture after 15 minutes was 0.5 indicating that no agglutination occurred, as expected. The optical density at 600 nm was reduced to 0.077 for *MAT***a** wild type x *MAT*α wild type and 0.071 for *MAT***a** *hpf1Δ hpf1'Δ* x *MAT*α *hpf1Δ hpf1'Δ* crosses. The agglutination assay showed that there was no significant difference ($P<0.05$) between the agglutination ability of wild type and *hpfΔ* strains in mating mixtures.

4.2.5 Mating projection formation

After pre-zygotic signalling and cell-cycle arrest, yeast respond to mating pheromone by polarising their growth along the pheromone concentration gradient. In doing this, the elongation of the cell forms a shape known as a 'shmoo.' The relative number of shmoos formed after α-factor treatment over time was not significantly different between wild type and *hpfΔ* mutant strains (data not shown). The shape of the mating projections was similar after treatment with α-factor (Figure 4.4 (a) and (b)). However, in mixes of mating cells, the shape of the mating projections of the wild type cells were found to be long and pointed compared to the *hpfΔ* mutants' projections which were blunt (broader and less focussed) as can be seen in Figure 4.4 (c) and (d). After five hours of mating, of 200 shmoos of each strain counted, 68% of shmoos were pointed in wild type x wild type matings, but only 18% were pointed in *hpfΔ* x *hpfΔ* matings (Figure 4.4 (e)). Interestingly, strains overexpressing *HPF1*, in the absence of pheromone, have a 'shmoo like' elongated morphology (Figure 4.5).

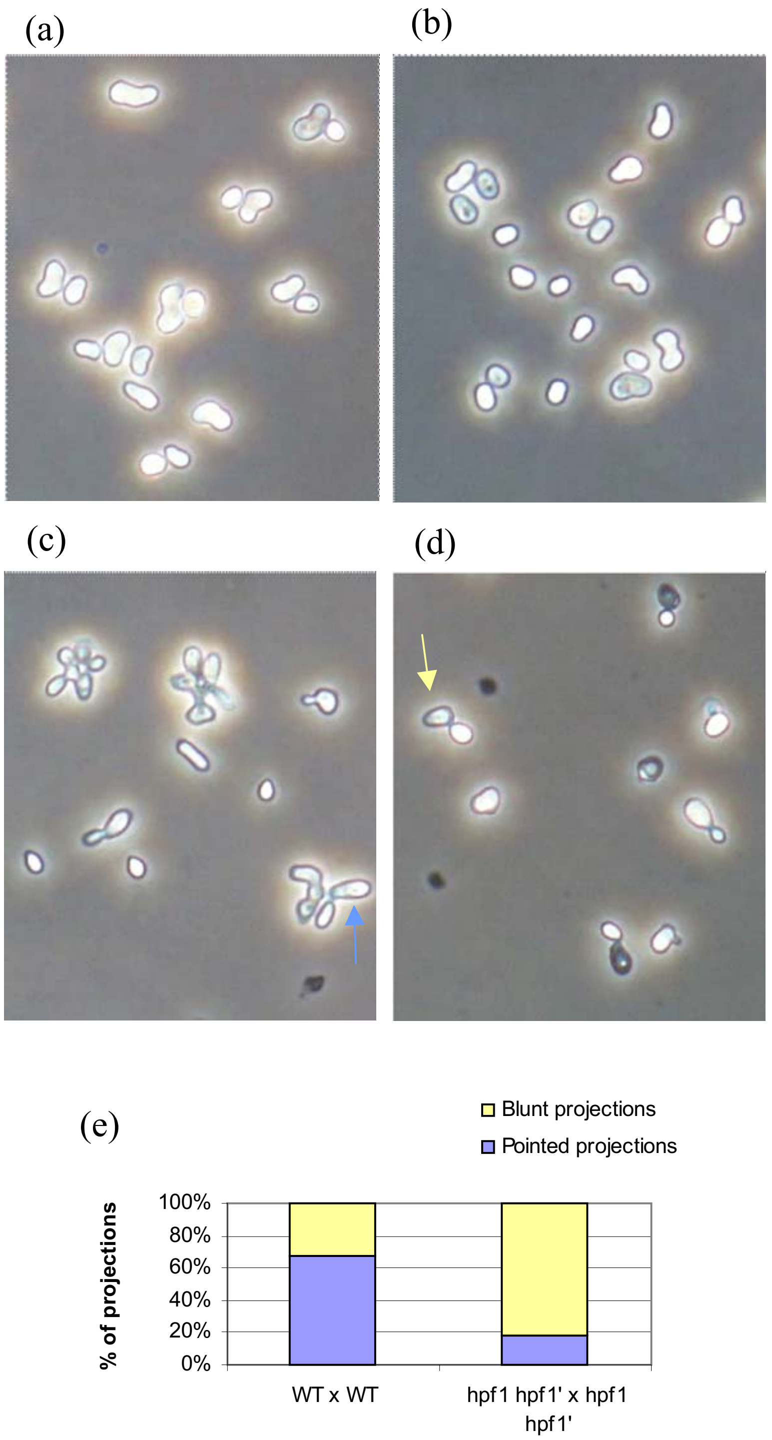


Figure 4.4 Mating projection formation of (a) wild type and (b) *hpf1Δ hpf1'Δ* cells after treatment with 5 μg/mL α-factor for two hours and mating (c) wild type x wild type and (d) *hpf1Δ hpf1'Δ* x *hpf1Δ hpf1'Δ* cells after two hours. The cells were visualised using phase contrast optics at 40x magnification. (e) The percentage of 'blunt' (→) and 'pointed' (→) mating projections in bilateral matings.

4.2.6 Persistence of a septum between *hpf1Δ hpf1'Δ* mating partners

The frequency of zygote formation was tested using both the liquid mating assay and the limited filter mating assay. The proportion of unpolarised, small/medium polarised, large polarised and zygotic cells visualised under the microscope over time was not noticeably different between the wild type and *hpf1Δ hpf1'Δ* bilateral matings when 500 cells of two cell preparations were counted. Although the number of zygotes was not altered in the deletion strains, a closer inspection of the mating cells using differential interference contrast (DIC) optics at high magnification (100x) demonstrated that the septum between the mating partners of an *hpf1Δ hpf1'Δ* bilateral cross persisted well into the mating process. After three hours of mating, of 200 zygotes counted, 6% of wild type bilateral mating zygotes had septums and for the *hpf1Δ hpf1'Δ* bilateral mating, 50% of zygotes had septums. This trend continued at four, five and six hours after the mating was initiated. These results point to a cell fusion defect in the *hpfΔ* strains (Figure 4.6).

Using transmission electron microscopy (TEM), a septum was seen between the mating partners in *hpf1Δ hpf1'Δ* bilateral crosses (Figure 4.7 (e)). The yeast cell surface consists of three layers, the membrane, and the inner and the outer layers of the cell wall (Figure 4.7 (e)). The electron-dense outer cell wall layer between the mating partners seems to have already degraded and rejoined. The single layer of electron-light material between the mating partners is likely to be the inner cell wall layer. The electron-dense membrane is present on either side of this inner cell wall layer and is not completely degraded. It is likely that the mating cells have recognized that they will not be able to mate because of this barrier and one of the mating partners has begun to bud near this septum (Figure 4.7 (d)).

4.2.6.1 Detection of cell fusion defects by a cytoplasmic mixing assay

Mating mixtures were prepared as described for the limited filter mating assay. *MAT***a** cells were transformed with a plasmid expressing soluble GFP under the control of the *GAL1* promotor. For all mating pairs examined, for both the wild type and *hpf1Δ hpf1'Δ* mutants, the GFP could be seen throughout the zygote suggesting that the barrier seen

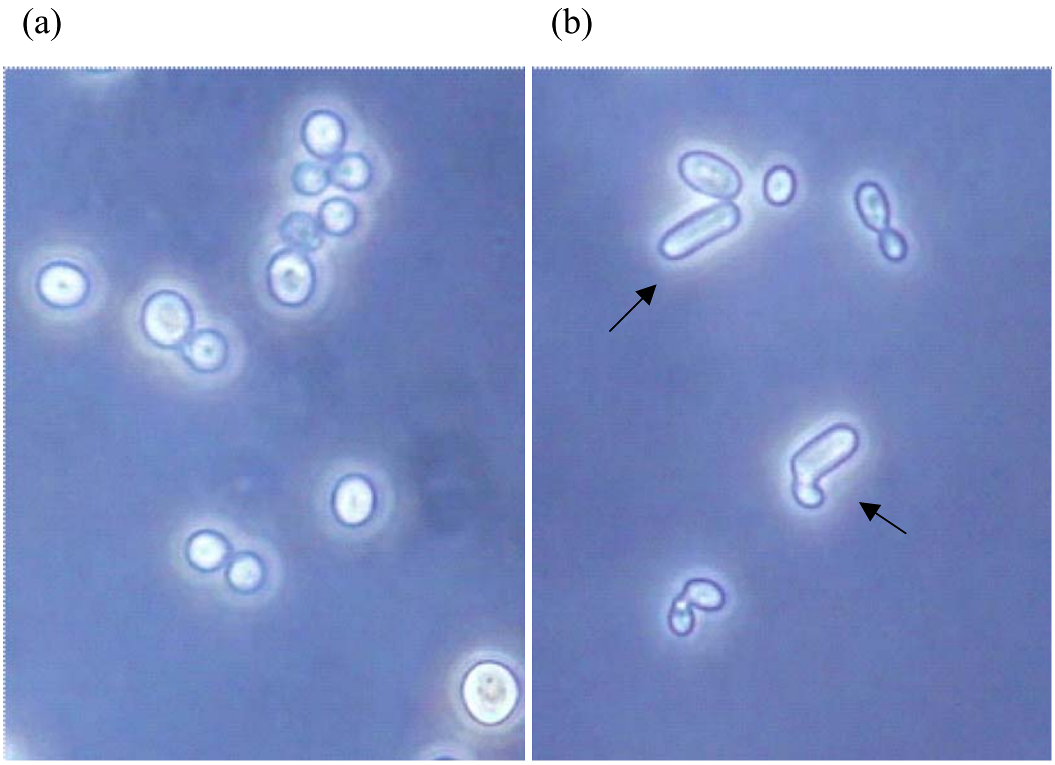


Figure 4.5 S288c *MAT***a** + p6xHis-HPF1 cells grown on (a) glucose (to repress expression of 6xHis-Hpf1p) and (b) galactose (to induce expression). The cells grown on galactose have an elongated morphology. These cells were visualised using phase contrast optics at 40x magnification. S288c *MAT***a** grown on glucose or galactose had similar morphology to cells shown in (a).

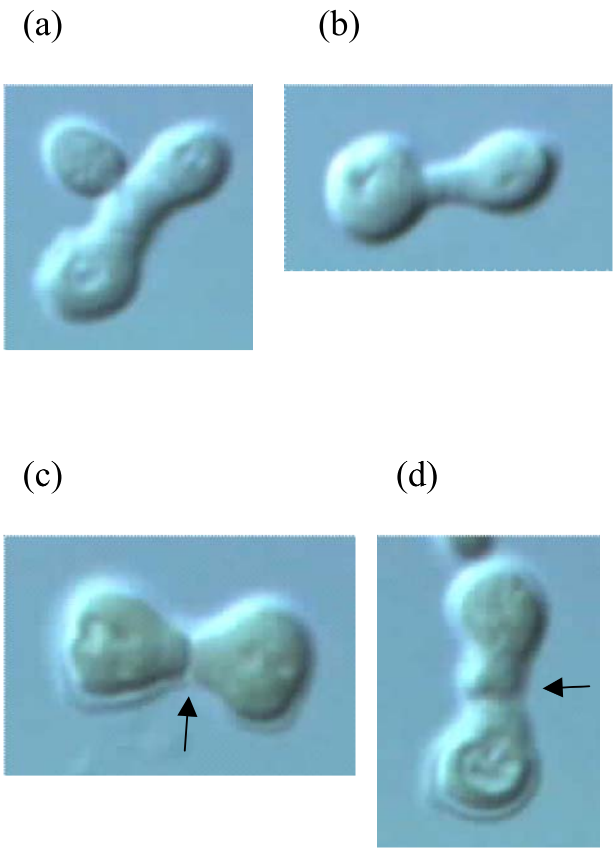
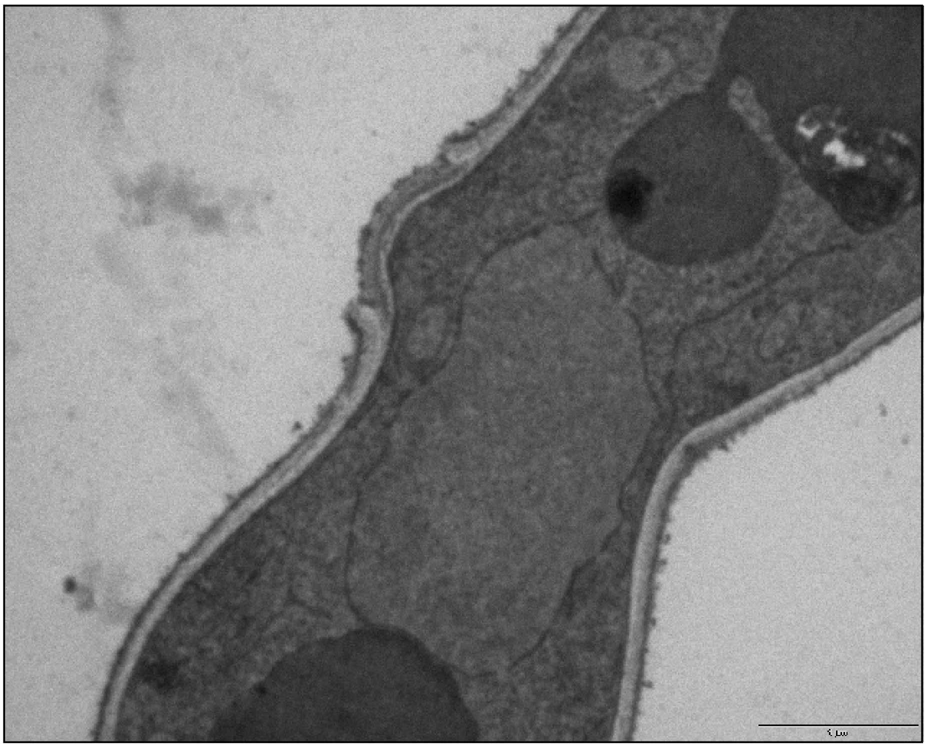


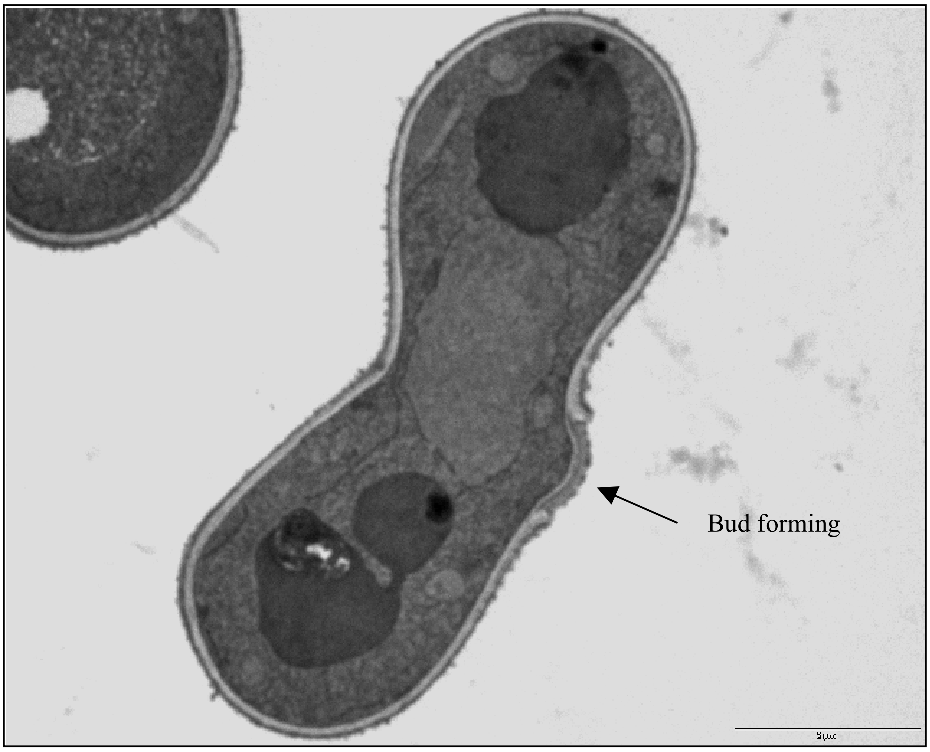
Figure 4.6 Morphological phenotype of cell fusion mutants. (a) and (b) wild type x wild type, (c) and (d) *hpf1Δ hpf1'Δ* x *hpf1Δ hpf1'Δ* matings were prepared on filters as described in the Chapter 2. Cells were visualised using Differential Interference Contrast optics at 100x magnification. A septum between the *hpf1Δ hpf1'Δ* mating partners is indicated by an arrow.

Figure 4.7 Transmission electron micrographs of bilateral mating cells, (a) and (b) wild type and (c), (d) and (e) *hpf1Δ hpf1'Δ*, after 3 hour limited filter matings. The scale bar for (a) is 1 μm, (b) is 2 μm, (c) is 2 μm, (d) is 1 μm and (e) is 500 nm.

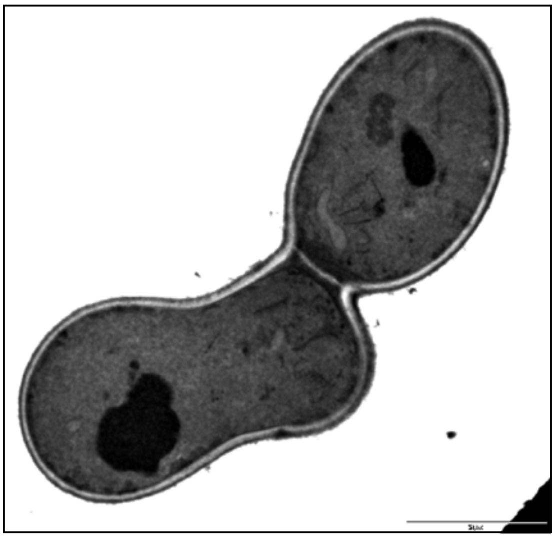
(a)



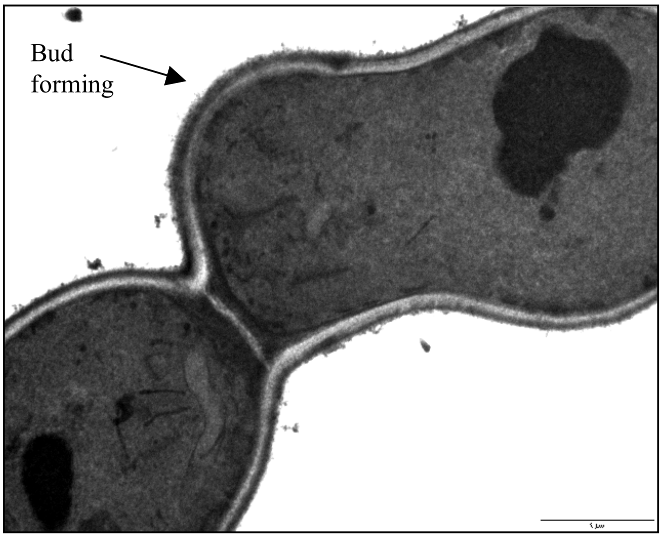
(b)



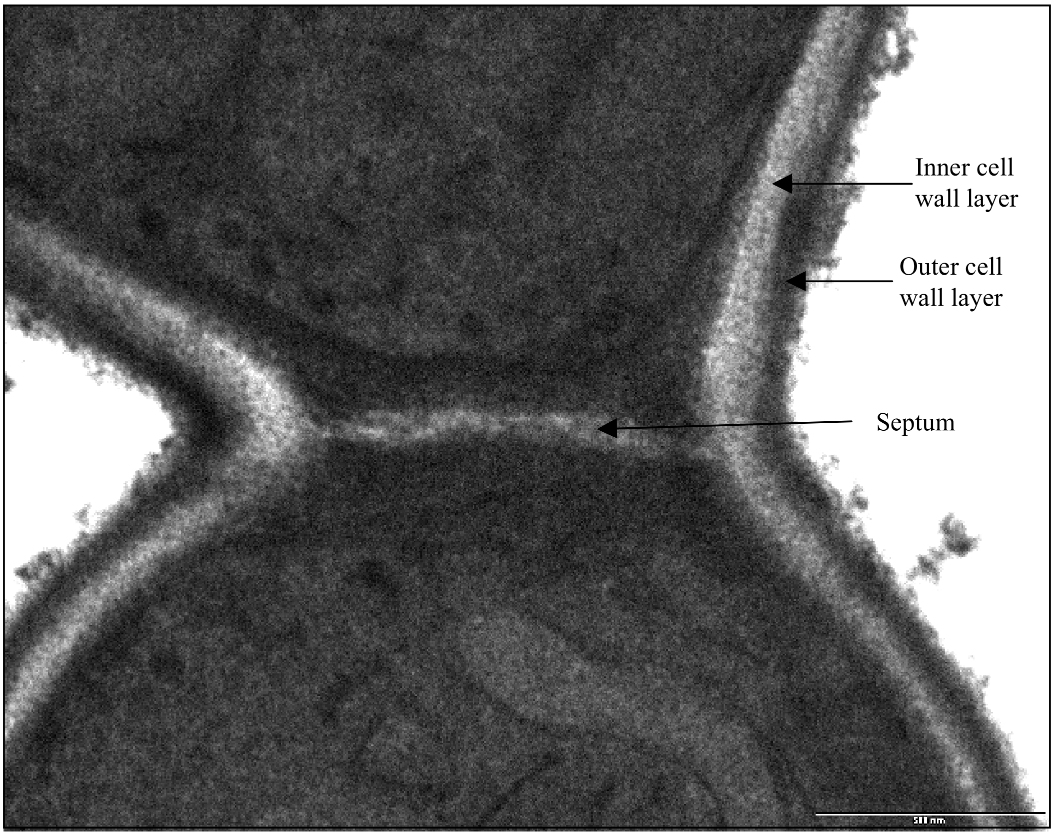
(c)


5µm

(d)


Bud
forming

(e)


Inner cell wall layer
Outer cell wall layer
Septum
500 nm

by DIC and TEM was unable to prevent GFP transfer between the mating partners (Figure 4.8).

4.2.7 Immunofluorescent localisation of Hpf in *S. cerevisiae*

The localisation of Hpf1p and Hpf1'p in yeast cells during mitotic growth and after exposure to α-factor was determined. This was done by localising the 6xHis tagged Hpf1p and Hpf1'p (described in Chapter 5) using a secondary goat anti-mouse antibody conjugated to FITC to bind to the primary mouse anti-6xHis antibody. The method of Guo *et al*., (2000), was followed for immunofluorescence of intact cells. Hpf1p and Hpf1'p were found to localise to the cell surface during mitotic growth and to the shmoo tip after cells have been treated with 5 μg/mL α-factor for 2 hours.

4.3 DISCUSSION

Two properties of Hpf1p and Hpf1'p, proposed cell wall localisation and induction of gene transcription by pheromone, led us to investigate the mating phenotype of the *HPF* deletion strains. Hpf1p and Hpf1'p have high serine and threonine content suggesting that they are likely to be highly *O*-glycosylated and they are predicted to have five and four *N*-glycosylation sites, respectively. Transcription of *HPF1* and *HPF1'* is upregulated 2.5-2.8 fold in response to the mating pheromones (Ren *et al*., 2000; Roberts *et al*., 2000). This activation requires Ste12p, the transcription factor regulating pheromone induced transcription (Ren *et al*., 2000). The absence of the PRE consensus sequence but presence of multiple sequences with similarity to the PRE in *HPF1* and *HPF1'* provide an indication that Ste12p is able to bind to variations of the PRE consensus sequence. This has also been seen for the pheromone induced *FIG* genes (Erdman *et al*., 1998).

Investigation of the wild type and *hpfΔ* mutant strains showed that there were significant differences in mating efficiencies. The mating efficiency of bilateral *hpf1Δ hpf1'Δ* matings and *MAT***a** wild type x *MAT*α *hpf1Δ hpf1'Δ* matings was 28% and 11% compared to wild type x wild type matings (100%) in liquid and filter matings, respectively. These findings are in contrast with the results published by Lafuente and

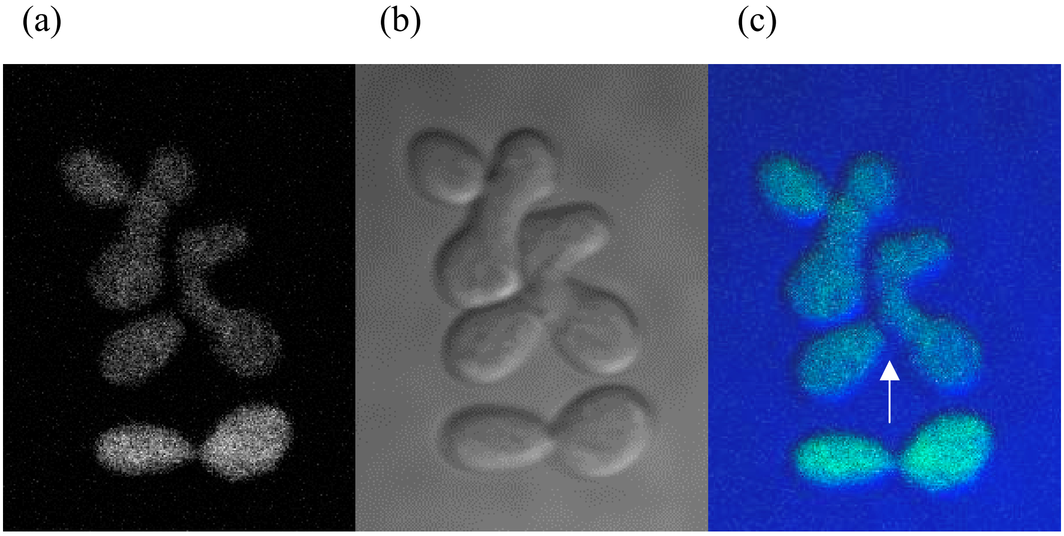


Figure 4.8 Detection of cell fusion defects by a cytoplasmic mixing assay. Expression of cytosolic GFP is induced when cells are grown on galactose. These photographs, taken using a BioRad confocal microscope at 60x magnification, are representative results from *hpf1Δ hpf1'Δ* x *hpf1Δ hpf1'Δ* matings. (a) GFP fluorescence; (b) light micrograph; (c) combined picture of (a) and (b). The arrow in (c) indicates a septum persisting between mating partners with GFP seen in both mating partners.

Gancedo, (1999), who suggested that the mating ability of these strains is unaffected. However, the authors do not describe how the mating assay was performed and it is possible that a quantitative mating assay was not employed.

Decreased mating efficiency has been seen for several other yeast deletion mutants. The decreased matings can result from defects in pheromone signalling, agglutination, cell polarity, osmotic regulation, cell wall removal, and membrane or nuclear fusion. The possible role of *HPF1* and *HPF1'* in these various mating functions has thus been investigated. The *hpfΔ* strains did not show a growth arrest difference in response to mating pheromone in the 'halo test.' It remains to be determined whether sensitivity to **a**-factor is altered in the *hpfΔ* mutant strains. Cells lacking *HPF1* and *HPF1'* showed no agglutination defect suggesting that these genes are not involved in the pre-zygotic signalling for agglutination that takes place when two mating partners meet.

The possible role of *HPF1* and *HPF1'* in cell polarity was explored by examining the 'shmoos' formed by *hpfΔ* mutants after exposure to isotropic concentrations of α-factor and in mating mixtures. In *hpf1Δ hpf1'Δ* x *hpf1Δ hpf1'Δ* matings, cells formed shmoos that were broader and less focussed than the wild type cells. Interestingly, *MAT***a** cells do not exhibit the mating defect so this difference in the shape of the shmoo may account for the decreased mating efficiency. The difference in shmoo shape was not significantly different in wild type and *hpf1Δ hpf1'Δ* cells when exposed to pheromone under isotropic conditions. Exposure to isotropic concentrations of α-factor may produce very different phenotypes to mating mixtures where pheromone gradients exist (Erdman *et al*., 1998; Dorer *et al*., 1997).

Degradation of the cell wall and plasma membrane fusion was examined for the *hpfΔ* mutants. A septum was formed between the mating partners in *hpf1Δ hpf1'Δ* cells, which was not observed in wild type matings. The reason for septum formation was likely to be caused by a defect in cell wall digestion and removal, or plasma membrane fusion. Transmission electron microscopy was used to look at the prezygotes more closely. The prezygotes viewed indicated that the cells were unable to fuse cell walls or plasma membranes correctly. However, a cytoplasmic GFP assay showed that GFP was able to pass from one mating partner to the other indicating that the septum was permeable.

A number of yeast gene products are necessary for proper cell fusion. Yeast lacking *SPA2* were unable to form shmoos upon exposure to mating pheromone and therefore had decreased mating efficiency (Gehrung and Snyder, 1990). Spa2p was found to have multiple functions in cell polarity (Arkowitz and Lowe, 1997), is required for efficient cell fusion (Dorer *et al*., 1997) and it facilitates clustering of vesicles in the cell fusion zone (Gammie *et al*., 1998). *FUS3* is the final MAPK in the pheromone response pathway as described in Section 4.1. When *FUS3* is deleted from yeast, the cells are sterile (Elion *et al*., 1990). *FUS3* overexpression results in increased pheromone sensitivity (Elion *et al*., 1990). Strains lacking *FIG1* possess a cell fusion defect and it was suggested that this defect could be attributed to the role of Fig1p in polarised growth sites during mating (Erdman *et al*., 1998). The mating projection of *fig2Δ* cells is narrower and longer than wild type cells and consequently, Erdman *et al*., (1998), showed that *fig2Δ* bilateral mating cells do not fuse to form zygotes because the conjugation bridge between the cells is narrower than wild type cells and the nuclei are unable to pass through (Zhang *et al*., 2002). Cells lacking *FIG4* were also found to have a mating defect. Fig4p has homology to Sac1p (phosphoinositide phosphatase), which, when deleted, results in altered actin cytoskeletal and secretory pathway function and inositol auxotrophy. Fig4p is potentially a regulator of effector molecules of the actin cytoskeleton (Erdman *et al*., 1998). *FUS1* encodes a membrane anchored glycoprotein with *N*- and *O*-linked sugars. A mutation in the *FUS1* gene, in one or both mating partners, resulted in the formation of a stable pre-zygote, as the mating process could not continue after the initial joining of the two mating partners (Trueheart *et al*., 1987). A septum dividing the mating partners was present several hours after prezygote formation (Trueheart *et al*., 1987). *FUS1* was later found to be required for normal vesicle localisation (Gammie *et al*., 1998). *FUS2* and *RVS161* have been shown to be essential for accurate cell wall removal (Brizzio *et al*., 1998; Dorer *et al*., 1997). Trueheart *et al*., (1987), showed that a partition dividing the mating partners of *fus2Δ* mutants was present several hours after prezygote formation. Rvs161p is required to interact with Fus2p for efficient cell fusion and appears to act after the vesicles are aligned in the cell fusion zone (Brizzio *et al*., 1998; Gammie *et al*., 1998). *PRM1* is necessary in one of the two mating partners for efficient plasma membrane fusion (Heiman and Walter, 2000). The majority of cells initiate zygote formation in matings between *prm1Δ* mutants, and continue to degrade the cell wall between the mating partners but fail to fuse, remaining eight nanometres apart. This suggests a role for Prm1p in membrane fusion (Heiman and Walter, 2000).

Hpf1p and Hpf1'p differ from all previously described proteins that affect fusion by being mating type specific. Interestingly, although the decrease in mating efficiency occurs in only one mating type (*MAT*α), transcription of *HPF1* and *HPF1'* is induced by mating pheromones similarly in both **a** and α mating types (Ren *et al.*, 2000; Roberts *et al.*, 2000). Roberts *et al.*, (2000), showed that *HPF1* transcription is increased 2.55 fold and *HPF1'* by 2.83 fold in wild type *MAT*α cells in response to **a**-factor. Ren *et al.*, (2000), showed that transcription of *HPF1* and *HPF1'* is activated in *MAT***a** cells in response to α-factor mating pheromone by 2.78 and 2.76 fold, respectively after 60 minutes exposure to 50 nM α-factor.

Several genes are expressed in a mating type-specific manner including the mating pheromone receptor genes, mating pheromone genes, pheromone maturation genes and mating specific-agglutinins. *STE2* and *STE3* encode the α-factor and **a**-factor pheromone receptors, respectively (Burkholder and Hartwell, 1985; Hagen *et al.*, 1986). Deletion of *STE2* from *MAT***a** cells causes sterility. *MAT***a** *ste3Δ* cells are able to mate but *MAT*α *ste3Δ* cells are sterile. *MFA1*, *MFA2*, *MFα1* and *MFα2* encode the mating pheromones **a**- and α-factor (Kurjan and Herskowitz, 1982; Michaelis and Herskowitz, 1988; Singh *et al.*, 1983; Yuan and Fields, 1991). **a**-factor must be present for cells to mate, however, the cells do not need to produce the mating pheromone, it may be supplied exogenously (Marcus *et al.*, 1991). Down regulation of *MFA1* causes cells to be defective in cell wall fusion, but not plasma membrane fusion (Brizzio *et al.*, 1996). *AXL1* and *RAM1* are required for proteolytic processing and prenylation of **a**-factor (Brizzio *et al.*, 1996; Elia and Marsh, 1998; Giot *et al.*, 1999). Brizzio *et al.*, (1996), reported that *axl1Δ* and *ram1Δ* mutants have mating defects due to their inability to produce the same quantity of **a**-factor as the wild type strain since high levels of pheromone are required to initiate cell fusion. *axl1Δ* and *ram1Δ* mutants are defective for cell wall remodeling and removal but not plasma membrane fusion (Brizzio *et al.*, 1996). Ste6p is an ABC-transporter required to transport **a**-factor (Elia and Marsh, 1996) but in a *ste6(cef)* mutant cell fusion is blocked at a late stage when mating partners were encased by a single cell wall and separated by only a thin layer of cell wall material termed the fusion wall (Elia and Marsh, 1996). Mating type-specific agglutination of *MAT***a** and *MAT*α cells depends on interaction of the cell surface glycoproteins Agα1p and Aga2p (Cappellaro *et al.*, 1994; Lipke *et al.*, 1989). Sag1p, a sexual agglutination protein, is expressed only in *MAT*α cells (Doi *et al.*, 1989). An agglutination defect by *fig2Δ* strain was found strongest in *MAT***a** wild type x *MAT*α *fig2Δ* compared to *MAT***a** *fig2Δ* x *MAT*α wild type, but the defect was seen most clearly when *FIG2* was deleted from both mating partners (Zhang *et al.*, 2002). Although all the above genes are mating-type specific, none of these genes are directly involved in cell wall fusion. It is possible that Hpf1p and Hpf1'p are mating type-specific gene products involved in controlling or regulating a fusion step of the perilous act of mating. More research is necessary to understand the exact function of Hpf1p and Hpf1'p, and why they are mating type specific.

Nuclear fusion is also required to form zygotes that are able to proliferate. *kar5Δ* x *kar5Δ* bilateral matings resulted in a quantitative mating defect of at least 77 fold (Erdman *et al.*, 1998). However, this mating generated small colonies, as did the *hpf1Δ hpf1'Δ* matings. These small colonies were found to be unstable heterokaryons brought about by unstable nuclear fusion (Erdman *et al.*, 1998). Further investigation is required to determine whether the small colonies produced in *hpf1Δ hpf1'Δ* matings are also unstable heterokaryons and if so, why this instability occurs.

Notably, *HPF1* and *HPF1'* transcription is upregulated 2.8 fold and 8.5 fold, respectively, in a *sok2Δ/sok2Δ* diploid strain (Pan and Heitman, 2000). Sok2p is a transcription factor that negatively regulates pseudohyphal differentiation *via* a transcription cascade that regulates cell-cell adhesion. A *sok2Δ/sok2Δ* diploid deletion mutant is hyperfilamentous. During filamentous growth, in response to nitrogen limitation, the cells elongate and adopt a unipolar budding pattern among other characteristics. The response of cells to levels of pheromone below a threshold can also induce a filamentous growth response by the filamentous growth pathway (Erdman and Snyder, 2001). The yeast adapt an elongated cell morphology and begin to spread over and into the agar searching for mating partners or nutrients. The term pseudohyphal is usually used for diploid cells and the term invasive is reserved for haploids, but this does not mean that diploids cannot invade or that haploids cannot form pseudohyphae (Gancedo, 2001). Two signalling pathways that regulate pseudohyphal growth have been identified, these being the pheromone response MAP kinase pathway and the cyclic AMP signalling pathway (Gancedo, 2001). It is possible that *HPF1* and *HPF1'* are involved in various stages of the yeast life cycle besides mating.

A comprehensive two-hybrid analysis has shown that Hpf1'p interacts with Fus3p, Mob1p and Ynr074p (Ito *et al*., 2001). Fus3p is a serine-threonine protein kinase that is required for cell cycle arrest, projection formation and fusion during mating (Elion, 2000; Elion *et al*., 1990). It acts in the pheromone response pathway as described in Section 4.1. If Hpf1'p does indeed interact with Fus3p, this may suggest a possible mechanism by which the action of Hpf1'p is controlled. The function of Mob1p remains unknown; however, it is known to interact with Dbf2p, a serine-threonine protein kinase that acts in the mitotic exit network (MEN) (Komarnitsky *et al*., 1998). Mob1p is required for the completion of mitosis and the maintenance of ploidy (Luca and Winey, 1998) as well as cytokinesis and mitotic exit (Luca *et al*., 2001). The MEN is similar to the pheromone response pathway in that it consists of several protein kinases connected with a scaffolding protein (Lee *et al*., 2001). The MEN includes a GTP-binding protein (Tem1p), protein kinases (Cdc15p, Cdc5p and Dbf2p), a protein of unknown function (Mob1p) and a phosphatase (Cdc14p). Ynr074p is an oxido-reductase that acts on NADH or NADPH with disulphide as an acceptor. It acts in response to singlet oxygen and is localised in the plasma membrane (Ververidis *et al*., 2001). The reason for the possible interactions between Hpf1'p and Mob1p and Ynr074p remain unknown at this time. Although these interactions are interesting and suggest possible additional roles for Hpf1'p, the two-hybrid results must be confirmed by co-immunoprecipitation or affinity chromatography followed by protein identification.

4.4 CONCLUSION

HPF1 and *HPF1'* are likely to be localised to the cell surface and their expression is induced by the mating pheromones (Ren *et al*., 2000; Roberts *et al*., 2000). Mating efficiency of *hpf1Δ hpf1'Δ* bilateral matings was 8.51 fold less than the wild type matings when assessed using the limited mating filter assay. The decrease in mating efficiency was found to be mating type specific as the reduction in mating efficiency occurred in *MAT***a** *hpf1Δ hpf1'Δ* x *MAT*α wild type unilateral crosses but not in *MAT***a** wild type x *MAT*α *hpf1Δ hpf1'Δ*. The reason for the decreased mating efficiency was the persistence of a septum between the mating partners. This septum was permeable as cytosolic GFP was able to transfer between the mating partners. This, to our knowledge, is the first example of gene products that are likely to be involved in cell fusion, which are mating type specific.

CHAPTER FIVE

HPFS PROVIDE HAZE PROTECTIVE ACTIVITY

5.1 INTRODUCTION

Several glycoproteins that exhibit haze protective activity have been isolated. These include yeast mannoproteins from wine (HPF) (Waters *et al*., 1994a), yeast invertase (McKinnon, 1996), a fragment of yeast invertase (Moine-Ledoux and Dubourdieu, 1999), a wine arabinogalactan protein (Waters *et al*., 1994b), an apple arabinogalactan protein and gum arabic (Pellerin *et al*., 1994), as described in Chapter 1. The use of these glycoproteins may be alternative or complementary to current methods of preventing protein haze formation by heat unstable proteins in white wine.

The first purification of HPF by Waters and colleagues was from wine fermented by an industrial winemaking yeast strain (Dupin *et al*., 2000b; Waters *et al*., 1993). Further work to purify HPF was done in chemically defined synthetic grape juice medium (Stockdale, 2000). Using partial amino acid sequence of the isolated HPF, putative genes, YOL155c, (E. Waters, unpublished) and YDR055w, (Stockdale, 2000) were identified by comparison to the *Saccharomyces* genome database (Cherry *et al*., 1997). Until the functions of these genes are confirmed, these genes will be named *HPF1* and *HPF2*, respectively. The homologue of *HPF1*, YIL169c, will be named *HPF1'*.

The 'heat test' was developed as a method to rapidly measure haze formation and thereby assess the haze protective activity of putative HPF fractions from yeast. Over several months to years, haze can form in wines stored under standard commercial conditions. A method to accelerate this haze formation in order to gain results that could be presented in a timely manner was required. It was known that heating wine at 80°C for 6 hours accelerated haze formation and simulated long-term haze formation (Pocock and Rankine, 1973). If, after 6 hours, no further components of the wine precipitated, the wine was considered stable to additional haze formation (Pocock and Rankine, 1973). This assay was scaled down in volume to reduce the amount of HPF material required for each assay (Dupin *et al*., 2000a; Dupin *et al*., 2000b; Stockdale, 2000). This test is considered very stringent by some practitioners and accordingly, other practices such as heating at 80°C for 30 minutes (Ledoux *et al*., 1992) or 49°C for two days (Moretti and Berg, 1965) are also employed.

With the knowledge that HPF could be isolated from chemically defined medium and the availability of a micro scale assay to test the activity of the material, the confirmation of the identity of the Hpfs and the contribution of each of the putative *HPF* gene products to haze protective activity could be assessed using *HPF* overexpression and deletion yeast strains. The putative *HPF* genes were both overexpressed and deleted in a laboratory yeast strain, as opposed to a wine yeast strain that previous HPF studies have used, because of the genetic tools available in laboratory yeast. It was known that the laboratory yeast strain, S288c, possessed genes that had similar sequence to the putative HPFs identified from the wine strain but it was not known if material isolated from ferments by S288c had haze protective activity.

The aims of this work presented in this chapter were to overexpress and delete the putative *HPF* genes in yeast to firmly establish whether these gene products indeed do possess haze protective activity and if so, to determine the contribution of each of the gene products to the total haze protective activity from yeast. This work involved isolating the haze protective mannoproteins from both overexpression and deletion strains and assessing their activity. The putative *HPF* genes were tagged with a 6xHis epitope, overexpressed and purified using IMAC. The purified proteins were assessed for haze protective activity.

5.2 RESULTS

5.2.1 The laboratory yeast strain, S288c, expresses haze protective factor

5.2.1.1 Comparative growth and glucose metabolism of the laboratory strain, S288c, and the wine strain, AWRI838, in chemically defined grape juice medium

The laboratory yeast, S288c, is unable to grow in chemically defined grape juice medium (CDGJM) without amino acid supplementation (M. de Barros Lopes, personal communication). The addition of 1.0 g/L of synthetic complete amino acid mix

(Appendix 2) enables growth and therefore all CDGJM used in this study were prepared in this way. The growth rate and glucose usage of the laboratory and wine strains were examined in this modified medium with initial glucose concentrations of 20, 40, 100, 150 and 200 g/L glucose, under anaerobic conditions. Two percent (w/v) glucose was selected as the minimum concentration as this is the normal glucose concentration used in laboratory yeast growth media and twenty percent glucose as the maximum concentration because this is similar to the sugar concentration in grape juice. The inoculation rate for all ferments was 1×10^6 cells/mL.

Before the full suite of conditions was examined, an alternative and faster method, using the spectrophotometer rather than the haemocytometer, to follow yeast growth was validated under these conditions (Figure 5.1). To ensure that the measure of optical density at 600nm using a spectrophotometer is correlated to the more standard procedure of counting yeast cells in a haemocytometer under a microscope to follow yeast growth, both measures were taken for fermentation samples every 15 minutes during a ferment of CDGJM with 40 g/L glucose by the laboratory yeast S288c *MAT***a** (Figure 5.1). The two measures gave good representations of the yeast growth curve although the measurements of the haemocytometer counts were more variable, because of the method. For example, assuming satisfactory technique, multiple determinations of a sample using two counting chambers can result in an accuracy of ±11% (Miale, 1972). Spectrophotometric methods have previously been shown to be less variable than use of a haemocytometer to determine the concentration of yeast in a culture (Pfaller *et al.*, 1988). These results suggest that, when performing multiple ferments, it would be reasonable to use a measure of OD_{600} to determine the growth curves of the ferments simultaneously and in a timely manner.

AWRI838 had a faster growth rate than S288c (Figure 5.2) in all the glucose concentrations tested and the rate of glucose usage between the two yeast was dramatically different (Figure 5.2) with the wine strain able to metabolise the sugar at a faster rate than the laboratory yeast.

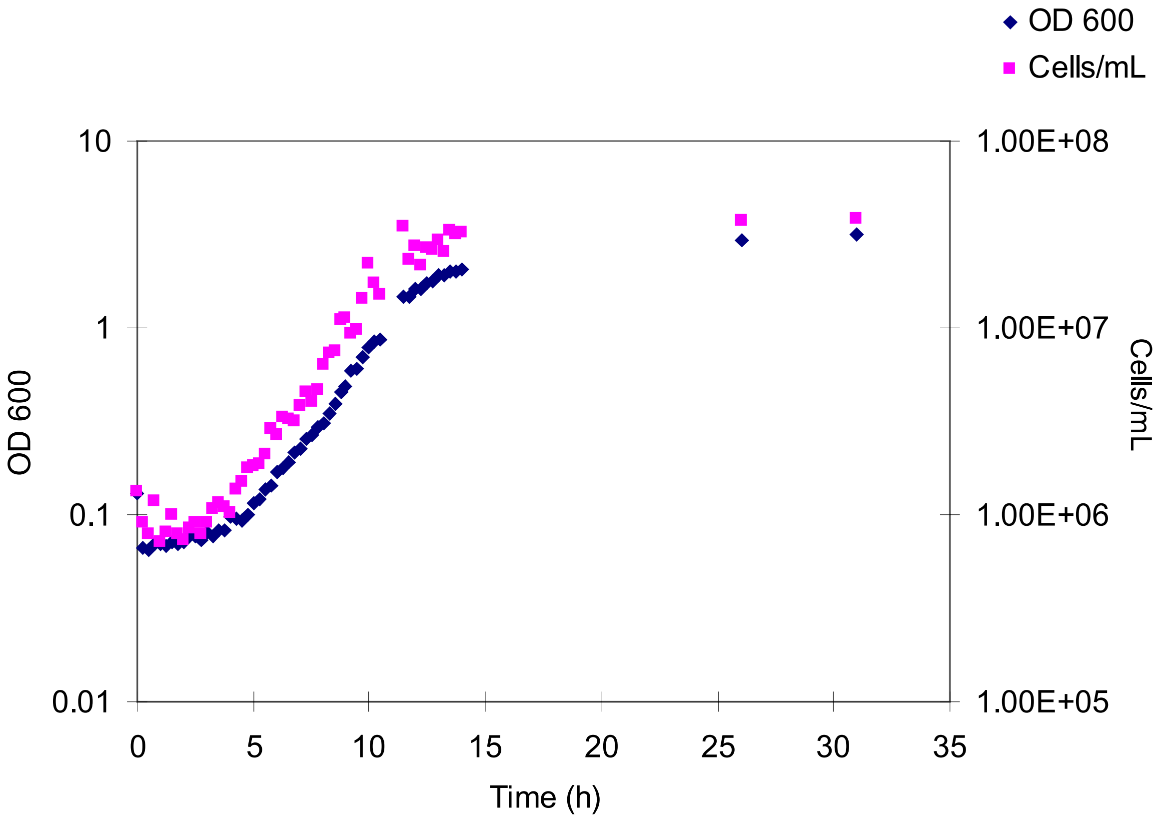
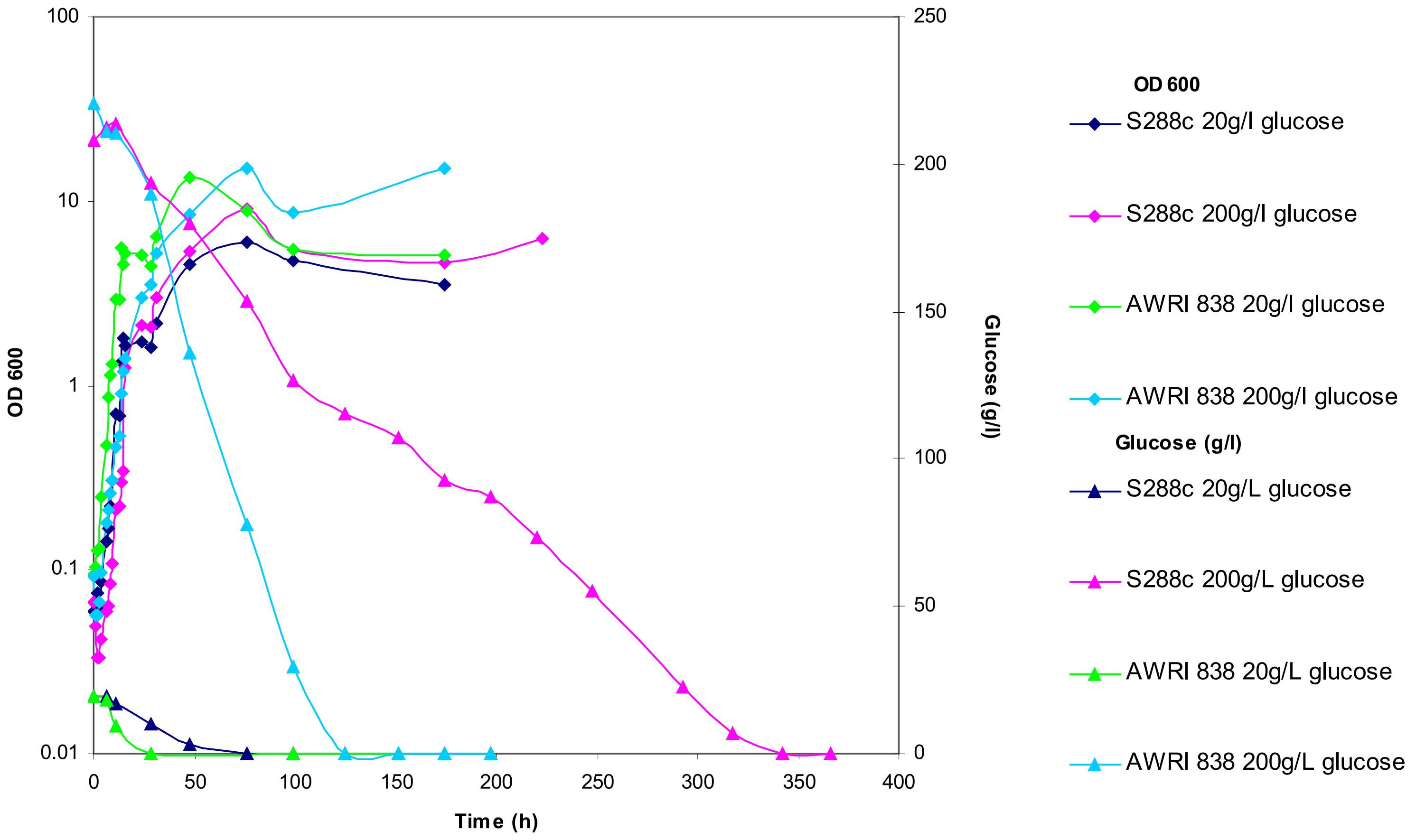


Figure 5.1 Fermentation of S288c *MAT***a** in CDGJM with 40g/l glucose. The increase in biomass was determined using both a haemocytometer to count cell number and spectrophotometer to measure increase in optical density at 600nm.

Figure 5.2 Growth curves and glucose usage of S288c (laboratory yeast) and AWRI838 (winemaking yeast) in CDGJM with 20 g/L or 200 g/L glucose.


OD 600
100
10
1
0.1
0.01
Time (h)
0
50
100
150
200
250
300
350
400
Glucose (g/l)
250
200
150
100
50
0
OD 600
S288c 20g/l glucose
S288c 200g/l glucose
AWRI 838 20g/l glucose
AWRI 838 200g/l glucose
Glucose (g/l)
S288c 20g/L glucose
S288c 200g/L glucose
AWRI 838 20g/L glucose
AWRI 838 200g/L glucose

5.2.1.2 Haze protective activity of material isolated from the laboratory yeast strain, S288c, ferments

CDGJM containing 200 g/L glucose was fermented either by the wine yeast, AWRI838, or by the laboratory yeast, S288c. Material precipitated by ethanol from the supernatant of these ferments was assayed for haze protective activity. Ferments were stopped 48 hours after inoculation, when the cultures were well into stationary phase, as this was found to be the time when maximal HPF is released into the supernatant (Dupin *et al*., 2000b).

The results of the heat test indicate that the haze protective activity of the ethanol precipitated material from the ferment supernatant from the lab yeast, S288c, was not significantly different ($P<0.05$) to that from the wine yeast, AWRI838 (Figure 5.3). This result suggested that it is reasonable to use S288c as a model yeast in which to study the effect of deleting and overexpressing the putative *HPF* genes.

5.2.2 Deletion and overexpression of *HPF* genes from yeast provides evidence that these genes code for Hpfs

5.2.2.1 Deletion of yeast *HPF* genes reduced the haze protective activity of the supernatant material

Haze protective activity of material precipitated by ethanol from the supernatant of wild type, *Δhpf1, Δhpf1', Δhpf2* single deletion mutants and an *Δhpf1 Δhpf1' Δhpf2* triple deletion mutant was examined. Fermentations were performed at 28^{o}C in CDGJM with 200 g/L glucose as the carbohydrate source, under anaerobic conditions, and were stopped 64 hours after inoculation. The fermentation curves of the mutant and wild type strains were similar (Figure 5.4). All strains were well into stationary growth phase at this point and were stopped at this time for reasons noted in Section 5.2.1.2.

At 0.1, 0.2 and 0.4 mg/mL levels of addition of material to the heat test assay mixture, the wild type yeast material reduced haze significantly more than any of the deletion mutants ($P<0.05$) (Figure 5.5). It was found that all the deletion mutants tested produced significantly fewer units of haze protective material than the wild type strain ($P<0.05$)

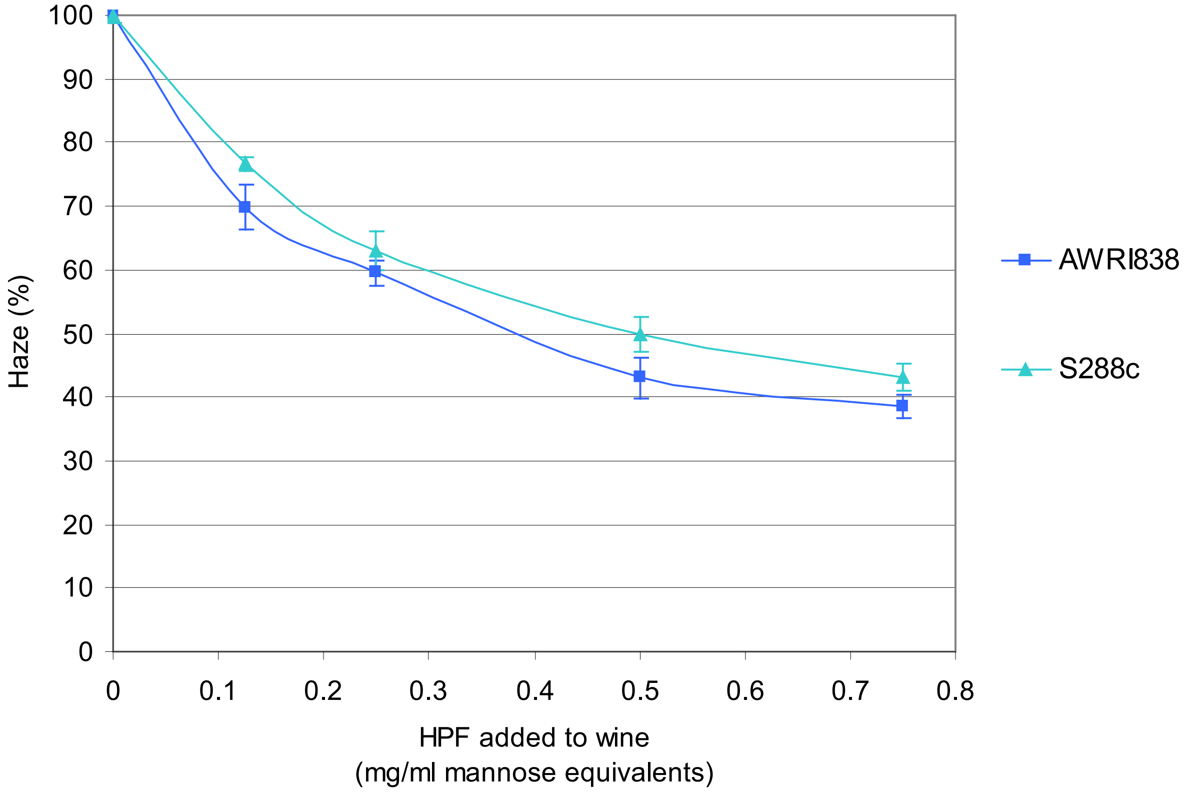


Figure 5.3 The effect of increasing concentrations of yeast macromolecules on heat induced protein haze in white wine. Ethanol precipitated material from supernatant of ferments by the laboratory yeast, S288c and the winemaking yeast, AWRI838, was tested according to the micro-heat test described in Chapter 2.

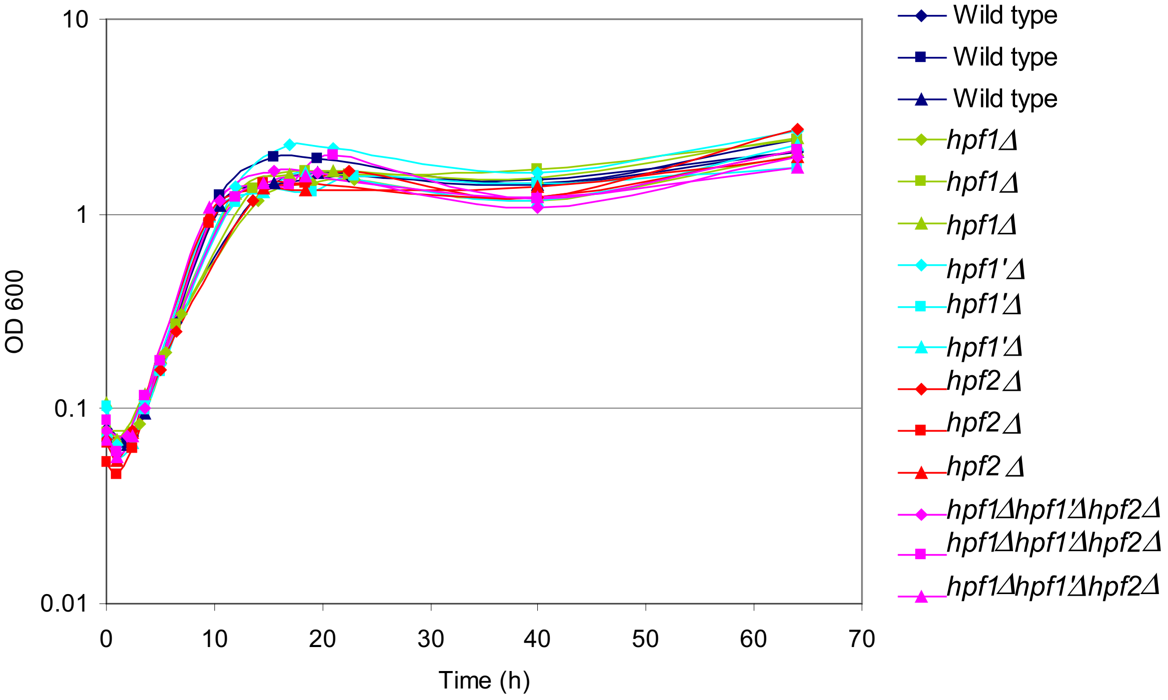


Figure 5.4 The growth of wild type and *hpfΔ* mutants in CDGJM at 30°C under anaerobic conditions. Triplicate fermentations of each strain are shown.

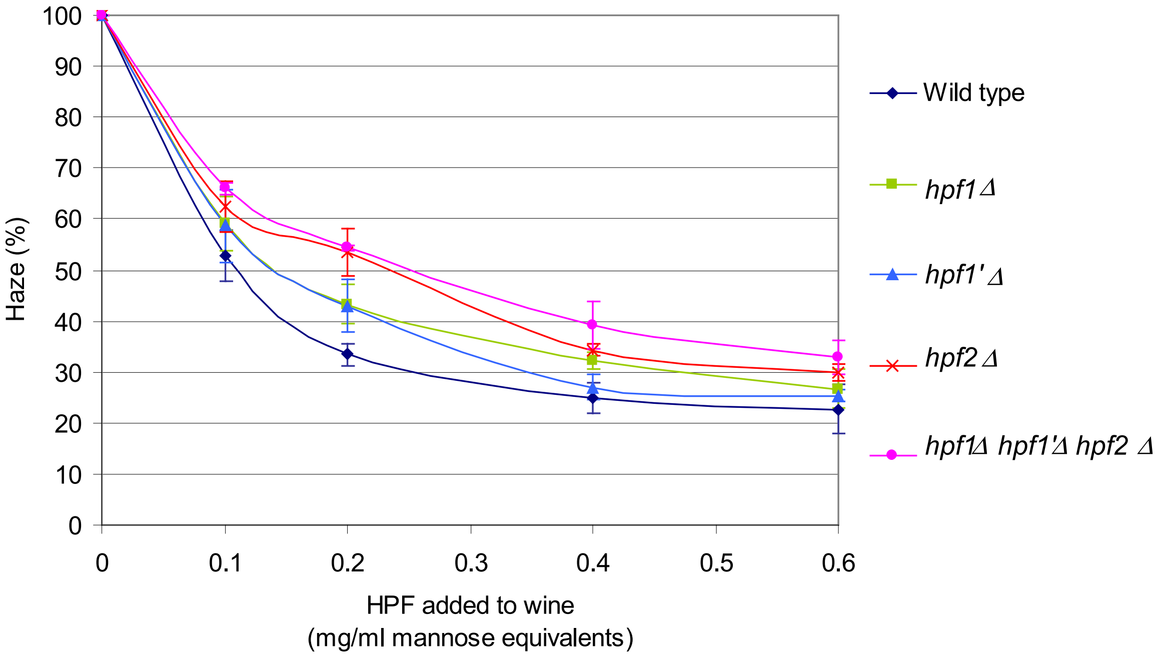


Figure 5.5 The effect of increasing concentrations of supernatant derived yeast macromolecules on heat induced protein haze in white wine. Ethanol precipitated material from supernatant of ferments by wild type and *hpfΔ* mutants was tested according to the micro-heat test. The mean and standard deviation of three replicate ferments are shown.

Table 5.1: Mannose and units of haze protective activity in ethanol precipitated material from ferments by *hpfΔ* strains. A unit of haze protective activity is defined as the amount of material, in milligrams, required to reduce the percent haze to 50% in standard conditions of the heat test (200µl, heated at 80°C for six hours, cooled overnight and optical density at 490 nm measured). *Not significantly different ($P>0.05$) Student's t-test; ^Significantly different from the wild type ($P<0.05$) Student's t-test.

Yeast strain	Mannose (mg) per litre of fermentation (Mean ± SD)	Units of activity per litre of fermentation (Mean ± SD)
S288c (Wild type)	9.99 ± 1.39*	499.73 ± 69.71
hpf1Δ	7.27 ± 0.76*	242.30 ± 25.48^
hpf1'Δ	10.28 ± 1.15*	342.77 ± 38.32^
hpf2Δ	8.58 ± 1.12*	171.69 ± 22.45^
hpf1Δ hpf1'Δ hpf2Δ	8.41 ± 1.33*	168.16 ± 26.55^

(Table 5.1), but the amount of mannose in the extracts was not significantly different (P>0.05) (Table 5.1).

5.2.2.2 Overexpression of *HPF* genes in yeast results in increased haze protective activity of supernatant material

Hpf1p, Hpf1'p and Hpf2p were overexpressed and the haze protective activity of the ethanol precipitated material from these ferments was studied. Fermentations using the *HPF1*, *HPF1'* and *HPF2* overexpressor yeast strains were performed in CDGJM without leucine (for selection of the overexpression plasmid) with 20 g/L galactose (to induce expression) at 28°C and 100 rpm under anaerobic conditions and were stopped 96 hours after inoculation. The fermentation curves of the control (plasmid, no insert) and overexpressor strains were similar (Figure 5.6). All strains were well into stationary growth phase at 96 hours and were stopped to extract the maximum possible amount of haze protective material as described in Section 5.2.1.2.

The haze protective activity of ethanol precipitated material from the *HPF1* overexpressing strain was found to be 10-fold more than the control (P<0.05), as shown in Table 5.2. The results also suggest that Hpf1'p has haze protective activity as the reduction in haze was more than the control at every addition level (P<0.05) (Figure 5.7), although the number of units of haze protective activity of material from the *HPF1'* overexpressor ferment was 5-fold more than the control, this was not statistically significant (P>0.05). The results did not provide evidence that Hpf2p had haze protective activity because the activity of material from the *HPF2* overexpressor was similar to the control.

The reason for this apparent lack of activity of Hpf2p was examined further, using 6xHis-tagged gene constructs (see Section 5.2.3 for details), since the result discussed here was somewhat unexpected given the results from the deletion strains (Section 5.2.2.1). Material extracted from ferments of the native (untagged) *HPF2* overexpressing strains was compared to material from tagged 6xHis-Hpf2p overexpression strain and the wild type by analysing the banding pattern on a Schiff-periodate stained SDS-PAGE gel (Figure 5.8). The absence of a band at about 178 kDa,

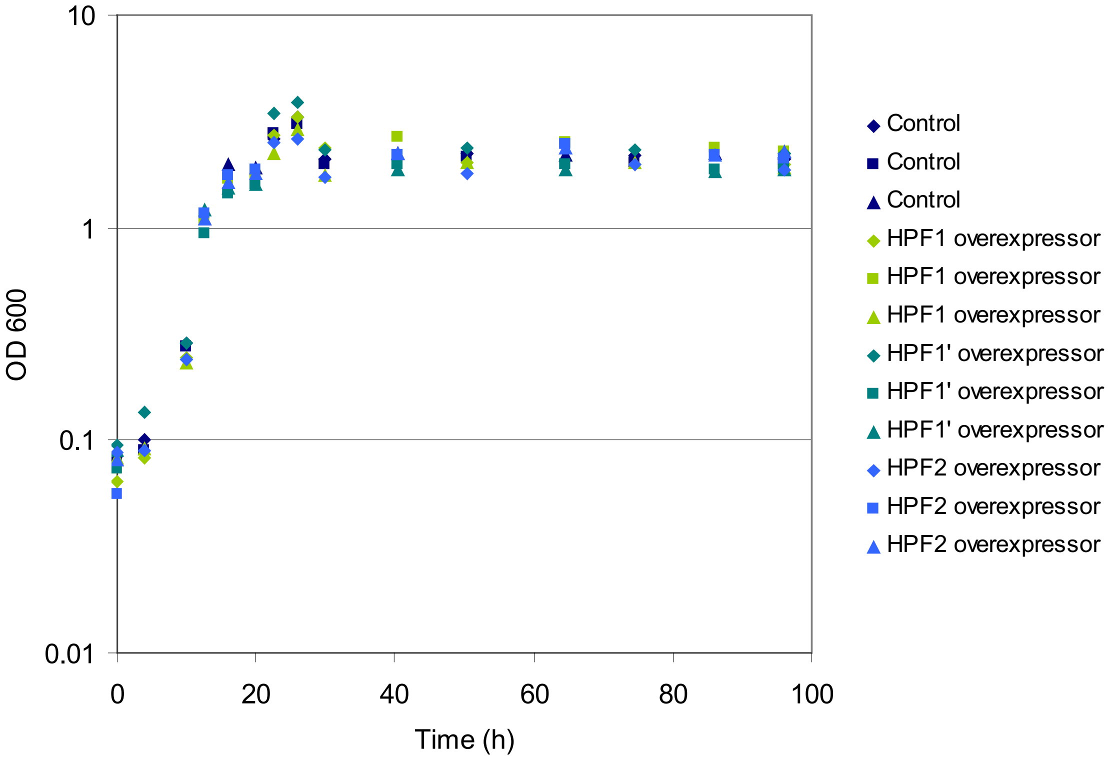


Figure 5.6 The growth of wild type and HPF overexpression strains in CDGJM with galactose at 30°C under anaerobic conditions. Triplicate fermentations of each strain are shown.

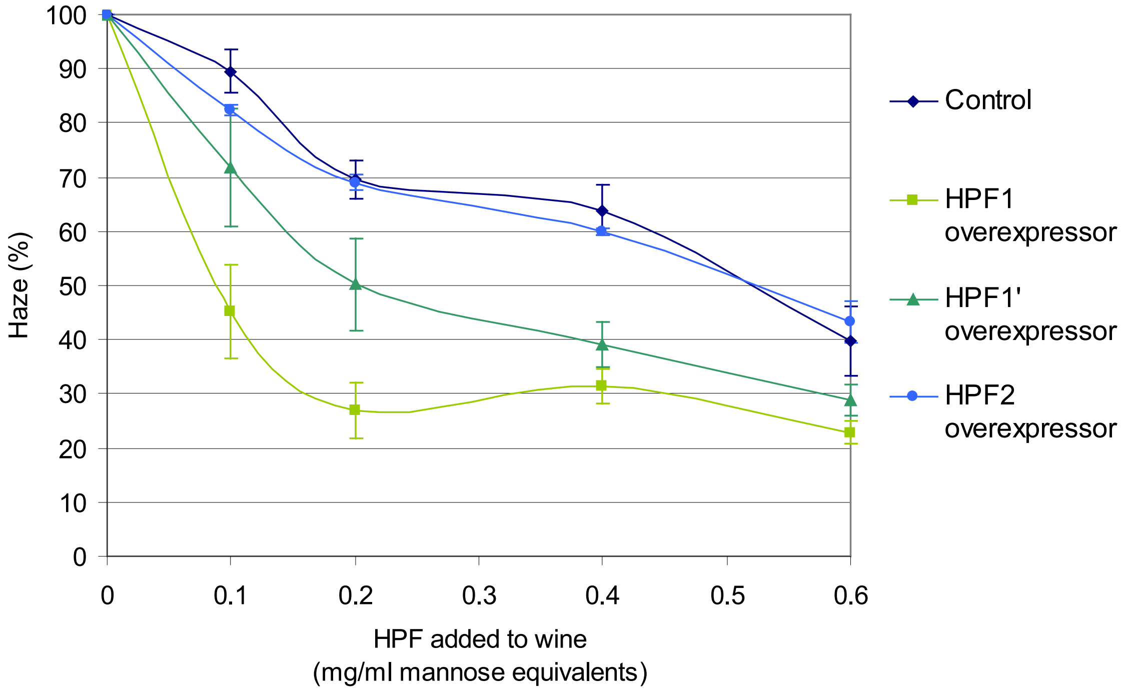


Figure 5.7 The effect of increasing concentrations of yeast macromolecules on heat induced protein haze in white wine. Ethanol precipitated material from the supernatant of ferments by wild type and HPF overexpression strains was tested according to the micro-heat test. The mean and standard deviation of three replicates are shown.

Table 5.2: Mannose and units of haze protective activity in ethanol precipitated material from ferments by *HPF* overexpression strains. A unit of haze protective activity is defined in Table 5.1. *Not significantly different (P>0.05) Student's t-test; ^Significantly different from the control (P<0.05) Student's t-test.

Yeast strain	Mannose (mg) per litre of fermentation (Mean ± SD)	Units of activity per litre of fermentation (Mean ± SD)
S288c + p415GAL1	12.26 ± 2.16*	122.63 ± 21.57
S288c + p415GAL1-HPF1	24.99 ± 14.14*	1249.72 ± 707.02^
S288c + p415GAL1-HPF1'	25.48 ± 13.42*	637.03 ± 335.53
S288c + p415GAL1-HPF2	31.09 ± 9.68*	310.89 ± 96.83

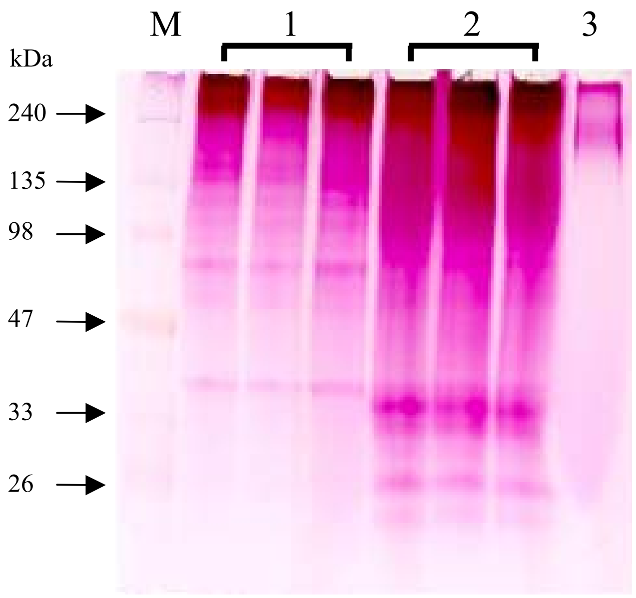


Figure 5.8 Schiff-periodate stained gel of material from control and *HPF2* overexpression ferments. M, Colour marker (Sigma); 1, Ethanol precipitated material from triplicate control overexpressor ferments; 2, Ethanol precipitated material from triplicate *HPF2* overexpressor ferments; 3, Ni-NTA purified 6xHis-Hpf2p.

the size of 6xHis-Hpf2p, in lane 2, and the presence of several bands of lower molecular weight, which were not present in the wild type samples, suggests that native Hpf2p expressed in the ferments may have been unstable.

5.2.3 Expression of 6xHis-*HPF* in yeast confirms that *HPF2* encodes a HPF

Fusion genes consisting of 6xHis-Hpf were constructed with the 6xHis epitope inserted into each of the *HPF* genes after the N-terminal secretion signal sequence (Figure 2.1). This was cloned into a plasmid (pYES2/GS) putting this fusion gene under the control of the *GAL1* promotor (Appendix 5). The resultant plasmids were transformed into a haploid S288c strain.

5.2.3.1 Expression levels of 6xHis-Hpf1p, 6xHis-Hpf1'p and 6xHis-Hpf2p

The relative amounts of 6xHis-Hpf1p, 6xHis-Hpf1'p and 6xHis-Hpf2p in the supernatant was estimated by subjecting both untreated and Endoglycosidase H (Endo H) treated (de-*N*-glycosylated) material to SDS-PAGE and Schiff-periodate staining the gel for carbohydrate and for protein with Coomassie blue (Figure 5.9). This clearly showed that 6xHis-Hpf1p and 6xHis-Hpf1'p cannot be seen on the gel, unless some glycosylated material is removed. This is probably because Hpf1p and Hpf1'p are large proteins and thus do not enter the gel. Faint bands in lane 4 at 183 kDa and in lane 6 at 208 kDa were assigned to Endo H treated 6xHis-Hpf1p and 6xHis-Hpf1'p, respectively. These bands were not present when analysing material from the wild type supernatant.

It was possible to detect bands assigned to 6xHis-Hpf2p in both the fully glycosylated and Endo H treated samples. The molecular weight calculated from the position on the gel for 6xHis-Hpf2p was 178 kDa and for Endo H treated 6xHis-Hpf2p, 58 kDa. It is also clear from the gel that the level of 6xHis-Hpf2p in the supernatant was far greater than that of 6xHis-Hpf1p or 6xHis-Hpf1'p (Figure 5.9).

In order to determine the best source of 6xHis-Hpfs, the location of the 6xHis-Hpfs in either cells or culture supernatant was determined (Figure 5.10). Yeast cultures were grown in triplicate for each overexpression strain on glucose (when *GAL1* is repressed)

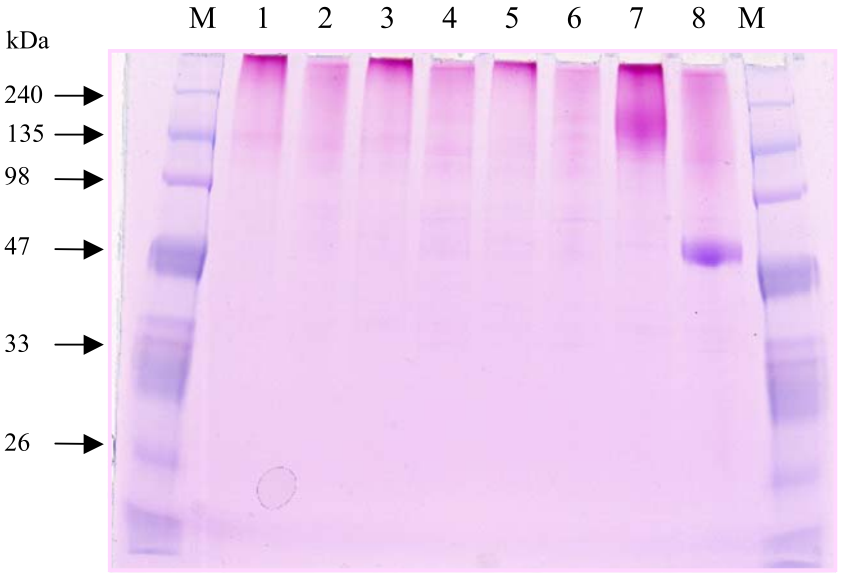


Figure 5.9 Schiff-periodate and Coomassie stained polyacrylamide gel showing the molecular weights of glycosylated and deglycosylated Hpf mannoproteins from concentrated supernatants. M, Sigma colour marker (wide range); 1, S288c - untreated; 2, S288c - Endo H treated; 3, 6xHis-Hpf1 overexpressor - untreated; 4, 6xHis-Hpf1 overexpressor - Endo H treated; 5, 6xHis-Hpf1' overexpressor - untreated; 6, 6xHis-Hpf1' overexpressor - Endo H treated; 7, 6xHis-Hpf2 overexpressor - untreated; 8, 6xHis-Hpf2 overexpressor - Endo H treated.

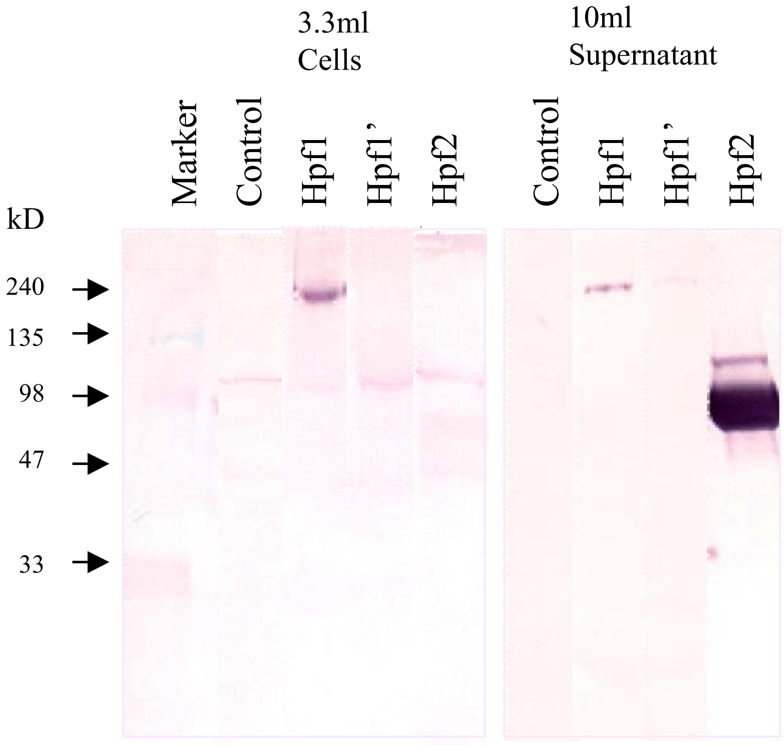


Figure 5.10 Western blot of 6xHis-tagged Hpf mannoproteins from cells and supernatant. The Western blot was probed with Anti-6xHis antibody as described in the Chapter 2. M, Sigma colour marker (wide range); Control, S288c; Hpf1, 6xHis-Hpf1; Hpf1', 6xHis-Hpf1'; Hpf2, 6xHis-Hpf2. Cell extract equivalent to cells from 3.3 mL and supernatant equivalent to 10 mL of culture was loaded on the gel. Extra bands in cell extracts are naturally occurring yeast proteins which may favourably interact with the IMAC resin and anti-6xHis antibody.

and on galactose (when *GAL1* is induced). After 24 hours, the cells were removed from the supernatant by centrifugation. Total protein was isolated from the cells and the protein in the supernatant was concentrated and desalted into water. Protein extract equivalent to 10 mL of culture supernatant or cells from 3.3 mL of the culture was deglycosylated by treating with Endo H, run on SDS-PAGE, and transferred to nitrocellulose for Western blotting. As the triplicates showed the same results, a representative of each is shown in Figure 5.10. The bands of deglycosylated 6xHis-Hpf1p and 6xHis-Hpf1'p were very faint in Figure 5.9. A Western blot with the anti-6xHis antibody rather than the general carbohydrate stain, Schiff-periodate, used previously, confirmed that the majority of 6xHis-Hpf1'p and 6xHis-Hpf2p is in the supernatant (Figure 5.10). The majority of 6xHis-Hpf1p is in the cells (Figure 5.10).

5.2.3.2 Purification of 6xHis-Hpf2p

6xHis-Hpf2p was overexpressed in S288c and using methods described in Section 2.11 and 2.12, 6xHis-Hpf2p was purified from the culture supernatant using affinity chromatography on Ni-NTA. Culture supernatant from a fermentation of S288c not carrying a plasmid was used as a control throughout the procedure.

Equivalent dry weight amounts of invertase and Ni-NTA purified 6xHis-Hpf2p were run on SDS-PAGE and the gel was Schiff-periodate stained (Figure 5.11). It was found that the two mannoproteins do not stain equivalently; in fact, invertase stains approximately five times more strongly than 6xHis-Hpf2p.

5.2.3.3 Addition of purified 6xHis-Hpf2p to wine in a heat test affords significant haze protective activity

Purified 6xHis-Hpf2p was added to wine and its haze protective activity was assayed using the heat test (Section 2.9.2). It was found that pure material added to wine protected the wine from haze formation and reduced haze to around 30% of that shown by wine alone (Figure 5.12) ($P<0.05$). The S288c wild type control had no haze protective activity.

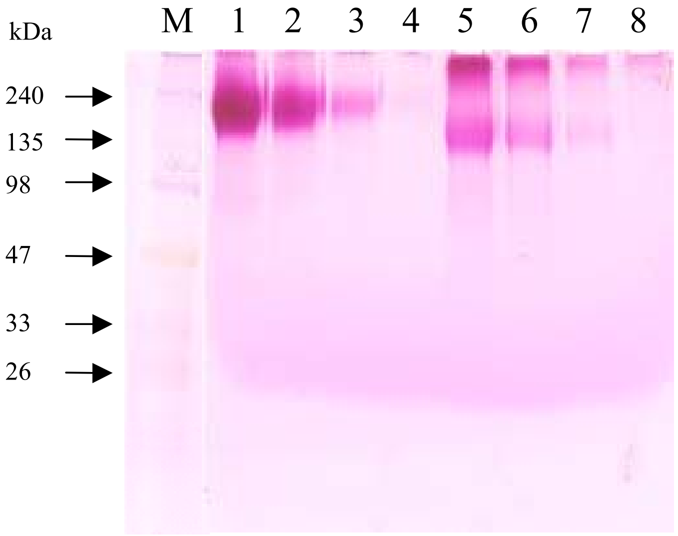


Figure 5.11 Invertase and 6xHis-Hpf2p subjected to SDS-PAGE and stained with Schiff-periodate stain. M, Sigma colour marker; 1, 100 µg invertase; 2, 50 µg invertase; 3, 10 µg invertase; 4, 1 µg invertase; 5, 100 µg 6xHis-Hpf2p; 6, 50 µg 6xHis-Hpf2p; 7, 10 µg 6xHis-Hpf2p; 8, 1 µg 6xHis-Hpf2p.

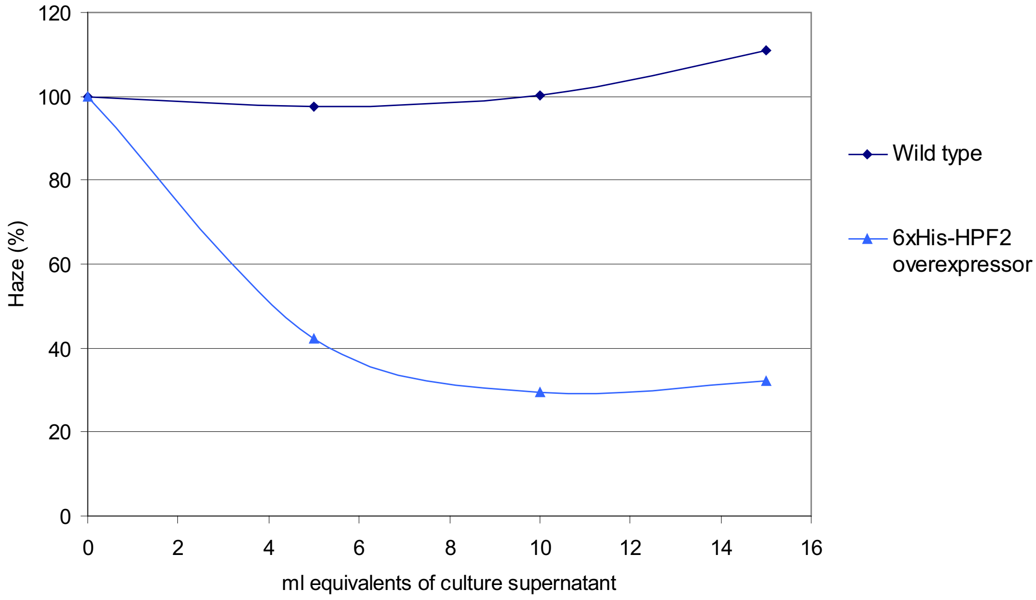


Figure 5.12 The effect of the addition of purified HPF on heat induced protein haze formation in white wine. The material purified by IMAC using Ni-NTA, from supernatant of an S288c (wild type) and SB59 (6xHis-Hpf2p overexpressor) were tested according to the micro-heat test as described in the Chapter 2.

5.2.4 Purification of 6xHis-Hpf1p and 6xHis-Hpf1'p

The 6xHis-Hpf1p and 6xHis-Hpf1'p were overexpressed and recovered from 5 L of supernatant using ethanol precipitation. The material was desalted into water and purification using Ni-NTA resin was attempted. These proteins did not bind strongly to the resin and were found in the unbound and wash fractions. The reason that the proteins did not bind was not known. The unbound fractions were desalted into water using a 10DG column (BioRad) and freeze-dried. The dry material was resuspended in 75 mM sodium acetate (pH 5.5) buffer, excess Endo H was added (0.06 U) and this was incubated at 37°C for 24 hours to deglycosylate the proteins under native conditions. The Endo H treated material was diluted 10-fold in Ni-NTA wash buffer (Appendix 2) and purified using Ni-NTA. The fractions were assessed for the presence of 6xHis-Hpf1p and 6xHis-Hpf1'p by Western blotting and it was found that the majority of the Endo H treated material bound to the column and was eluted with elution buffer (Appendix 2) (Figure 5.13). The dry weight of Endo H treated 6xHis-tagged material from five litres of supernatant was 2.8 mg of 6xHis-Hpf1p and 2.7 mg of 6xHis-Hpf1'p.

The de-*N*-glycosylated 6xHis-Hpf1p and 6xHis-Hpf1'p were assessed for haze protective activity using the heat test. Neither 6xHis-Hpf1p nor 6xHis-Hpf1'p exhibited haze protective activity (Figure 5.14). The requirement for Endo H treatment in order to purify 6xHis-Hpf1p and 6xHis-Hpf1'p was a major flaw to testing the hypothesis that Hpf1p and Hpf1'p have haze protective activity. It was not possible to purify native 6xHis-Hpf1p or 6xHis-Hpf1'p by Ni-NTA chromatography therefore the deletion data (Section 5.2.2.1) and overexpression data (Section 5.2.2.2) is the best available at this time. However, the results do indicate that if 6xHis-Hpf1p and 6xHis-Hpf1'p are HPFs, then the mechanism for haze protective activity may be different to 6xHis-Hpf2p.

5.3 DISCUSSION

The experiments described in this chapter were designed to establish whether the S288c genes suspected to encode haze protective factors did indeed code for material with haze protective activity in wine.

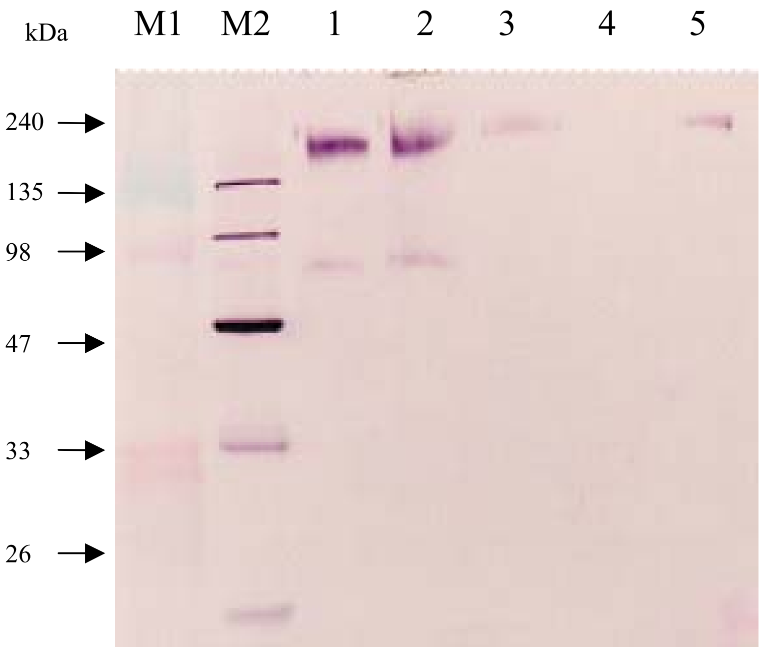


Figure 5.13 Purification of Endo H treated 6xHis-Hpf1'p under non-denaturing conditions by Ni-NTA. M1, Colour marker (Sigma); M2, 6xHis ladder (QIAGEN); 1, Unbound fraction of 6xHis-Hpf1'p loaded on to Ni-NTA. The fraction was treated with Endo H under denaturing conditions after chromatography; 2, Material shown in lane 1 that has been desalted and treated with Endo H under non-denaturing conditions. This is the starting material for the Ni-NTA purification of Endo H treated 6xHis-Hpf1'p (non-denaturing conditions); 3, Unbound fraction of Ni-NTA purification of Endo H treated 6xHis-Hpf1'p (non-denaturing conditions); 4, Wash fraction of Ni-NTA purification of Endo H treated 6xHis-Hpf1'p (non-denaturing conditions); 5, Eluate fraction of Ni-NTA purification of Endo H treated 6xHis-Hpf1'p (non-denaturing conditions).

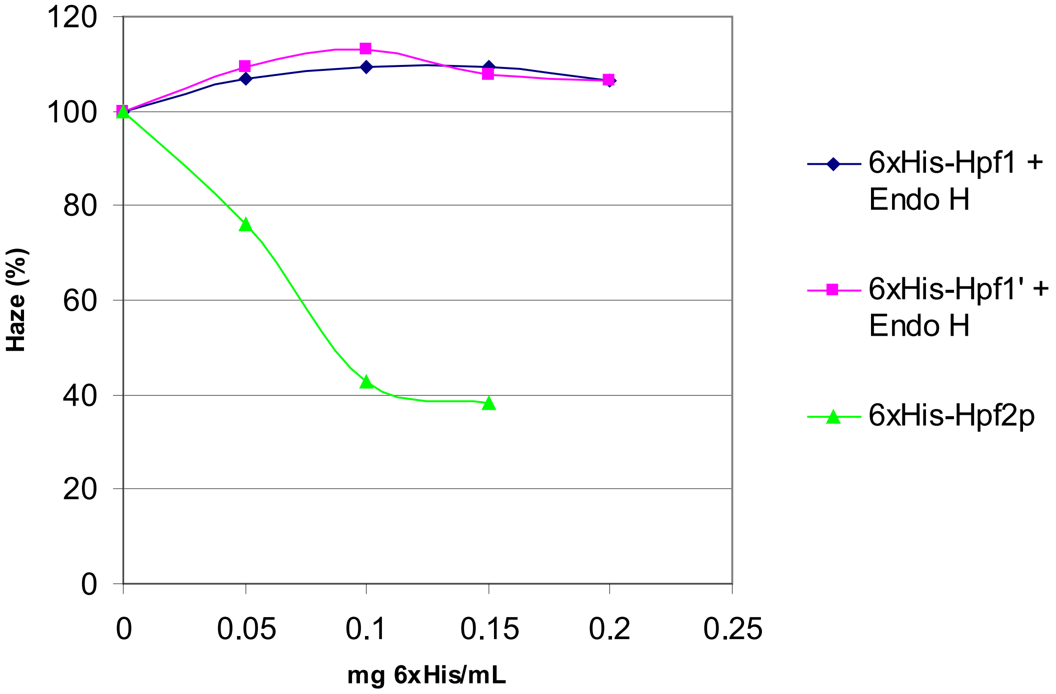


Figure 5.14 The effect of the addition of purified Endo H treated 6xHis-Hpf1p and 6xHis-Hpf1’p on heat induced protein haze formation in white wine. The Endo H treated material purified from the supernatant of SB39 (6xHis-Hpf1p overexpresssor) and SB57 (6xHis-Hpf1’p overexpressor) was tested according to the heat test as described in the Chapter 2.

It was known that S288c was unable to grow in CDGJM without amino acids; therefore synthetic complete amino acid mix (Appendix 2) was added to CDGJM, for both S288c and AWRI 838. The wine yeast, AWRI 838 was able to grow and metabolise glucose at a faster rate at all of the five glucose concentrations tested. Not surprisingly, this suggests that the wine yeast was able to cope with the simulated winemaking conditions better than the laboratory strain; however, the laboratory strain reached a similar cell density in stationary growth phase and eventually metabolised all the glucose available.

HPF had been purified originally from wine and there was no guarantee that laboratory strains were capable of synthesising HPF. The next experiment was therefore aimed at determining whether S288c indeed produced HPF and thus if it could be used as a model yeast strain for putative *HPF* gene product analysis. It was expected that S288c had the putative *HPF* genes since the *Saccharomyces* Genome Database was used to identify the genes; however, it was not known whether S288c would express haze protective forms of these proteins. Figure 5.3 shows that material extracted from ferment supernatants of the laboratory yeast, S288c and the wine yeast, AWRI 838, have similar levels of haze protective activity. Since there was no significant difference in their haze protective activity, this indicated that it was reasonable to continue the deletion and overexpression studies using this laboratory yeast strain.

To determine if any, or all, of the putative *HPF* gene products are involved in haze protective activity, a series of *hpfΔ* strains were constructed and the material extracted from ferment supernatants tested for its haze protective activity. A decrease in haze protective activity by *hpf1Δ*, *hpf1'Δ* and *hpf2Δ* strains indicated that these proteins have some contribution to total haze protective activity. Moreover, material extracted from ferments by the triple deletion strain had less haze protective activity than the wild type strain. Clearly, material from the triple deletion strain did have some haze protective activity. This indicates that other haze protective factors exist in the laboratory yeast, S288c, and that they are also found in the ferment supernatant. As the active component of the supernatant from the triple deletion strain was not determined in this work, it is not possible to predict which other yeast proteins or mannoproteins have haze protective activity.

Each putative *HPF* gene was also overexpressed individually in yeast. Material extracted from the ferment supernatant of the *HPF1* and *HPF1'* overexpression strains had enhanced haze protective activity but the material from the *HPF2* overexpressor strain was not significantly different from the control strain. The result seemingly contradicts the findings with the *hpf2Δ* deletion strain. Initially it was considered that this might have been due to accumulation of Hpf2p in the yeast cell. Hpf2p is predicted to be GPI-anchored and localised in the plasma membrane (Caro *et al*., 1997), and it may be that the secretory pathway was ‘blocked’ by excess Hpf2p. The extraction of Hpf2p from overexpressor cells compared to wild type cells may give an indication of the location of the excess Hpf2p. Other possible explanations for the lack of haze protective activity in the ethanol-precipitated material from the *HPF2* overexpressor include loss of plasmid, weak expression, unfavourable growth conditions or degradation of expressed product. Since the yeast were able to grow in medium lacking leucine, to select for the p415GAL1-HPF2 plasmid, it is unlikely that the explanation was a loss of plasmid. It is possible that the growth conditions (20 g/L galactose, anaerobic) make it difficult for the yeast to overexpress this protein, but again, this is doubtful because the yeast were able to overexpress Hpf1p and Hpf1’p under the same conditions. There is evidence to suggest that the overexpressed protein may have been degraded. There are several bands present in all Hpf2p overexpression ferment extracts that are not present in the wild type ferment extracts (Figure 5.8). Hpf2p may have been overexpressed to the same level as Hpf1p and Hpf1’p, but over the time of the ferment been degraded. The ferment for the untagged Hpf2p was allowed to continue for 96 hours before spinning the cells out but in the 6xHis-Hpf2p culture, the culture was stopped after 24 hours, so there was less opportunity for degradation to occur. Results of Stockdale, (2000), found that generally Hpf2p ran as a series of bands on SDS-PAGE, indicating that Hpf2p may be prone to degradation during long fermentations.

To clarify if Hpf2p does indeed have haze protective activity as suggested by Stockdale, (2000) and the *hpf2Δ* deletion strain material, a pure sample of Hpf was prepared by 6xHis-tagging Hpf2p at the amino-terminus of the mature protein. Purification of 6xHis-tagged proteins from culture supernatant was performed using Ni-NTA IMAC resin. The heat test results of purified 6xHis-Hpf2p clearly established that 6xHis-Hpf2p is indeed a haze protective factor as it reduced haze to 30% whereas the equivalent material from the wild type strain did not show haze protective activity (Figure 5.12). 6xHis-tagged versions of *HPF1* and *HPF1'* were also produced to confirm the data from the overexpression and deletion material. Since the 6xHis-Hpf1p and 6xHis-Hpf1'p from the culture supernatant did not bind to the Ni-NTA resin until they had been treated with Endo H to remove the *N*-glycosylation, it was likely that the *N*-linked sugars were interfering with the accessibility of the 6xHis tag to the resin. The need for Endo H treatment before purification prevented assessment of haze protective activity of pure glycosylated 6xHis-Hpf1p and 6xHis-Hpf1'p. The Ni-NTA purified Endo H treated 6xHis-Hpf1p and 6xHis-Hpf1'p did not have haze protective activity (Figure 5.14). This is further discussed in Chapter 6.

Relative amounts and location of the 6xHis-Hpfs was determined by Schiff-periodate staining and Western blotting, respectively. The amount of 6xHis-Hpf2p was much higher than 6xHis-Hpf1p or 6xHis-Hpf1'p, and the majority of it was found in the supernatant. The supernatant was the preferred source of the Hpf mannoprotein for purification purposes because there are fewer contaminating proteins present and these are not likely to naturally contain six consecutive histidine residues (Figure 5.10). There are 15 proteins in yeast that contain 6xHis (Cherry *et al*., 1997). These naturally expressed 6xHis containing proteins may be co-purified with the 6xHis-tagged Hpf. However, no 6xHis containing proteins were detected in the S288c wild type supernatant (Figure 5.10), although some intracellular proteins that cross-react with the anti-6xHis antibody and that bind to Ni-NTA resin were seen in the cell wall extract (Figure 5.10).

Hpf2p has been shown to be secreted by regenerating protoplasts using a two-dimensional polyacrylamide gel approach (Pardo *et al*., 1999). A spot of 123 kD and pI 5.6 was isolated and the amino-terminal was sequenced; this was found to be YDR055w (*HPF2*) and was renamed *PST1* for protoplasts secreted. The protein shows similarity to members of the Sps2p family as described in Section 3.1 (Caro *et al*., 1997). YDR055p has been proven to undergo some post-translational modification because its experimental M_r (123 kD) is higher than its predicted weight (45.7 kD) and its pI is 5.6 as opposed to the predicted 9.4 (Pardo *et al*., 1999).

During the purification process for Hpf2p, comparison of 6xHis-Hpf2p with another yeast mannoprotein, invertase was made. It was noted that invertase stained considerably more strongly than 6xHis-Hpf2p with Schiff-periodate stain. Schiff's reagent is prepared by reacting one molecule of pararosanaline (fuchsin dye) with three molecules of sulphur dioxide to form a colourless reagent. When hydroxyls in carbohydrates of glycoproteins are exposed to periodic acid, they are oxidised to aldehydes. Schiff's reagent reacts with an aldehyde resulting in an addition product that loses sulphurous acid to form a new coloured compound. If the reaction exhibits simple stoichiometry, the difference in staining intensity between 6xHis-Hpf2p and invertase is not likely to be because 6xHis-Hpf2p contains less mannose than invertase on a weight basis. Based on the observed M_r for 6xHis-Hpf2p (178 kDa, Figure 5.10) and predicted M_r from the gene sequence (45.8 kDa, (Cherry *et al.*, 1997)) it is likely that 6xHis-Hpf2p is approximately 75% mannose on a weight basis. This is greater than invertase, which is 50% mannose (Reddy *et al.*, 1988). However, the reaction may not have simple stoichiometry, since formaldehyde gives three to seven different reaction products depending on the relative amounts of dye, formaldehyde and sulphur dioxide (Noller, 1965).

5.4 CONCLUSION

The strong haze protective activity of 6xHis-Hpf2p (Figure 5.12) along with the decreased activity of the material from the *hpf2Δ* strain (Figure 5.5) convincingly confirms that Hpf2p has haze protective activity. As suggested earlier, it was likely that the Hpf2p expressed by the untagged overexpression strain was degraded during fermentation. The results of the deletion and overexpression studies strongly support the suggestion that Hpf1p and Hpf1'p have haze protective activity (Figures 5.5 and 5.7), however, the 6xHis-tagged forms could not be purified unless the proteins were treated with Endo H.

CHAPTER SIX

A PRELIMINARY STUDY TO ASSIST IDENTIFICATION OF THE ACTIVE COMPONENT OF HPF

6.1 INTRODUCTION

The experiments described in this chapter aimed to determine whether the protein or polysaccharide component of Hpf, or a combination of both, is responsible for haze protective activity. The 6xHis-tagged Hpf2p, described in Chapter 5, was used for experiments to identify the active component. Identification of the active component was approached with two strategies. In the first, the *N*-glycans of 6xHis-Hpf2p were removed with the enzyme Endoglycosidase H (Endo H), and the haze protective activity of partially de-*N*-glycosylated 6xHis-Hpf2p was assessed. In the second, 6xHis-Hpf2p was expressed in several glycosylation mutants and the haze protective activity of the resultant differentially glycosylated 6xHis-Hpf2p was assessed.

Endo H cleaves *N*-linked glycans between the *N*-acetylglucosamine residues of the core structure, removing almost the entire chain (Figure 1.6). Endo H has no activity against *O*-glycosylated carbohydrate units. An Hpf2p preparation has previously been treated with PNGase F (Stockdale, 2000). PNGase F de-*N*-glycosylates proteins by hydrolysing the amide bond of the asparagine linked carbohydrate. PNGase F treated Hpf2p exhibited less haze protective activity than glycosylated Hpf2p as treated Hpf2p was only able to decrease haze to 45% whereas glycosylated Hpf2p reduced haze to 35% at addition rates of 0.12 mg/mL (Stockdale, 2000). Similar trends were seen with PNGase F treated invertase (Stockdale, 2000), suggesting that the carbohydrate component of the HPFs plays a role in their activity. This interpretation of the data, however, can only be tentative, because the conditions of the experiment did not allow removal of the PNGase F or the released *N*-glycans before haze protective activity was determined. By using 6xHis-Hpf2p and including appropriate controls, de-*N*-glycosylation experiments and subsequent haze protective activity tests could be performed with greater rigour. The results from such experiments are reported in this chapter.

A great deal is known about the mechanism of *N*- and *O*- glycosylation in yeast, as described in Chapter 1. Strains mutated in many of the genes that catalyse and regulate glycosylation are available. Some of the genes encoding components that are involved in early steps of the glycosylation are essential. Several of the viable mutants, *mnn1Δ, mnn2Δ, mnn4Δ, mnn5Δ* and *van1Δ* were used in this work. It is possible that these deletion mutants will have multiple effects resulting in unanticipated glycan structures, particularly if the proteins are components of a complex (Dean, 1999). These changes in glycan structure can be characterised and the role of the glycosylation for haze protective activity determined. The role of each of the respective gene products in yeast glycosylation is described in Sections 1.5.3, 1.5.5 and below.

Van1p is a mannosyltransferase that is located in the Golgi complex and is involved in *N*-linked mannosylation maturation of the mannan backbone. It is a subunit of mannan polymerase I (M-Pol I) along with Mnn9p (Figure 1.6). According to (Kanik-Ennulat *et al*., 1995), deletion of *VAN1* results in complete loss of the mannan backbone, although (Ballou *et al*., 1991) have proposed that Van1p acts only on a subset of mannoproteins.

Mnn2p is a putative α-1,2-mannosyltransferase and thus mannan from a *mnn2Δ* strain lacks the main α-1,2-linked branches of wild type *N*-glycans (Figure 1.6) (Rayner and Munro, 1998). A single α-1,2-linked mannosyl residue is found at the end of an unbranched chain in *mnn2Δ* strains suggesting that Mnn2p may have a capping function (Ballou *et al*., 1989).

Mnn5p is a Golgi α-1,2-mannosyltransferase that acts sequentially after Mnn2p (Figure 1.6) (Rayner and Munro, 1998). Its null mutant is viable, but it is defective in the addition of the α-1,2-linked mannose branches to the mannan structure found in *N*-glycans.

Mnn1p is an α-1,3-mannosyltransferase localised in the Golgi apparatus and is active in both *N*- and *O*-mannosylation (Figure 1.6) (Munro, 2001; Wiggins and Munro, 1998). Mnn1p adds terminal mannose to the outer chain branches of *N*- and *O*-glycosylation, in some cases masking mannosylphosphate.

Mnn4p is likely to function as a positive regulator of Mnn6p, a mannosylphosphate transferase (Figure 1.6) (Odani *et al.*, 1996). The extent of mannosylphosphorylation catalysed by Mnn6p is dependant on Mnn4p function (Odani *et al.*, 1997; Wang *et al.*, 1997). The level of mannosyl phosphorylation of cell wall proteins increases at the late exponential and stationary phase of cell growth. It has been suggested that Mnn4p and Mnn6p act on both *N*- and *O*-linked glycans (Nakayama *et al.*, 1998).

This chapter will describe the use of glycosylation mutants and Endo H to alter glycosylation to examine changes in haze protective activity of 6xHis-Hpf2p.

6.2 RESULTS

6.2.1 Removal of *N*-linked oligosaccharides from 6xHis-Hpf2p affects its haze protective activity

6.2.1.1 QIAGEN Ni-NTA is the optimal IMAC resin for purifying native and partially deglycosylated 6xHis-Hpf2p

Several immobilised metal affinity chromatography (IMAC) resins were assessed for their ability to bind the glycosylated (native) and Endo H treated (de-*N*-glycosylated) forms of 6xHis-Hpf2p. TALON (BD Biosciences Clontech), a cobalt-based resin and Ni-NTA (QIAGEN) and ProBond Ni-NTA (Invitrogen) were assessed. Neither the glycosylated nor the Endo H treated forms (Figure 6.1) of 6xHis-Hpf2p bound to TALON. It was possible to bind and elute the Endo H treated form correctly from the ProBond Ni-NTA resin (Figure 6.1), but not the native glycosylated form. Both the glycosylated and Endo H treated forms bound and eluted from the Ni-NTA (QIAGEN) resin (Figure 6.1) and thus it was used for all further purification.

6.2.1.2 6xHis-Hpf2p is de-*N*-glycosylated equally under native and denaturing conditions

The Ni-NTA purified 6xHis-Hpf2p and invertase (Sigma) were de-*N*-glycosylated using Endo H under native and denaturing conditions to compare the efficiency of the

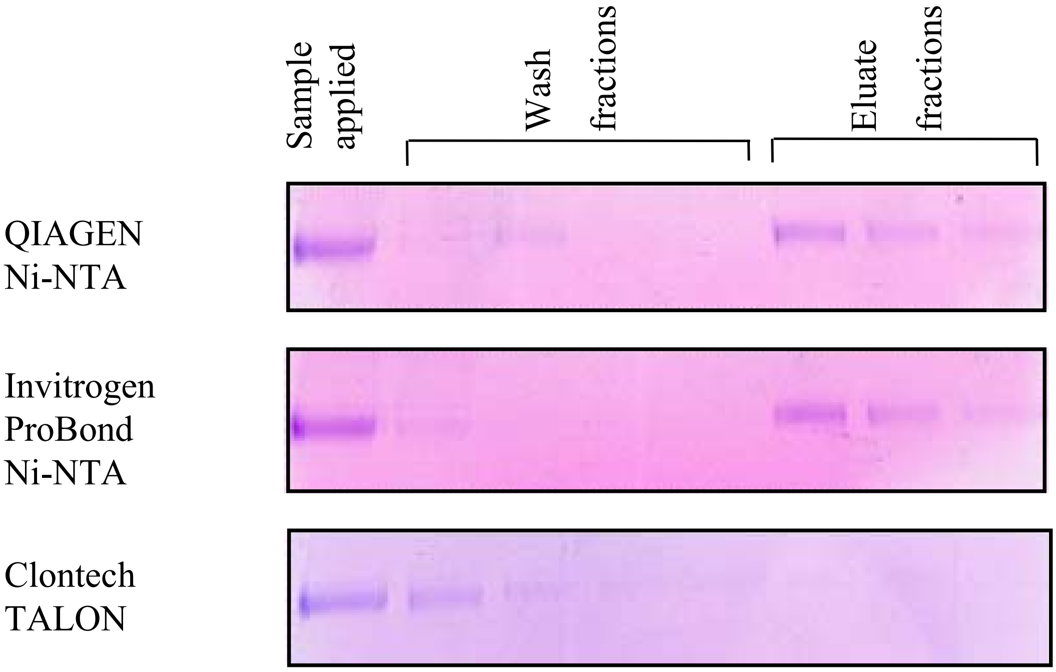


Figure 6.1 The binding and elution of Endo H treated 6xHis-Hpf2p to various Immobilised Metal Affinity Chromatography (IMAC) resins. The gels have been stained with Schiff's-periodate stain and Coomassie blue stain.

treatment. De-*N*-glycosylation under native conditions involved buffering the protein solution in 75 mM sodium acetate to pH 5.5, adding 0.006 U Endo H, and incubating the mixture at 37°C for the appropriate time. Under denaturing conditions, 5x SDS-PAGE sample buffer (Appendix 2) was added to the protein solution at a final concentration of 1x. The mixture was boiled for five minutes, allowed to cool to room temperature and 300 mM sodium acetate pH 5.5 was added to a final concentration of 75 mM. Endo H (0.006 U) was added and the sample was incubated at 37°C for two hours. The samples were boiled again before loading onto the SDS gels.

Upon SDS-PAGE, 6xHis-Hpf2p produced a broad band at 180 kDa (Figure 6.2, (a)). Following the native de-*N*-glycosylation treatment, bands were present at 180 kDa and at 73 kDa. The band at 180 kDa (presumably material that had not been deglycosylated) stained primarily with Schiff-periodate stain and the 73 kDa band with both Schiff-periodate and Coomassie blue. This Schiff-periodate and Coomassie blue double staining suggests that the de-*N*-glycosylated material is still partially glycosylated. A smaller band produced at 26 kDa stained with increasing intensity with increased incubation time and this is likely to be a degradation product. The amount of material de-*N*-glycosylated under non-denaturing conditions did not change greatly with incubation time. After de-*N*-glycosylation treatment under denaturing conditions, the main species observed migrated with an apparent molecular weight of 73 kDa. A larger band, stained primarily with Schiff-periodate stain, was present at 127 kDa. This band was not seen in any of the native treatments. It is likely that this band was partially de-*N*-glycosylated 6xHis-Hpf2p. No band can be seen at 180 kDa (Lane 7, Figure 6.2), suggesting that Endo H treatment of 6xHis-Hpf2p was more efficient after denaturation.

Invertase was also treated with Endo H under native and denaturing conditions (Figure 6.2 (b)) as a control to compare the treatment conditions. Untreated invertase produced a broad band at around 120 kDa. Invertase that was de-*N*-glycosylated under non-denaturing conditions produced a band of 96 kDa. This band was stained primarily with Coomassie blue. A second Schiff periodate stained broad band at 120 kDa is likely to be material that Endo H has not acted upon. The amount of material de-*N*-glycosylated under non-denaturing conditions was similar at all incubation times (Figure 6.2). Denaturing de-*N*-glycosylation produced a band of 90 kDa, which is smaller than that

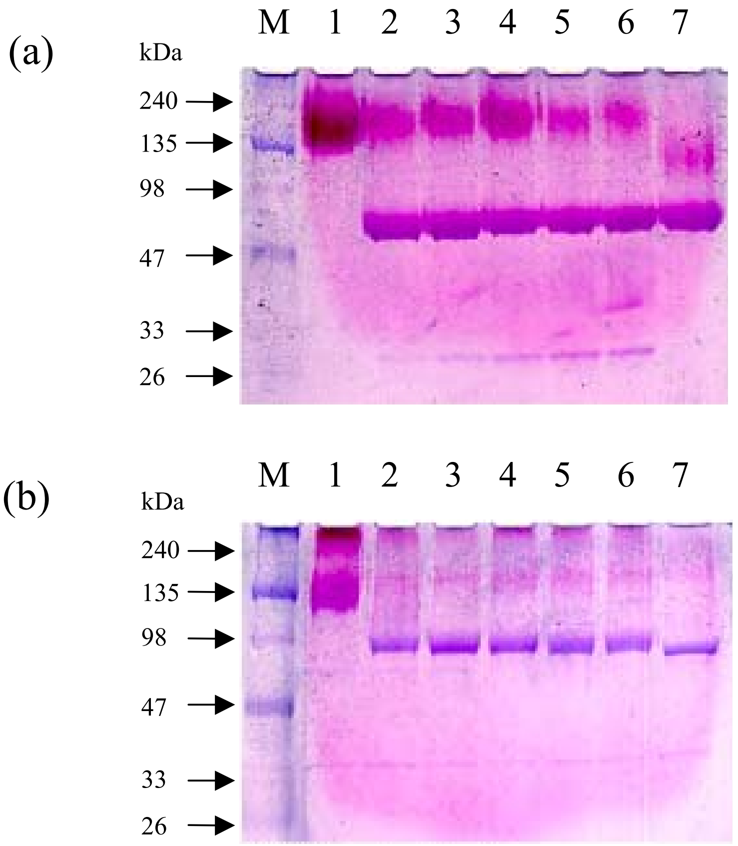


Figure 6.2 Comparison of efficiency of Endo H treatment under non-denaturing conditions to denaturing conditions of (a) 6xHis-Hpf2p and (b) invertase (Sigma). For both gels, M, Colour marker (Sigma); 1, Untreated protein; 2, Endo H treatment under non-denaturing conditions, 2 hours; 3, Endo H treatment under non-denaturing conditions, 4 hours; 4, Endo H treatment under non-denaturing conditions, 8 hours; 5, Endo H treatment under non-denaturing conditions, 20 hours; 6, Endo H treatment under non-denaturing conditions, 24 hours; 7, Endo H treatment under denaturing conditions, 2 hours. Sixty μg of protein was loaded in each lane. The gels were stained with Schiff periodate stain and then Coomassie blue.

produced by the native treatment (96 kDa). This suggests that more *N*-glycans are removed during the denaturing treatment because of improved access to the site of action. A band at 36 kDa can be seen in all lanes and is an artefact of the commercial preparation (Sigma).

6.2.1.3 The haze protective activity of Endo H treated 6xHis-Hpf2p is less than that of native 6xHis-Hpf2p

IMAC purified 6xHis-Hpf2p was de-*N*-glycosylated with Endo H for two hours under non-denaturing conditions. 6xHis-Hpf2p without Endo H and water with and without Endo H were also prepared as controls. The 6xHis-tagged proteins in these samples were purified using Ni-NTA resin as described in Section 2.12. The eluate was desalted using a 10DG column (BioRad) and freeze-dried. Equivalent amounts of 6xHis-Hpf2p were added to wine under heat test conditions as determined by a Western blot probed with anti-6xHis antibody. Haze protective activity of Endo H treated 6xHis-Hpf2p was significantly less ($P<0.05$) than material that had not been treated with Endo H, at 0.15 and 0.2 mg/mL (Figure 6.3).

6.2.2 6xHis-Hpf2p expressed in glycosylation mutants has altered haze protective activity compared to wild type

The importance of the glycosylation for activity of the 6xHis-Hpf2p was examined further by overexpressing 6xHis-Hpf2p in several yeast mutants with glycosylation defects. The glycosylation mutants used in this experiment were *mnn1Δ*, *mnn2Δ*, *mnn4Δ*, *mnn5Δ* and *van1Δ*. These strains were sourced from the EUROSCARF deletion mutant collection (Appendix 4) and transformed with p6xHis-Hpf2 following the method described in Section 2.1.5. Glycosylation mutants overexpressing 6xHis-Hpf2p were grown in SC-Ura+GAL+RAF for 24 hours at 30°C.

The 6xHis-Hpf2p was purified from the culture supernatant using Ni-NTA as described in Section 2.12. When 6xHis-Hpf2p was overexpressed in these mutants with glycosylation defects, slight alterations to the mobility of the major band of 6xHis-Hpf2p in a SDS gel could be seen (Figure 6.4). Material from the wild type cells

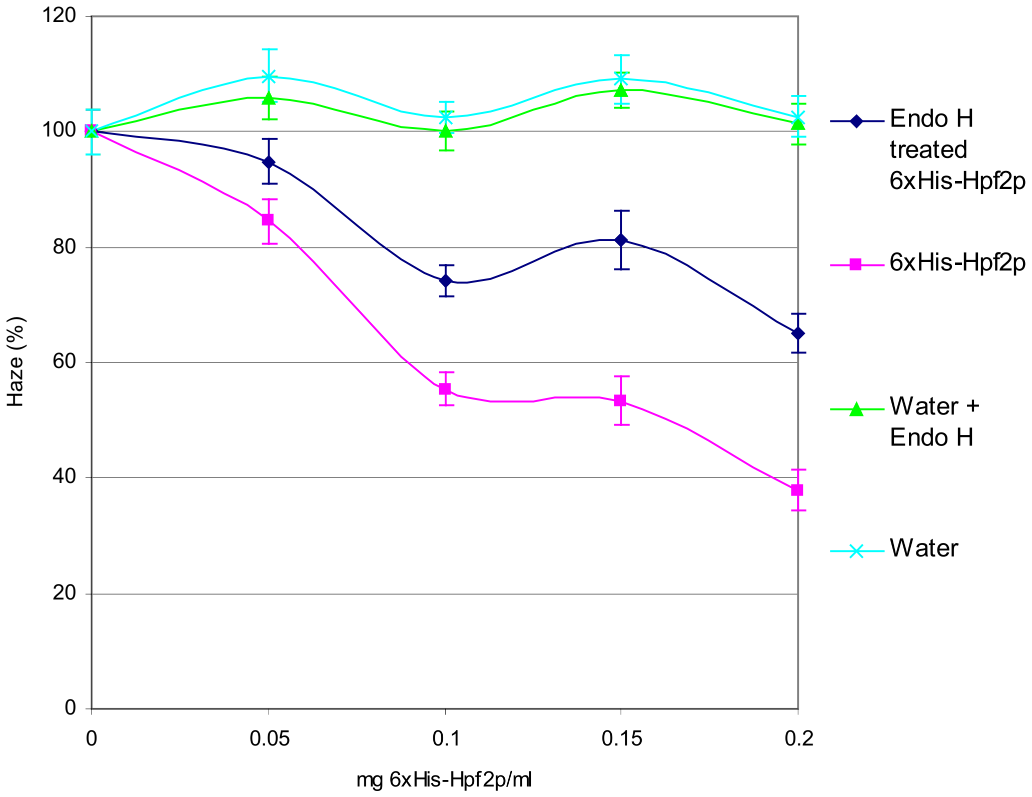


Figure 6.3 The effect of increasing concentration of 6xHis-Hpf2p treated with Endo H on heat induced protein haze formation compared to material that has not been treated, but has endured the same purification processes. IMAC purified 6xHis-Hpf2p was treated with Endo H under non-denaturing conditions and then purified by IMAC again to remove the enzyme and released *N*-glycans. Water controls are also presented to show that any Endo H that may remain in the sample has no haze protective activity.

produced a band of 180 kDa. Material from all the glycosylation mutants had greater mobility than that from the wild type strain.

6.2.3 Glycosylation mutants and their effect of haze protective activity of HPF

The 6xHis-Hpf2p material isolated from the glycosylation mutants was added to wine under heat test conditions to determine the effects of altered glycosylation on haze protective activity of 6xHis-Hpf2p. Material from *mnn2Δ* and *van1Δ* was found to have no activity; (this may have been because the protein degraded under the harsh conditions of the heat test. Due to time constraints, this has not yet been tested). Material from *mnn4Δ* had significantly less (P<0.05) activity than the wild type at every addition, except for at the 0.15 mg/mL addition level (Figure 6.7). Material from *mnn1Δ* and *mnn5Δ* had significantly more (P<0.05) activity than the wild type at every addition level (Figure 6.5).

6.3 DISCUSSION

The experiments described in this chapter constitute preliminary investigations to identify the active component of Hpf in relation to haze protection. Hpfs are putatively both *N*- and *O*-glycosylated. In order to determine whether the *N*-linked oligosaccharide component was responsible for haze protective activity, the *N*-linked oligosaccharides were cleaved from 6xHis-Hpf2p using Endo H. 6xHis-Hpf2p was also overexpressed in several glycosylation mutants and Ni-NTA purified before assessment of haze protective activity by the heat test.

In order to determine the active component of Hpf, it was essential to have a pure source. This was achieved, for the first time, as described in Chapter 5, by integrating a sequence encoding a 6xHis epitope into the *HPF* genes, so that following post-translational modification, the 6xHis epitope was positioned at the predicted N-terminus of the secreted protein. As described in Chapter 5, 6xHis-Hpf1p and 6xHis-Hpf1'p did not exhibit haze protective activity following Endo H treatment. This may have been because the conformation of the proteins was changed when the glycosylation was removed, altering the active site or stability of the protein, so that haze protective

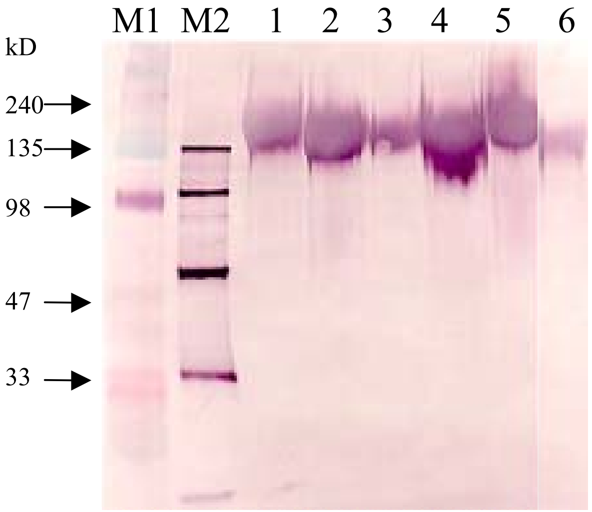


Figure 6.4 Western blot of 6xHis-Hpf2p purified from supernatants of glycosylation mutants overexpressing 6xHis-Hpf2p. The equivalent of 100 µg of dry weight material was run on the gel and transferred to nitrocellulose for Western blotting and probed with mouse anti-6xHis antibody (Sigma). Lane M1, Colour marker (Sigma), band sizes shown on left of blot; M2, 6xHis ladder (QIAGEN); 1, Wild type; 2, *mnn1Δ*; 3, *mnn2Δ*; 4, *mnn4Δ*; 5, *mnn5Δ*; 6, *van1Δ*. The bands in lane M2 contain the following amounts of 6xHis tagged protein: 100 kDa, 75 ng; 75 kDa, 60 ng; 50 kDa, 50 ng; 30 kDa, 50 ng; 15 kDa, 75 ng.

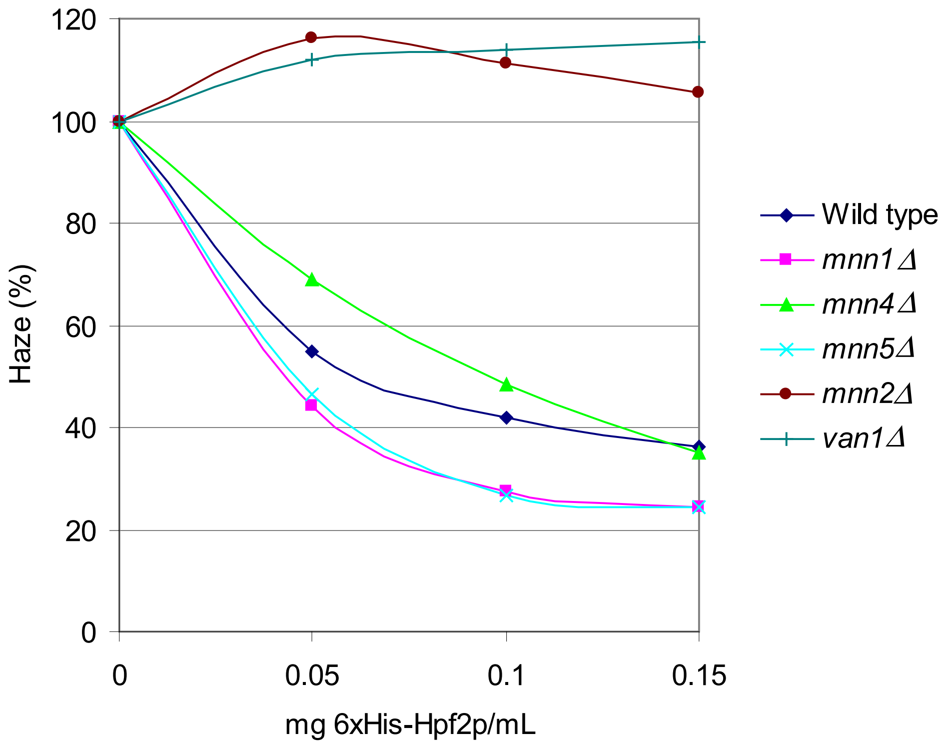


Figure 6.5 The effect of increasing concentration of 6xHis-Hpf2p expressed by glycosylation mutants on heat induced protein haze in white wine. IMAC purified 6xHis-Hpf2p from the supernatant of cultures by wild type and glycosylation mutants was tested according to the micro-heat test described in Chapter 2.

activity was lost. Results from glycosylated 6xHis-Hpf1p and 6xHis-Hpf1'p are necessary controls to make conclusions about these results; thus these proteins must be purified by other means.

The 6xHis tag was selected because it is small relative to other commonly used tags such as glutathione-S-transferase or GFP, meaning that the tag is less likely to interfere with the structure or function of the protein and does not necessarily require removal from the mature protein. 6xHis-tagged proteins can generally be purified using IMAC with a Ni-NTA resin, and are easily detectable using an anti-6xHis antibody during the process. However, in the case of Hpf1p and Hpf1'p, the selection of the 6xHis tag may nevertheless have been disadvantageous since the 6xHis tag was concealed by glycosylation in the 6xHis-Hpf1p and 6xHis-Hpf1'p overexpressors. Other constructs with the 6xHis tag located elsewhere in the protein may allow purification of 6xHis-Hpf1p and 6xHis-Hpf1'p. The 6xHis tag could be inserted in the middle of the sequence or at the C-terminus, before the GPI anchor signal sequence but this may interfere with the function of the protein or correct cleavage of the GPI anchor signal sequence and attachment of the GPI anchor. The use of a different, larger tag may be more exposed allowing the protein to be affinity purified using an antibody to the tag.

6xHis-Hpf2p was purified in both its native and Endo H treated forms, by IMAC, using Ni-NTA (QIAGEN). TALON bound neither the native nor Endo H treated forms and ProBond did not bind the native form, hence QIAGEN Ni-NTA was used for all purification of 6xHis-Hpf2p. The conformation of the protein may have prevented the 6xHis tag from being exposed thus interfering with binding to the TALON and ProBond resins. The TALON resin, consisting of a cobalt reactive core, has strict requirements for the spatial positioning of the histidine residues. In the Nickel based resins, these requirements are said to be less strict (BD Biosciences Clontech). The ProBond resin, a nickel-iminodiacetic acid resin, has only three metal-chelating sites as opposed to four for the Ni-NTA resin (Porath *et al*., 1975). Ni-NTA retains metal ions more stably and the 6xHis-tag binds strongly to the immobilised nickel ion (Hochuli *et al*., 1987). High molecular weight proteins generally do not bind to resins as well as low molecular weight proteins because the 'bulk' or glycosylation of larger proteins may interfere with the binding of the 6xHis tag (BD Biosciences Clontech).

Both 6xHis-Hpf2p and invertase were treated for two hours with Endo H under denaturing and non-denaturing conditions to compare the level of de-*N*-glycosylation that was achieved. A time course of Endo H treatment under non-denaturing conditions was conducted. Similar levels of de-*N*-glycosylation of 6xHis-Hpf2p were seen at all time points assessed, however, the amount of a degradation product (26 kDa) increased with time. No band was seen at 180 kDa following Endo H treatment under denaturing conditions, and a band at 127 kDa was present, which indicates that de-*N*-glycosylation is more efficient under denaturing conditions (Trimble and Maley, 1984). To purify the 6xHis-Hpf2p using the Ni-NTA resin, Endo H treatment had to be performed under native conditions as the resin is incompatible with high concentrations of β-mercaptoethanol and SDS used in the denaturing buffers. Endo H treatment of invertase is also apparently more efficient under denaturing conditions as a 90 kDa de-*N*-glycosylated product is generated under denaturing conditions and a 96 kDa product is generated under non-denaturing conditions (Trimble and Maley, 1984).

Haze protective activity of Endo H treated 6xHis-Hpf2p was found to be significantly less than the untreated material at 0.15 and 0.20 mg/mL ($P<0.05$). This confirmed the result observed by (Stockdale, 2000), namely that a Hpf2p preparation treated with PNGase F had less haze protective activity than untreated material. The experiment described here allowed removal of the 6xHis-tagged Hpf2p from the released *N*-glycans by purification of the protein using Ni-NTA after Endo H treatment. The amount of glycosylated and Endo H treated 6xHis-Hpf2p that was added to the heat test was determined to be equivalent by a Western blot probed with an anti-6xHis antibody. Therefore, equivalent molar amounts of 6xHis-Hpf2p were added to the heat test so the only difference was the glycosylation of 6xHis-Hpf2p. The decrease in haze protective activity after partial de-*N*-glycosylation could be because the carbohydrate has a direct role in haze protective activity or because the carbohydrate is required for thermal stability (Figure 6.4). To test this hypothesis, the released *N*-glycans from the 6xHis-Hpf2p should be isolated and their haze protective activity assessed.

Deletion mutants of gene products involved in glycosylation were used to examine the requirement of glycosylation for haze protective activity. The 6xHis-Hpf2p purified from the glycosylation mutants was assessed for changes in electrophoretic mobility compared to the wild type. Slight changes in M_r were seen in the major band but not the dramatic decrease in M_r seen from 180 to 73 kDa following treatment with Endo H as might have been expected for some mutants. However, the preliminary results of linkage analysis of 6xHis-Hpf2p purified from these strains suggest that the mannoproteins have altered glycosylation (Filomena Pettolino, University of Melbourne) (see below). It is possible that other glycosylation enzymes with related activities are offsetting the deficiencies of these mutants. For example, Goto *et al.*, (1999), suggested that Ktr1p and Ktr3p, both α-1,2-mannosyltransferases, have increased accessibility to the oligosaccharides in a *kre2Δ/mnt1Δ* mutant as the chain length is shortened in this mutant, thus, they are able to increase mannosylation. Similarly, Kre2p/Mnt1p, Ktr1p and Ktr3p, may aid during loss of function of other genes encoding α-1,2-mannosyltransferases such as *MNN2* and *MNN5*.

The 6xHis-Hpf2p purified from *mnn1Δ* and *mnn5Δ* mutants had significantly more haze protective activity than that from the wild type. This increase in activity reduced haze to 24%, the greatest reduction in haze seen to this time. The *MNN5* gene product adds the second 1,2-linked mannosyl residue to the extending *N*-linked branches (Figure 1.6) (Rayner and Munro, 1998). As predicted, 6xHis-Hpf2p in this mutant had decreased 1,2-linked mannosylation and thus the decreased amount of 1,2-linked mannosylation observed here was expected. The 1,3-linked mannosylation was increased and 1,6-linked mannosylation was halved for 6xHis-Hpf2p expressed in the *mnn5Δ* mutant. Addition of 1,6-linked mannosylation may occur normally but degrade more rapidly in the absence of correct 1,2-linked mannosylation to cap and stabilise it. With decreased 1,2-linked mannosylation, the cell may increase 1,3-linked mannosylation to balance the mannose pool in the cell, as described above. 6xHis-Hpf2p expressed in a *mnn1Δ* mutant, which had similar haze protective activity to that from the *mnn5Δ* mutant, had less 1,3-linked mannosylation than the wild type. This was not surprising since Mnn1p is responsible for the addition of 1,3-linked mannosyl residues of both *N*- and *O*-glycosylation (Figure 1.6) (Wiggins and Munro, 1998). This increased haze protective activity may have been because the change in glycosylation has increased either the stability of the protein or the haze protective activity. It is unlikely that the increase in activity is simply related to 1,3-linked mannosylation as the proportion of 1,3-linked glycans of 6xHis-Hpf2p purified from the *mnn1Δ* increased and from the *mnn5Δ*, decreased.

The linkage types in the material from the *mnn4Δ* mutant were similar to that in the wild type. Mnn4p regulates phosphorylation of the mannosyl chains (Figure 1.6) and therefore the linkage of the chains was not expected to be significantly different from the wild type. It is notable that material from the *mnn4Δ* mutant had less activity than from the wild type and was of lower M_r. The importance of phosphorylation for haze protective activity should be further examined.

The 6xHis-Hpf2p purified from *mnn2Δ* and *van1Δ* mutants, which had no apparent haze protective activity, migrated with apparent molecular weights of 160 and 170 kDa, respectively, when subjected to SDS-PAGE. Therefore the amount of glycosylation does not appear to be considerably less than the wild type. The linkage of the glycosylation has been suggested to be different to the wild type from initial results from Filomena Pettolino, The University of Melbourne. The 6xHis-Hpf2p expressed in a *van1Δ* strain had slightly decreased 1,6-linked mannosylation as well as increased 1,3-linked mannosylation. Van1p, as a component of M-Pol I, adds the first 1,6-linked mannosyl residues to those attached by Och1p, thus extending the *N*-linked backbone (Figure 1.6). The oligosaccharide structures from the *van1Δ* mutant are inconsistent with a simple loss of 1,6-mannosyltransferase activity. As these proteins form a complex, one mutant component may have multiple effects resulting in unanticipated structures (Dean, 1999). The altered glycosylation linkage may have still occurred however, because Mnn9p cannot act correctly unless Van1p is present (Dean, 1999). Van1p may only act on a subset of proteins (Ballou *et al*., 1991), but as the apparent molecular weight was not significantly reduced on a SDS gel, it may not act on Hpf2p. Significantly, altered oligosaccharide structures were found on 6xHis-Hpf2p expressed in a *mnn2Δ* mutant. The *MNN2* gene product adds the first 1,2-linked mannosyl residue to the 1,6-linked backbone (Figure 1.6). There was less terminal, 1,2-linked and 1,2,6-linked mannose and more 1,3-linked and 1,6-linked mannose on 6xHis-Hpf2p expressed in the *mnn2Δ* mutant. This result suggests that the glycans have an extended backbone that is decorated with more 1,3-linked mannose residues that may have occurred to compensate for the excess mannose in the cell. Mnn2p is responsible for the first addition of the 1,2-linked mannosylation to the 1,6-linked backbones (Figure 1.6). These backbones may be unstable without the short chain extensions, and the final 1,2-linked residue to cap it.

The glycosylation may have a direct role in haze protective activity as the active site, an indirect role in maintaining or enhancing protein stability or a shared role with the protein backbone forming an active site. It may be difficult to separate the requirement for glycosylation to maintain the active site or the stability of Hpf, as the glycosylation necessary for the active site may also be essential for stability. A change in protein stability due to altered glycosylation has been observed previously (Cipollo and Trimble, 2002). External invertase overexpressed in an *alg12Δ* (asparagine linked glycosylation) mutant was shown to have reduced glycosylation and to be rapidly destroyed proteolytically. It is known that oligosaccharides are involved in protein structural maintenance during heat, pH and pressure stress (Lis and Sharon, 1993). The oligosaccharides may not only be important for function of haze protective factors but also structure and stability of the protein, particularly under acidic conditions of wine (pH 3.0-3.8). If haze protective factors were used commercially in the wine industry, the stability of the mannoproteins would be highly important for their reliability. Glycosylation has been shown to be important for not only stability of certain proteins, but also function. (Cappellaro *et al.*, 1994) showed that oligosaccharides of agglutinins are not essential for the mating type specific cell-cell interaction but the glycosylated peptides are four to five times more active than non-glycosylated ones. In addition, Axl2p, an *O*-glycosylated protein required for axial budding and mating, requires glycosylation for stability and localisation (Sanders *et al.*, 1999).

6.4 CONCLUSION

The work described in this chapter, to determine the active component of Hpf are preliminary experiments. A clear association between altered glycosylation and haze protective activity was not gained from these results. The decrease in haze protective activity of Endo H treated 6xHis-Hpf2p and 6xHis-Hpf2p purified from the *mnn4Δ* strain as well as the lack of haze protective activity exhibited by 6xHis-Hpf2p purified from the *mnn2Δ* and *van1Δ* mutants suggest that the presence of glycosylation is critical for haze protection. The 6xHis-Hpf1p and 6xHis-Hpf1'p could only be purified by IMAC after the *N*-glycans were removed by Endo H, and these de-*N*-glycosylated proteins did not exhibit haze protective activity. The data shown in Figure 6.7 that 6xHis-Hpf2p purified from the *mnn1Δ* and *mnn5Δ* mutants has increased haze protective activity relative to material from the wild type strain may aid identification of the structure of the glycan required for this phenomenon. The results indicate that the glycans of 6xHis-Hpf2p are important for haze protective activity. The mechanism of action for haze protection of 6xHis-Hpf1p and 6xHis-Hpf1'p may be different to 6xHis-Hpf2p as the former proteins did not have any activity after Endo H treatment whereas 6xHis-Hpf2p did. Whether their role is direct, involved in an active site, or indirect, through improving protein stability, could not be elucidated. It is possible that by altering the glycosylation, both the activity and stability of the protein are affected making it difficult to determine the active component of Hpf. All the materials that are known to exhibit haze protective activity (Section 5.1) are glycoproteins or proteoglycans, suggesting that both the protein and polysaccharide components are important for haze protective activity.

Further investigation to remove glycans from Hpfs will necessitate removal of *O*-glycosylation from the protein backbone using β-elimination, since no enzymes are commercially available for specific removal of *O*-linked mannosylation. β-elimination involves treating the mannoprotein with alkali in the presence of sodium borohydride. This may determine if the *O*-linked sugars are responsible for haze protective activity. This may also be done in combination with Endo H treatment, to remove all the glycosylation. The glycans should be recovered and assessed for haze protective activity. Unglycosylated material could be produced by expressing the Hpfs in *E. coli*. There is a risk that the protein will be inactive when all the glycosylation is removed because of instability or loss of the active site. The protein may be digested using specific proteases to examine which section is responsible for haze protective activity, both with and without glycosylation. When the active site of Hpf is determined, peptides, glycopeptides or oligosaccharides could be synthesised or purified from an alternate source and assessed for haze protective activity. This may constitute a non-genetically modified source of material that exhibits haze protective activity, which could be used in industry.

CHAPTER SEVEN

SUMMARY AND PERSPECTIVE FOR FUTURE WORK

Heat induced protein haze is a common problem in white wine. Pathogenesis related proteins from grapes slowly denature and aggregate resulting in a light dispersing haze. The use of the haze protective factors, Hpf1p and Hpf2p, specific mannoproteins from *Saccharomyces cerevisiae*, may be an alternative or complementary treatment to current methods to prevent protein haze formation. *HPF1* has a homologue (71% positional sequence identity) identified in the *Saccharomyces* genome database. The research described in this thesis aims to firmly establish Hpf1p and Hpf2p as haze protective factors and to elucidate the biological function of these proteins in yeast.

Since the *HPF* genes were likely to be localised to the cell surface, cell wall related phenotypes were investigated. It was found that the *hpfΔ* strains are not affected by compounds that alter cell wall structure or cell wall signalling events. No ‘marginal benefit’ of these genes was found when investigated using a competition assay.

The *hpf1Δ hpf1’Δ* strain is cold and ethanol tolerant

Transcription of *HPF1* was induced more than 70 fold when grown on oleic acid compared to glucose as the sole carbon source (Kal *et al.*, 1999), however, no difference in growth between *hpf1Δ hpf1’Δ* and wild type yeast on oleic acid was observed. Oleic acid is required for maintenance of membrane fluidity in yeast, thus the effect of deleting *HPF1* and *HPF1’* was investigated under conditions that alter membrane fluidity. The *hpf1Δ hpf1’Δ* strain was found to be more tolerant to cold temperature and ethanol than the wild type strain.

The serendipitous finding that an *hpf1Δ hpf1’Δ* strain is cold and ethanol tolerant may have beneficial applications to the wine industry. During alcoholic fermentation, yeast are exposed to stressful conditions, and high ethanol concentrations are recognised as the major stress to yeast cells during fermentation. However, if the conditions become too harsh during fermentation, the yeast may slow or stop fermenting resulting in a 'stuck fermentation.' With further research to understand the function and regulation of the *HPF* genes, knowledge that may lead to decreased incidence of stuck ferments may be achieved. Knowledge to improve fermentation capacity of yeast in colder conditions may also be advantageous to minimise the loss, during fermentation, of volatile sensory compounds.

HPF1 **and** ***HPF1'*** **are essential for efficient mating**

Ren *et al*., (2000) and Roberts *et al*., (2000), showed that both *HPF1* and *HPF1'* are induced 2.5-2.8 fold in response to mating pheromones. Given that the Hpf1p and Hpf1'p are likely to be localised to the cell surface, their involvement in mating was investigated. Mating efficiency of a *hpf1Δ hpf1'Δ* bilateral mating decreased 8.51 fold in the limited mating filter assay. This mating defect was found to be mating type specific as it occurred in *hpfΔ* bilateral matings but only *MAT***a** *hpfΔ* x *MAT*α wild type unilateral matings. Degradation of the cell wall and plasma membrane fusion was incomplete and a septum was found to persist between the mating partners.

Hpf1p, Hpf1'p and Hpf2p do indeed have haze protective activity

The results of the deletion and overexpression studies strongly support the suggestion that Hpf1p and Hpf2p are haze protective factors. The haze protective activity of material extracted from the supernatant of *hpf1Δ*, *hpf1'Δ* and *hpf2Δ* single deletion strains was reduced. Material from a triple deletion mutant (*hpf1Δ hpf1'Δ hpf2Δ*) had the least activity, but still had activity suggesting that other HPFs are present in yeast. Overexpression of *HPF1* and *HPF1'* resulted in increased haze protective activity of material extracted from fermentation supernatants. Surprisingly, material from the *HPF2* overexpression ferment did not exhibit increased activity. However, IMAC purified 6xHis-Hpf2p had strong haze protective activity. This confirmed beyond doubt that Hpf2p is a haze protective factor. IMAC purification of 6xHis-Hpf1p and 6xHis-Hpf1'p was unsuccessful as the proteins were unable to be purified unless the *N*-glycans were removed.

Removal of *N*-linked glycans from 6xHis-Hpf2p reduces its haze protective activity

The loss of *N*-linked glycans as a result of Endo H treatment significantly reduced haze protective activity of 6xHis-Hpf2p. The 6xHis-Hpf2p purified from glycosylation mutants had altered haze protective activity compared to the wild type, but no clear association was seen between the initial glycosylation linkage data and the haze protective activity. The role of the glycosylation may be direct, involved in an active site, or indirect, through improving protein stability.

A relationship between HPFs and PR proteins?

A relationship between PR proteins and haze protective factors is suggested. This was first implied by the fact that PR proteins cause protein haze formation in wine and that haze protective factors have the ability to prevent that haze formation. A second possible connection are the findings of Yun *et al*., (1998), that osmotin, a PR5 protein, uses a signal transduction pathway to weaken defensive cell wall barriers and increase its cytotoxic efficacy. For full sensitivity to the PR5 protein, osmotin, *S. cerevisiae* requires phosphomannans to bind the protein to the cell wall (Ibeas *et al*., 2000). At this point, these relationships have not been clearly defined, but further research may show a link between PR proteins and HPFs, which may give an indication to their mechanism of action.

Concluding remarks

The work described in this thesis has laid the groundwork for new outcomes. The finding of the mating defect of the *hpf1Δ hpf1'Δ* strain and persistence of a septum between the mating cells adds knowledge to further define and understand the mating process in yeast. The cold and ethanol tolerance of the *hpf1Δ hpf1'Δ* strain could be used in further understanding the cold and ethanol tolerance mechanisms, and from this tolerant yeast strains could be developed that could be used, not only by the wine industry, but for the brewing and ethanol fuel production industries. The use of haze protective factors in the wine industry may reduce or replace the need for bentonite as a fining agent to remove heat unstable proteins. Peptides, glycopeptides and the oligosaccharides removed from Hpf must be recovered and assessed for haze protective activity. This testing may result in an alternative source of material that exhibits haze protective activity, which is non-genetically modified.

APPENDIX ONE

Appendix 1: DNA sequences of *HPF* genes and corresponding translation

Blue sequence signifies putative secretion signal sequence

Red sequence signifies putative ω-site for GPI anchor attachment

HPF1

```
           10           20           30            40           50           60
ATG TTC AAT CGC TTT AAT AAA CTT CAA GCC GCT TTG GCT TTG GTC CTT TAC TCC CAA AGT
 M   F   N   R   F   N   K   L   Q   A   A   L   A   L   V   L   Y   S   Q   S

           70           80           90           100          110          120
GCA TTG GGC CAA TAT TAT ACC AAC AGT TCC TCA ATC GCT AGT AAC AGC TCC ACC GCC GTT
 A   L   G   Q   Y   Y   T   N   S   S   S   I   A   S   N   S   S   T   A   V

          130          140          150           160          170          180
TCG TCA ACT TCA TCA GGT TCC GTT TCC ATC AGT AGT TCT ATT GAG TTG ACC TCA TCT ACT
 S   S   T   S   S   G   S   V   S   I   S   S   S   I   E   L   T   S   S   T

          190          200          210           220          230          240
TCT GAT GTC TCG AGC TCT CTC ACT GAG TTA ACG TCA TCC TCC ACC GAA GTC TCG AGC TCC
 S   D   V   S   S   S   L   T   E   L   T   S   S   S   T   E   V   S   S   S

          250          260          270           280          290          300
ATT GCT CCA TCA ACC TCG TCC TCT GAA GTC TCG AGC TCT ATT ACT TCA TCA GGC TCT TCA
 I   A   P   S   T   S   S   S   E   V   S   S   S   I   T   S   S   G   S   S

          310          320          330           340          350          360
GTC TCC GGC TCA TCT TCT ATT ACT TCA TCA GGC TCA TCA GTC TCC AGT TCA TCT TCT GCC
 V   S   G   S   S   S   I   T   S   S   G   S   S   V   S   S   S   S   S   A

          370          380          390           400          410          420
ACT GAA TCA GGC TCA TCC GCC TCC GGT TCA TCC TCC GCC ACT GAA TCG GGC TCA TCA GTC
 T   E   S   G   S   S   A   S   G   S   S   S   A   T   E   S   G   S   S   V

          430          440          450           460          470          480
TCC GGT TCA TCT ACT TCC ATT ACA TCA GGC TCA TCC TCC GCC ACT GAA TCG GGC TCA TCA
 S   G   S   S   T   S   I   T   S   G   S   S   S   A   T   E   S   G   S   S

          490          500          510           520          530          540
GTC TCC GGT TCA ACT TCT GCC ACT GAA TCA GGC TCA TCC GCC TCC GGT TCA TCT TCT GCC
 V   S   G   S   T   S   A   T   E   S   G   S   S   A   S   G   S   S   S   A

          550          560          570           580          590          600
ACT GAA TCA GGC TCA TCC GCC TCC GGT TCA TCT TCT GCC ACA GAA TCA GGC TCA TCA GTC
 T   E   S   G   S   S   A   S   G   S   S   S   A   T   E   S   G   S   S   V

          610          620          630           640          650          660
TCC GGT TCA TCT TCT GCC ACA GAA TCA GGC TCA TCA GTC TCC GGT TCA TCT TCT GCC ACA
 S   G   S   S   S   A   T   E   S   G   S   S   V   S   G   S   S   S   A   T

          670          680          690           700          710          720
GAA TCA GGC TCC GCT TCT TCG GTT CCT AGC TCA TCC GGT TCT GTC ACA GAA TCA GGC TCA
 E   S   G   S   A   S   S   V   P   S   S   S   G   S   V   T   E   S   G   S

          730          740          750           760          770          780
TCC TCA TCA GCA TCT GAA TCA TCT ATC ACA CAA TCT GGT ACC GCT TCC GGT TCA TCA GCC
 S   S   S   A   S   E   S   S   I   T   Q   S   G   T   A   S   G   S   S   A

          790          800          810           820          830          840
TCC AGC ACG TCC GGT TCT GTT ACA CAA TCT GGT TCC TCC GTT TCC GGT TCA TCA GCT TCT
 S   S   T   S   G   S   V   T   Q   S   G   S   S   V   S   G   S   S   A   S

          850          860          870           880          890          900
TCT GCT CCA GGT ATC TCG AGT TCA ATT CCT CAA TCA ACC TCA TCG GCT TCC ACT GCC TCT
 S   A   P   G   I   S   S   S   I   P   Q   S   T   S   S   A   S   T   A   S

          910          920          930           940          950          960
GGT TCT ATC ACC TCC GGT ACC TTA AGT TCT ATT ACC TCT TCG GCT TCT AGT GCA ACT GCA
 G   S   I   T   S   G   T   L   S   S   I   T   S   S   A   S   S   A   T   A
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          970          980          990         1000         1010         1020
ACT GCT TCC AAC TCT CTT TCT TCC AGC GAT GGT ACT ATT TAT TTG CCT TCT ACA ACC ATC
 T   A   S   N   S   L   S   S   S   D   G   T   I   Y   L   P   S   T   T   I

         1030          1040         1050          1060         1070         1080
AGT GGT GAC ATC ACA CTC ACC GGT TCA GTC ATT GCA ACT GAA GCT GTC GAA GTC GCT GCA
 S   G   D   I   T   L   T   G   S   V   I   A   T   E   A   V   E   V   A   A

         1090          1100         1110          1120         1130         1140
GGT GGT AAG TTG ACC CTA CTT GAT GGT GAC AAA TAC GTT TTC TCT GCT GAT TTC ATA ATC
 G   G   K   L   T   L   L   D   G   D   K   Y   V   F   S   A   D   F   I   I

         1150          1160         1170          1180         1190         1200
CAT GGT GGC GTT TTC GTA GAA AAG TCT AAG CCA ACT TAC CCA GGT ACC GAA TTC GAC ATT
 H   G   G   V   F   V   E   K   S   K   P   T   Y   P   G   T   E   F   D   I

         1210          1220         1230          1240         1250         1260
TCT GGT GAA AAC TTT GAT GTA TCT GGT ACC TTT AAC GCT GAA GAG CCT GCT GCT TCT TCC
 S   G   E   N   F   D   V   S   G   T   F   N   A   E   E   P   A   A   S   S

         1270          1280         1290          1300         1310         1320
GCA TCT GCC TAC TCC TTC ACT CCA GGC TCT TTC GAT AAC AGT GGT GAT ATT TCT TTG AGT
 A   S   A   Y   S   F   T   P   G   S   F   D   N   S   G   D   I   S   L   S

         1330          1340         1350          1360         1370         1380
CTA TCA GAG TCC ACA AAG GGC GAA GTC ACA TTC TCT CCC TAC TCT AAC TCT GGT GCT TTC
 L   S   E   S   T   K   G   E   V   T   F   S   P   Y   S   N   S   G   A   F

         1390          1400         1410          1420         1430         1440
TCT TTC TCA AAT GCT ATT CTC AAT GGT GGT TCC GTC TCT GGT TTG CAG CGT AGA GCT GAA
 S   F   S   N   A   I   L   N   G   G   S   V   S   G   L   Q   R   R   A   E

         1450          1460         1470          1480         1490         1500
TCA GGT TCT GTC AAC AAC GGT GAG ATA AAT CTT GAG AAT GGC AGT ACC TAC GTC GTT GTC
 S   G   S   V   N   N   G   E   I   N   L   E   N   G   S   T   Y   V   V   V

         1510          1520         1530          1540         1550         1560
GAA CCA GTT TCT GGA AGT GGT ACA ATC AAC ATC ATC TCT GGC AAC CTT TAC TTG CAC TAT
 E   P   V   S   G   S   G   T   I   N   I   I   S   G   N   L   Y   L   H   Y

         1570          1580         1590          1600         1610         1620
CCA GAC ACC TTT ACT GGC CAA ACT GTT GTA TTC AAG GGT GAA GGT GTT CTT GCC GTT GAC
 P   D   T   F   T   G   Q   T   V   V   F   K   G   E   G   V   L   A   V   D

         1630          1640         1650          1660         1670         1680
CCA ACC GAA ACC AAC ACT ACT CCT ATC CCT GTG GTT GGA TAC ACT GGT GAA AAC CAA ATC
 P   T   E   T   N   T   T   P   I   P   V   V   G   Y   T   G   E   N   Q   I

         1690          1700         1710          1720         1730         1740
GCC ATT ACA GCA GAT GTC ACT GCT CTT TCT TAC GAC AGT GCT ACT GGT GTT TTA ACT GCA
 A   I   T   A   D   V   T   A   L   S   Y   D   S   A   T   G   V   L   T   A

         1750          1760         1770          1780         1790         1800
ACA CAA GGC AAC TCA CAA TTC TCC TTC TCT ATT GGT ACT GGG TTC TCC AGT TCT GGC TTC
 T   Q   G   N   S   Q   F   S   F   S   I   G   T   G   F   S   S   S   G   F

         1810          1820         1830          1840         1850         1860
AAC GTC TCC GAA GGA ACA TTT GCT GGT GCC TAT GCT TAT TAT CTA AAT TAC GGA GGT GTT
 N   V   S   E   G   T   F   A   G   A   Y   A   Y   Y   L   N   Y   G   G   V

         1870          1880         1890          1900         1910         1920
GTT GCT TCC AGC GCT ACA CCC TCA TCC ACA TCT ACC ACA TCA GGG GCT ACC AAC TCT ACT
 V   A   S   S   A   T   P   S   S   T   S   T   T   S   G   A   T   N   S   T

         1930          1940         1950          1960         1970         1980
TCC GGT TCC ACT TCA TTC GGT GCT TCC GTA ACT GGT TCA ACC GCT TCC ACT TCA TTC GGT
 S   G   S   T   S   F   G   A   S   V   T   G   S   T   A   S   T   S   F   G

         1990          2000         2010          2020         2030         2040
GCT TCC GTG ACT GGT TCA ACG GCT TCC ACC TTG ATT TCC GGC TCC CCA TCT GTT TAT ACC
 A   S   V   T   G   S   T   A   S   T   L   I   S   G   S   P   S   V   Y   T

         2050          2060         2070          2080         2090         2100
ACA ACA TTA ACA TAT GCA ACA ACC ACA AGC ACA GTA GTT GTC TCC TGT TCA GAA ACA ACT
 T   T   L   T   Y   A   T   T   T   S   T   V   V   V   S   C   S   E   T   T
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         2110         2120         2130          2140         2150         2160
GAT TCG AAC GGT AAC GTC TAT ACC ATT ACC ACA ACC GTA CCA TGT TCA TCT ACC ACC GCC
 D   S   N   G   N   V   Y   T   I   T   T   T   V   P   C   S   S   T   T   A

         2170         2180         2190          2200         2210         2220
ACT ATC ACT TCT TGC GAT GAG ACC GGA TGT CAT GTA ACT ACG TCT ACC GGT ACC GTC GCC
 T   I   T   S   C   D   E   T   G   C   H   V   T   T   S   T   G   T   V   A

         2230         2240         2250          2260         2270         2280
ACT GAA ACC GTT TCT TCC AAA TCA TAC ACC ACT GTT ACC GTC ACC CAC TGT GAC AAC AAT
 T   E   T   V   S   S   K   S   Y   T   T   V   T   V   T   H   C   D   N   N

         2290         2300         2310          2320         2330         2340
GGC TGT AAC ACC AAG ACT GTC ACT TCT GAA TGT CCT GAA GAA ACT TCA GCA ACT ACT ACT
 G   C   N   T   K   T   V   T   S   E   C   P   E   E   T   S   A   T   T   T

         2350         2360         2370          2380         2390         2400
TCT CCA AAA TCA TAC ACT ACT GTT ACC GTT ACT CAC TGT GAC GAC AAC GGC TGT AAC ACT
 S   P   K   S   Y   T   T   V   T   V   T   H   C   D   D   N   G   C   N   T

         2410         2420         2430          2440         2450         2460
AAG ACT GTC ACC TCT GAG GCC CCT GAA GCC ACA ACC ACT ACT GTT TCT CCA AAG ACA TAC
 K   T   V   T   S   E   A   P   E   A   T   T   T   T   V   S   P   K   T   Y

         2470         2480         2490          2500         2510         2520
ACT ACC GCT ACT GTT ACT CAG TGC GAT GAC AAT GGA TGT AGC ACC AAG ACT GTC ACT TCT
 T   T   A   T   V   T   Q   C   D   D   N   G   C   S   T   K   T   V   T   S

         2530         2540         2550          2560         2570         2580
GAA GCT CCT AAA GAA ACT TCA GAA ACT TCA GAA ACC AGT GCT GCC CCT AAG ACA TAC ACT
 E   A   P   K   E   T   S   E   T   S   E   T   S   A   A   P   K   T   Y   T

         2590         2600         2610          2620         2630         2640
ACT GCC ACT GTT ACT CAA TGT GAT GAC AAT GGT TGT AAC GTC AAG ATA ATC ACC TCT CAA
 T   A   T   V   T   Q   C   D   D   N   G   C   N   V   K   I   I   T   S   Q

         2650         2660         2670          2680         2690         2700
ATA CCT GAA GCT ACT TCA ACC GTC ACC GCA ACT AGT GCT TCT CCA AAG TCA TAC ACT ACT
 I   P   E   A   T   S   T   V   T   A   T   S   A   S   P   K   S   Y   T   T

         2710         2720         2730          2740         2750         2760
GTC ACT TCT GAG GGT TCT AAA GCA ACC TCA TTG ACT ACT GCC ATT TCC AAG GCT TCT AGT
 V   T   S   E   G   S   K   A   T   S   L   T   T   A   I   S   K   A   S   S

         2770         2780         2790          2800         2810         2820
GCA ATT TCC ACA TAC TCC AAA TCT GCA GCT CCA ATA AAG ACC TCT ACT GGT ATC ATT GTC
 A   I   S   T   Y   S   K   S   A   A   P   I   K   T   S   T   G   I   I   V

         2830         2840         2850          2860         2870         2880
CAG TCC GAG GGT ATT GCC GCA GGT TTG AAT GCC AAT ACT TTG AAT GCA TTG GTC GGT ATT
 Q   S   E   G   I   A   A   G   L   N   A   N   T   L   N   A   L   V   G   I

         2890         2900
TTC GTT CTT GCT TTC TTT AAC TAA
 F   V   L   A   F   F   N   *
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           10           20           30            40           50           60
ATG TTC AAT CGT TTA AAC AAA TTC CAA GCT GCT TTA GCT TTG GCC CTT TAC TCT CAA AGT
 M   F   N   R   L   N   K   F   Q   A   A   L   A   L   A   L   Y   S   Q   S

           70           80           90           100          110          120
GCA TTG GGT CAG TAC TAT AGC AAT AGC ACC TCA ATT TCA AGC AAC AGC TCA TCC ACC TCT
 A   L   G   Q   Y   Y   S   N   S   T   S   I   S   S   N   S   S   S   T   S

          130          140          150           160          170          180
GTG GTA TCA AGT TCC TCT GGA TCC GTT TCG ATC AGT AGT TCT ATT GCT GAG ACA TCC TCG
 V   V   S   S   S   S   G   S   V   S   I   S   S   S   I   A   E   T   S   S

          190          200          210           220          230          240
TCC GCT ACC GAT ATC TTA AGT TCT ATC ACA CAA TCA GCT TCA TCC ACT TCT GGT GTC TCG
 S   A   T   D   I   L   S   S   I   T   Q   S   A   S   S   T   S   G   V   S

          250          260          270           280          290          300
AGC TCT GTC GGT CCA TCC TCT TCC TCT GTT GTC TCA AGC TCT GTC AGC CAA TCC TCT TCA
 S   S   V   G   P   S   S   S   S   V   V   S   S   S   V   S   Q   S   S   S

          310          320          330           340          350          360
TCC GTT TCT GAT GTA TCA AGC TCT GTC AGT CAA TCT TCT TCT TCA GCT TCT GAT GTC TCA
 S   V   S   D   V   S   S   S   V   S   Q   S   S   S   S   A   S   D   V   S

          370          380          390           400          410          420
AGC TCT GTC AGT CAA TCA GCT TCC TCT ACT TCT GAT GTC TCA AGC TCT GTC AGT CAA TCT
 S   S   V   S   Q   S   A   S   S   T   S   D   V   S   S   S   V   S   Q   S

          430          440          450           460          470          480
TCT TCT TCA GCT TCT GAT GTA TCA AGC TCT GTC AGT CAA TCT TCT TCC TCA GCT TCT GAT
 S   S   S   A   S   D   V   S   S   S   V   S   Q   S   S   S   S   A   S   D

          490          500          510           520          530          540
GTC TCA AGC TCT GTC AGT CAA TCA GCT TCC TCA GCT TCT GAT GTC TCA AGC TCT GTC AGT
 V   S   S   S   V   S   Q   S   A   S   S   A   S   D   V   S   S   S   V   S

          550          560          570           580          590          600
CAA TCA GCT TCC TCT ACT TCT GAT GTC TCA AGC TCT GTC AGT CAA TCT TCT TCT TCA GCT
 Q   S   A   S   S   T   S   D   V   S   S   S   V   S   Q   S   S   S   S   A

          610          620          630           640          650          660
TCT GAT GTA TCA AGC TCT GTC AGT CAA TCT TCT TCC TCA GCT TCT GAT GTC TCA AGC TCT
 S   D   V   S   S   S   V   S   Q   S   S   S   S   A   S   D   V   S   S   S

          670          680          690           700          710          720
GTC AGT CAA TCA GCT TCC TCT ACT TCT GAT GTC TCA AGC TCT GTC AGT CAA TCA GCT TCC
 V   S   Q   S   A   S   S   T   S   D   V   S   S   S   V   S   Q   S   A   S

          730          740          750           760          770          780
TCT ACT TCT GGT GTC TCA AGC TCT GGC AGC CAA TCA GTC TCA TCC GCT TCT GGT AGC TCA
 S   T   S   G   V   S   S   S   G   S   Q   S   V   S   S   A   S   G   S   S

          790          800          810           820          830          840
AGC TCA TTC CCT CAA TCA ACC TCA TCC GCT TCT ACT GCC TCC GGT TCT GCC ACT TCC AAT
 S   S   F   P   Q   S   T   S   S   A   S   T   A   S   G   S   A   T   S   N

          850          860          870           880          890          900
TCC TTG AGT TCC ATT ACT TCT TCA GCA TCT AGT GCA AGC GCA ACT GCT TCC AAC TCC CTT
 S   L   S   S   I   T   S   S   A   S   S   A   S   A   T   A   S   N   S   L

          910          920          930           940          950          960
TCT TCC AGC GAT GGT ACT ATC TAT CTA CCA ACT ACT ACA ATC AGT GGT GAT CTA ACT CTT
 S   S   S   D   G   T   I   Y   L   P   T   T   T   I   S   G   D   L   T   L

          970          980          990          1000         1010         1020
ACT GGT AAA GTA ATT GCA ACA GAA GGT GTT GTG GTC GCA GCT GGT GCC AAA TTG ACT CTA
 T   G   K   V   I   A   T   E   G   V   V   V   A   A   G   A   K   L   T   L

         1030         1040         1050          1060         1070         1080
CTT GAC GGT GAC AAA TAT TCT TTC TCA GCT GAC CTA AAA GTC TAC GGT GAC TTG CTT GTG
 L   D   G   D   K   Y   S   F   S   A   D   L   K   V   Y   G   D   L   L   V
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         1090         1100         1110          1120         1130         1140
AAA AAG TCC AAA GAA ACC TAT CCA GGT ACC GAA TTC GAC ATC TCC GGT GAA AAC TTT GAC
 K   K   S   K   E   T   Y   P   G   T   E   F   D   I   S   G   E   N   F   D

         1150         1160         1170          1180         1190         1200
GTG ACC GGT AAC TTC AAC GCT GAA GAA TCC GCT GCC ACC TCT GCA TCC ATC TAC TCC TTC
 V   T   G   N   F   N   A   E   E   S   A   A   T   S   A   S   I   Y   S   F

         1210         1220         1230          1240         1250         1260
ACT CCA AGT TCT TTT GAC AAC AGT GGT GAC ATT TCC TTA AGT CTA TCA AAG TCC AAG AAG
 T   P   S   S   F   D   N   S   G   D   I   S   L   S   L   S   K   S   K   K

         1270         1280         1290          1300         1310         1320
GGT GAA GTC ACT TTC TCT CCA TAC TCC AAT TCT GGT GCC TTC TCT TTC TCG AAC GCT ATT
 G   E   V   T   F   S   P   Y   S   N   S   G   A   F   S   F   S   N   A   I

         1330         1340         1350          1360         1370         1380
CTC AAC GGT GGT TCT GTT TCC GGT CTA CAA CGT AGA GAC GAC ACT GAA GGT TCA GTA AAC
 L   N   G   G   S   V   S   G   L   Q   R   R   D   D   T   E   G   S   V   N

         1390         1400         1410          1420         1430         1440
AAC GGT GAA ATT AAC CTA GAC AAT GGA AGT ACC TAT GTT ATT GTC GAA CCA GTT TCT GGA
 N   G   E   I   N   L   D   N   G   S   T   Y   V   I   V   E   P   V   S   G

         1450         1460         1470          1480         1490         1500
AAA GGT ACA GTC AAC ATC ATT TCC GGT AAC CTA TAC TTA CAC TAC CCT GAC ACA TTT ACT
 K   G   T   V   N   I   I   S   G   N   L   Y   L   H   Y   P   D   T   F   T

         1510         1520         1530          1540         1550         1560
GGC CAA ACT GTT GTA TTC AAG GGT GAA GGT GTC CTT GCT GTT GAC CCA ACC GAA ACC AAT
 G   Q   T   V   V   F   K   G   E   G   V   L   A   V   D   P   T   E   T   N

         1570         1580         1590          1600         1610         1620
GCC ACT CCT ATT CCT GTT GTT GGC TAC ACT GGT AAG AAC CAA ATT GCC ATT ACC GCT GAC
 A   T   P   I   P   V   V   G   Y   T   G   K   N   Q   I   A   I   T   A   D

         1630         1640         1650          1660         1670         1680
ATC ACT GCT CTT TCT TAT GAC GGT ACT ACC GGT GTT CTA ACT GCA ACC CAA GGT AAC AGA
 I   T   A   L   S   Y   D   G   T   T   G   V   L   T   A   T   Q   G   N   R

         1690         1700         1710          1720         1730         1740
CAA TTC TCT TTT GCT ATT GGT ACC GGA TTC TCC AGC TCT GAC TTC AGC GTC TCT GAA GGA
 Q   F   S   F   A   I   G   T   G   F   S   S   S   D   F   S   V   S   E   G

         1750         1760         1770          1780         1790         1800
ATC TTT GCA GGT GCT TAC GCT TAC TAC CTA AAC TAC AAT GGT GTT GTC GCT ACT AGT GCC
 I   F   A   G   A   Y   A   Y   Y   L   N   Y   N   G   V   V   A   T   S   A

         1810         1820         1830          1840         1850         1860
GCT TCT TCA TCC ACT GCA TCT GGT GCT TCC GCT TCC GTT ACC GGT TCT ACT TCA TTC GGT
 A   S   S   S   T   A   S   G   A   S   A   S   V   T   G   S   T   S   F   G

         1870         1880         1890          1900         1910         1920
GCT TCC GTT ACC GGT TCA ACT GCT TCC ACT TCA TTC GGT GCT TCC GTT ACC GGT TCA ACT
 A   S   V   T   G   S   T   A   S   T   S   F   G   A   S   V   T   G   S   T

         1930         1940         1950          1960         1970         1980
GCT TCC ACT TCA TTT GGT GCT TCC GTT ACC GGT TCA ACA TCT GTT TAC ACT ACA ACA CTA
 A   S   T   S   F   G   A   S   V   T   G   S   T   S   V   Y   T   T   T   L

         1990         2000         2010          2020         2030         2040
GAC TAT GTA AAT GCC ACA AGC ACA GTC GTA GTT TCT TGT TCA GAG ACA ACT GAC TCC AAT
 D   Y   V   N   A   T   S   T   V   V   V   S   C   S   E   T   T   D   S   N

         2050         2060         2070          2080         2090         2100
GGT AAC GTC TAT ACC ATT ACC ACA ACC GTT CCA TGC TCA TCC ACT ACC GCC ACT ATT ACT
 G   N   V   Y   T   I   T   T   T   V   P   C   S   S   T   T   A   T   I   T

         2110         2120         2130          2140         2150         2160
TCT TGT GAT GAA ACT GGA TGT CAC GTT AGT ACA TCA ACC GGT GCT GTT GTA ACT GAA ACC
 S   C   D   E   T   G   C   H   V   S   T   S   T   G   A   V   V   T   E   T
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         2170         2180         2190          2200         2210         2220
GTT TCT TCC AAA TCA TAC ACA ACT GCC ACT GTA ACT CAC TGT GAC GAT AAT GGC TGT AAC
 V   S   S   K   S   Y   T   T   A   T   V   T   H   C   D   D   N   G   C   N


         2230         2240         2250          2260         2270         2280
ACC AAG ACT GTC ACT TCT GAA TGT TCC AAA GAA ACA TCA GCA ACA ACT GCT TCT CCA AAA
 T   K   T   V   T   S   E   C   S   K   E   T   S   A   T   T   A   S   P   K

         2290         2300         2310          2320         2330         2340
TCA TAC ACC ACT GTC ACC GTA ACC CAC TGT GAC GAC AAT GGC TGT AAC ACC AAG ACT GTC
 S   Y   T   T   V   T   V   T   H   C   D   D   N   G   C   N   T   K   T   V

         2350         2360         2370          2380         2390         2400
ACC TCT GAA GCT CCT GAA GCT ACC ACC ACA ACT ACT GTT TCT TCT CAA TCG TAC ACC ACT
 T   S   E   A   P   E   A   T   T   T   T   T   V   S   S   Q   S   Y   T   T

         2410         2420         2430          2440         2450         2460
GCC ACC GTC ACC CAC TGT GAT GAC AAT GGA TGT AAG ACC AAG ACT GTC ACT TCT GAA GCT
 A   T   V   T   H   C   D   D   N   G   C   K   T   K   T   V   T   S   E   A

         2470         2480         2490          2500         2510         2520
CCT GAA GCC ACA ACC ACT ACT GTT TCT CCA AAG ACA TAC ACC ACC GCT ACT GTT ACT CAG
 P   E   A   T   T   T   T   V   S   P   K   T   Y   T   T   A   T   V   T   Q

         2530         2540         2550          2560         2570         2580
TGT GAT GAC AAT GGA TGT AGC ACC AAG ACT GTC ACT TCT GAA TGT CCT GAA GAA ACT TCA
 C   D   D   N   G   C   S   T   K   T   V   T   S   E   C   P   E   E   T   S

         2590         2600         2610          2620         2630         2640
GCA ACT ACT ACT TCT CCA AAA TCA TAC ACT ACT GTT ACC GTT ACT CAC TGT GAC GAC AAC
 A   T   T   T   S   P   K   S   Y   T   T   V   T   V   T   H   C   D   D   N

         2650         2660         2670          2680         2690         2700
GGC TGT AAC ACT AAG ACT GTC ACC TCT GAG GCC CCT GAA GCC ACA ACC ACT ACT GTT TCT
 G   C   N   T   K   T   V   T   S   E   A   P   E   A   T   T   T   T   V   S

         2710         2720         2730          2740         2750         2760
CCA AAG ACA TAC ACC ACC GCT ACT GTT ACT CAG TGC GAT GAC AAT GGA TGT AGC ACC AAG
 P   K   T   Y   T   T   A   T   V   T   Q   C   D   D   N   G   C   S   T   K

         2770         2780         2790          2800         2810         2820
ACT GTC ACT TCT GAA GCT CCT AAA GAA ACT TCA GAA ACT TCA GAA ACC AGT GCT GCC CCT
 T   V   T   S   E   A   P   K   E   T   S   E   T   S   E   T   S   A   A   P

         2830         2840         2850          2860         2870         2880
AAG GAC ATA CAC TAC TGC CAC TGG TTA CTC AAT GGT GAT GAC AAT GGT TGT AAC GTC AAG
 K   D   I   H   Y   C   H   W   L   L   N   G   D   D   N   G   C   N   V   K

         2890         2900         2910          2920         2930         2940
ATA ATC ACC TCT AAA ATA CCT GAA GCT ACT TCA ACC GTC ACG CAA CTA GTG CTT CTC CAA
 I   I   T   S   K   I   P   E   A   T   S   T   V   T   Q   L   V   L   L   Q

         2950         2960         2970          2980
AGT CAT ACA CTA CTG TCA CTT CTG AGG GTT CTA AAG CAA CCT CAT TGA
 S   H   T   L   L   S   L   L   R   V   L   K   Q   P   H   *
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HPF2

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           10           20           30            40           50           60
ATG CAA TTA CAT TCA CTT ATC GCT TCA ACT GCG CTC TTA ATA ACG TCA GCT TTG GCT GCT
 M   Q   L   H   S   L   I   A   S   T   A   L   L   I   T   S   A   L   A   A

           70           80           90           100          110          120
ACT TCC TCT TCT TCC AGC ATA CCC TCT TCC TGT ACC ATA AGC TCA CAT GCC ACG GCC ACA
 T   S   S   S   S   S   I   P   S   S   C   T   I   S   S   H   A   T   A   T

          130          140          150           160          170          180
GCT CAG AGT GAC TTA GAT AAA TAT AGC CGC TGT GAT ACG TTA GTC GGG AAC TTA ACT ATT
 A   Q   S   D   L   D   K   Y   S   R   C   D   T   L   V   G   N   L   T   I

          190          200          210           220          230          240
GGT GGT GGT TTG AAG ACT GGT GCT TTG GCT AAT GTT AAA GAA ATC AAC GGG TCT CTA ACT
 G   G   G   L   K   T   G   A   L   A   N   V   K   E   I   N   G   S   L   T

          250          260          270           280          290          300
ATA TTT AAC GCT ACA AAT CTA ACC TCA TTC GCT GCT GAT TCC TTG GAG TCC ATC ACA GAT
 I   F   N   A   T   N   L   T   S   F   A   A   D   S   L   E   S   I   T   D

          310          320          330           340          350          360
TCT TTG AAC CTA CAG AGT TTG ACA ATC TTG ACT TCT GCT TCA TTT GGG TCT TTA CAG AGC
 S   L   N   L   Q   S   L   T   I   L   T   S   A   S   F   G   S   L   Q   S

          370          380          390           400          410          420
GTT GAT AGT ATA AAA CTG ATT ACT CTA CCC GCC ATC TCC AGT TTT ACT TCA AAT ATC AAA
 V   D   S   I   K   L   I   T   L   P   A   I   S   S   F   T   S   N   I   K

          430          440          450           460          470          480
TCT GCT AAC AAC ATT TAT ATT TCC GAC ACT TCG TTA CAA TCT GTC GAT GGA TTC TCA GCC
 S   A   N   N   I   Y   I   S   D   T   S   L   Q   S   V   D   G   F   S   A

          490          500          510           520          530          540
TTG AAA AAA GTT AAC GTG TTC AAC GTC AAT AAC AAT AAG AAA TTA ACC TCG ATC AAA TCT
 L   K   K   V   N   V   F   N   V   N   N   N   K   K   L   T   S   I   K   S

          550          560          570           580          590          600
CCA GTT GAA ACA GTC AGC GAT TCT TTA CAA TTT TCG TTC AAC GGT AAC CAG ACT AAA ATC
 P   V   E   T   V   S   D   S   L   Q   F   S   F   N   G   N   Q   T   K   I

          610          620          630           640          650          660
ACC TTC GAT GAC TTG GTT TGG GCA AAC AAT ATC AGT TTG ACC GAT GTC CAC TCT GTT TCC
 T   F   D   D   L   V   W   A   N   N   I   S   L   T   D   V   H   S   V   S

          670          680          690           700          710          720
TTC GCT AAC TTG CAA AAG ATT AAC TCT TCA TTG GGT TTC ATC AAC AAC TCC ATC TCA AGT
 F   A   N   L   Q   K   I   N   S   S   L   G   F   I   N   N   S   I   S   S

          730          740          750           760          770          780
TTG AAT TTC ACT AAG CTA AAC ACC ATT GGC CAA ACC TTC AGT ATC GTT TCC AAT GAC TAC
 L   N   F   T   K   L   N   T   I   G   Q   T   F   S   I   V   S   N   D   Y

          790          800          810           820          830          840
TTG AAG AAC TTG TCG TTC TCT AAT TTG TCA ACC ATA GGT GGT GCT CTT GTC GTT GCT AAC
 L   K   N   L   S   F   S   N   L   S   T   I   G   G   A   L   V   V   A   N

          850          860          870           880          890          900
AAC ACT GGT TTA CAA AAA ATT GGT GGT CTC GAC AAC CTA ACA ACC ATT GGC GGT ACT TTG
 N   T   G   L   Q   K   I   G   G   L   D   N   L   T   T   I   G   G   T   L

          910          920          930           940          950          960
GAA GTT GTT GGT AAC TTC ACC TCC TTG AAC CTA GAC TCT TTG AAG TCT GTC AAG GGT GGC
 E   V   V   G   N   F   T   S   L   N   L   D   S   L   K   S   V   K   G   G

          970          980          990          1000         1010         1020
GCA GAT GTC GAA TCA AAG TCA AGC AAT TTC TCC TGT AAT GCT TTG AAA GCT TTG CAA AAG
 A   D   V   E   S   K   S   S   N   F   S   C   N   A   L   K   A   L   Q   K

         1030         1040         1050          1060         1070         1080
AAA GGG GGT ATC AAG GGT GAA TCT TTT GTC TGC AAA AAT GGT GCA TCA TCC ACA TCT GTT
 K   G   G   I   K   G   E   S   F   V   C   K   N   G   A   S   S   T   S   V
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         1090         1100         1110          1120         1130         1140
AAA CTA TCG TCC ACT TCC AAA TCT CAA TCA AGC CAA ACT ACT GCC AAG GTT TCC AAG TCA
 K   L   S   S   T   S   K   S   Q   S   S   Q   T   T   A   K   V   S   K   S

         1150         1160         1170          1180         1190         1200
TCT TCT AAG GCC GAG GAA AAG AAG TTC ACT TCT GGC GAT ATC AAG GCT GCT GCT TCT GCC
 S   S   K   A   E   E   K   K   F   T   S   G   D   I   K   A   A   A   S   A

         1210         1220         1230          1240         1250         1260
TCT AGT GTT TCT AGT TCT GGC GCT TCC AGC TCT AGC TCT AAG AGT TCC AAA GGC AAT GCC
 S   S   V   S   S   S   G   A   S   S   S   S   S   K   S   S   K   G   N   A

         1270         1280         1290          1300         1310         1320
GCT ATC ATG GCA CCA ATT GGC CAA ACA ACC CCT TTG GTC GGT CTT TTG ACG GCA ATC ATC
 A   I   M   A   P   I   G   Q   T   T   P   L   V   G   L   L   T   A   I   I

         1330
ATG TCT ATA ATG TAA
 M   S   I   M   *
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Alignment of DNA sequences of *HPF1* and *HPF1'*

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HPF1      1 ATGTTCAATCGCTTTAATAAACTTCAAGCCGCTTTGGCTTTGGTCCTTTACTCCCAAAGT
HPF1'     1 ATGTTCAATCGTTTAAACAAATTCCAAGCTGCTTTAGCTTTGGCCCTTTACTCTCAAAGT


HPF1     61 GCATTGGGCCAATATTATACCAACAGTTCCTCAATCGCTAGTAACAGCTC---CACCGCC
HPF1'    61 GCATTGGGTCAGTACTATAGCAATAGCACCTCAATTTCAAGCAACAGCTCATCCACCTCT


HPF1    118 GTTTCGTCAACTTCATCAGGTTCCGTTTCCATCAGTAGTTCTATTGAGTTGAC---CTCA
HPF1'   121 GTGGTATCAAGTTCCTCTGGATCCGTTTCGATCAGTAGTTCTATTGCTGAGACATCCTCG


HPF1    175 TCTACTTCTGATGTCTCGAGCTCTCTCACTGAGTTAACGTCATCCTCCACCGAAGTCTCG
HPF1'   181 TCCGCTACCGATATCTTAAGTTCTATCACACAATCAGCTTCATCCACTTCTGGTGTCTCG


HPF1    235 AGCTCCATTGCTCCATCAACCTCGTCCTCTGAAGTCTCGAGCTCTATTACTTCATCAGGC
HPF1'   241 AGCTCTGTCGGTCCATC---CTCTTCCTCTGTTGTCTCAAGCTCTGTCAGCCAATC---C


HPF1    295 TCTTCAGTCTCCGGCTCATCTTCTATTA--CTTCATCAGGCTCATCAGTCTCCAGTTCAT
HPF1'   295 TCTTCA---TCCGTTTC-TGATGTATCAAGCTCTGTCAG--TCAATCTTCTTC--TTCAG


HPF1    353 CTTCTGCCACTGAATCAGGCTCA-TCCGCC--TCCGGTTCATCCTCCGCCACTGAATCGG
HPF1'   347 CTTCTGA---TGTCTCAAGCTCTGTCAGTCAATCAGCTTCCTCTACTTCTGATGTCTCAA


HPF1    410 GCTCA-TCAGTCTCCGGTTCATCTACTTCCATTACATCAGGCTCATCCTCCGCCACTGAA
HPF1'   404 GCTCTGTCAGTC----AATCTTCTTCTTCAGCTTCTGATGTATCAAGCTCTGTCAGTCAA


HPF1    469 TCGGGCTCATCAGTCTCCGGT---TCAACTTCTGCCACTGAATCAGGCTCATCCGCCTCC
HPF1'   460 TCTTCTTCCTCAGCTTCTGATGTCTCAAGCTCTGTCAGTCAATCAGCTTCCTCAGCTTCT


HPF1    526 GGTTCATCTTCTGCCACTGAATCAGGCTCATCCGCCTCCGGTTCATCTTCTGCCACAGAA
HPF1'   520 GATG--TCTCAAGCT-CTG--TCAGTCA-ATCAGCTTCCTCTACTTCTGATGTCTCAAGC
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HPF1    586 TCAGGCTCA-TCAGTCTCCGG-TTCATCTTCTGCCACAGAATCAGGCTCA-TCAGTCTCC
HPF1'   574 TCTG--TCAGTCAATCTTCTTCTTCAGCTTCTGA---TGTATCAAGCTCTGTCAGTC---

HPF1    643 GGTTCATCTTCTGCCACAGAATCAGGCTCCGCTTCTTCGGTTCCTAGCTCATCCGGTTCT
HPF1'   626 ----AATCTTCTTCC------TCAG------CTTCTGATGTCTCAAGCTC---------T

HPF1    703 GTCACAGAATCAGGCTCATCCTCATCAGCA-TCTGAATCATCTATCACACAATCTGGTAC
HPF1'   661 GTCAGTCAATCAGCTTCCTCTACTTCTGATGTCTCAAGC-TCTGTCAGTCAATCAGCTTC

HPF1    762 CGCTTCCGGTTCATCAGCCTCCAGCACGTCCGGTTCTGTTACACAATCTGGTTCCTCCGT
HPF1'   720 CTCTAC---TTCTGGTGTCTCAAGC--------T-CTGGCAGCCAATCAG-----TC---

HPF1    822 TTCCGGTTCATCAGCTTCTTCTGCTCCAGGTATCTCGAGTTCAATTCCTCAATCAACCTC
HPF1'   760 -------TCATCCGCTTCT---------GGTAGCTCAAGCTCATTCCCTCAATCAACCTC

HPF1    882 ATCGGCTTCCACTGCCTCTGGTTCTATCACCTCCGGTACCTTAAGTTCTATTACCTCTTC
HPF1'   804 ATCCGCTTCTACTGCCTCCGGTTCTGCCACTTCCAATTCCTTGAGTTCCATTACTTCTTC

HPF1    942 GGCTTCTAGTGCAACTGCAACTGCTTCCAACTCTCTTTCTTCCAGCGATGGTACTATTTA
HPF1'   864 AGCATCTAGTGCAAGCGCAACTGCTTCCAACTCCCTTTCTTCCAGCGATGGTACTATCTA

HPF1   1002 TTTGCCTTCTACAACCATCAGTGGTGACATCACACTCACCGGTTCAGTCATTGCAACTGA
HPF1'   924 TCTACCAACTACTACAATCAGTGGTGATCTAACTCTTACTGGTAAAGTAATTGCAACAGA

HPF1   1062 AGCTGTCGAAGTCGCTGCAGGTGGTAAGTTGACCCTACTTGATGGTGACAAATACGTTTT
HPF1'   984 AGGTGTTGTGGTCGCAGCTGGTGCCAAATTGACTCTACTTGACGGTGACAAATATTCTTT

HPF1   1122 CTCTGCTGATTTCATAATCCATGGTGGCGTTTTCGTAGAAAAGTCTAAGCCAACTTACCC
HPF1'  1044 CTCAGCTGACCTAAAAGTCTACGGTGACTTGCTTGTGAAAAAGTCCAAAGAAACCTATCC
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HPF1  1182 AGGTACCGAATTCGACATTTCTGGTGAAAACTTTGATGTATCTGGTACCTTTAACGCTGA
HPF1' 1104 AGGTACCGAATTCGACATCTCCGGTGAAAACTTTGACGTGACCGGTAACTTCAACGCTGA

HPF1  1242 AGAGCCTGCTGCTTCTTCCGCATCTGCCTACTCCTTCACTCCAGGCTCTTTCGATAACAG
HPF1' 1164 AGAATCCGCTGCCACCTCTGCATCCATCTACTCCTTCACTCCAAGTTCTTTTGACAACAG

HPF1  1302 TGGTGATATTTCTTTGAGTCTATCAGAGTCCACAAAGGGCGAAGTCACATTCTCTCCCTA
HPF1' 1224 TGGTGACATTTCCTTAAGTCTATCAAAGTCCAAGAAGGGTGAAGTCACTTTCTCTCCATA

HPF1  1362 CTCTAACTCTGGTGCTTTCTCTTTCTCAAATGCTATTCTCAATGGTGGTTCCGTCTCTGG
HPF1' 1284 CTCCAATTCTGGTGCCTTCTCTTTCTCGAACGCTATTCTCAACGGTGGTTCTGTTTCCGG

HPF1  1422 TTTGCAGCGTAGAGCTGAATC---AGGTTCTGTCAACAACGGTGAGATAAATCTTGAGAA
HPF1' 1344 TCTACAACGTAGAGACGACACTGAAGGTTCAGTAAACAACGGTGAAATTAACCTAGACAA

HPF1  1479 TGGCAGTACCTACGTCGTTGTCGAACCAGTTTCTGGAAGTGGTACAATCAACATCATCTC
HPF1' 1404 TGGAAGTACCTATGTTATTGTCGAACCAGTTTCTGGAAAAGGTACAGTCAACATCATTTC

HPF1  1539 TGGCAACCTTTACTTGCACTATCCAGACACCTTTACTGGCCAAACTGTTGTATTCAAGGG
HPF1' 1464 CGGTAACCTATACTTACACTACCCTGACACATTTACTGGCCAAACTGTTGTATTCAAGGG

HPF1  1599 TGAAGGTGTTCTTGCCGTTGACCCAACCGAAACCAACACTACTCCTATCCCTGTGGTTGG
HPF1' 1524 TGAAGGTGTCCTTGCTGTTGACCCAACCGAAACCAATGCCACTCCTATTCCTGTTGTTGG

HPF1  1659 ATACACTGGTGAAAACCAAATCGCCATTACAGCAGATGTCACTGCTCTTTCTTACGACAG
HPF1' 1584 CTACACTGGTAAGAACCAAATTGCCATTACCGCTGACATCACTGCTCTTTCTTATGACGG

HPF1  1719 TGCTACTGGTGTTTTAACTGCAACACAAGGCAACTCACAATTCTCCTTCTCTATTGGTAC
HPF1' 1644 TACTACCGGTGTTCTAACTGCAACCCAAGGTAACAGACAATTCTCTTTTGCTATTGGTAC
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HPF1  1779 TGGGTTCTCCAGTTCTGGCTTCAACGTCTCCGAAGGAACATTTGCTGGTGCCTATGCTTA
HPF1' 1704 CGGATTCTCCAGCTCTGACTTCAGCGTCTCTGAAGGAATCTTTGCAGGTGCTTACGCTTA


HPF1  1839 TTATCTAAATTACGGAGGTGTTGTTGCTTCCAGCGCTACACCCTCATCCACATCTACCAC
HPF1' 1764 CTACCTAAACTACAATGGTGTTGTCGCTACTAGTGC------CGCTTCTTCATCCACTGC


HPF1  1899 ATCAGGGGCTACCAACTCTACTTCCGGTTCCACTTCATTCGGTGCTTCCGTAACTGGTTC
HPF1' 1818 ATCTGGTGCTTCCGCTTCCGTTACCGGTTCTACTTCATTCGGTGCTTCCGTTACCGGTTC


HPF1  1959 AACCGCTTCCACTTCATTCGGTGCTTCCGTGACTGGTTCAACGGCTTCCACCTTGATTT-
HPF1' 1878 AACTGCTTCCACTTCATTCGGTGCTTCCGTTACCGGTTCAACTGCTTCCAC-TTCATTTG


HPF1  2018 ------------CCGGCTCCCCATCTGTTTATACCACAACATTAACATATGCAACAACCA
HPF1' 1937 GTGCTTCCGTTACCGGTTCAACATCTGTTTACACTACAACACTAGACTATGTAAATGCCA


HPF1  2066 CAAGCACAGTAGTTGTCTCCTGTTCAGAAACAACTGATTCGAACGGTAACGTCTATACCA
HPF1' 1997 CAAGCACAGTCGTAGTTTCTTGTTCAGAGACAACTGACTCCAATGGTAACGTCTATACCA


HPF1  2126 TTACCACAACCGTACCATGTTCATCTACCACCGCCACTATCACTTCTTGCGATGAGACCG
HPF1' 2057 TTACCACAACCGTTCCATGCTCATCCACTACCGCCACTATTACTTCTTGTGATGAAACTG


HPF1  2186 GATGTCATGTAACTACGTCTACCGGTACCGTCGCCACTGAAACCGTTTCTTCCAAATCAT
HPF1' 2117 GATGTCACGTTAGTACATCAACCGGTGCTGTTGTAACTGAAACCGTTTCTTCCAAATCAT


HPF1  2246 ACACCACTGTTACCGTCACCCACTGTGACAACAATGGCTGTAACACCAAGACTGTCACTT
HPF1' 2177 ACACAACTGCCACTGTAACTCACTGTGACGATAATGGCTGTAACACCAAGACTGTCACTT
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HPF1  2306 CTGAATGTCCTGAAGAAACTTCAGCAACTACTACTTCTCCAAAATCATACACTACTGTTA
HPF1' 2237 CTGAATGTTCCAAAGAAACATCAGCAACAACTGCTTCTCCAAAATCATACACCACTGTCA

HPF1  2366 CCGTTACTCACTGTGACGACAACGGCTGTAACACTAAGACTGTCACCTCTGAGGCCCCTG
HPF1' 2297 CCGTAACCCACTGTGACGACAATGGCTGTAACACCAAGACTGTCACCTCTGAAGCTCCTG

HPF1  2426 AAGCCACAACCAC---TACTGTTTCTCCAAAGACATACACTACCGCTACTGTTACTCAGT
HPF1' 2357 AAGCTACCACCACAACTACTGTTTCTTCTCAATCGTACACCACTGCCACCGTCACCCACT

HPF1  2483 GCGATGACAATGGATGTAGCACCAAGACTGTCACTTCTGAAGCTCCTAAAGAAACTTCAG
HPF1' 2417 GTGATGACAATGGATGTAAGACCAAGACTGTCACTTCTGAAGCTCCTGAAGC-----CAC

HPF1  2543 AAACTTCAGAAACCAGTGCTGCCCCTAAGACATACACTACTGCCACTGTTACTCAATGTG
HPF1' 2472 AACC-------ACTACTGTTTCTCCAAAGACATACACCACCGCTACTGTTACTCAGTGTG

HPF1  2603 ATGACAATGGTTGTAACGTCAAGATAATCACCTCTCAAATACCTGAAGCTACTTCAACCG
HPF1' 2525 ATGACAATGGATGTAGCACCAAGACTGTCACTTCTGAATGTCCTGAAGAAACTTCA----

HPF1  2663 TCACCGCAACTAGTGCTTCTCCAAAGTCATACACTACTGTCACTTCTGAGGGTTCTAAAG
HPF1' 2581 -----GCAACTACTACTTCTCCAAAATCATACACTACTGTTACCGTTACTCACTGTGACG

HPF1  2723 -CAACCTCATTGACT-----ACTGCCATTTCCAAGGCTTCTAGTGCAATTTCCACATACT
HPF1' 2636 ACAACGGCTGTAACACTAAGACTGTCACCTCTGAGGCCCCTGAAGCCACAACCAC-TACT

HPF1  2777 CCAAATCTGCAGCTCCAATAAAGACCTCTACTGGTA-TCATTGTCCAGTCCGAGGGTATT
HPF1' 2695 GTT--TCTCCAAAGACATACACCACCGCTACTGTTACTCAGTGCGATGACAATGGATGTA

HPF1  2836 GCCGCAGGTTTG--AATGCCAATACTTTGAATGCATTGGTCGGTATTTTCGTTCTTGCTT
HPF1' 2753 GCACCAAGACTGTCACTTCTGAAGCTCCTAAAGAAACT-TCAGAAACTTCAGAAACCAGT
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HPF1   2894 TCTTTAACTAA-------------------------------------------------
HPF1'  2812 GCTGCCCCTAAGGACATACACTACTGCCACTGGTTACTCAATGGTGATGACAATGGTTGT

HPF1        ------------------------------------------------------------
HPF1'  2872 AACGTCAAGATAATCACCTCTAAAATACCTGAAGCTACTTCAACCGTCACGCAACTAGTG

HPF1        ---------------------------------------------------------
HPF1'  2932 CTTCTCCAAAGTCATACACTACTGTCACTTCTGAGGGTTCTAAAGCAACCTCATTGA
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Amino acid sequence alignment of Hpf1p and Hpf1'p

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Hpf1p      1 MFNRFNKLQAALALVLYSQSALGQYYTNSSSIASNSS-TAVSSTSSGSVSISSSIELTSS
Hpf1'p     1 MFNRLNKFQAALALALYSQSALGQYYSNSTSISSNSSSTSVVSSSSGSVSISSSIAETSS


Hpf1p     60 -TSDVSSSLTELTSSSTEVSSSIAPSTSSSEVSSSITSSGSSVSGSSSITSSGSSVSSSS
Hpf1'p    61 SATDILSSITQSASSTSGVSSSVGPSSSS------------VVSSSVSQSSSSVSDVSSS


Hpf1p    119 SATESGSSASGSSSATESGSSVSGSSTSITSGSSSATESGSSVSGSTSATESGSSASGSS
Hpf1'p   109 VSQSSSSASDVSSSVSQSASSTSDVSSSVSQSSSSASDVSSSVS-----------QSSSS


Hpf1p    179 SATESGSSASGSSSATESGSSVSGSSSATESGSSVSGSSSATESGSASSVPSSSGSVTES
Hpf1'p   158 ASDVSSSVSQSASSASDVSSSVSQSASSTSDVSSSVSQSSSSASDVSSSVSQSSSSASDV


Hpf1p    239 GSSSSASESSITQSGTASGSSASSTSGSVTQSGSSVSGSSASSAPGISSSIPQSTSSAST
Hpf1'p   218 SSSVSQSASSTSDVSSSVSQSASSTSG-----VSSSGSQSVSSASGSSSSFPQSTSSAST


Hpf1p    299 ASGSITSGTLSSITSSASSATATASNSLSSSDGTIYLPSTTISGDITLTGSVIATEAVEV
Hpf1'p   273 ASGSATSNSLSSITSSASSASATASNSLSSSDGTIYLPTTTISGDLTLTGKVIATEGVVV


Hpf1p    359 AAGGKLTLLDGDKYVFSADFIIHGGVFVEKSKPTYPGTEFDISGENFDVSGTFNAEEPAA
Hpf1'p   333 AAGAKLTLLDGDKYSFSADLKVYGDLLVKKSKETYPGTEFDISGENFDVTGNFNAEESAA


Hpf1p    419 SSASAYSFTPGSFDNSGDISLSLSESTKGEVTFSPYSNSGAFSFSNAILNGGSVSGLQRR
Hpf1'p   393 TSASIYSFTPSSFDNSGDISLSLSKSKKGEVTFSPYSNSGAFSFSNAILNGGSVSGLQRR


Hpf1p    479 -AESGSVNNGEINLENGSTYVVVEPVSGSGTINIISGNLYLHYPDTFTGQTVVFKGEGVL
Hpf1'p   453 DDTEGSVNNGEINLDNGSTYVIVEPVSGKGTVNIISGNLYLHYPDTFTGQTVVFKGEGVL


Hpf1p    538 AVDPTETNTTPIPVVGYTGENQIAITADVTALSYDSATGVLTATQGNSQFSFSIGTGFSS
Hpf1'p   513 AVDPTETNATPIPVVGYTGKNQIAITADITALSYDGTTGVLTATQGNRQFSFAIGTGFSS
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Hpf1p   598 SGFNVSEGTFAGAYAYYLNYGGVVASSATPSSTSTTSGATNSTSGSTSFGASVTGSTAST
Hpf1'p  573 SDFSVSEGIFAGAYAYYLNYNGVVATSAASS--STASGASASVTGSTSFGASVTGSTAST


Hpf1p   658 SFGASVTGSTAST----LISGSPSVYTTTLTYATTTSTVVVSCSETTDSNGNVYTITTTV
Hpf1'p  631 SFGASVTGSTASTSFGASVTGSTSVYTTTLDYVNATSTVVVSCSETTDSNGNVYTITTTV


Hpf1p   714 PCSSTTATITSCDETGCHVTTSTGTVATETVSSKSYTTVTVTHCDNNGCNTKTVTSECPE
Hpf1'p  691 PCSSTTATITSCDETGCHVSTSTGAVVTETVSSKSYTTATVTHCDDNGCNTKTVTSECSK


Hpf1p   774 ETSATTTSPKSYTTVTVTHCDDNGCN----------------------------------
Hpf1'p  751 ETSATTASPKSYTTVTVTHCDDNGCNTKTVTSEAPEATTTTTVSSQSYTTATVTHCDDNG


Hpf1p   800 --TKTVTSEAPEATTTTVSPKTYTTATVTQCDDNGCSTKTVTSEAPKETSETSETSAAPK
Hpf1'p  811 CKTKTVTSEAPEATTTTVSPKTYTTATVTQCDDNGCSTKTVTSECPEETSATTTS--P-K


Hpf1p   858 TYTTATVTQCDDNGCNVKIITSQIPEATS-----------TVTATSASPKSYTTVTSEGS
Hpf1'p  868 SYTTVTVTHCDDNGCNTKTVTSEAPEATTTTVSPKTYTTATVTQCDDNGCSTKTVTSEAP


Hpf1p   907 KATSLTTAISKASSAISTYSKSAAPIKTSTGIIVQSEGIAAGLNANTLNALVGIFVLAFF
Hpf1'p  928 KETSETSETSAAPKDIHYCHWLLNGDDNGCNVKIITSKIPEATSTVTQLVLLQSHTLLSL


Hpf1p   967 N-------
Hpf1'p  988 LRVLKQPH
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APPENDIX TWO

SOB medium

Dissolve in 950 mL deionised water:

20 g tryptone

5 g yeast extract

0.5 g NaCl

Adjust to pH 7.0, adjust volume to 1000 mL. Autoclave.

Before use, add 5 mL of sterile 2 M $MgCl_2$.

SOC medium

To 1 litre of SOB medium, add:

20 mL of sterile 1M glucose

LB medium

Dissolve in 950 mL deionised water:

10 g tryptone

5 g yeast extract

10 g NaCl

Adjust to pH 7.0, adjust volume to 1000 mL. Autoclave.

Synthetic dropout medium

Dissolve in 950 mL deionised water:

6.67 g Difco Yeast Nitrogen Base

20 g glucose, raffinose or galactose

0.67 g Synthetic Complete Dropout Mix

20 g Difco Agar (for solid medium)

Add one pellet of NaOH, adjust volume to 1000 mL. Autoclave.

Synthetic Complete Dropout Mix

2 g Adenine hemisulphate
2 g Arginine HCl
2 g Histidine HCl
2 g Isoleucine
4 g Leucine
2 g Lysine HCl
2 g Methionine
3 g Phenylalanine
2 g Serine
2 g Threonine
3 g Tryptophan
2 g Tyrosine
1.2 g Uracil
9 g Valine
Omit appropriate components to prepare required dropout mix.

Chemically Defined Grape Juice Medium

For 1 litre of medium:

Carbon:	Glucose at the appropriate concentration
Salts:	2.5 g Potassium hydrogen tartrate
	3.0 g L-Malic acid
	1.23 g $MgSO_4.7H_2O$
	1.14 g K_2HPO_4
	0.2 g Citric acid
	0.33 g Calcium chloride (anhydrous)
	1.7 g Ammonium chloride
	1.0 g Complete amino acid mix
	0.1 g *Myo*-inositol
Minerals:	1 mL of 1000x Mineral stock solution
Vitamins:	1 mL of 1000x Vitamin stock solution

Dissolve 2.5 g potassium hydrogen tartrate in 100 mL of water at 80°C with constant stirring. Add glucose at appropriate concentration in small amounts along with 900 mL of water. Add the remaining salts, minerals and vitamins. Adjust the pH to 3.2 with 5 M tartaric acid and filter through a 0.2 µm membrane to sterilise.

1000x Mineral stock solution

200 mg/L $MnCl_2$
135 mg/L $ZnCl_2$
30 mg/L $FeCl_2$
15 mg/L $CuCl_2$
5 mg/L H_3BO_3
30 mg/L $Co(NO_3)_2.6H_2O$
25 mg/L $NaMoO_4.2H_2O$
10 mg/L KIO_3

1000x Vitamins stock solution

2 g/L Pyridoxine hydrochloride
2 g/L Nicotinic acid
1 g/L D-Pantothenic acid (Hemi-calcium salt)
500 mg/L Thiamin hydrochloride
200 mg/L PABA.K
200 mg/L Riboflavin
125 mg/L Biotin
200 mg/L Folic acid

Sporulation medium

Dissolve in 950 mL deionised water:
10 g potassium acetate
1 g glucose
20 g agar
Adjust volume to 1000 mL. Autoclave.

Low pH Blue plates

Dissolve in 533 mL deionised water:

6 g yeast extract

12 g peptone

12 g glucose

12 g agar

Autoclave the above ingredients and add the following solutions:

Methylene blue in sterile water	5 mL
Phosphate-citrate buffer	67 mL

Methylene blue in sterile water

Dissolve 20 mg of methylene blue in 5 mL of sterile deionised water.

Phosphate-citrate buffer for low pH medium

Dissolve 14.07 g of citric acid and 18.96 g of K_2HPO_4 in 67 mL of deionised water. Adjust the pH to 4.5 using solid K_2HPO_4 or citric acid. Sterilise by autoclaving.

Yeast Extract / Peptone / Dextrose (YPD) Medium

Dissolve in 950 mL deionised water:

10 g yeast extract

20 g peptone

20 g glucose

20 g agar (for solid medium)

Adjust volume to 1000 mL. Autoclave.

Oleic acid induction medium

0.12% (w/v) oleic acid

0.2% (v/v) Tween 20

0.3% (w/v) yeast extract

0.5% (w/v) bactopeptone

0.3% (w/v) glucose

Auxotrophic requirements

Autoclave.

Selective oleic acid medium
0.1% (w/v) oleic acid
0.4% (w/v) Tween 20
0.1% (w/v) yeast extract
0.67% (w/v) yeast nitrogen base without amino acids
2% (w/v) agar
Auxotrophic requirements
Autoclave.

Plasmid solution 1 – *E. coli* (Sambrook and Russell, 2001)
50 mM glucose
25 mM Tris.Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Plasmid solution 2 – *E. coli* (Sambrook and Russell, 2001)
0.2 M NaOH
1% (w/v) SDS

Plasmid solution 3 – *E. coli* (Sambrook and Russell, 2001)
5 M potassium acetate, 60 mL
Glacial acetic acid, 11.5 mL
Water, 28.5 mL

Breaking buffer – Yeast chromosomal DNA
2% (v/v) Triton X-100
1% (w/v) SDS
100 mM NaCl
10 mM Tris.Cl (pH 8.0)
1 mM EDTA (pH 8.0)

SDS-PAGE running buffer

15.5 g Tris base
71.3 g glycine
5 g SDS
Dissolve in 5 litres of water

SDS-PAGE sample buffer (2x strength)

0.5% (v/v) β-mercaptoethanol
4% (w/v) SDS
20% (v/v) glycerol
125 mM Tris.Cl (pH 6.8)
0.01% (w/v) bromophenol blue

SDS-PAGE sample buffer (5x strength)

500 mM dithiothreitol
10% (w/v) SDS
40% (v/v) glycerol
300mM Tris.Cl (pH 6.8)
0.025% (w/v) bromophenol blue

Coomassie blue stain

40% (v/v) ethanol
7% (v/v) acetic acid
0.1% (w/v) Brilliant blue R-250

Destain solution / Fixative solution

20% (v/v) ethanol
7% (v/v) acetic acid

Periodate solution – Schiff stain

0.7% (w/v) periodic acid
5% (v/v) acetic acid

Metabisulphite solution – Schiff stain

0.2% (w/v) potassium metabisulphite

5% (v/v) acetic acid

Western transfer buffer

25 mM Tris base (pH 8.0)

192 mM glycine

20% (v/v) methanol

The pH should be 8.3. Do not add acid or base to adjust the pH.

TBS-T (Tris buffered saline with Tween 20)

To prepare 500 mL of 10x stock solution:

137 mM NaCl

2.7 mM KCl

25 mM Tris base (pH 8.0)

Add 5 mL of Tween 20, adjust the pH to 8.0 with HCl and the volume to 500 mL with deionised water.

Sarkosyl wash reagent

0.4% (v/v) sarkosyl

50 mM Tris base (pH 8.0)

1 M NaCl

5 mM EDTA (pH 8.0)

Ponceau S solution

0.1% (w/v) Ponceau S

5% (v/v) acetic acid

Blocking buffer

5% (w/v) dry skim milk powder in 1x TBS-T

Alkaline phosphatase buffer

100 mM Tris-HCl (pH 9.0)

150 mM NaCl

1 mM $MgCl_2$

Wash buffer – Ni-NTA resin

50 mM NaH_2PO_4

300 mM NaCl

pH 8.0

Elution buffer – Ni-NTA resin

50 mM NaH_2PO_4

300 mM NaCl

250 mM imidazole

pH 8.0

Electron microscopy fixative solution

4% (v/v) paraformaldehyde

1.25% (v/v) glutaraldehyde

in phosphate buffered saline (pH 7.2)

Electron microscopy wash buffer

4% (w/v) sucrose in phosphate buffered saline (pH 7.2)

Phosphate buffered saline (PBS)

10 mM Na_2HPO_4

2 mM KH_2PO_4

0.15 M NaCl

3 mM KCl

pH 7.4

Autoclave.

APPENDIX THREE

Appendix 3: Oligonucleotide primers used in this study

SLB2	**AGCCGTTGTCGTCACAGTGAGTAACGGTAACAGTAGTGTATGATTTTGGAGAAGTAGTAG***CCTCGAGGTCGACGGTATC*	**HPF1' homology reverse (+2645 bp after ATG) +** HIS5 *gene homology*
SLB3	**TGCCTTTTCTGGTTCTCAAGTCAGTAACAACAAATACCCATCGTTGATTCTTTTTGGTAA***GTGATTCTGGGTAGAAGATC*	**HPF1 homology forward (-79 bp before ATG) +** URA3 *gene homology*
SLB4	**GTAGTGTATGACTTTGGAGAAGCACTAGTTGCGGTGACGGTTGAAGTAGCTTCAGGTATT***TTGCTTCTTTTCTTATCCGG*	**HPF1 homology reverse (+2699 bp after ATG) +** URA3 *gene homology*
SLB5	**GTGTTGTTTTTATTTCGAGTAAATTTAAGAAACAGCAAGCCTCATCATTGCAAATTTGGT***ACGAGCTCGTTTAAACTGG*	**HPF1' homology forward (-82 bp before ATG) +** HIS5 *gene homology*
SLB6	**CAGTACATCACGCTGCAATAGATCTAATCCAAGAGAGAAGCAAAAAAAAAAAGCTCGCTA***CGAGCTCGTTTAAACTGG*	**HPF2 homology forward (-70 bp before ATG) +** HIS5 *gene homology*
SLB7	**GGAAACCTTGGCAGTAGTTTGGCTTGATTGAGATTTGGAAGTGGACGATAGTTTAACAGA***CCTCGAGGTCGACGGTATC*	**HPF2 homology reverse (+1134 bp after ATG) +** HIS5 *gene homology*
SLB11	GC*TCTAGA*GTAAGATCTCGCCTGTCAAAGAC	HPF1 overexpression forward (includes *Xba*I restriction site) (-23 bp before ATG)
SLB12	GC*GTCGAC*CCTATCTCGTTTCAGAGGAATAAAC	HPF1 overexpression reverse (includes *Sal*I restriction site), HPF1 deletion confirmation reverse (+2952 bp after ATG)
SLB13	CGGCAACCATTGCGTTT	HPF1' deletion confirmation forward (-317 bp before ATG)
SLB14	TGGCATTCAAACCTGCG	HPF1' deletion confirmation reverse (+3101 bp after ATG)
SLB15	TGATTGGGCTGGGTTTT	HPF2 deletion confirmation forward (-241 bp before ATG)
SLB16	CAATTTTGTTGCTTTCT	HPF2 deletion confirmation reverse (+1566 bp before ATG)

SLB17	GC*TCTAGA*GCAAATTTGGTAAGATCTCGCCT	HPF1’ overexpression forward (includes *Xba*I restriction site) (-34 bp before ATG)
SLB18	GC*GTCGAC*TAGAAGCCTTGGAAATGGCA	HPF1’ overexpression reverse (includes *Sal*I restriction site) (+3012 bp after ATG)
SLB19	GC*TCTAGA*GAAGCAAAAAAAAAAAGCTCGC	HPF2 overexpression forward (includes *Xba*I restriction site) (-34 bp before ATG)
SLB20	GC*GTCGAC*TGAATGACAGGTACTAGTTATCA	HPF2 overexpression reverse (includes *Sal*I restriction site) (+1384 bp after ATG)
SLB21	CGCTTTAATAAACTTCAAGCCG	Sequencing primer #1, HPF1 coding strand, Starting base #10 (of YOL155c)
SLB22	TCCGGTTCATCTTCTGCC	Sequencing primer #2, HPF1 coding strand, Starting base #484
SLB23	CCATCAGTGGTGACATCAC	Sequencing primer #3, HPF1 coding strand, Starting base #1016
SLB24	ACCAGTTTCTGGAAGTGG	Sequencing primer #4, HPF1 coding strand, Starting base #1503
SLB25	TCCACCTTGATTTCCGGC	Sequencing primer #5, HPF1 coding strand, Starting base #2005
SLB26	GCACCAAGACTGTCACTTC	Sequencing primer #6, HPF1 coding strand, Starting base #2501
SLB27	GCAAGAACGAAAATACCGACC	Sequencing primer #1, HPF1 non-coding strand, Starting base #2888
SLB28	GTGTTACAGCCGTTGTCG	Sequencing primer #2, HPF1 non-coding strand, Starting base #2396
SLB29	GGTAGATGTGGATGAGGG	Sequencing primer #3, HPF1 non-coding strand, Starting base #1893
SLB30	TGAGAAAGAGAAAGCACC	Sequencing primer #4, HPF1 non-coding strand, Starting base #1386
SLB31	AACCAGAGGCAGTGGAAG	Sequencing primer #5, HPF1 non-coding strand, Starting base #901
SLB32	TCAGTGGCGGAGGATGAA	Sequencing primer #6, HPF1 non-coding strand, Starting base #401
SLB33	AAACAAATTCCAAGCTGC	Sequencing primer #1, HPF1’ coding strand, Starting base # 15 (of YIL169c)
SLB34	CAGCTTCCTCAGCTTCTG	Sequencing primer #2, HPF1’

		coding strand, Starting base #503
SLB35	GACTCTACTTGACGGTGAC	Sequencing primer #3, HPF1' coding strand, Starting base #1014
SLB36	TCAAGGGTGAAGGTGTCC	Sequencing primer #4, HPF1' coding strand, Starting base #1517
SLB37	CTTGTTCAGAGACAACTGAC	Sequencing primer #5, HPF1' coding strand, Starting base #2015
SLB38	CCACCGCTACTGTTACTC	Sequencing primer #6, HPF1' coding strand, Starting base #2501
SLB39	GAGGTTGCTTTAGAACCC	Sequencing primer #1, HPF1' non-coding strand Starting base #2980
SLB40	AGTGGTTGTGGCTTCAGG	Sequencing primer #2, HPF1' non-coding strand, Starting base #2688
SLB41	CATAGTCTAGTGTTGTAGTG	Sequencing primer #3, HPF1' non-coding strand, Starting base #1984
SLB42	GTGTAAGTATAGGTTACCGG	Sequencing primer #4, HPF1' non-coding strand, Starting base #1479
SLB43	CTGTTGCAATTACTTTACC	Sequencing primer #5, HPF1' non-coding strand, Starting base #979
SLB44	GAGACATCAGAAGCTGAGG	Sequencing primer #6, HPF1' non-coding strand, Starting base #650
SLB45	CACCAGAAGTGGATGAAGC	Sequencing primer #7, HPF1' non-coding strand, Starting base #232
SLB46	ATGCAATTACATTCACTT	Sequencing primer #1, HPF2 coding strand, Starting base #1 (of YDR055w)
SLB47	CAATAAGAAATTAACCTCG	Sequencing primer #2, HPF2 coding strand, Starting base #513
SLB48	GCTTTGCAAAAGAAAGGGGG	Sequencing primer #3, HPF2 coding strand, Starting base #1009
SLB49	TAGACATGATGATTGCCG	Sequencing primer #1, HPF2 non-coding strand, Starting base #1324
SLB50	CAACGACAAGAGCACCAC	Sequencing primer #2, HPF2 non-coding strand, Starting base #832
SLB51	GATTGTCAAACTCTGTAGG	Sequencing primer #3, HPF2 non-coding strand, Starting base #324
SLB52	GCATAACCACTTTAACTA	Sequencing primer 5' region of MCS in p415GAL1 coding strand
SLB53	AGCGTCCCAAAACCTTCT	Sequencing primer 5' region of MCS in p415GAL1 non-coding

		strand
SLB54	CTGAATCAGGCTCATCCG	Sequencing primer #7, HPF1 coding strand, Starting base #362
SLB55	GGTATAGACGTTACCGTT	Sequencing primer #8, HPF1 non-coding strand, Starting base #2124
SLB56	GAGGCGGATGAGCCTGAT	Sequencing primer #9, HPF1 non-coding strand, Starting base #524
SLB57	GC*CAGCTG*GAG**ATG**TTCAATCGCTTTAATAAACTTCAAGCCGCTTTGGCTTTGGTCCTT**CATCATCATCATCATCAT**TACTCCCAAAGTGCATTG	Forward primer for 6xHis HPF1 cloning in yeast, *PvuII restriction site*, Signal sequence including **ATG**, **6xHis sequence**
SLB58	GC*TCTAGA*CCTATCTCGTTTCAGAGGAATAAAC	Reverse primer for 6xHis HPF1 cloning in yeast, *XbaI restriction site* (+2952 bp after ATG)
SLB62	GC*CAGCTG*TAT**ATG**TTCAATCGTTTAAACAAATTCCAAGCTGCTTTAGCTTTGGCCCTT**CATCATCATCATCATCAT**TACTCTCAAAGTGCA	Forward primer for 6xHis HPF1' cloning in yeast, *PvuII restriction site*, Signal sequence including **ATG**, **6xHis sequence**
SLB63	GC*TCTAGA*TAGAAGCCTTGGAAATGGCA	Reverse primer for 6xHis HPF1' cloning in yeast, *XbaI restriction site* (+3012 bp after ATG)
SLB64	GC*CAGCTG*GAG**ATG**CAATTACATTCACTTATCGCTTCAACTGCGCTCTTAATAACGTCAGCTTTGGCT**CATCATCATCATCATCAT**GCTACTTCCTCTTC	Forward primer for 6xHis HPF2 cloning in yeast, *PvuII restriction site*, Signal sequence including **ATG**, **6xHis sequence**
SLB65	GC*TCTAGA*TGAATGACAGGTACTAGTTATCA	Reverse primer for 6xHis HPF2 cloning in yeast, *XbaI restriction site* (+1384 bp after ATG)

APPENDIX FOUR

Appendix 4: Yeast strains developed and used in this study

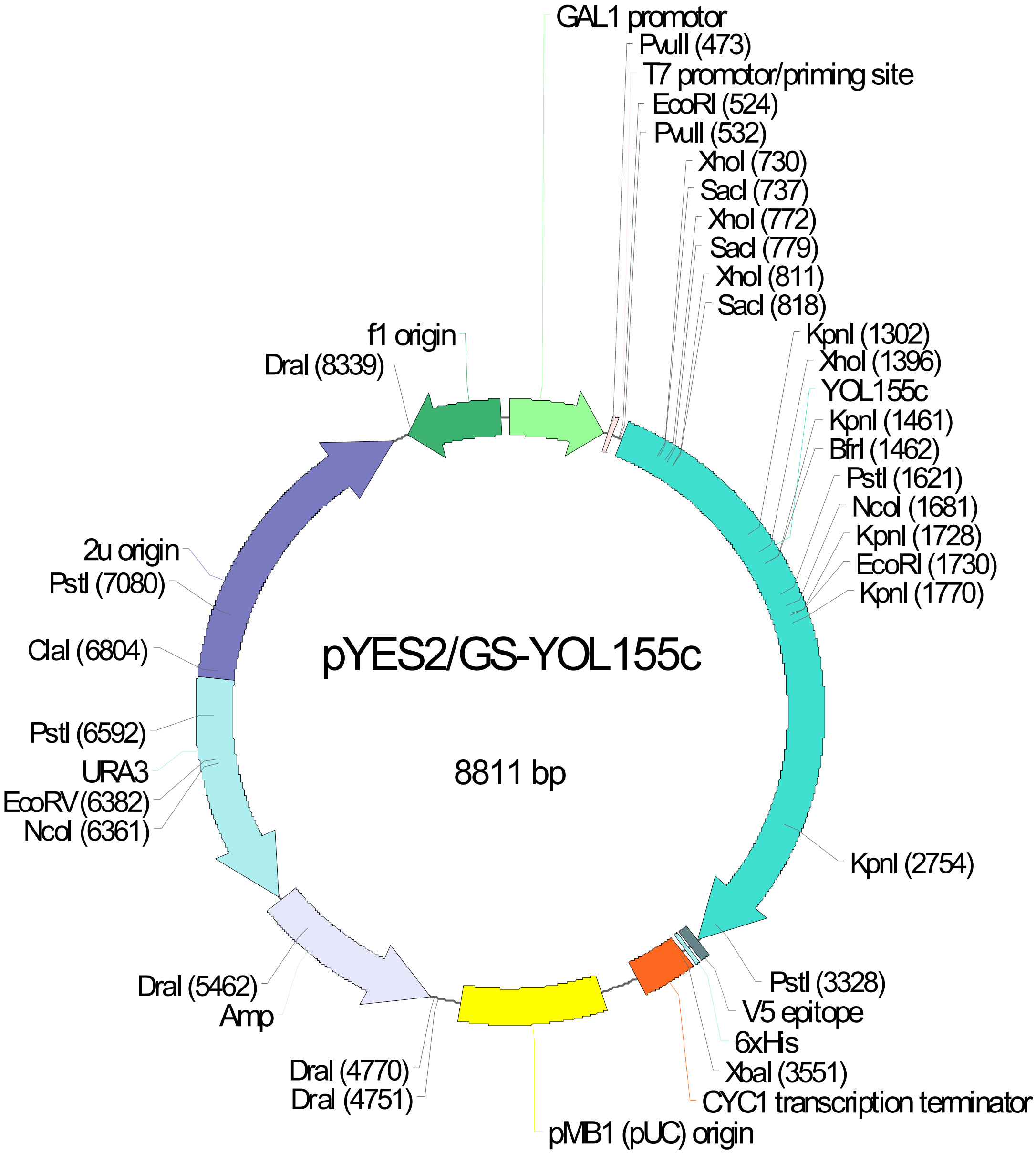
Strain	**Genotype**	**Reference, source or derivation**
FY833	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$	Winston *et al*., (1995)
FY834	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$	Winston *et al*., (1995)
S288c diploid	*MAT***a**/α *his3Δ200/his3Δ200 ura3-52/ ura3-52 leu2Δ1/ leu2Δ1 lys2Δ202/ lys2Δ202 trp1Δ63/ trp1Δ63 GAL2*$^+$*/ GAL2*$^+$	This study
SB1	*MAT***a**/α *his3Δ200/his3Δ200 ura3-52/ ura3-52 leu2Δ1/ leu2Δ1 lys2Δ202/ lys2Δ202 trp1Δ63/ trp1Δ63 GAL2*$^+$*/ GAL2*$^+$ *yol155c::URA3*/*YOL155c*	This study
SB2	*MAT***a**/α *his3Δ200/his3Δ200 ura3-52/ ura3-52 leu2Δ1/ leu2Δ1 lys2Δ202/ lys2Δ202 trp1Δ63/ trp1Δ63 GAL2*$^+$*/ GAL2*$^+$ *yil169c::HIS5*/*YIL169c*	This study
SB3	*MAT***a**/α *his3Δ200/his3Δ200 ura3-52/ ura3-52 leu2Δ1/ leu2Δ1 lys2Δ202/ lys2Δ202 trp1Δ63/ trp1Δ63 GAL2*$^+$*/ GAL2*$^+$ *ydr055w::HIS5*/*YDR055w*	This study
SB4	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ *yol155c::URA3*	This study
SB5	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ *yol155c::URA3*	This study
SB6	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ *yil169c::HIS5*	This study
SB7	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ *yil169c::HIS5*	This study
SB8	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ *ydr055w::HIS5*	This study
SB9	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ *ydr055w::HIS5*	This study
SB10	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ p415*GAL1-HPF1*	This study
SB11	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ p415*GAL1-HPF1*	This study
SB12	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ p415*GAL1-HPF1'*	This study
SB13	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ p415*GAL1-HPF1'*	This study
SB14	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ p415*GAL1-HPF2*	This study

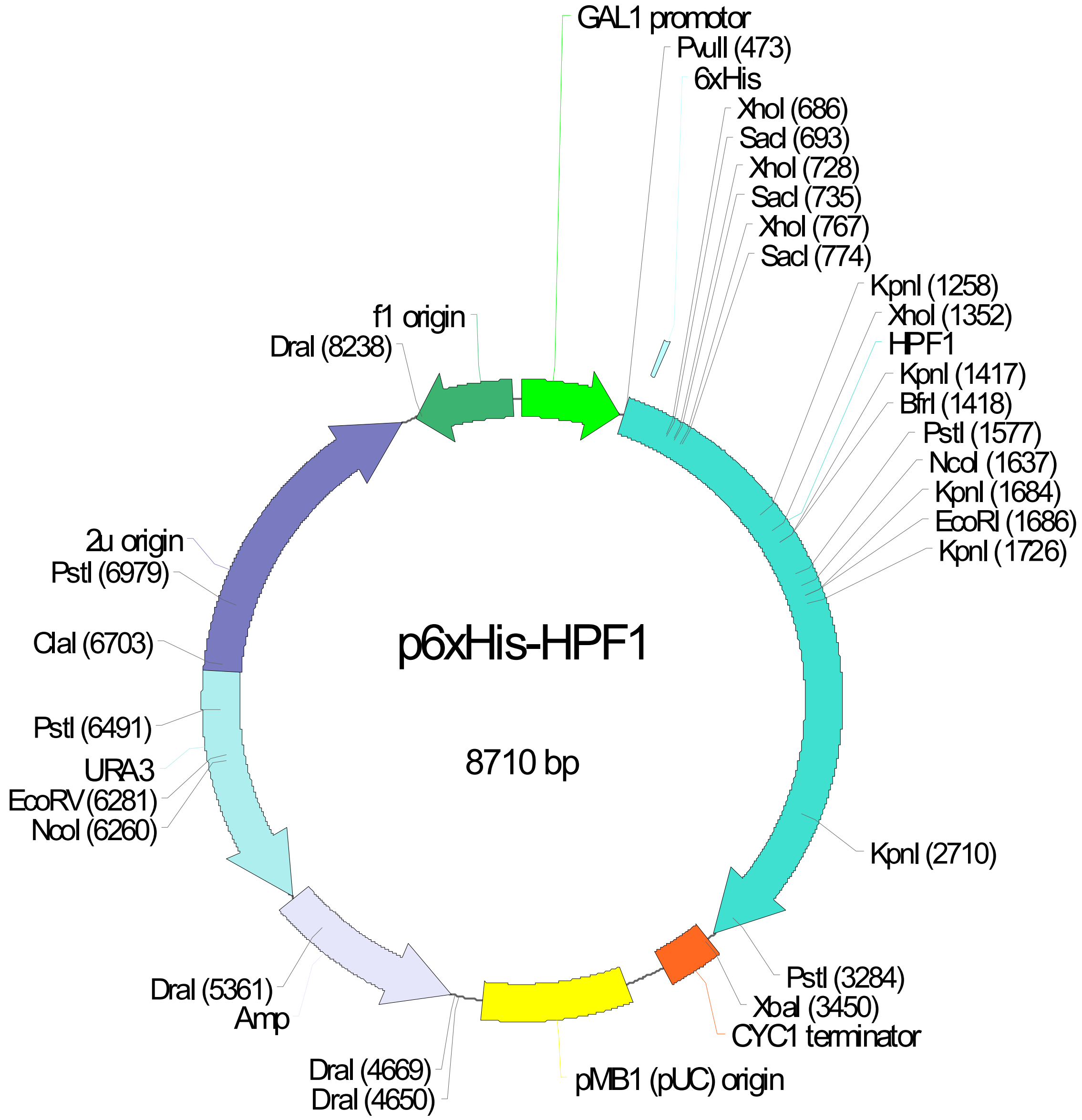
SB15	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ p415*GAL1-HPF2*	This study
SB16	*MAT**a**/α his3Δ200/his3Δ200 ura3-52/ ura3-52 leu2Δ1/ leu2Δ1 lys2Δ202/ lys2Δ202 trp1Δ63/ trp1Δ63 GAL2*$^{+}$*/ GAL2*$^{+}$ *yol155c::URA3/ yol155c::URA3*	This study
SB17	*MAT**a**/α his3Δ200/his3Δ200 ura3-52/ ura3-52 leu2Δ1/ leu2Δ1 lys2Δ202/ lys2Δ202 trp1Δ63/ trp1Δ63 GAL2*$^{+}$*/ GAL2*$^{+}$ *yil169c::HIS5/ yil169c::HIS5*	This study
SB18	*MAT**a**/α his3Δ200/his3Δ200 ura3-52/ ura3-52 leu2Δ1/ leu2Δ1 lys2Δ202/ lys2Δ202 trp1Δ63/ trp1Δ63 GAL2*$^{+}$*/ GAL2*$^{+}$ *ydr055w::HIS5/ ydr055w::HIS5*	This study
SB19	*MAT**a** his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ *yol155c::URA3 yil169c::HIS5*	This study
SB20	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ *yol155c::URA3 yil169c::HIS5*	This study
SB21	*MAT**a** his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ *yol155c::URA3 ydr055w::HIS5*	This study
SB22	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ *yol155c::URA3 ydr055w::HIS5*	This study
SB23	*MAT**a** his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ p415*GAL1*	This study
SB24	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ p415*GAL1*	This study
SB25	*MAT**a** his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ *yol155c::URA3 yil169c::HIS5 ydr055w::HIS5*	This study
SB35	*MAT**a** his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ pYES2/GS-*YOL155c*	This study
SB39	*MAT**a** his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^{+}$ p6xHis-*HPF1*	This study
SB41	*MAT**a** his3Δ200 ura3-52 LEU2*$^{+}$ *lys2Δ202 trp1Δ63 GAL2*$^{+}$	This study
SB42	*MAT**a** his3Δ200 ura3-52 leu2Δ1 lys2Δ202 TRP1*$^{+}$ *GAL2*$^{+}$	This study
SB43	*MATα his3Δ200 ura3-52 LEU2*$^{+}$ *lys2Δ202 trp1Δ63 GAL2*$^{+}$	This study
SB44	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 TRP1*$^{+}$ *GAL2*$^{+}$	This study
SB45	*MAT**a** his3Δ200 ura3-52 LEU2*$^{+}$ *lys2Δ202 trp1Δ63 GAL2*$^{+}$ *yol155c::URA3*	This study
SB46	*MAT**a** his3Δ200 ura3-52 leu2Δ1 lys2Δ202 TRP*$^{+}$ *GAL2*$^{+}$ *yol155c::URA3*	This study
SB47	*MATα his3Δ200 ura3-52 LEU2*$^{+}$ *lys2Δ202*	This study

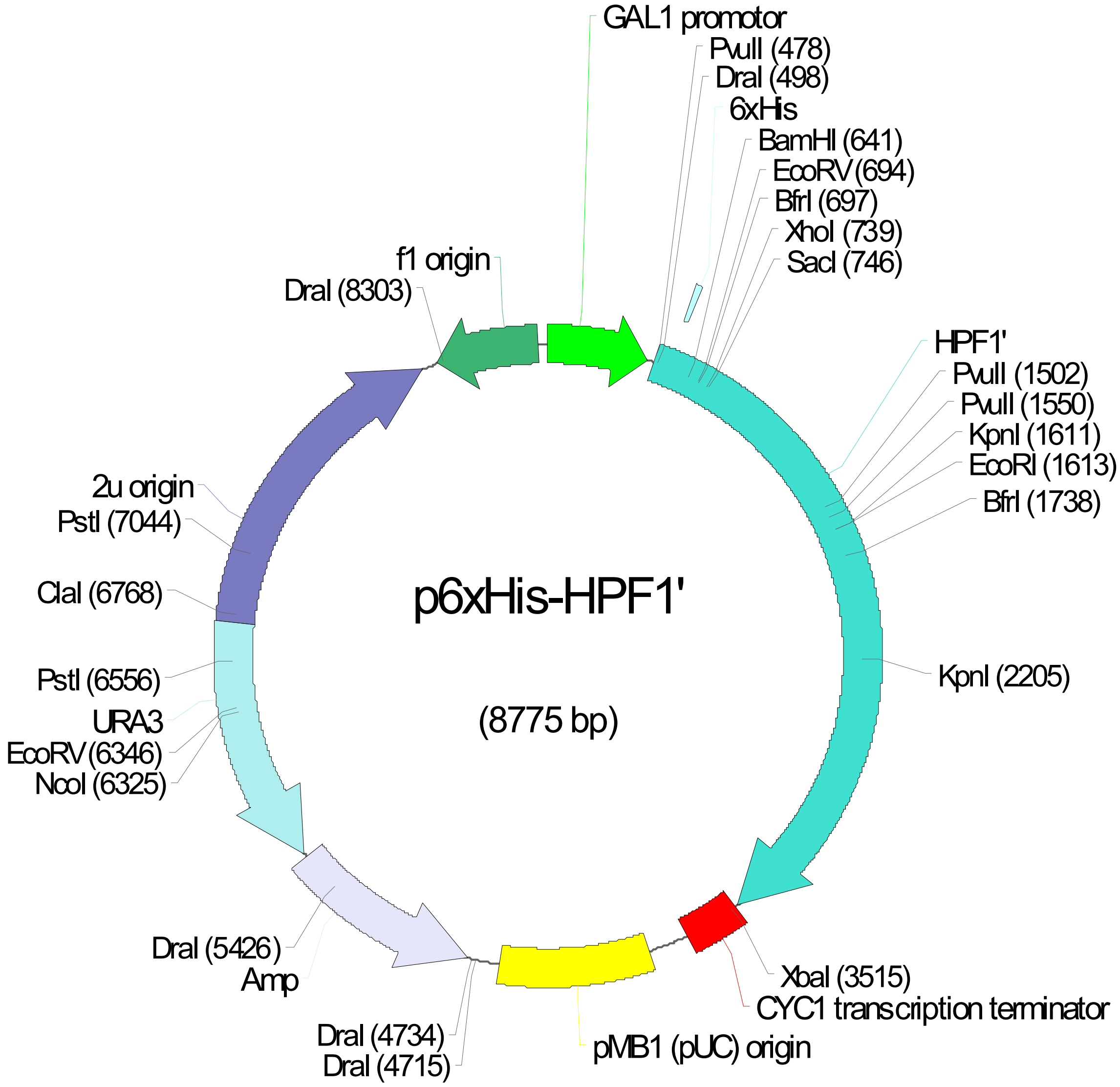
	trp1Δ63 GAL2$^+$ *yol155c::URA3*	
SB48	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 TRP1*$^+$ *GAL2*$^+$ *yol155c::URA3*	This study
SB49	*MAT***a** *his3Δ200 ura3-52 LEU2*$^+$ *lys2Δ202 trp1Δ63 GAL2*$^+$ *yil169c::HIS5*	This study
SB50	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 TRP1*$^+$ *GAL2*$^+$ *yil169c::HIS5*	This study
SB51	*MATα his3Δ200 ura3-52 LEU2*$^+$ *lys2Δ202 trp1Δ63 GAL2*$^+$ *yil169c::HIS5*	This study
SB52	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 TRP1*$^+$ *GAL2*$^+$ *yil169c::HIS5*	This study
SB53	*MAT***a** *his3Δ200 ura3-52 LEU2*$^+$ *lys2Δ202 trp1Δ63 GAL2*$^+$ *yol155c::URA3 yil169c::HIS5*	This study
SB54	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 TRP1*$^+$ *GAL2*$^+$ *yol155c::URA3 yil169c::HIS5*	This study
SB55	*MATα his3Δ200 ura3-52 LEU2*$^+$ *lys2Δ202 trp1Δ63 GAL2*$^+$ *yol155c::URA3 yil169c::HIS5*	This study
SB56	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 TRP1*$^+$ *GAL2*$^+$ *yol155c::URA3 yil169c::HIS5*	This study
SB57	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ p6xHis-*HPF1'*	This study
SB59	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ p6xHis-*HPF2*	This study
SB61	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn1Δ::kanMX4* p6xHis-*HPF1*	Euroscarf/This study
SB62	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn2Δ::kanMX4* p6xHis-*HPF1*	Euroscarf/This study
SB63	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn4Δ::kanMX4* p6xHis-*HPF1*	Euroscarf/This study
SB64	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn5Δ::kanMX4* p6xHis-*HPF1*	Euroscarf/This study
SB65	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn10Δ::kanMX4* p6xHis-*HPF1*	Euroscarf/This study
SB66	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pmt1Δ::kanMX4* p6xHis-*HPF1*	Euroscarf/This study
SB67	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 och1Δ::kanMX4* p6xHis-*HPF1*	Euroscarf/This study
SB68	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 van1Δ::kanMX4* p6xHis-*HPF1*	Euroscarf/This study
SB69	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn1Δ::kanMX4* p6xHis-*HPF1'*	Euroscarf/This study
SB70	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn2Δ::kanMX4* p6xHis-*HPF1'*	Euroscarf/This study
SB71	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn4Δ::kanMX4* p6xHis-*HPF1'*	Euroscarf/This study
SB72	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf/This study

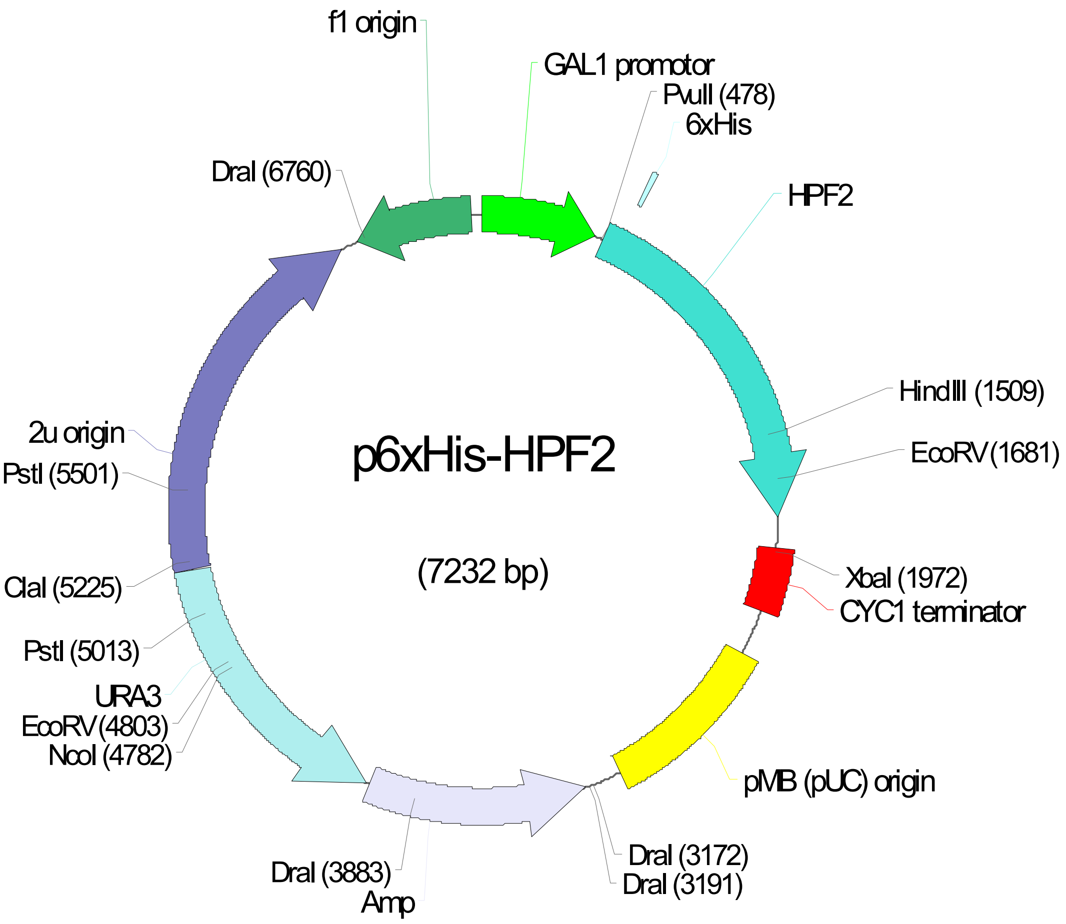
	mnn5Δ::kanMX4 p6xHis-*HPF1'*	
SB73	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn10Δ::kanMX4* p6xHis-*HPF1'*	Euroscarf/This study
SB74	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pmt1Δ::kanMX4* p6xHis-*HPF1'*	Euroscarf/This study
SB75	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 och1Δ::kanMX4* p6xHis-*HPF1'*	Euroscarf/This study
SB76	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 van1Δ::kanMX4* p6xHis-*HPF1'*	Euroscarf/This study
SB77	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn1Δ::kanMX4* p6xHis-*HPF2*	Euroscarf/This study
SB78	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn2Δ::kanMX4* p6xHis-*HPF2*	Euroscarf/This study
SB79	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn4Δ::kanMX4* p6xHis-*HPF2*	Euroscarf/This study
SB80	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn5Δ::kanMX4* p6xHis-*HPF2*	Euroscarf/This study
SB81	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn10Δ::kanMX4* p6xHis-*HPF2*	Euroscarf/This study
SB82	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pmt1Δ::kanMX4* p6xHis-*HPF2*	Euroscarf/This study
SB83	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 och1Δ::kanMX4* p6xHis-*HPF2*	Euroscarf/This study
SB84	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 van1Δ::kanMX4* p6xHis-*HPF2*	Euroscarf/This study
SB85	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ pGAL-GFP	This study
SB86	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ pGAL-GFP	This study
SB87	*MAT***a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ *yol155c::URA3 yil169c::HIS5* pGAL-GFP	This study
SB88	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 GAL2*$^+$ *yol155c::URA3 yil169c::HIS5* pGAL-GFP	This study
TB1	*MAT***a** *ade2-1 his3-11 leu2-3 leu2-112 trp1-1 ura3 yil169c::LEU2*	Traude Beilharz and Trevor Lithgow
TB2	*MATα ade2-1 his3-11 leu2-3 leu2-112 trp1-1 ura3*	Traude Beilharz and Trevor Lithgow
TB3	*MAT***a** *ade2-1 his3-11 leu2-3 leu2-112 trp1-1 ura3 yol155c::URA3*	Traude Beilharz and Trevor Lithgow
TB4	*MATα ade2-1 his3-11 leu2-3 leu2-112 trp1-1 ura3 yol155c::URA3 yil169c::LEU2*	Traude Beilharz and Trevor Lithgow

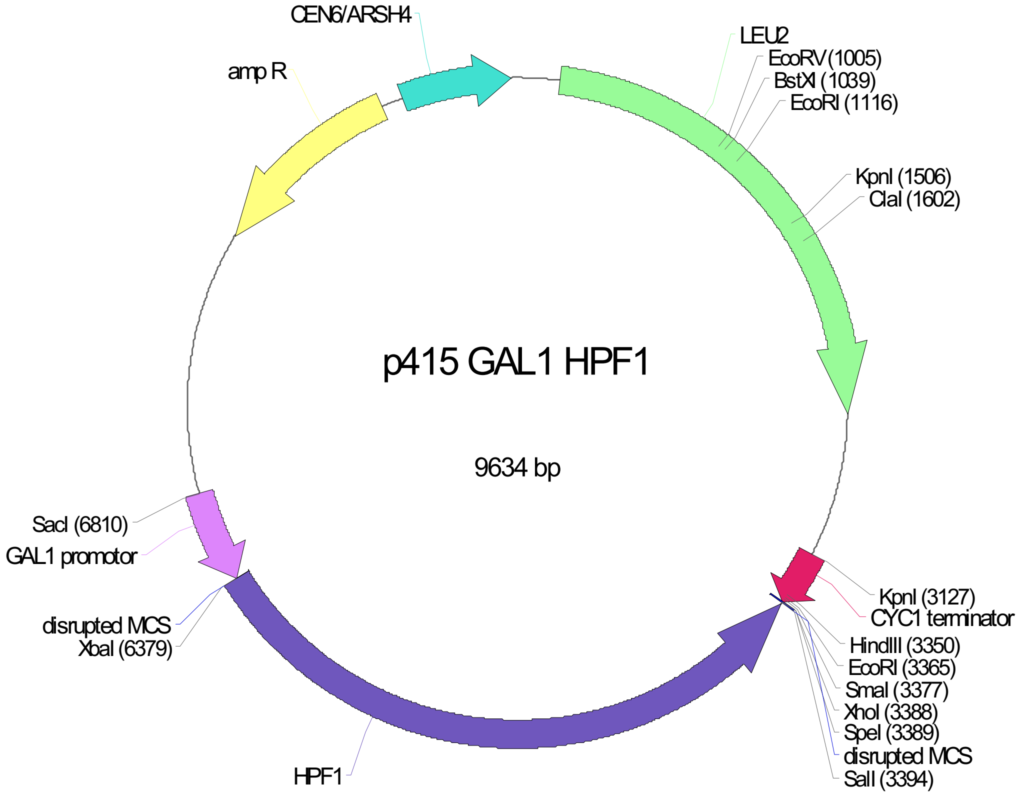
APPENDIX FIVE




GAL1 promotor
PvuII (473)
6xHis
XhoI (686)
SacI (693)
XhoI (728)
SacI (735)
XhoI (767)
SacI (774)
KpnI (1258)
XhoI (1352)
HPF1
KpnI (1417)
BfrI (1418)
PstI (1577)
NcoI (1637)
KpnI (1684)
EcoRI (1686)
KpnI (1726)
f1 origin
DraI (8238)
2u origin
PstI (6979)
ClaI (6703)
p6xHis-HPF1
8710 bp
PstI (6491)
URA3
EcoRV (6281)
NcoI (6260)
KpnI (2710)
DraI (5361)
Amp
PstI (3284)
XbaI (3450)
CYC1 terminator
DraI (4669)
DraI (4650)
pMB1 (pUC) origin


GAL1 promotor
PvuII (478)
DraI (498)
6xHis
BamHI (641)
EcoRV (694)
BfrI (697)
XhoI (739)
SacI (746)
f1 origin
DraI (8303)
HPF1'
PvuII (1502)
PvuII (1550)
KpnI (1611)
EcoRI (1613)
BfrI (1738)
2u origin
PstI (7044)
ClaI (6768)
p6xHis-HPF1'
(8775 bp)
KpnI (2205)
PstI (6556)
URA3
EcoRV (6346)
NcoI (6325)
DraI (5426)
Amp
XbaI (3515)
CYC1 transcription terminator
DraI (4734)
DraI (4715)
pMB1 (pUC) origin


f1 origin
GAL1 promotor
PvuII (478)
6xHis
HPF2
DraI (6760)
HindIII (1509)
EcoRV (1681)
p6xHis-HPF2
(7232 bp)
2u origin
PstI (5501)
ClaI (5225)
XbaI (1972)
CYC1 terminator
PstI (5013)
URA3
EcoRV (4803)
NcoI (4782)
pMB (pUC) origin
DraI (3172)
DraI (3191)
DraI (3883)
Amp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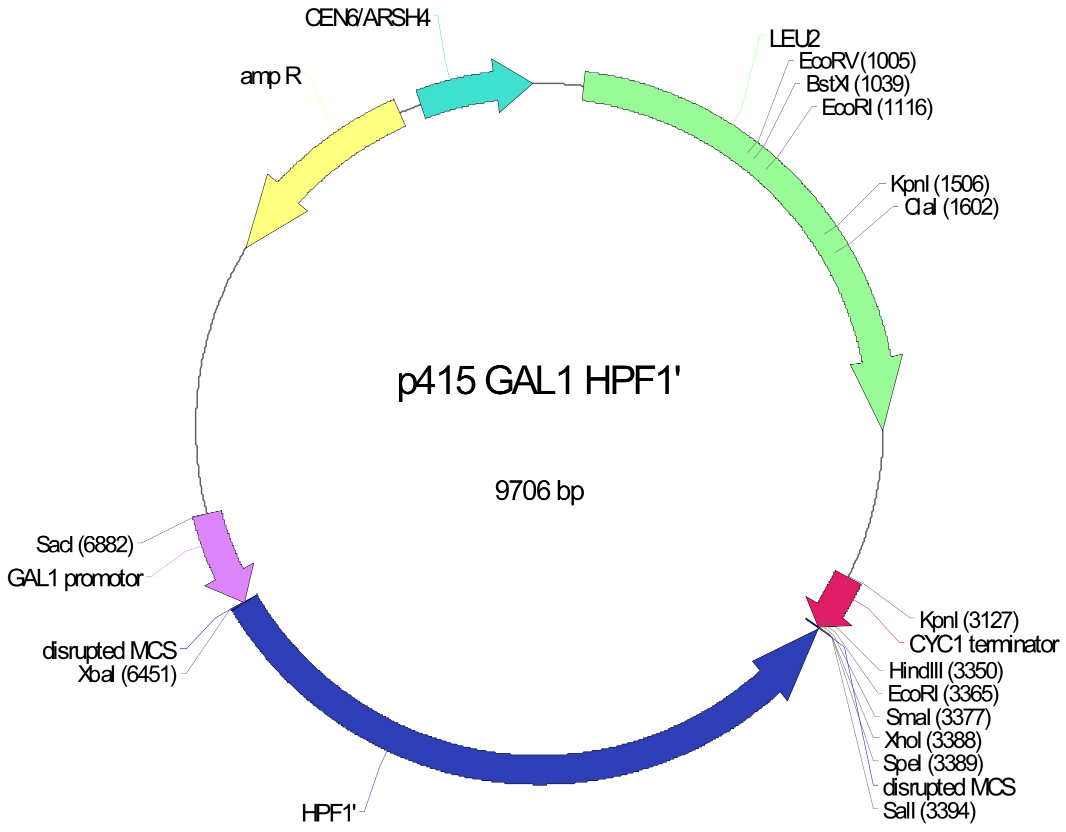
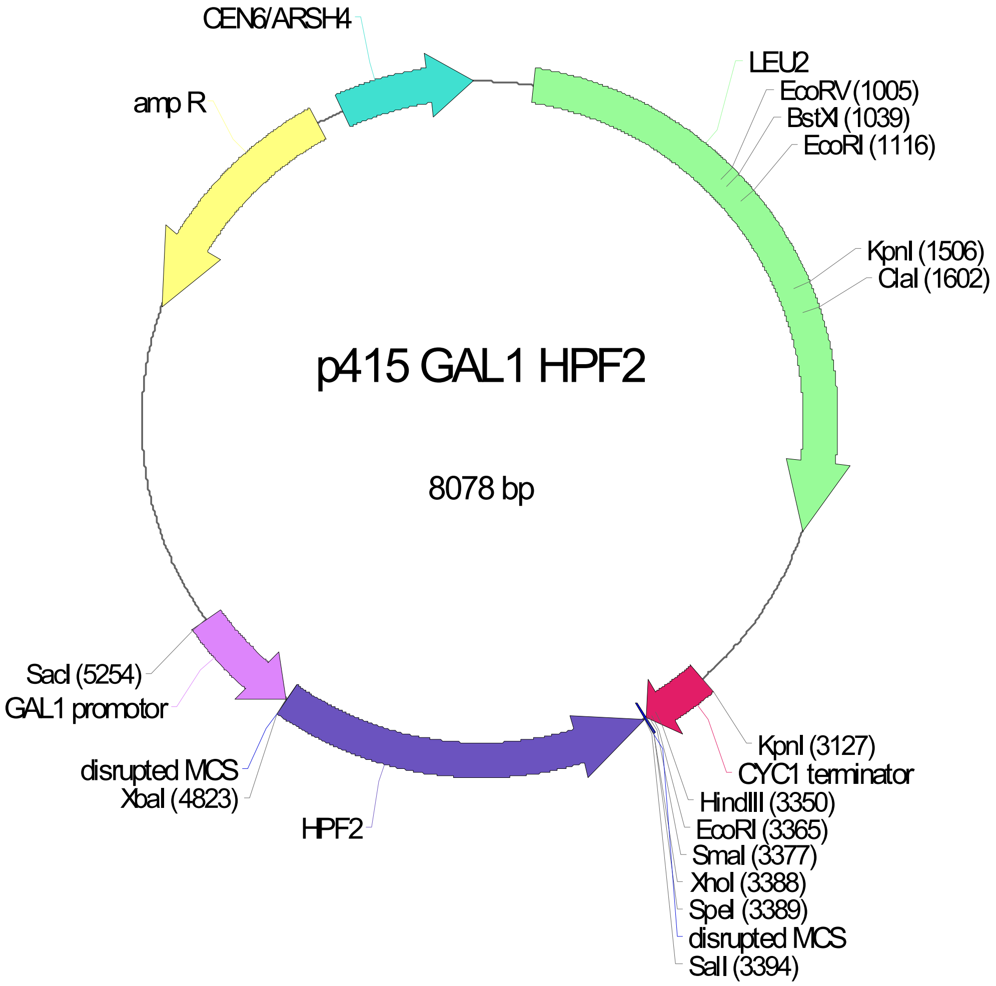
9634 bp
CEN6/ARSH4
amp R
LEU2
EcoRV (1005)
BstXI (1039)
EcoRI (1116)
KpnI (1506)
ClaI (1602)
KpnI (3127)
CYC1 terminator
HindIII (3350)
EcoRI (3365)
SmaI (3377)
XhoI (3388)
SpeI (3389)
disrupted MCS
SalI (3394)
HPF1
disrupted MCS
XbaI (6379)
SacI (6810)
GAL1 promotor


CEN6/ARSH4
amp R
LEU2
EcoRV (1005)
BstXI (1039)
EcoRI (1116)
KpnI (1506)
ClaI (1602)
p415 GAL1 HPF1'
9706 bp
SacI (6882)
GAL1 promotor
disrupted MCS
XbaI (6451)
HPF1'
KpnI (3127)
CYC1 terminator
HindIII (3350)
EcoRI (3365)
SmaI (3377)
XhoI (3388)
SpeI (3389)
disrupted MCS
SalI (3394)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8078 bp
CEN6/ARSH4
LEU2
EcoRV (1005)
BstXI (1039)
EcoRI (1116)
amp R
KpnI (1506)
ClaI (1602)
SacI (5254)
GAL1 promotor
KpnI (3127)
CYC1 terminator
disrupted MCS
XbaI (4823)
HindIII (3350)
HPF2
EcoRI (3365)
SmaI (3377)
XhoI (3388)
SpeI (3389)
disrupted MCS
SalI (3394)

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