Nutrient regulation of oligopeptide transport in *Saccharomyces cerevisiae*

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Small peptides (2–5 amino acid residues) are transported into *Saccharomyces cerevisiae* via two transport systems: PTR (Peptide TRansport) for di-/tripeptides and OPT (OligoPeptide TRansport) for oligopeptides of 4–5 amino acids in length. Although regulation of the PTR system has been studied in some detail, neither the regulation of the OPT family nor the environmental conditions under which family members are normally expressed have been well studied in *S. cerevisiae*. Using a *lacZ* reporter gene construct fused to 1 kb DNA from upstream of the genes *OPT1* and *OPT2*, which encode the two *S. cerevisiae* oligopeptide transporters, the relative expression levels of these genes were measured in a variety of environmental conditions. Uptake assays were also conducted to measure functional protein levels at the plasma membrane. It was found that *OPT1* was up-regulated in sulfur-free medium, and that Ptr3p and Ssy1p, proteins involved in regulating the di-/tripeptide transporter encoding gene *PTR2* via amino acid sensing, were required for *OPT1* expression in a sulfur-free environment. In contrast, as measured by response to toxic tetrapeptide and by real-time PCR, *OPT1* was not regulated through Cup9p, which is a repressor for *PTR2* expression, although Cup9p did repress *OPT2* expression. In addition, all of the 20 naturally occurring amino acids, except the sulfur-containing amino acids methionine and cysteine, up-regulated *OPT1*, with the greatest change in expression observed when cells were grown in sulfur-free medium. These data demonstrate that regulation of the OPT system has both similarities and differences to regulation of the PTR system, allowing the yeast cell to adapt its utilization of small peptides to various environmental conditions.

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INTRODUCTION

During studies of peptide transport in *Candida albicans*, it was noted that the yeast was able to transport peptides consisting of two to five amino acid residues. The cloning of a peptide transporter gene, *CaPTR2*, accounted for the phenotype of di-/tripeptide transport (Basrai *et al.*, 1995), but clearly a second transporter responsible for tetra-/pentapeptide uptake was present in this organism. It was this conclusion that led Lubkowitz *et al.* (1997) to clone *CaOPT1*, the tetra-/pentapeptide transporter from *C. albicans*. It was shown that CaOpt1p was not a member of either the ABC or the PTR membrane transport families: CaOpt1p contained neither the nucleotide-binding domain of the ABC family nor the conserved PTR family motifs. Two *Saccharomyces cerevisiae* ORFs, *YJL212C* and *YPR194C*, later named *OPT1* and *OPT2*, respectively, were subsequently cloned and

identified by function as encoding oligopeptide transporters (Hauser *et al.*, 2000). The phenotype of strains expressing either *S. cerevisiae* *OPT* gene was similar to that of a *S. cerevisiae* strain expressing *CaOPT1*, in that they were able to transport tetra-/pentapeptides (Hauser *et al.*, 2000; Lubkowitz *et al.*, 1997). *OPT1* was also cloned in another study and given the name *HGT1*, since it was also identified as a GSH transporter (Bourbouloux *et al.*, 2000). Little information was obtained regarding the regulation of these *OPT* genes, except that their expression was very low under normal growth conditions, requiring expression under a constitutive promoter to reveal their function as oligopeptide transporters (Hauser *et al.*, 2000; Lubkowitz *et al.*, 1998). Some information regarding expression of *OPT1* and *OPT2* may be extracted from the *S. cerevisiae* microarray expression database, as mentioned below. There is an additional putative *S. cerevisiae* *OPT* gene that was identified by homology (Yen *et al.*, 2001). This ORF, named *YGL114w*, has been examined rigorously now by sequence comparison and the protein it encodes does not appear to be a true member of the OPT family, despite having slight sequence similarity to *S. cerevisiae* Opt1p and Opt2p (Wiles *et al.*, 2006).

Abbreviations: ABC, ATP-binding cassette; OPT family, oligopeptide transport family; PTR family, peptide transport family.

Tables showing media formulations and amino acids added to media are available as supplementary data with the online version of this paper.

The number of identified genes encoding oligopeptide transporters has increased recently. Plants such as *Arabidopsis thaliana* (Osawa *et al.*, 2006) and *Oryza sativa* (rice) (Cagnac *et al.*, 2004) have been shown to contain multiple *OPT* homologues, and some information has been generated regarding expression of *OPT* genes in plants. Koh *et al.* (2002) found that *AtOPT3* expression is required for embryonic growth of *A. thaliana*, which has nine *OPT* genes. The same group have also found that the other *AtOPT* genes are expressed in different plant tissues during different developmental stages (Osawa *et al.*, 2006). The genome of the pathogenic yeast *C. albicans* contains seven *OPT* genes as determined by sequence homology. Lorenz *et al.* (2004) observed up to 30-fold induction of three of these *OPT*s upon phagocytosis by macrophages, but no induction of the other four family members in the same species. Thus, having a multiplicity of *OPT* genes in a single species provides the opportunity for differential expression of the individual genes under various environmental or developmental conditions.

Although there appear to be only two members of the *OPT* family in *S. cerevisiae*, a few microarray studies have shown differential regulation of *ScOPT1* and *ScOPT2* under stress or nutrient limitation conditions. In response to nitrogen depletion, *OPT1* was induced (Gasch *et al.*, 2000); another group observed that *OPT1* was up-regulated when cells were grown in a limited source of sulfur but not when cells were grown in a limited source of nitrogen (Boer *et al.*, 2003). These two results seem to conflict with each other with respect to the regulation of *OPT1* by nitrogen starvation, although the media, strains and growth conditions were different in these two studies.

While these microarray data have been gathered for only a few environmental conditions that regulate *OPT* expression in *S. cerevisiae*, the regulation of *PTR2*, the gene encoding the di-/tripeptide transporter in *S. cerevisiae*, has been well characterized. Earlier studies indicated that dipeptide transport was increased when cells were grown on the sole, poor nitrogen source allantoin (Island *et al.*, 1987). When cells are grown on a poor nitrogen source, *PTR2* expression is induced by the nitrogen catabolite repression pathway (Magasanik & Kaiser, 2002). Levels of Ptr2p at the membrane drop dramatically within 30 min of addition of a rich nitrogen source, such as ammonium sulfate (S. Minkin and J. M. Becker, unpublished). In addition, expression of both *PTR2* and Ptr2p is increased when cells are exposed to micromolar levels of amino acids, with tryptophan and leucine being the best inducers; *PTR2* mRNA levels peak at 40 min when induced with leucine (Perry *et al.*, 1994) and dipeptide transport peaks at 4 h when induced with tryptophan (Island *et al.*, 1987). This amino acid induction of *PTR2* is regulated through the amino acid receptor Ssy1p (Didion *et al.*, 1998).

Ssy1p is an integral membrane protein that interacts with Ptr3p and Ssy5p to form the SPS complex (Forsberg *et al.*, 2001). Ssy1p is also required for the induction of genes

encoding amino acid transporters, *AGP1*, *BAP1*, *BAP2*, *GAP1*, *GNP1*, *TAT1* and *TAT2* in *S. cerevisiae* (Kodama *et al.*, 2002). Upon receptor activation by amino acids, the signal is transduced by the SPS complex that processes the transcription factors Stp1p and Stp2p, which then migrate to the nucleus (Andreasson *et al.*, 2006). In addition, dipeptides initiate the degradation of the repressor Cup9p, which results in transcription of *PTR2* (Hauser *et al.*, 2001) by an SPS-independent pathway (Byrd *et al.*, 1998; Turner *et al.*, 2000). Our lab has recently completed a comprehensive study of *S. cerevisiae* genes involved in dipeptide utilization that has revealed a total of 42 genes involved in the expression of *PTR2* (Cai *et al.*, 2006).

Because expression of genes encoding the amino acid and di-/tripeptide transporters in *S. cerevisiae* is affected by nitrogen supply, we tested the effect of nitrogen constraints on *OPT* expression. We also tested the effect of sulfur and carbon constraints on expression because of findings by both Gasch *et al.* (2000) and Boer *et al.* (2003). Additionally, the roles of Ptr3p and Ssy1p in *OPT1* expression were investigated, since these proteins are known to play a role in the gene expression of amino acid and di-/tripeptide transporters. We were also interested in whether each individual *OPT* or YGL114wp protein had an effect on the expression of *OPT1* or *OPT2*, and if the genes were autoregulated. Our results demonstrated that the absence of sulfur, but not nitrogen or carbon, and the addition of all naturally occurring amino acids, except the sulfur-containing amino acids methionine and cysteine, led to SPS-dependent induction of *OPT1* and increased transport of a pentapeptide.

METHODS

Media. Strains were maintained on synthetic complete (SC) medium made using the original recipe for yeast nitrogen base with 2% glucose without amino acids (Wickerham, 1946, 1951). Modifications were made to Wickerham's formulation to create media with specific nutrient reductions as shown in Supplementary Table S1, available with the online journal. Reduced carbon medium contained 25% of the amount of glucose found in SC. Reduced nitrogen medium contained 10% of the amount of ammonium sulfate found in SC, with the sulfate added back via sodium sulfate. Reduced sulfur medium contained no ammonium sulfate and 10% of the amount of magnesium sulfate found in SC, with the ammonium and magnesium added back via the corresponding chloride salts. Furthermore, reduced nitrogen and sulfur medium had no ammonium sulfate and 10% of the magnesium sulfate found in SC; 10% of the ammonium and 90% of the magnesium were added via their chloride salts. Modifications were also made to SC to create media with no carbon, no nitrogen, no sulfur, and with neither nitrogen nor sulfur. In media with either no nitrogen or no sulfur, the corresponding chloride salts were added to maintain the correct concentration of other nutrients. Allantoin SC medium was made from 1.7 g yeast nitrogen base l⁻¹ (Difco) without (NH₄)₂SO₄ or amino acids, 20% glucose, and 1% allantoin as nitrogen source. As needed, 20 µg uracil ml⁻¹ or 30 µg leucine ml⁻¹ were supplemented.

Strain construction. Single gene knock-outs of *OPT1*, *OPT2*, *YGL114W*, *PTR3* and *SSY1*, and double knock-outs of *PTR3* and *SSY1*, and *PTR3* and *OPT1*, were created in the *S. cerevisiae* FY2

strain background (*MATa ura3-52*) (Winston *et al.*, 1995) using the drug resistance cassettes KanMX4, providing resistance to G418, HphMX3, providing resistance to hygromycin B, and NatMX3, providing resistance to clonNAT (nourseothricin) (Goldstein & McCusker, 1999; Guldener *et al.*, 1996). YEPD (1%, w/v, yeast extract; 2%, w/v, peptone; 2% glucose) was used in the construction of knock-out strains, with final concentrations of 200 µg G418 ml⁻¹, 300 µg hygromycinB ml⁻¹ or 100 µg clonNAT ml⁻¹ (Werner BioAgents) added when appropriate. FY2 was used throughout these experiments as the wild-type control.

Cassettes with ends homologous to the genomic region to be knocked-out were amplified by PCR. The reaction mixture consisted of 2 units RedTaq (Sigma-Aldrich), 1 pmol each primer (Table 1), 200 µM dNTPs, 1 × Sigma RedTaq buffer with MgCl₂ and 2 ng plasmid DNA; 7% DMSO was included in the PCR for amplifying the NatMX3 cassette due to its high G/C content. The components were combined on ice and immediately placed at 95 °C for 5 min to begin the reaction. The reaction conditions were as follows: 40 cycles of 1 min at 95 °C, 1 min at 52 °C for KanMX4 (Guldener *et al.*, 1996), 55 °C for HphMX3 or 52 °C for NatMX3, and 3 min at 72 °C. An additional 7 min at 72 °C were added for extension, followed by a hold at 4 °C. The PCR products were visualized on a 1% agarose/TAE gel with subsequent staining by ethidium bromide, and purified using a SOPE column (Open Biosystems).

The amplified knock-out cassettes were transformed into the required strain following a well-established protocol (Gietz & Woods, 2002). In brief, cells to be transformed were harvested in exponential phase and made competent with lithium acetate. They were allowed to recover for 2 h in YEPD at 30 °C, with shaking at 200 r.p.m., before being plated on YEPD containing the appropriate drug. Drug-resistant (presumed knock-out) colonies were picked and streaked for isolation after 4 days of incubation.

Gene knock-outs were confirmed by PCR and Southern blotting using yeast genomic DNA. For purifying the DNA, 5 ml cultures were grown overnight at 30 °C, with shaking at 200 r.p.m., in YEPD. The cells were harvested in a tabletop centrifuge at top speed for 5 min, and resuspended in 500 µl sterile water. The cells were again pelleted at top speed, this time in a microfuge, and resuspended in 300 µl breaking buffer (2%, w/v, Triton X-100, 1%, w/v, SDS, 100 mM NaCl, 10 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.0), and 450 mg glass beads and 300 µl phenol/chloroform/isoamyl alcohol (24:24:1, by vol.) were added to the tube. The tube was vortexed at top speed for 3 min, then 100 µl TE buffer was added, and the tube vortexed briefly. The sample was again microfuged at top speed for 5 min, and the aqueous layer was transferred to a new tube. The genomic DNA was ethanol precipitated and resuspended in 100 µl sterile water.

For PCR confirmation of the strains, primers were designed to anneal to sequences flanking the knocked-out region in the genomic DNA (Table 1). The PCR mixtures and conditions were as described above except that 8 ng genomic DNA was used as the template and the annealing step was carried out at 54 °C.

Southern blotting was carried out using 20 µg genomic DNA digested overnight in a volume of 200 µl with 10 µl restriction enzyme, one of two enzymes chosen for each gene, one cutting only flanking the ORF, the other cutting internally. *Bam*HI flanked *OPT1*, *OPT2* and *YGL114W*, while *Kpn*I cut *OPT1*, *Fsp*I cut *OPT2* and *Bgl*II cut *YGL114W*. *PTR3* was flanked by *Fsp*I and cut by *Sac*I, and *SSY1* was flanked by *Xho*I and cut by *Hind*III. Enzymes were used as recommended by New England BioLabs. Also included was genomic DNA from the parent strain FY2. After digestion, DNA was ethanol precipitated, resolved on a 0.7% agarose/TAE gel and blotted.

Probes were constructed by PCR amplification of the ORF from genomic DNA using probe primers specific for each gene (Table 1). The PCR mixtures and conditions were as described for PCR analysis of the knock-outs except that the annealing temperature was 55 °C. Purified PCR product was labelled using a random-prime labelling technique as follows: 50 ng DNA was incubated for 1 h at 37 °C with 3 µg random 9-mer or 5 µg random 6-mer, 1 × label buffer (900 µM HEPES, 100 µM Mg²⁺), 50 nM dNTP (no ATP), 10 mM DTT, 3 µl [³²P]dATP (50 µCi = 1.85 MBq) and 1 µl Klenow enzyme (Fisher Scientific) in 20 µl total volume. A spin-column purification was performed to purify the radioactive probe, using either Bio-Spin chromatography columns (Bio-Rad) or ProbeQuant micro columns (Amersham/GE Healthcare). Hybridization was carried out at 65 °C overnight. The blot was then placed against a Molecular Dynamics phosphor screen (Amersham/GE Healthcare) overnight, and the screen analysed on a STORM 840 densitometer with ImageQuant (Amersham/GE Healthcare).

Plasmid constructions containing a reporter gene. The promoters of *OPT1*, *OPT2* and *YGL114W* were cloned into pYLZ6 (Hermann *et al.*, 1992) upstream of the gene encoding β-galactosidase. The region 1 kb upstream of each ORF, including the start codon, was amplified by PCR. Primers (Table 1) were designed for homologous recombination into the plasmid, which had been linearized at the *Hind*III site. PCR conditions to amplify the promoters were as follows: 2 units *Pfu* DNA polymerase (Stratagene), 1 pmol each primer, 200 µM dNTPs, 1 × buffer (supplied with enzyme), 2.5 mM MgCl₂ and 8 ng genomic DNA. The components were combined on ice and immediately placed at 95 °C for 5 min to begin the reaction. Forty cycles of PCR were carried out as follows: 45 s at 95 °C, 45 s at 53 °C, and 5 min at 72 °C. An additional 7 min at 72 °C was added for extension, followed by a hold at 4 °C. The reaction products were visualized and purified as described for strain construction. Each product and the linearized plasmid were used to co-transform FY2 following the protocol by Gietz & Woods (2002). Plasmid transformants and cells transformed to make use of homologous recombination were picked after 2–3 days of growth on SC medium. Plasmids were recovered with a Zymoprep kit (Zymo Research) and sequenced. These new plasmids were named pYLZ6-*OPT1*, pYLZ6-*OPT2* and pYLZ6-*YGL114W* after the genes whose promoters they contained.

Escherichia coli was used for amplification of plasmids. Transformations were carried out using DH5α heat-shock competent cells (Invitrogen). The manufacturer's protocol was followed, with 10 ng plasmid DNA. For plasmid preps, Wizard Plus SV miniprep DNA purification spin columns (Promega) were used. *E. coli* containing plasmid was maintained on LB (1%, v/w, tryptone, 0.5%, w/w, yeast extract, 1%, w/v, sodium chloride) with 100 µg ampicillin ml⁻¹.

Growth curve assays. To determine growth curves or gene expression levels in these media, cells were first grown overnight in SC, harvested at top speed in a tabletop centrifuge, and washed twice with cold water. The various media were then inoculated at 5 × 10⁵ cells ml⁻¹, and the OD₆₀₀ of each culture was measured every 2 h for 12 h and compared to a standard curve constructed for each knock-out strain.

β-galactosidase assays. Assays for β-galactosidase activity were conducted using a protocol modified from that of Hoffman *et al.* (2002). Cells containing the empty vector pYLZ6 or the vector containing 1 kb DNA from upstream of the gene of interest (pYLZ6-*OPT1*, pYLZ6-*OPT2* or pYLZ6-*YGL114W*) were scraped from a fresh plate and grown overnight in 25 ml SC. They were harvested in a tabletop centrifuge at high speed at 4 °C, and washed three times with cold water. Cells were inoculated into fresh media at 5 × 10⁵ cells ml⁻¹ and grown for 2–12 h. If amino acids were added, this was carried out simultaneously to the addition of the cells, at

Table 1. Primers used in the construction of plasmids and strains or real-time RT-PCR

Primer	Sequence (5' to 3')	Comment
KO_029_F	GGTACGAAATACACAACCTGATAGGCGTTCATGCACTCTCACAGCTGAAGCTTCGTACGC	<i>YFR029W</i> (<i>PTR3</i>) knock-out
KO_029_R	GTGGAATATTTGCTTACATTTGGTGATTTTCGCGAGATTGGGCATAGGCCACTAGTGGATCTG	<i>YFR029W</i> (<i>PTR3</i>) knock-out
KO_114_F	CCGTTAAAGATTCTAAATCGGTACTGTAAATACTTTGAAACAGCTGAAGCTTCGTACGC	<i>YGL114W</i> knock-out
KO_114_R	CCTAATAGTGAGGGACATTTAAGCAGATGAAGAGCATGTTGCATAGGCCACTAGTGGATCTG	<i>YGL114W</i> knock-out
KO_160_F	GGGAAGTTCCTTGAGGAATATGAGTTCTGTCAACCAAATACAGCTGAAGCTTCGTACGC	<i>YDR160W</i> (<i>SSY1</i>) knock-out
KO_160_R	CCCTAAATCAGCCAGGTAACCAACTTCTTCGCTCTTTCCCGCATAGGCCACTAGTGGATCTG	<i>YDR160W</i> (<i>SSY1</i>) knock-out
KO_194_F	GGGTTTGTATACGCAATATTGCTGTTGAATTATTAGAAAACAGCTGAAGCTTCGTACGC	<i>YPR194C</i> (<i>OPT2</i>) knock-out
KO_194_R	GCGTTCTAAGAAAAGTTCTAGTCATGGATAGTGTCTGGAGCATAGGCCACTAGTGGATCTG	<i>YPR194C</i> (<i>OPT2</i>) knock-out
KO_212_F	GGCAGAAAAATAACCGCAACAATTATATAACGTCACAGAACCAGCTGAAGCTTCGTACGC	<i>YJL212C</i> (<i>OPT1</i>) knock-out
KO_212_R	CCACCATTTATCATAACCAAATGTCTCACCTTTCTTTAAGGCATAGGCCACTAGTGGATCTG	<i>YJL212C</i> (<i>OPT1</i>) knock-out
212prom_pYLZ_F	GCAGCTGAATTCAAAGCTTAGGATCCCTAAATTAACCCATTTGTACAGC	Creation of pYLZ6-OPT1
212prom_pYLZ_R	CCCAGTCACGACGTTGTAACGACCATAATTTCTAATAATTCAACAGC	Creation of pYLZ6-OPT1
194prom_pYLZ_F	GCAGCTGAATTCAAAGCTTAGGATCCCGTGTCAACTTTGCATTAACGCG	Creation of pYLZ6-OPT2
194prom_pYLZ_R	CCCAGTCACGACGTTGTAACGACCATAATTTCTAATAATTCAACAGC	Creation of pYLZ6-OPT2
ACT1 (F)	CCACCATGTTCCCAGGTATT	Real-time PCR for amplification of <i>ACT1</i> amplicon
ACT1 (R)	CCAATCCAGACGGAGTACTT	Real-time PCR for amplification of <i>ACT1</i> amplicon
OPT1 (F)	CCACCAAGCACACCTTATAAC	Real-time PCR for amplification of <i>OPT1</i> amplicon
OPT1 (R)	ATTGCCACACCTGCTTCAACA	Real-time PCR for amplification of <i>OPT1</i> amplicon
OPT2 (F)	CCTGATGCTGTGACCTACTAT	Real-time PCR for amplification of <i>OPT2</i> amplicon
OPT2 (R)	ATGCATGCGCCTATCAACCAA	Real-time PCR for amplification of <i>OPT2</i> amplicon
PTR2 (F)	CAGTGACCGTTGATCCTAAAT	Real-time PCR for amplification of <i>PTR2</i> amplicon
PTR2 (R)	CTGAAGCACAACCAGAACAAA	Real-time PCR for amplification of <i>PTR2</i> amplicon

concentrations required to fulfil auxotrophic requirements (see Supplementary Table S2 available with the online journal). When needed, GSH was added at 250 μM . Aliquots were harvested as described above for the growth assay. Using a black 96-well plate, 100 μl cells at 1×10^8 cells ml^{-1} were added to each well. A fluorescein di- β -D-galactopyranoside (FDG; Molecular Probes/Invitrogen) stock solution was made with 100 μM FDG in 25 mM PIPES (pH 7.2). The stock solution was combined 1:1 with a 5% Triton X-100, 250 mM PIPES (pH 7.2) solution; 20 μl of this final solution was added to each well. The 96-well plate was incubated at 37 °C in the dark for 4 h. It was then read with a Wallac Victor² fluorescence multi-well plate reader (PerkinElmer) with excitation at 485 nm and emission at 530 nm. Background levels were determined using FY2 transformed with the empty vector pYLZ6 grown in SC.

Leu-enkephalin uptake assays. Cells containing the empty plasmid pYLZ6 were grown in SC or no sulfur medium, with or without amino acids or GSH, as described for β -galactosidase assays. Cultures were harvested at 4 °C by centrifugation at top speed in a tabletop centrifuge, washed twice with cold 2% glucose, and resuspended at 2×10^8 cells ml^{-1} . These cells were then combined with an equal part of 2 $\times$ uptake medium [2% glucose, 400 μM citric acid/potassium phosphate buffer pH 5.5, 250 μM [³H]Leu-enkephalin at 1 $\mu\text{Ci ml}^{-1}$ (37 kBq ml^{-1})] and incubated for 10 min at 30 °C, after which the cells were filtered using a vacuum manifold fitted with 0.45 μm nitrocellulose filters (Millipore). The filters were then washed with 4 ml ice cold water, and were dissolved with CytoScint (MP Biomedicals) and counted with a scintillation counter. Preliminary experiments showed that uptake was linear for at least 10 min.

Toxic halo assays. The sensitivity of various strains to the toxic dipeptide Ala-Eth (AE) or toxic tetrapeptide Lys-Leu-Ala-Eth (KLAE) was measured as previously described (Island *et al.*, 1987). Eth (ethionine) is a toxic analogue of methionine, and its intracellular accumulation results in cell death. Yeast cells capable of transporting the toxic peptide die as a result of uptake of the peptide and subsequent intracellular release of ethionine. Cells with defective dipeptide or tetrapeptide transport will survive as indicated by a reduced halo or no halo in the halo assay system. Strains with the genetic background FY2, which is considered to be wild-type with respect to this assay, and FY2 strains carrying deletions in *OPT1*, *OPT2* and *CUP9*, were grown overnight at 30 °C in allantoin SC+Ura medium. Cells were harvested and washed three times with sterile, distilled water, counted by haemocytometer, and adjusted to 5×10^6 cells ml^{-1} . One millilitre cell suspension was added to 0.8% noble agar (3 ml) and plated onto solid (2% agar) allantoin SC+Ura medium with or without Leu. Sterile paper disks (6 mm) containing KLAE (0.2 or 0.4 μmoles) or AE (0.1 or 0.05 μmoles) were placed on the lawn of cells. The halo size was measured after 1 or 2 days of incubation at 30 °C.

Real-time RT-PCR. Approximately 3×10^8 cells of the tested deletion strains *opt1*, *opt2* and *cup9*, and wild-type (FY2), were grown overnight in allantoin SC+Ura+Leu medium. The overnight cultures were harvested, transferred into the same fresh medium, and grown for 3 h. Yeast total RNA was isolated using a RiboPure-yeast extraction kit (Ambion) and then treated to eliminate genomic DNA contamination (TURBO DNA-free kit; Ambion). The amount of total RNA was quantified by monitoring absorbance at 260 nm. To check that the genomic DNA had been eliminated, the obtained total RNA was used as the template to PCR amplify the target genes, and no PCR product was obtained. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen; 400 U) in a reaction mixture containing 1 μg oligo(dT), 1 mM deoxynucleoside triphosphates, 14 U anti-RNase and 1 μg total RNA at 42 °C for 45 min, and the reaction was stopped after 5 min by incubation at 72 °C.

Real-time PCR analysis was performed using the Opticon DNA engine (MJ Research) with the QuantiTect SYBR Green PCR kit (Qiagen). The primers used for detecting the expression of *OPT1*, *OPT2*, *PTR2* and *ACT1* are shown in Table 1. The resulting PCR products were about 100 bp. The reaction contained 12.5 μl 2 $\times$ quantitative PCR reaction mix, 15 pmol primers, run with 1 cycle of 50 °C for 2 min, 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 54 °C for 30 s and 72 °C for 15 s. The Ct value, the cycle when the sample fluorescence exceeded the threshold above background fluorescence, was determined using the DNA engine software program. The copy number of *OPT1*, *OPT2*, *PTR2* and *ACT1* genes in the tested strains was calculated based on a standard curve. The ratio was calculated by the fold-change of the target genes (*OPT1*, *OPT2* or *PTR2*) in the *cup9* strain compared to that of the wild-type, divided by the fold-change of *ACT1* in the *cup9* strain compared to that of the wild-type. The ratio of the fold-change in the wild-type was standardized to 1.0.

RESULTS

Determination of growth curves in media with reduced nutrients or without nutrients

The growth of wild-type (FY2) cells was observed in several media variations in order to determine at what point growth slowed due to nutrient restriction. SC medium (Wickerham yeast nitrogen base with 2% glucose, without amino acids) was varied by reducing or eliminating the source of carbon, nitrogen or sulfur. Wild-type cells, inoculated at 5×10^5 cells ml^{-1} from a primary culture grown in SC, were grown in these media for 12 h to determine exponential-phase growth. When grown in SC medium, exponential growth of wild-type cells continued throughout the 12 h incubation (Fig. 1). Growth of cells in media without a nitrogen source, or without nitrogen and sulfur, slowed after a few hours compared to the growth of cells in SC. In the sulfur-free medium, growth was the same as that of wild-type for the first 8 h and then growth slowed considerably, while cells grown with no carbon did not divide at all. There were no apparent differences among the growth curves of wild-type cells grown in SC and cells grown in media with reduced carbon, nitrogen or sulfur. Under the conditions used for these experiments, 10% of the sulfur or nitrogen and 25% of the carbon were sufficient for full growth during the 12 h incubation period.

Growth curves of each of the mutant strains carrying deletions of the *OPT* genes, singly or in combination, were also examined to determine if the deletions affected growth under these nutrient constraints (data not shown). All deletion strains except for the *ysl114w* Δ strain grew at the same rate as wild-type in the various media. When the *ysl114w* Δ strain was grown in SC, its growth was compromised and was comparable to wild-type grown without nitrogen. Not only did the *ysl114w* Δ strain have limited growth in liquid media, but also its colony and culture colour on SC agar plates was white in comparison to wild-type *S. cerevisiae*, which was cream coloured. There was no visible microscopic difference between *ysl114w* Δ and wild-type under growth conditions used in this study (data not

Fig. 1. Growth curves in reduced nutrient media and media lacking nutrients. Wild-type cells (FY2) were grown for 12 h in SC, reduced nitrogen, no nitrogen, reduced sulfur, no sulfur, reduced nitrogen and reduced sulfur, no nitrogen or sulfur, reduced carbon, or no carbon media. Cell density was measured every 2 h. The growth curves were conducted in quadruplicate, with nearly identical growth patterns obtained. ▼, SC; ■, reduced nitrogen or reduced sulfur or reduced carbon or reduced nitrogen and sulfur; △, no sulfur; ○, no nitrogen; +, no nitrogen and no sulfur; ▽, no carbon.

shown). Under these conditions, experiments showed that *OPT1* and *OPT2* deletion did not affect growth, and that starvation for sulfur appeared to manifest itself at 8 h whereas nitrogen starvation manifested itself at an earlier time, and the cells did not tolerate growth without a carbon source. However, the concentration used in this study for reduced carbon, nitrogen and sulfur did not affect growth noticeably. These conditions formed the basis for further studies of *OPT1* and *OPT2* expression.

OPTp-lacZ expression under limited nutrient conditions

To create reporter systems for measuring transcription of the oligopeptide transporter genes in response to various environmental conditions, the *lacZ* coding region was placed under the control of the promoters of *OPT1*, *OPT2* or *YGL114W*. We chose to use the low-copy-number plasmid pYLZ6 that was constructed specifically for the study of promoters by measurement of β -galactosidase activity in *S. cerevisiae* (Hermann *et al.*, 1992). The segment of DNA 1 kb upstream of the ORFs of *OPT1*, *OPT2* and *YGL114W*, including the start codon, was amplified and cloned into pYLZ6, immediately upstream of the *lacZ* gene.

Cells of the wild-type strain FY2, harbouring pYLZ6-*OPT1*, pYLZ6-*OPT2* or pYLZ6-*YGL114W*, were grown for 12 h in each of the previously tested media. Cells were

harvested every 2 h after inoculation of SC-grown cells into fresh media and tested for β -galactosidase activity. Cells containing pYLZ6-*OPT1* grown without sulfur showed increased β -galactosidase activity by 6 h, indicating that *OPT1* was up-regulated in response to lack of this nutrient (Fig. 2). *OPT2* and *YGL114W* were not up-regulated by sulfur starvation (data not shown). In addition, no changes in *OPT1*, *OPT2* or *YGL114W* gene expression were observed when cells were incubated without carbon or grown without nitrogen, or when grown in any of the reduced nutrient media.

OPT1p-lacZ expression in the presence of amino acids

In *S. cerevisiae*, the gene encoding the di-/tripeptide transporter, *PTR2*, is up-regulated in a SPS-dependent fashion by the addition of leucine to the growth medium. We tested whether leucine also induced *OPT1* and whether this was influenced by the sulfur content of the medium. Because the cell growth slowed after 8 h when cells were grown with no sulfur, cells of the strain FY2 bearing pYLZ6-*OPT1* were grown for 8 h in either SC or no sulfur media containing leucine or no added amino acids. Cells grown overnight in SC were placed into fresh medium with or without sulfur and with or without leucine and assessed for β -galactosidase activity every 2 h for 8 h (Fig. 3). Leucine addition resulted in increased expression of *OPT1* in the presence or absence of sulfur at the 4, 6 and 8 h time points; the highest induction of leucine was at the 6 h time point. Because of these results and because an appreciable increase of *OPT1* expression was seen at 6 h growth without sulfur (threefold

Fig. 2. Sulfur-free medium induces *OPT1* expression. Wild-type cells (FY2) harbouring pYLZ6-*OPT1*, pYLZ6-*OPT2* or pYLZ6-*YGL114W* were grown in SC medium. Additionally, cells containing pYLZ6-*OPT1* were grown in medium lacking sulfur. Aliquots were taken every 2 h for 12 h, and assayed for β -galactosidase activity, in quadruplicate. Error bars represent the SEM. β -Galactosidase activity was reported as relative fluorescence. ●, *OPT1-lacZ* no sulfur; ■, *YGL114W-lacZ* SC; □, *OPT2-lacZ* SC; ○, *OPT1-lacZ* SC.

Fig. 3. Time-course of *OPT1* induction by leucine. Wild-type cells (FY2) harbouring pYLZ6-*OPT1* were grown in SC (+ on abscissa) or no sulfur medium (– on abscissa) without supplemented amino acid (white bars) or with leucine (black bars). Aliquots were taken every 2 h for 8 h, and assayed for β -galactosidase activity, in quadruplicate. β -Galactosidase activity was reported as relative fluorescence.

increase, Fig. 2), the 6 h time point was chosen for all further experiments.

The induction of *OPT1p-lacZ* was then assayed after growth in SC and no sulfur media with each of the 20 naturally occurring amino acids individually present (Fig. 4). Leucine and tryptophan induced *OPT1* about threefold when cells were grown in SC in comparison to the no amino acid addition control. In contrast, when cells were grown in sulfur-free medium, most amino acids, except cysteine and methionine, greatly induced *OPT1* expression. The acidic amino acids – aspartic acid and glutamic acid – were the best inducers of *OPT1*, followed by tyrosine, when added to medium lacking sulfur. The sulfur-containing amino acids, cysteine and methionine, did not induce *OPT1* under these conditions; methionine even down-regulated *OPT1* about twofold in both SC and no sulfur media.

Fig. 4. Amino acids modulate *OPT1* expression. Wild-type cells (FY2) bearing pYLZ6-*OPT1* were grown for 6 h in SC (white bars) or no sulfur medium (black bars), without supplemented amino acids (none) or with each amino acid added, as indicated by one-letter abbreviations. β -Galactosidase activity was reported as relative fluorescence. This was calculated by dividing the fluorescence of β -galactosidase activity in the experimental sample by the fluorescence of β -galactosidase activity produced in the strain FY2 grown in SC medium without amino acids. The experiment was conducted in quadruplicate.

OPT1p-lacZ expression in deletion strains

Because both Ptr3p and Ssy1p are known to induce gene expression of amino acid and di-/tripeptide transporters by sensing amino acids, including leucine, we tested whether these proteins were also involved in the regulation of the gene encoding the oligopeptide transporter Opt1p. Additionally, we tested whether Opt1p autoregulated its gene expression and whether expression of *OPT2* affected the expression of *OPT1*. Cells of the six knock-out strains, *opt1Δ*, *opt2Δ*, *ptr3Δ*, *ssylΔ*, *ptr3Δ ssylΔ* and *ptr3Δ opt1Δ*, and the wild-type strain FY2, were transformed with pYLZ6-*OPT1* and grown for 6 h in the presence or the absence of: leucine; methionine or cysteine (which did not induce *OPT1* expression); aspartic acid, glutamic acid or tyrosine (the best inducers of *OPT1* when cells were grown without sulfur); or GSH (γ -Glu-Cys-Gly). GSH was tested because Opt1p is the sole transporter of GSH into *S. cerevisiae* (Bourbouloux *et al.*, 2000). These strains were then assayed for β -galactosidase activity (Fig. 5).

When knock-out strains were grown in either SC or no sulfur medium supplemented with leucine, *OPT1p-lacZ* expression in the strains with the single gene knock-outs *opt1Δ* and *opt2Δ* was generally slightly higher in the absence of sulfur, with or without leucine addition, compared to the response of the wild-type (Fig. 5A). In contrast, *OPT1p-lacZ* expression in strains containing deletions of *ptr3* and *ssyl*, genes encoding proteins in the SPS sensor complex, was not up-regulated in the presence of leucine. In fact, *OPT1p-lacZ* was not induced in any strain containing a knock-out of either *ptr3* or *ssyl*, regardless of whether cells were grown in either SC or no sulfur media, with or without any addition of leucine.

When the sulfur-containing amino acids, methionine and cysteine, were added to SC or no sulfur medium, *OPT1p-lacZ* was not up-regulated in any of the deletion mutants or in wild-type cells (Fig. 5B). Similarly, the effect of no sulfur medium was not seen when the medium was supplemented

Fig. 5. *OPT1* induction is dependent on *PTR3*, *SSY1* and *OPT2*. Wild-type cells (FY2) and the deletion mutant strains *opt1Δ*, *opt2Δ*, *ptr3Δ*, *ssy1Δ*, *ptr3Δ ssy1Δ* and *ptr3Δ opt1Δ* harbouring pYLZ6-*OPT1* were grown for 6 h in SC or no sulfur medium, with or without: leucine (A); methionine, cysteine or GSH (B); or aspartic acid, glutamic acid or tyrosine (C). β -Galactosidase activity was reported as relative fluorescence. This was calculated by dividing the fluorescence of β -galactosidase activity in the experimental sample by the fluorescence of β -galactosidase activity produced in the strain FY2 grown in SC medium without additions. For clarity, (A), (B) and (C) have different ordinate scales, and the values for no additions are repeated in each. These experiments were conducted in quadruplicate.

with GSH with one exception. The only strain to retain the up-regulation of *OPT1* in this instance was *opt1Δ* (Fig. 5B).

The effect of *PTR3*, *SSY1*, *OPT1* and *OPT2* on the expression of *OPT1p-lacZ* was also examined in the presence of the inducing amino acids aspartic acid, glutamic acid and tyrosine (Fig. 5C). Similar to results observed upon the addition of leucine (Fig. 5A), the absence of *PTR3* and *SSY1* eliminated the induction of *OPT1* in the presence of the acidic amino acids and tyrosine when cells were grown in sulfur-free medium. Unlike induction by leucine, the absence of *OPT1* and *OPT2* affected *OPT1p-lacZ* expression when cells grown without sulfur were supplemented with aspartic acid, glutamic acid or tyrosine (Fig. 5C). Without *OPT1* or *OPT2*, *OPT1p-lacZ* expression was reduced by 25 % upon addition of aspartic acid, as was *OPT1p-lacZ* expression when *opt1Δ* cells were supplemented with glutamic acid. *OPT1* was only slightly induced in *opt2Δ* cells when glutamic acid was added. When either *OPT* was knocked-out, *OPT1p-lacZ* expression was reduced by more than 60 % when sulfur-free medium was supplemented with tyrosine.

The effect of the ORF *YGL114W* was also investigated as to its potential role in *OPT1* expression. Because *ygl114wΔ* cells grew poorly even in SC (data not shown), the yield of cells from 2 l primary culture was only enough to investigate the effect of *YGL114W* on *OPT1p-lacZ* expression in SC and medium lacking sulfur, each with or without leucine or cysteine. Under all six conditions, these cells showed *OPT1* expression that was barely above background levels. Thus, the result of the influence of *YGL114W* on expression of *OPT1* could not be calculated with this strain.

Leu-enkephalin uptake under different nutrient conditions

To complement the *OPT1* gene expression data, the initial rate of uptake of Leu-enkephalin, a known pentapeptide substrate of Opt1p (Hauser *et al.*, 2000), was measured to assess the functionality of Opt1p. Cells grown under the same conditions used in the *lacZ* study were assayed for their ability to accumulate [³H]Leu-enkephalin over 10 min (Fig. 6). FY2 cells harbouring the empty vector pYLZ6 were grown in either SC or no sulfur medium, with or without amino acids and GSH (Fig. 6A). Consistent with the *lacZ* assay results, growth in the presence of either cysteine or GSH did not increase Leu-enkephalin uptake, and the addition of methionine reduced transport of Leu-enkephalin to half of that observed in FY2 cells grown in

SC. Growth in sulfur-free medium with the addition of aspartic acid, glutamic acid, or tyrosine increased the ability of the cells to transport the pentapeptide (Fig. 6B). Leucine increased the transport capability of FY2 when grown without sulfur, but did not increase transport when cells had been grown in SC supplemented with leucine, even though β -galactosidase activity suggested leucine increased *OPT1* expression in this medium.

When *opt2Δ* cells were tested for their ability to accumulate [³H]Leu-enkephalin (Fig. 6B), results were consistent with those of the *lacZ* assay; compared to FY2, Leu-enkephalin uptake was reduced when aspartic acid was supplemented and similar to unsupplemented SC when tyrosine was added. The effect of the absence of *PTR3*, *SSY1* and *OPT1* on Leu-enkephalin uptake was tested after cells were grown without sulfur and supplemented with leucine (Fig. 6C). As expected, leucine did not lead to increased uptake in *ptr3Δ* or *ssy1Δ* cells. The *opt1Δ* strain showed background to be half that of the wild-type strain grown in SC.

Regulation of peptide transport by the addition of leucine and by *CUP9*

To test the effect of Cup9p and the addition of leucine on peptide utilization, the sensitivity of wild-type and *cup9* strains to AE and KLAЕ was examined with or without the addition of leucine (Fig. 7). Deletion of *CUP9* increased the sensitivity to AE and KLAЕ in the presence or absence of leucine (compare A to B, C to D, E to F, and G to H). We also found that the sensitivity of wild-type to AE and KLAЕ was regulated by leucine (compare A to E, and C to G) but that leucine did not markedly affect AE and KLAЕ sensitivity in the *cup9* deletion strain (compare B to F, and D to H). The *opt1* deletion strain showed the same sensitivity to AE and KLAЕ as the wild-type, and the *opt2* deletion strain showed the same sensitivity to AE as the wild-type but no sensitivity to KLAЕ (data not shown), demonstrating that KLAЕ is transported by Opt2p and not by Opt1p.

To further determine whether Cup9p regulated the expression of *OPT1* and *OPT2*, the expression level of these genes was measured by real-time RT-PCR in the wild-type and *cup9* deletion strain (Fig. 8). *ACT1* was used as the internal control for gene expression, and *PTR2* was measured as well due to its known regulation by Cup9p. The expression of *PTR2* in the *cup9* strain was more than 500-fold higher than the wild-type, while the expression of *OPT2* was 12-fold higher in the *cup9* strain. There was no

Fig. 6. [^3H]Leu-enkephalin uptake. Cells were grown for 6 h with or without sulfur, the media supplemented with various amino acids, GSH or nothing. To be consistent with the media used in the lacZ assays, all cells harboured the vector pYLZ6. Cells were incubated for 10 min with 250 μM [^3H]Leu-enkephalin, in quadruplicate. Uptake is represented as the accumulation of [^3H]Leu-enkephalin [$\text{nmol (mg cell dry weight)}^{-1} (10 \text{ min})^{-1}$]. (A) Accumulation of Leu-enkephalin in the wild-type strain (FY2) after growth in either SC (white bars) or sulfur-free medium (black bars) supplemented with methionine, cysteine or GSH. (B) Accumulation of Leu-enkephalin in FY2 after growth in either SC (white bars) or sulfur-free medium (black bars) and in *opt2Δ* after growth in sulfur-free medium (checked bars). These media were supplemented with leucine, aspartic acid, glutamic acid, tyrosine or nothing. (C) Accumulation of Leu-enkephalin in FY2, *opt2Δ*, *ptr3Δ*, *ssy1Δ* and *opt1Δ* after growth in medium lacking sulfur and supplemented with leucine. Also included is the control of FY2 grown in SC with leucine added.

Fig. 7. Toxic peptide (halo) assay. The dipeptide (AE) or the tetrapeptide (KLAE) was placed on disks (visible as white rings) on top of cell lawns. The amount of AE added was 0.1 and 0.05 μmoles per disk on the left and right disks, respectively, of plates A, B, E and F, whereas KLAE was added at 0.4 and 0.2 μmoles per disk on the left and right disks, respectively, of plates C, D, G and H. Both a wild-type strain (plates A, C, E and G) and a *cup9* deletion mutant strain (B, D, F and H) were tested.

difference in *OPT1* expression level in the wild-type strain in comparison to that of the *cup9* strain.

DISCUSSION

This paper represents an advancement in the understanding of the regulation of *OPT1* in *S. cerevisiae*. Previously, limited information was known about the transcriptional regulation of the *OPT* genes in *S. cerevisiae*, although some evidence was available regarding native conditions for transcription for members of the *OPT* family in other species (Hauser *et al.*, 2001). Here we show that absence of sulfur in the medium up-regulates expression of *OPT1-lacZ*, determined by β -galactosidase assays, and Opt1p, determined by uptake assays. Boer *et al.* (2003) found similar up-regulation

Fig. 8. The expression of *OPT1*, *OPT2* and *PTR2* in the wild-type and *cup9* strains grown in allantoin SC medium+Ura+Leu. The expression level was measured by real-time RT-PCR analysis and shown as a ratio calculated by the fold-change of the target gene (*OPT1*, *OPT2* or *PTR2*) in the *cup9* strain compared to that of the wild-type, divided by the fold-change of *ACT1* in the *cup9* strain compared to that of the wild-type. The ratio of the fold-change in the wild-type was standardized to 1.0. Black bars, *OPT1/ACT1*; hatched bars, *OPT1/ACT1*; white bars, *PTR2/ACT1*.

of *OPT1* transcription in microarray studies of chemostat cultures grown with limited sulfur. Unlike these authors, we observed no change in *OPT1* expression in the absence of carbon for up to 10 h in batch culture, whereas they observed the down-regulation of *OPT1* under conditions limiting carbon. Gasch *et al.* (2000) detected a great increase in *OPT1* expression by microarray analysis when cells were depleted of nitrogen in batch culture, yet our study, as well as that of Boer *et al.* (2003), did not observe this. All three studies used different strain backgrounds considered to be wild-type, which may explain the variations in results obtained. *PTR2*, the gene encoding the di-/tripeptide transporter in *S. cerevisiae*, is also regulated by limited nutrient conditions. The expression of this transporter gene is up-regulated when cells are grown on a poor nitrogen source (Perry *et al.*, 1994).

The substrates of Ptr2p are similar to those of Opt1p in that both permeases transport small peptides, but perhaps the physiological roles of these peptide transporters are more different than initially thought. Ptr2p, which transports dipeptides and tripeptides, is likely present to scavenge amino acids as a nitrogen source, whereas the studies presented herein indicate that a function of Opt1p, which transports GSH and tetra-/pentapeptides, may be to obtain sulfur for the cell. Opt1p does not transport all tetrapeptides, however, as indicated by the observation that KLAЕ was equally toxic to the *opt1* deletion strain and the wild-type, whereas KLAЕ was not toxic to the *opt2*-deletion strain (data not shown). These results suggest that KLAЕ is not a

substrate of Opt1p but is transported by Opt2p. The role of Opt1p as the yeast GSH transporter (Bourbouloux *et al.*, 2000) may be primary with peptide transport perhaps serving a secondary function as an amino acid and sulfur scavenger from sulfur-containing peptides. This conclusion is based on the observation explored herein that sulfur regulates *OPT1* expression in *S. cerevisiae*. However, it can be inferred from the regulation of *OPT1* by amino acids that there is a role for Opt1p in yeast amino acid metabolism as well.

Our data indicate that *OPT1* is differentially regulated by various amino acids when starved for sulfur. Of the 20 naturally occurring amino acids, the acidic amino acids – aspartic acid and glutamic acid – up-regulate *OPT1* to the greatest degree, followed by tyrosine. Only the sulfur-containing amino acids do not up-regulate *OPT1* expression in sulfur-free medium. Additionally, GSH does not up-regulate *OPT1*. We were not able to analyse the effect of leucine on the regulation of Opt1p function in the KLAЕ halo assay since the *opt2* deletion strain showed no sensitivity to KLAЕ, indicating that KLAЕ is not a substrate of Opt1p (data not shown). It is likely that when these sulfur-containing compounds are transported into the cell, they supply it with enough sulfur to negate the effect of the sulfur-free medium. When *opt1Δ* cells are grown in medium lacking sulfur that has been supplemented with GSH, *OPT1p-lacZ* expression is up-regulated to the same extent as if it were grown in sulfur-free medium without any additions (Fig. 5B). Since Opt1p, which also functions as the GSH transporter, is absent, the cell is not able to take up the sulfur supplied GSH. Thus *OPT1p-lacZ* is up-regulated since it is essentially still in a sulfur-free environment. Unlike cysteine and GSH, however, methionine down-regulated *OPT1p-lacZ* and *Opt1p* when cells were grown in sulfur-free media. Perhaps in a natural environment the presence of certain amino acids indicates to the cell that particular peptides are available for scavenging. Another explanation for the regulatory phenomena observed is that no sulfur in the environment is a signal to the cell that there is a lack of, or at least very few, amino acids available. The presence of extracellular methionine is a signal that there is enough sulfur, and therefore sufficient amino acids being taken into the cell, which results in *OPT1* down-regulation.

Of all the 20 amino acids tested, leucine and tryptophan up-regulated *OPT1p-lacZ* to the highest level when cells were grown in SC medium. Similarly, the addition of leucine to the medium caused a significant increase in sensitivity to KLAЕ in the wild-type and *opt1*-deletion strains, from which it can be inferred that there is a role for leucine in the regulation of *OPT2* expression. This is similar to *PTR2* and *BAP2* induction – leucine and tryptophan are the best inducers (Didion *et al.*, 1998). Yet the fold induction of these genes due to these amino acids is dramatically different. *PTR2p-lacZ* is up-regulated 100-fold upon the addition of tryptophan or leucine (V. Narita and J. M. Becker,

unpublished data), whereas *OPT1* is up-regulated about 10-fold. Leucine and tryptophan induce *OPT1* when cells are grown without sulfur, but only moderately when compared to the other amino acids.

The amino acid sensor Ssy1p and its associated protein Ptr3p are responsible for the regulation of genes encoding amino acid transporters and the regulation of *PTR2*. The SPS system has the greatest response to leucine and tryptophan (Didion *et al.*, 1998) but is able to sense all other amino acids except proline and arginine in assays measuring *AGP1p-lacZ* expression (Iraqi *et al.*, 1999). Here we show that both *SSY1* and *PTR3* are also required for induction of *OPT1*. A microarray study investigating the effects of the SPS sensor system responding to leucine (Forsberg *et al.*, 2001) found less than a twofold change in *OPT1* expression, but the medium in which the cells were grown contained sulfur, so increased expression would not be expected. Our data show that the presence of both Ssy1p and Ptr3p is required for expression of *OPT1p-lacZ* when cells are grown in a sulfur-free environment – even in the absence of inducing amino acids. This suggests the possibility that the SPS system has the additional role of a sulfur sensor.

When cells are grown in sulfur-free medium, *OPT2* is required for the induction of *OPT1p-lacZ* by glutamic acid, and *OPT1* and *OPT2* are required for the induction of *OPT1p-lacZ* by tryptophan (Fig. 5C). If one of the OPT genes is not present at the plasma membrane, perhaps the yeast cell is able to recognize the need for a greater number of oligopeptide transporters and up-regulate either OPT gene to compensate for this deficiency. This may suggest that intracellular peptides, although rapidly degraded upon entry (Hauser *et al.*, 2000), may be involved in signalling to regulate the expression of the OPT family. Indeed, the expression of the gene encoding the di-/tripeptide transporter, *PTR2*, is regulated by dipeptides binding to Ptr3p/Ubr1p, which then degrades the *PTR2* repressor Cup9p (Turner *et al.*, 2000).

The sensitivity of the *cup9* deletion strain to KLAЕ did not change upon addition of leucine, suggesting that the regulation of *OPT2* by Cup9p is independent of leucine induction. A similar observation was made for the Cup9p regulation of *PTR2* (Byrd *et al.*, 1998). In addition, the sensitivity to AE and KLAЕ was increased in the *cup9* deletion strain, which suggests that Cup9p regulates *PTR2* and *OPT2* expression (Fig. 7). In the direct analysis of gene expression by RT-PCR, the expression of *OPT1* was not affected by *cup9* deletion, whereas the expression of *OPT2* increased 12-fold and *PTR2* increased over 500-fold (Fig. 8). The observation that the halo assay does not directly reflect the large differences in gene expression indicates that regulatory interactions are superimposed upon gene expression that control the ability of the cell to accumulate the toxic peptides (Cai *et al.*, 2006). Nevertheless, these studies show that Cup9p contributes to regulation of *OPT2* expression, but not *OPT1* expression. More studies will be needed to understand the regulator network of *OPT1* and *OPT2*

expression in the detail now known for *PTR2* expression (Cai *et al.*, 2006).

No difference was seen in either *OPT2* or *YGL114W* expression when cells were grown either without various nutrients or with reduced amounts of the nutrients, as compared to growth in SC (data not shown). These genes are, therefore, regulated by different conditions than those tested in this study. Perhaps there is a different physiological state in which Opt2p is the preferred oligopeptide transporter, and its favoured substrate is something other than a peptide. It may be that Opt2p transports a variety of biological molecules, some of which include peptides such as KLAЕ. For instance, Opt1p transports both Leu-enkephalin and GSH, but has a higher affinity for the latter, which is not a true peptide, yet Opt1p does not transport KLAЕ. Also the PTR family is able to transport β -lactams (Meredith & Boyd, 2000) even though its primary role is di-/tripeptide transport.

The role of the protein Ygl114wp is currently unknown. We could not detect *OPT1p-lacZ* expression in the *ygl114w* Δ strain, but conditions required to grow the strain were different than those required to grow cultures of all other deletion mutants and wild-type. Therefore, we were unable to draw any conclusions from the experiment. Yen *et al.* (2001) have postulated that *YGL114W* encodes a transporter that is a member of the OPT family. When we overexpressed *YGL114W* in a strain with the knock-outs *opt1* Δ , *opt2* Δ and *leu2* Δ , we observed no growth of cells when grown in the presence of either Leu-enkephalin, a substrate for Opt1p, or KLL, a substrate for Opt2p (A. M. Wiles and J. M. Becker, unpublished). Nevertheless, cells lacking *YGL114W* double only every 12 h when grown in SC medium, and culture colour is different than that of wild-type.

We have presented direct evidence that *OPT1* and Opt1p expression is dependent on the sulfur content of the medium. Additionally, we show for the first time that expression is induced by all but the sulfur-containing amino acids, and that expression is dependent upon the presence of *PTR3* and *SSY1*. This extends the roles of Ptr3p and Ssy1p to regulation of all known peptide and amino acid permeases in yeast. The differential regulation of the PTR and OPT systems allows yeast to adapt its utilization of small peptides to obtain optimal growth in a variety of environmental conditions.

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