Control of Nitrogen Regulated Virulence Traits of the Human Fungal Pathogen *Candida albicans*

Steuerung von stickstoffregulierten Virulenzeigenschaften des human-pathogenen Pilzes *Candida albicans*

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I hereby declare that my thesis entitled "Control of Nitrogen Regulated Virulence Traits of the Human Fungal Pathogen *Candida albicans*" is the result of my own work. I did not receive any help or support from third parties, i.e., commercial consultants or others. All sources and / or materials applied are listed and specified in the thesis.

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Dedicated to

My mother

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TABLE OF CONTENTS

1 Summary

The yeast *Candida albicans* is a member of the normal microflora on the mucosal surfaces of the gastrointestinal and urogenital tract in healthy persons. However, it is an opportunistic pathogen that can cause a range of infections from superficial to disseminated, in response to perturbation of the normal microflora or alterations in the host immunity. *C. albicans* exhibits a variety of characteristics such as adhesion, morphogenetic switching and secreted aspartic protease production that contribute to its virulence. Expression of many of these virulence factors is controlled by the availability of essential element, nitrogen. *C. albicans* undergoes morphogenetic transition to form filaments under nitrogen starvation conditions and this switch is controlled by the ammonium permease Mep2p. However, little is known about how this signaling function of Mep2p is regulated.

Mutational analysis of Mep2p was carried out to identify the residues that confer signaling activity to this permease. The C-terminal cytoplasmic tail of Mep2p contains a signaling domain that is dispensable for ammonium transport but essential for the signaling activity of Mep2p. In this work, progressive C-terminal truncations analysis demonstrated that a $MEP2^{\Delta C433}$ allele was still able to induce filamentation while nitrogen starvation-induced filamentous growth was abolished in cells expressing a $MEP2^{\Delta C432}$ allele. Therefore, tyrosine at position 433 (Y433) is the last amino acid in Mep2p that is essential for signaling. To gain insights into how the signaling activity of Mep2p is regulated by ammonium availability and transport, conserved residues that have been implicated in ammonium binding or uptake were mutated. Mutation of D180, which has been proposed to mediate initial contact with extracellular ammonium, or the pore-lining residues H188 and H342 abolished Mep2p expression, indicating that these residues are important for protein stability. Mutation of F239, which together with F126 is predicted to form an extracytosolic gate to the conductance channel, abolished both ammonium uptake and Mep2p-dependent filamentation, despite proper localization of the protein. On the other hand, mutation of W167, which is assumed to participate along with Y122, F126, and S243 in the recruitment and coordination of the ammonium ion at the extracytosolic side of the cell membrane, also abolished filamentation without having a strong impact on ammonium transport, demonstrating that extracellular alterations in Mep2p can affect intracellular signaling. Mutation of Y122 reduced ammonium uptake much more strongly than mutation of W167 but still allowed efficient filamentation, indicating that the signaling activity of Mep2p is not directly correlated with its transport activity.

An important aspect in the ability of Mep2p to stimulate filamentation in response to nitrogen limitation is its high expression levels. The *cis*-acting sequences and *trans*-acting regulators that mediate *MEP2* induction in response to nitrogen limitation were identified. Promoter analysis revealed that two putative binding sites for GATA transcription factors have a central role in *MEP2* expression, as deletion of the region containing these sites or mutation of the GATAA sequences in the full-length *MEP2* promoter strongly reduced *MEP2* expression. To elucidate the roles of the GATA transcription factors *GLN3* and *GAT1* in regulating *MEP2* expression, mutants lacking one or both of these transcription factors were constructed. Mep2p expression was strongly reduced in *gln3Δ* and *gat1Δ* single mutants and virtually abolished in *gln3Δ gat1Δ* double mutants. Deletion of *GLN3* strongly inhibited filamentous growth under limiting nitrogen conditions, which could be rescued by constitutive expression of *MEP2* from the *ADH1* promoter. In contrast, inactivation of *GAT1* had no effect on filamentation. Surprisingly, filamentation became partially independent of the presence of a functional *MEP2* gene in the *gat1Δ* mutants, indicating that the loss of *GAT1* function results in the activation of other pathways that induce filamentous growth. These findings demonstrated that the GATA transcription factors Gln3p and Gat1p control expression of the *MEP2* ammonium permease and that *GLN3* is also an important regulator of nitrogen starvation-induced filamentous growth in *C. albicans*.

C. albicans mutants lacking both the GATA transcription factors Gln3p and Gat1p were unable to grow in a medium containing an alternative nitrogen source, bovine serum albumin (BSA) as the sole nitrogen source. The ability to utilize proteins as sole source of nitrogen for growth of *C. albicans* is conferred by the secreted aspartic protease Sap2p, which degrades the proteins, and oligopeptide transporters that mediate uptake of the proteolytic products into cell. The growth defect of *gln3Δ gat1Δ* mutants was mainly caused by their inability to express the *SAP2* gene, as *SAP2* expression from the constitutive *ADH1* promoter restored the ability of the mutants to grow on BSA. Expression of *STP1*, which encodes a transcription factor that is required for *SAP2* induction in the presence of proteins, was regulated by Gln3p and Gat1p. Forced expression of *STP1* from a tetracycline-inducible promoter bypassed the requirement of the GATA transcription factors for growth of *C. albicans* on proteins. When preferred nitrogen sources are available, *SAP2* is repressed and this nitrogen catabolite repression of *SAP2* was correlated with downregulation of *STP1* under these conditions. Tetracycline-induced *STP1* expression abolished nitrogen catabolite repression of *SAP2*, demonstrating that regulation of *STP1* expression levels by the GATA transcription factors is a key aspect of both positive and negative regulation of *SAP2*

expression. Therefore, by using a regulatory cascade in which expression of the specific transcription factor Stp1p is controlled by the general regulators Gln3p and Gat1p, *C. albicans* places *SAP2* expression under nitrogen control and ensures proper expression of this virulence determinant.

In summary, the present study illustrated how GATA factors, Gln3p and Gat1p, play partially overlapping, but distinct roles, in mediating the appropriate responses of *C. albicans* to the availability of different nitrogen sources. These responses are also determinants of pathogenicity of the fungus. The relative contributions of Gln3p and Gat1p vary with their target genes and the availability of nitrogen source. Overall, these findings provide us with a better understanding of the molecular basis of some of the important processes that help in adaptation of *C. albicans* to various environmental conditions.

1 Zusammenfassung

Der Hefepilz *Candida albicans* ist ein harmloser Kommensale auf den Schleimhäuten des Gastrointestinal- und Urogenitaltrakts der meisten gesunden Menschen. Bei einer Störung der natürlichen Mikroflora oder des Wirtsimmunsystems kann der Pilz jedoch auch oberflächliche und sogar systemische Infektionen verursachen. *C. albicans* weist eine Reihe von Eigenschaften auf, die zur Virulenz des Erregers beitragen. Dazu gehören die Adhärenz an unterschiedliche Wirtsoberflächen, die morphologische Variabilität des Pilzes und die Sekretion von Aspartatproteasen. Die Expression vieler dieser Virulenzfaktoren wird unter anderem durch die Verfügbarkeit von Stickstoff reguliert. Unter Stickstoffmangelbedingungen wechselt *C. albicans* vom Wachstum als sprossende Hefe zum filamentösen Wachstum, und dieser Wechsel wird durch die Ammoniumpermease Mep2p reguliert. Wie die Induktion des filamentösen Wachstums durch Mep2p kontrolliert wird, ist jedoch weitgehend unbekannt.

In der vorliegenden Arbeit wurde eine Mutationsanalyse von Mep2p durchgeführt, um Aminosäuren zu identifizieren, die an der Signalfunktion dieser Permease beteiligt sind. Die C-terminale cytoplasmatische Domäne von Mep2p wird für den Ammoniumtransport nicht benötigt, ist jedoch essentiell für die Signaltransduktion. Progressive C-terminale Verkürzungen von Mep2p zeigten, dass ein $MEP2^{\Delta C433}$-Allel immer noch in der Lage war, das filamentöse Wachstum zu induzieren, wohingegen die Deletion einer weiteren Aminosäure die Morphogenese blockierte. Das Tyrosin an Position 433 (Y433) ist deshalb die letzte Aminosäure, die für die Signalfunktion von Mep2p essentiell ist. Um besser zu verstehen, wie die Signalaktivität von Mep2p durch die Verfügbarkeit und den Transport von Ammonium reguliert wird, wurden verschiedene hochkonservierte Aminosäuren mutiert, die vermutlich an der Bindung oder dem Transport von Ammonium in die Zelle beteiligt sind. Die Mutation von D180, von dem postuliert wurde, dass es den initialen Kontakt mit extrazellulärem Ammonium ermöglicht, oder der im Transportkanal lokalisierten Histidine H188 und H342 hatte zur Folge, dass Mep2p nicht mehr exprimiert wurde, so dass diese Aminosäuren vermutlich für die Proteinstabilität wichtig sind. Die Mutation von F239, das zusammen mit F126 eine extracytosolische Pforte zur Transportpore bildet, verhinderte trotz korrekter Membranlokalisation sowohl den Ammoniumtransport als auch das filamentöse Wachstum. Allerdings führte auch die Mutation von W167, das vermutlich zusammen mit Y122, F126 und S243 an der Rekrutierung des Ammoniumions an der extrazellulären Seite der Membran beteiligt ist, zur Blockierung des filamentösen Wachstums, obwohl der

4

Ammoniumtransport kaum beeinflusst war. Dies zeigte, dass die intrazelluäre Signaltransduktion durch extrazelluläre Veränderungen in Mep2p beeinflusst werden kann. Die Mutation von Y122 reduzierte die Ammoniumaufnahme weitaus starker als die Mutation von W167, erlaubte jedoch immer noch ein effizientes filamentöses Wachstum. Die Signalaktivität von Mep2p ist deshalb offensichtlich nicht direkt mit der Transportaktivität des Proteins korreliert.

Ein wichtiger Aspekt in der Fähigkeit von Mep2p, die Morphogenese zu stimulieren, ist die vergleichsweise starke Expression des Proteins. Um die Regulation der *MEP2*-Expression aufzuklären, wurden die *cis*-regulatorischen Sequenzen und die *trans*-aktivierenden Faktoren, die die *MEP2*-Induktion unter Stickstoffmangel vermitteln, identifiziert. Eine Promotoranalyse zeigte, dass zwei mutmaßliche Bindungsstellen für GATA-Transkriptionsfaktoren eine zentrale Rolle in der *MEP2*-Expression haben, da die Deletion oder Mutation dieser GATAA-Sequenzen die Expression von *MEP2* stark reduzierte. Um die Rolle der GATA-Transkriptionsfaktoren Gln3p und Gat1p bei der Regulation der *MEP2*-Expression zu untersuchen, wurden Mutanten hergestellt, in denen die entsprechenden Gene deletiert waren. Die Expression von Mep2p war in *gln3*Δ und *gat1*Δ Einzelmutanten stark verringert und in *gln3*Δ *gat1*Δ Doppelmutanten nicht mehr nachweisbar. Die Deletion von *GLN3* hatte auch eine starke Reduktion des filamentösen Wachstums zur Folge, die durch die konstitutive Expression von *MEP2* unter Kontrolle des *ADH1*-Promotors aufgehoben wurde. Dagegen hatte die Deletion von *GAT1* keinen Einfluss auf das filamentöse Wachstum. Überraschenderweise war das filamentöse Wachstum in den *gat1*Δ Mutanten teilweise unabhängig von Mep2p, was darauf hinwies, dass in Abwesenheit von *GAT1* andere Signalwege aktiviert werden, die die Morphogenese stimulieren. Diese Ergebnisse zeigten, dass die GATA-Transkriptionsfaktoren Gln3p und Gat1p die Expression der Ammoniumpermease *MEP2* kontrollieren und dass Gln3p auch ein wichtiger Regulator des durch Stickstoffmangel induzierten filamentösen Wachstums von *C. albicans* ist.

Mutanten, in denen die beiden GATA-Transkriptionsfaktoren Gln3p und Gat1p fehlten, waren nicht mehr in der Lage, in einem Medium zu wachsen, das bovines Serumalbumin (BSA) als einzige Stickstoffquelle enthält. Die Fähigkeit von *C. albicans*, Proteine als einzige Stickstoffquelle zum Wachstum zu verwenden, wird durch die sekretierte Aspartatprotease Sap2p, die die Proteine zu Peptiden abbaut, und durch Oligopeptidtransporter, die diese Peptide in die Zelle aufnehmen, vermittelt. Der Wachstumsdefekt der *gln3*Δ *gat1*Δ Doppelmutanten war hauptsächlich durch einen Defekt in der *SAP2*-Expression verursacht, da die Expression von *SAP2* unter Kontrolle des

konstitutiven *ADH1*-Promotors die Fähigkeit zum Wachstum auf BSA wieder herstellte. Es zeigte sich, dass Gln3p und Gat1p die Expression des Transkriptionsfaktors *STP1*, der für die Induktion von *SAP2* in Gegenwart von Proteinen notwendig ist, regulieren. Bei einer Expression von *STP1* unter Kontrolle des induzierbaren Tet-Promotors waren Gln3p und Gat1p nicht mehr notwendig für das Wachstum auf Proteinen. Wenn bevorzugte Stickstoffquellen verfügbar sind, wird *SAP2* auch in Gegenwart von Proteinen reprimiert, und diese Stickstoff-Katabolitrepression korrelierte mit einer reduzierten *STP1*-Expression. Die Expression von *STP1* unter Kontrolle des Tet-Promotors hob diese Repression auf, was zeigte, dass die Regulation der *STP1*-Expression durch die GATA-Transkriptionsfaktoren eine Schlüsselrolle sowohl bei der positiven als auch bei der negativen Kontrolle der *SAP2*-Expression spielt. Eine regulatorische Kaskade, in der die Expression des spezifischen Transkriptionsfaktors Stp1p durch die allgemeinen Regulatoren Gln3p und Gat1p kontrolliert wird, stellt die Expression von *SAP2* in *C. albicans* deshalb unter Stickstoffkontrolle und gewährleistet eine angepasste Expression dieses Virulenzfaktors.

Die Ergebnisse dieser Arbeit illustrieren, dass die GATA-Faktoren Gln3p und Gat1p zum Teil überlappende aber auch spezifische Funktionen in der Anpassung von *C. albicans* an die Verfügbarkeit verschiedener Stickstoffquellen haben. Diese Anpassungsmechanismen spielen auch eine Rolle in der Pathogenität des Pilzes, wobei die relative Bedeutung von Gln3p und Gat1p vom Zielgen und der Stickstoffquelle abhängt. Diese Erkenntnisse geben einen vertieften Eiblick in die molekularen Grundlagen der Anpassung von *C. albicans* an unterschiedliche Umweltbedingungen.

2 Introduction

The fungal kingdom is incredibly diverse, and its members can inhabit an extraordinarily wide range of niches. Surprisingly, out of 1.5 million (approximate) existing fungal species only around 150 are known to be associated with human infections. Some of these occasionally infect humans, but normally grow in the environment, e.g., *Aspergillus fumigatus*, *Cryptococcus neoformans* or *Histoplasma capsulatum*. However, there are a few fungal species that seem to be extremely successful in their adaptation to the human host. These are the dermatophytes, such as members of the genus *Microsporum* or *Trychophyton*, which frequently cause skin infections, and those fungi that belong to the normal microbial flora such as some *Candida* species (Hube, 2006). Although the genus *Candida* comprises about 150 yeast species, the majority of them, ~65%, are nonpathogenic as they are unable to grow at 37°C, a trait that is certainly a prerequisite for being a successful human pathogen (Calderone, 2002). The majority of all *Candida* infections are caused by *Candida albicans*, the most frequently encountered species in clinical practice. However, the incidences of non-albicans infections such as the ones caused by *C. glabrata*, *C. tropicalis* and *C. parapsilosis* species are steadily increasing (Calderone, 2002).

Pathogenicity of *Candida albicans*

Candida albicans can be found as a harmless commensal of the human oral, gastrointestinal and vaginal mucosal surfaces. Under certain predisposing conditions, the delicate balance between the host and this otherwise normally commensal fungus may turn into a parasitic relationship, resulting in the development of infection called candidiasis. The nature and extent of the impairment of normal host defense influence the manifestation and severity of infection. In general, superficial mucocutaneous candidiasis is frequent in individuals with T-cell deficiencies, such as AIDS patients. The more serious, life-threatening, deep-seated or disseminated candidiasis is normally found in a spectrum of severely immunocompromised patients (Odds, 1988). *C. albicans* has become an ever increasing medical problem as the number of immunocompromised patients is steadily increasing either due to rising number of HIV infections or use of more aggressive medical procedures such as organ transplants and cancer chemotherapy.

    C. albicans as a well-evolved opportunistic pathogen does not rely on any single attribute to enable its conversion from a passive commensal to a life-threatening invasive

pathogen. As expected, a multitude of genes involved in diverse biological functions have been implicated in various roles in the development of the pathogenic state of *C. albicans*

2.1 Determinants of pathogenicity: Virulence factors of *C. albicans*

There are several predisposing factors for *C. albicans* to alter it from a state of a relatively quiescent commensalism to an aggressive pathogenic lifestyle. *C. albicans* acts as an alert opportunist in the presence of these factors. Host factors, like infectious, congenital and other debilitating diseases or a digression from the natural physiological status inclusive of a hormonal variation can cause an impaired state of immune function which is a prerequisite for candidiasis. Dietary factors, like excess or deficiency of certain nutrients may alter the endogenous microbial flora; mechanical factors, like trauma or occlusive injury can alter the microenvironment; medical factors such as drugs used to suppress immune activity after surgery, and medication, which alters the host defenses against specific infections are all causes for this predisposition towards candidiasis (Odds, 1988). The fungus is not a mere passive participant in the infectious process and uses several attributes which are potential pathogenicity parameters. These fungal attributes include adherence, antigenic variability as a result of change in expression pattern of cell surface composition, dimorphic transition, production of secreted hydrolytic enzymes and phenotypic switching (Calderone & Fonzi, 2001). *C. albicans* virulence is a function of a multiplicity of factors working jointly to overcome the host defenses. A lack or debility in any of these parameters will reflect negatively on its infectivity and make it difficult for *Candida* to establish itself, particularly in a healthy individual (Ghannoum & Abu-Elteen, 1990).

2.1.1 Adhesins

The cell wall of *C. albicans* is an essential and highly dynamic structure which is involved not only in several physiological functions such as maintaining the cellular morphology and osmotic protection of cell, but also in adherence, and for being antigenic it modulates the immunological response against the infection (Navarro-García *et al.*, 2001). The pathogenicity of *C. albicans* is correlated with its ability to adhere to host surfaces, an early but essential step in the establishment of infection, which is an outcome of both fungal and host cell properties. Expectedly, adherent strains of *C. albicans* are more pathogenic than strains with a less adherent phenotype (Sundstrom, 2002). Adherence of Candida cells is mediated by adhesins which include the *ALS* family of proteins and the Hwp1p surface

8

protein, which recognize specific ligands on the host surfaces. The *ALS* (agglutinin-like sequence) family includes eight genes (*ALS1* to *ALS7* and *ALS9*) that encode large cell-surface glycoproteins (Hoyer, 2001). Each Als protein consist of three domains, relatively conserved N-terminal domain which is believed to have adhesive function, a central domain consisting of tandem copies of a highly conserved 108 base pair unit of a repeated motif, and a serine-threonine rich C-terminal domain that is relatively variable in length and sequence across the family. Als proteins are uniformly distributed across the cellular surface rather than focally clumped. The cell surface localization of Als proteins is attributable to amino- and carboxy-terminal hydrophobic sequences, which are reported to function as a secretory signal sequence and glycosylphosphatidylinositol (GPI) anchor addition site, respectively. Expression profiling and mutational analysis have shown enormous diversity within the Als family either due to differential regulation of *ALS* genes or to variability in size because of differences in the number of 108 bp tandem repeat copies present in the central domain. This provides *C. albicans* with an array of cell wall proteins capable of recognizing and interacting with a wide range of host constituents during infection (Hoyer, 2001). *HWP1* (hyphal wall protein) is an important developmentally regulated adhesin found only on surfaces of hyphae but not yeasts or pseudohyphae of *C. albicans*. Hwp1p is a cell surface glycoprotein that acts as substrate for mammalian transglutaminases. These enzymes are thought to generate covalent cross links between Hwp1p on the fungal hyphal surface and proteins on the mammalian mucosa (Sundstrom, 2002).

2.1.2 Extracellular hydrolytic enzymes

C. albicans, like other microbial pathogens uses secretion of hydrolytic enzymes as a strategy to invade the host and cause infection. Secreted aspartyl proteases (Saps), lipases and phospholipases are the most significant extracellular hydrolytic enzymes produced by the pathogen. The proteolytic activity of *C. albicans* due to Sap production was one of the first recognized virulence attributes of this fungus (Remold *et al.*, 1968; Staib, 1965; 1969). Proteolytic activity has also been found *in vitro* in most isolates of other pathogenic *Candida* species *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis*. Less pathogenic or nonpathogenic *Candida* species do not appear to produce significant amounts of proteases, suggesting that virulence is correlated with level of Sap production. Similarly, isolates of *C. albicans* from symptomatic patients with candidiasis are reported to be significantly more proteolytic than those from asymptomatic carriers (Naglik *et al.*, 2003).

C. albicans possesses a family of ten genes, *SAP1-10,* encoding secreted aspartic proteases (Albrecht *et al.*, 2006; Monod *et al.*, 1994; 1998). While eight members of the family, Sap1p-Sap8p, are secreted into the extracellular space, the other two, Sap9p and Sap10p, are membrane anchored GPI proteins. On the basis of amino acid sequence homology two Sap subfamilies, Sap1p-Sap3p and Sap4p-Sap6p are formed. These isoenzymes are inhibited by protease inhibitor, pepstatin A, and most of them have an optimal activity at low pH values ('acid proteases').

All ten Saps of *C. albicans* are synthesized as precursors in preproprotein forms with approximately 60-200 amino acids longer than the mature protein which are processed when transported via the secretory pathway. The N- terminal secreting signal peptide (prepeptide) is removed by the signal peptidase complex in the rough endoplasmic reticulum. Active enzyme is obtained after further processing of the propetide by Kex2 proteinase in Golgi apparatus. However, alternative processing pathways for Saps, yet unidentified, must exist because a Kex2 deficient mutant secrete abnormally processed but active Sap2p (Newport & Agabian, 1997). The mature enzymes are in the range of 35-48kDa, contain sequence motifs typical for all aspartic proteases, including the two conserved aspartate residues of the active site and the conserved cysteine residues that are implicated in maintenance of its three-dimensional structure.

Most biochemical properties of Saps have been deduced from the studies on Sap2p since it is the major *in vitro* secreted protease of *C. albicans*. Sap2p acts mainly at acidic pH and has very broad spectrum of activity. It is known to degrade many human proteins at mucosal sites including extracellular matrix and host surface proteins such as keratin, collagen, laminin, fibronectin and mucin (Colina *et al.*, 1996; Morschhäuser *et al.*, 1997; Ray & Payne, 1990). The efficient removal of host barriers by the proteolytic activity *in vivo* would not only provide essential nutrients for growth (Staib, 1965), but would also reveal potential binding sites to enhance adherence of *C. albicans* (Watts *et al.*, 1998). Sap2p can also digest several host defense molecules like immunoglobulins (including IgA) and complement proteins (Kaminishi *et al.*, 1995; Rüchel, 1981) which may help in evasion of host defense by the pathogen. It is not yet clear whether the digestion of substrates by Sap2p *in vivo* is similar to that shown *in vitro* or if other Saps utilize the same substrates as Sap2p. Different Saps have different pH optima, while Sap1p-Sap3p have the highest activity at low pH, Sap4p-Sap6p have at higher pH values. This empower *C. albicans* with a proteolytic activity in pH range 2-7, which may be vital to survival and infection of the fungus in different host tissues such as vaginal mucosa (acidic pH) or oral cavity (neutral pH).

The presence of 10 Sap isoenzymes suggests that their expression may be differentially regulated depending on environmental conditions, thus providing growth advantage to *C. albicans*. *In vitro* expression analyses have revealed that a variety of conditions affect the expression of Sap genes. *SAP2* is the major proteinase which is expressed in yeast cells at 30-37ºC in media containing proteins as the sole source of nitrogen (Hube *et al.*, 1994). Interestingly, even the two alleles of *SAP2* are differentially regulated under these conditions (Staib *et al.*, 2002). Expression of *SAP1* and *SAP3* is regulated by phenotypic switching and these genes are expressed only in the opaque form of WO-1 strain (Morrow *et al.*, 1992; White *et al.*, 1993). Surprisingly, although members of acid proteinase family, *SAP4-6* genes are almost exclusively expressed during hyphal transition at neutral pH (Hube *et al.*, 1994; White & Agabian, 1995). *SAP8* expression is temperature regulated with increased levels at 25ºC than at 37ºC (Monod *et al.*, 1998). *SAP9* and *SAP10* are constitutively expressed in both yeast and hyphal growth forms and their expressions are independent of the environmental conditions (Felk *et al.*, 2002; Schaller *et al.*, 2003). Therefore, protease expression in *C. albicans* is a highly regulated process and possibly different members of the Sap family might also be regulated differentially *in vivo*. These ideas were the basis for lots of studies in which expressions of Saps were analyzed on reconstituted human epithelium (*in vitro*) and animal (*in vivo*) experimental models. Various *in vitro* models of infection (oral, vaginal) demonstrated that the *SAP1-3* subfamily is mainly expressed in the initial stages of epithelial colonization (Schaller *et al.*, 1998; 2003). In animal models, *SAP* gene expression and regulation is shown to be dependent on type and stage of *C. albicans* infections. For instance, in a mouse model of esophageal candidiasis *SAP5* and *SAP6* were strongly activated and in intravenous model *SAP4-6* were activated, while *SAP2* gene was activated only in the late stages of disseminated candidiasis (Staib *et al.*, 2000). *SAP2, SAP4-6* and *SAP9* transcripts were detected continuously in mouse model of intraperitoneal infection (Felk *et al.*, 2002). Importance of Saps in virulence of *C. albicans* is implicated by the studies of *SAP* null mutants. It seems that during mucosal infections Sap1-3 are probably the most important of Saps. While *SAP2* null mutants are reported to be avirulent in rat vaginitis model of infection, the virulence of *SAP1* and *SAP*3 null mutants was compromised (De Bernardis *et al.*, 1999). Sap4p-Sap6p appeared critical for systemic infections, as virulence of a *SAP4-SAP6* triple null mutant was strongly attenuated in both guinea pig and murine models of systemic infections while a weak attenuation was observed for *sap1Δ*, *sap2Δ* and *sap3Δ* single mutants (Hube *et al.*, 1997; Sanglard *et al.*, 1997).

Naglik *et al*. (1999; 2003) demonstrated that *SAP1, SAP3* and *SAP7* transcripts were predominantly expressed in oral candidiasis patients as opposed to carriers, while *SAP2* and *SAP5* were the most commonly expressed genes. High titre of anti-*SAP* antibodies from the sera of candidiasis patients suggest that Saps are expressed and secreted during human mucosal and systemic infections. With the information that *C. albicans* has 10 Saps which are differentially expressed and differ in their enzymatic characteristics and substrate specificities, it is likely that individual proteases may fulfill various functions during infections and help in optimal adaptation of the pathogen to various host niches. Despite being one of the most studied virulence determinant of *C. albicans*, knowledge regarding molecular basis of *SAP* gene regulation is still in infancy.

Phospholipases:

Phospholipases (PL) hydrolyse one or more ester linkages in glycerophospholipids, hence play important role in damaging cell membranes and invading host cells. High phospholipase production is correlated with increased adherence and higher mortality rate in animal models (Mayser *et al.*, 1996). Though four subclasses A, B, C and D of phospholipase have been identified in *C. albicans,* it is the PL B class encoded by genes *PLB1* and *PLB2* which is extracellular in nature. Expression of *PLB1* is regulated by nutritional supplementation, environmental factors, and the growth phase of *C. albicans* cells. The differential expression of *PLB1* in response to environmental factors may be correlated to host-specific components available to *C. albicans* (Mukherjee *et al.*, 2003). The virulence of *PLB1* null mutants was significantly attenuated for systemic and intragastric model of candidiasis (Ghannoum, 2000; Leidich *et al.*, 1998) providing the most convincing evidence for role of PLs in pathogenesis of *C. albicans* infections.

2.1.3 Morphogenetic switching

A striking feature of *C. albicans* biology is its ability to grow in variety of morphological forms. The fungus is not as usually described dimorphic, but with its ability to adopt a spectrum of morphologies, is more properly considered polymorphic or pleomorphic. *C. albicans* can reversibly convert unicellular budding yeast growth form (blastospores) to either pseudohyphal or hyphal form, collectively referred as filamentous growth form. Primary hyphae emerging from the blastospores are referred to as germ tubes until it forms a septum. Hyphae exhibit both tip-growth and cross branching, and extensive filamentous growth leads to formation of mycelium which displays hyphae with branches and lateral buds. The

formation of pseudohyphae occurs by polarized cell division when yeast cells growing by budding have elongated without detaching from the parent cells. Pseudohyphal cells are elliptical in shape and have constrictions at cell junctions whereas hyphal cells have parallel sides and true septa at cell junctions (Sudbery *et al.*, 2004). These morphological transitions often represent a response of the fungus to changing environmental conditions and this plasticity in forms allows adaptation of the fungus to different biological niches.

Each morphological form of *C. albicans* has certain unique characteristics which provide each cell type with distinct roles in the infectious process of this pathogen. Yeast form is proposed to be the one that initiate as well disseminate infection, since blastospores are generally found at the epithelial cell surface and in between epithelial cells. Filamentous form is associated with adhesion, invasion of host tissues and escape from the phagocytic immune cells (Kumamoto & Vinces, 2005). Tissues infected with *C. albicans* typically contain a mixture of yeast and filamentous cells implicating both growth forms are essential for pathogenic lifestyle (Odds, 1988). Diminished virulence of *C. albicans* mutant strains which are unable to undergo morphogenetic switching, highlighted the importance of morphogenesis in the pathogenicity of this organism (Lo *et al.*, 1997; Saville *et al.*, 2003; Zheng & Wang, 2004).

C. albicans yeast-to-filament transition can be triggered by a wide variety of *in vitro* conditions, such as presence of serum, N-acetyl glucosamine (GlcNAc), higher temperature, neutral pH, nutrient starvation e.g. carbon or nitrogen, growth under 5% CO_2 (physiological relevant concentration) (Odds, 1988). Hyphal development is stated to be dependent on two factors; the nature, number and intensity of environmental signals (outside cues), and the activity of signaling pathways (cellular response machinery) (Ernst, 2000). The environmental control of dimorphism in *C. albicans* is a huge topic, and information gathered till now suggest that depending on the environmental cues, different signal transduction pathways are activated which then regulate the targets that are required to initiate hyphal growth (Biswas *et al.*, 2007; Brown & Gow, 1999; Ernst, 2000; Whiteway & Oberholzer, 2004). Though the components of the signaling networks have been well characterized, knowledge of receptors/sensors that mediate the environmental responses is limited.

Molecular basis of morphogenesis in *C. albicans*

Molecular dissection of morphogenesis in *C. albicans* has greatly benefited from the studies on the model yeast *S. cerevisiae* which switches to a filamentous, pseudohyphal growth form, on nutrient poor media. Most of the signal transduction genes in *C. albicans* have been identified through complementation of the defects of the corresponding homolog mutants in

S. cerevisiae. Networks of signaling pathways regulate the yeast to hyphal transition in *C. albicans*, out of which the Cph1-mediated MAPK pathway and the Efg1-mediated cAMP-PKA pathway are well characterized (Fig. 1).

Environmental sensing: Upstream components of the signal transduction networks

Gpr1p, a G-protein coupled receptor along with Gpa2p, a Gα protein, is reported to act upstream of the cAMP-PKA pathway and regulate morphogenesis in *C. albicans* (Maidan *et al.*, 2005a). However, there have been conflicting reports regarding the nature of the ligand for Gpr1p. Miwa *et al.*, (2004) reported that, as in *S. cerevisiae*, Gpr1p is required for glucose induced increase in cAMP, but Maidan *et al.*, (2005b) showed that Gpr1 directly or indirectly senses the amino acid methionine. Mechanism(s) involved in activation of cAMP-PKA pathway by Gpr1p, and role of its ligand in this mechanism is not known. Gpa2p is also reported to act upstream of the MAPK pathway to regulate filamentous growth in *C. albicans* (Sanchez-Martinez & Perez-Martin, 2002).

Under limiting nitrogen conditions, an ammonium permease Mep2p is assigned a regulatory role in the induction of filamentous growth in *C. albicans* and pseudohyphal growth in *S. cerevisiae* (Biswas & Morschhäuser, 2005; Lorenz & Heitman, 1998). In *C. albicans*, Mep2p activates both the cAMP-PKA and MAPK pathway in a Ras1p dependent manner via its cytoplasmic C-terminal tail, which is essential for signaling but not for ammonium transport (Biswas & Morschhäuser, 2005). However, so far, it is not understood how Mep2p activate these signaling networks.

Intracellular signaling: Signal transduction cascades and downstream effectors

MAP Kinase Pathway

The first morphogenetic signaling components to be identified in *C. albicans* were members of a mitogen-activated protein kinase (MAPK) pathway. The MAPK cascade includes the kinases Cst20p (homolog of the p21-activated kinase [PAK] kinase Ste20p), Hst7p (homolog of the MAPK kinase [MAPKK] Ste7p), Cek1p (homolog of the Fus3 and Kss1 MAPKs) and Cph1p (homolog of transcription factor Ste12p) (Csank *et al.*, 1998; Köhler & Fink, 1996; Leberer *et al.*, 1996; Liu *et al.*, 1994). Studies have shown that Cst20p, Hst7p, Cek1p and Cph1p act sequentially in the order of a canonical MAPK cascade to induce the *C. albicans* yeast to hyphal transition in response to nutritional starvation. Null mutations at each of the loci of MAPK cascade confer hyphal defect only on certain solid starvation type media (Spider or SLAD), all these mutants filament normally in response to pH and serum, indicating that this pathway is not the only mechanism of hyphal development (Csank *et al.*,

1998; Köhler & Fink, 1996; Leberer *et al.*, 1996; Liu *et al.*, 1994). Virulence of each of *cst20Δ* and *hst7Δ* single mutants is partially affected in a mouse model of systemic candidiasis. The phosphorylation status of Cek1p, hence the activity of this pathway as a whole, is negatively regulated by a MAP kinase phosphatase, Cpp1p (Csank *et al.*, 1997). Inactivation of *CPP1* leads to constitutive hyerfilamentation phenotype which is suppressed by deletion of the Cek1p. The *cpp1Δ* mutant strains are reduced for virulence in systemic model of candidiasis (Csank *et al.*, 1997).
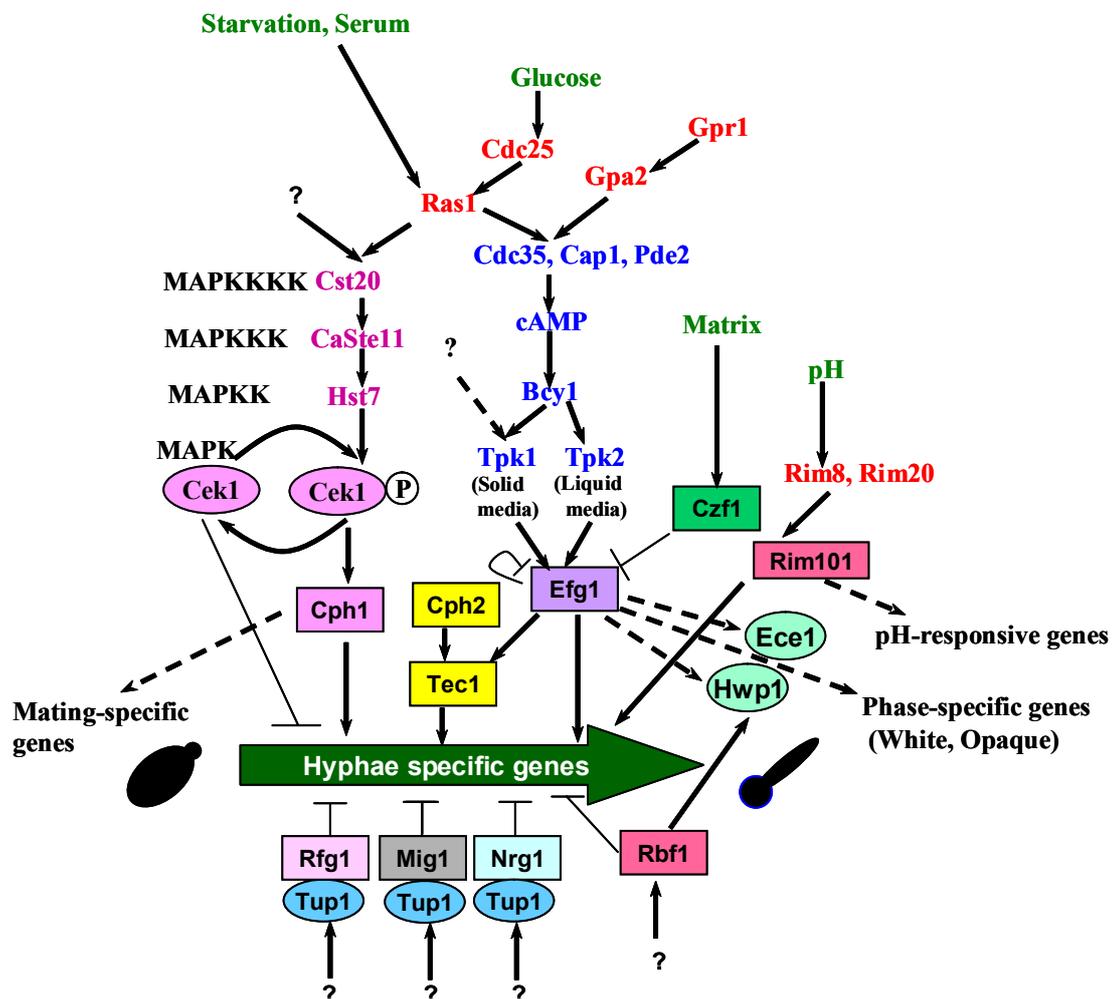

Fig. 1. Regulation of dimorphism in *C. albicans* by multiple signaling pathways (adopted and modified from Biswas *et al.,* 2007)

cAMP-PKA Pathway

A second well characterized morphogenesis pathway that acts independently but in parallel to MAPK cascade is the cyclic AMP-dependent protein kinase A (cAMP-PKA) pathway.

Homologs of *S. cerevisiae* included in the cAMP-PKA pathway are, Ras1p, Cyr1p/Cdc35p, protein kinase A and the transcription factor Efg1p (Bockmühl *et al.*, 2001; Feng *et al.*, 1999; Rocha *et al.*, 2001; Sonneborn *et al.*, 2000). In *C. albicans, ras1Δ* mutants are viable but have a severe defect in hyphal growth in response to serum and other conditions (Feng *et al.*, 1999). These defects in hyphal morphogenesis can be rescued by either exogenous cAMP or overexpressing components of the MAPK cascade, demonstrating that Ras1p functions upstream of both the cAMP as well as the MAPK pathway (Leberer *et al.*, 2001). Like Ras1p, adenylate cyclase gene *CDC35/CYR1*, is not essential for growth but is required for hyphal development in *C. albicans* (Rocha *et al.*, 2001). External cues elevate intracellular cAMP levels, whose binding to Bcy1 (the regulatory subunit of PKA) liberates it from Tpk1 and Tpk2 (the catalytic subunits of PKA), and thereby results in the activation of the catalytic subunits. Although both *C. albicans* Tpk isoforms act positively to regulate hyphal formation, they have different functional specificities, while Tpk1 seems to mediate filamentation on solid media, Tpk2 plays a prominent role in liquid medium (Sonneborn *et al.*, 2000). The transcription factor Efg1p, a basic helix-loop-helix protein, is a downstream component of the cAMP-PKA pathway. In presence of serum or GlcNAc the *efg1Δ* mutant is unable to undergo hyphal formation (Lo *et al.*, 1997; Stoldt *et al.*, 1997). On the other hand, under microaerophilic or embedded conditions hyphal formation in *efg1Δ* mutant is rather stimulated. It seems, depending on environmental cues, Efg1p has a dual role as a transcriptional activator and repressor of morphogenesis. The *efg1Δ* mutant has strong reduction in virulence, the *cph1Δ* mutant has no defect, whereas *efg1Δ cph1Δ* double mutant, with extreme filamentous growth defect, are avirulent in mouse model of infection (Lo *et al.*, 1997).

Apart from these two major signal transduction pathways, other genes like *TEC1* or *CPH2* have also been found to regulate hyphal development in *C. albicans*. Epistatis analysis has suggested that *TEC1* is one of the downstream effectors of both Efg1p and Cph2p (Lane *et al.*, 2001; Schweizer *et al.*, 2000). Morphogenesis is also regulated by a conserved pH response pathway mediated by transcription factor Rim101p. Rim8p and Rim20p are the upstream components of this pathway and are involved in proteolytic activation of Rim101p which is required for high pH induced filamentation and expression of alkaline responsive gene *PHR1* and repression of acid expressed gene *PHR2* (Davis *et al.*, 2000; El Barkani *et al.*, 2000; Porta *et al.*, 1999; Ramon *et al.*, 1999). Czf1p, a zinc-finger containing protein, is important for hyphal development in response to matrix embedding conditions (Brown *et al.*, 1999). Besides positive control, *C. albicans* hyphal development is subjected to negative

regulation by Tup1p (Braun & Johnson, 1997). Although Tup1p itself has no DNA binding activity, it is recruited to promoter of target genes through interaction with sequence specific DNA binding proteins Nrg1, Mig1, Rfg1 (Braun *et al.*, 2001; Kadosh & Johnson, 2001; Khalaf & Zitomer, 2001; Murad *et al.*, 2001a; 2001b)

These different environmental signaling pathways ultimately bring about the expression of not only hyphae specific genes such as *HWP1*, *ALS1*, *ALS3*, *SAP4-6* but also those of pathway specific genes e.g. *PHR1* and *PHR2,* all of which are important virulence determinants of *C. albicans*.

2.1.4 Phenotypic switching

Phenotypic switching in *C. albicans* is a reversible high-frequency phenomenon that is readily detectable as changes in cell or colony morphology. Common strains of *C. albicans* could be induced to switch reversibly and at high frequency between a number of variant phenotypes. Strain WO-1 undergoes 'white-opaque' switching, in which cells switched between a smooth, white colony phenotype containing round budding yeast cells to an opaque colony phenotype containing elongated oblong cells with surface pimples (Slutsky *et al.*, 1987). With the discovery of mating in *C. albicans* in late 1990s, the importance of conversion from white to opaque physiological phase was realized. Only strains that are homozygous or hemizygous at the mating-type-like (MTL) locus, i.e., **a** and α cells can undergo white-opaque switch; furthermore, only the opaque cells mate at high efficiency (Johnson, 2003; Noble & Johnson, 2007). However, there is more to white-opaque switching than just mating efficiency. Although white cells are more virulent than opaque cells in the mouse systemic model of infections, the opposite pattern is seen in a mouse skin model (Kvaal *et al.*, 1999; 1997). Several virulence attributes of *C. albicans*, including expression of adhesins, Sap production and hyphal formation have been associated with the switching phenomenon (Soll, 2002). White-opaque switching provides *C. albicans* with an extraordinary phenotypic variability and mating competence to the cells, which in turn could help in generation of genetic diversity within an individual host, thus contributing to successful adaptation of the fungus in different environments.

2.2 Nitrogen regulation in *C. albicans*

Apparently, *C. albicans* virulence is a quantitative trait caused by the contributions of a number of genes. However, a complete understanding of its pathogenicity requires insights

into how this pathogen meets its nutritional need within the host. It is not known what nutrients, e.g., nitrogen sources, are exactly utilized by *C. albicans* during infectious growth in host, though there are two obvious and abundant nitrogen sources i.e., amino acids and host proteins. Under *in vitro* conditions, the quantity and quality of nitrogen sources in growth medium governs the responses of *C. albicans*. Nitrogen starvation induces morphogenetic switching that require a functional ammonium permease Mep2p (Biswas & Morschhäuser, 2005), and availability of proteins as the sole nitrogen source results in production of Saps (Hube *et al.*, 1994; White & Agabian, 1995), and both these features have been implicated in virulence of *C. albicans*. However, not much is known about the regulatory factors that control these important nitrogen responses in *C. albicans*.

Nitrogen is an essential element for living organisms for being a major constituent of complex macromolecules such as proteins, purines, pyrimidines, enzyme co-factors, some carbohydrates and lipids, all of which are central to growth processes. Hence, nitrogen metabolism and its regulation that ensures constant supply of nitrogen in diverse environments are fundamental to growth and survival of microorganisms. For pathogenic microbes the ability to infect and cause disease is often dependent on a proper response to the nitrogen environment of the host niche due either to the need to acquire adequate nutrients from nutrient poor host sites or to coordinate the expression of virulence determinants. Numbers of studies have highlighted the importance of nitrogen regulation in virulence of human fungal pathogens. *Aspergillus fumigatus* mutants lacking the *areA* gene, a key regulator of nitrogen metabolism, are partially attenuated in murine model of pulmonary aspergillosis (Hensel *et al.*, 1998). Recent work has revealed the importance of *GAT1* (homolog of *areA*) which is required for proper expression of alternative nitrogen source utilization pathways in the virulence of *C. albicans* (Limjindaporn *et al.*, 2003). *SAP2* mutants of *C. albicans* exhibit reduced virulence in animal models of candidiasis (De Bernardis *et al.*, 1999; Hube *et al.*, 1997; Staib *et al.*, 2002), suggesting that the ability to utilize proteins as a nitrogen source is important for growth of the fungus in certain host niches. Similarily, importance of the capacity to take up amino acids for growth in mammalian hosts is demonstrated by the reduced virulence of *csh3Δ* mutants in murine disseminated disease model (Martinez & Ljungdahl, 2004). Csh3p, an endoplasmic reticulum localized chaperone, is specifically required for proper localization of amino acid permeases (AAPs), consequently the most upstream component of extracellular amino acids sensing and uptake mechanisms (Martinez & Ljungdahl, 2004). These studies emphasize the importance of utilizing available

nitrogen sources in infection process of *C. albicans,* and bring focus into significance of the factors that control nitrogen regulation.

2.2.1 Nitrogen regulatory genes: GATA transcription factors

Fungi are able to use a wide variety of compounds as nitrogen sources. Use of any compound as nitrogen source requires permeases for the transport of these compounds into the cell and enzymes for generation of ammonia by their metabolism. Once inside the cell, ammonia in the presence of NADP-linked glutamate dehydrogenase, can react with α-ketoglutarate, provided by the metabolism of carbon source of the growth medium, to produce glutamate. Glutamine synthetase catalyzes the incorporation of ammonium into glutamate to form glutamine. Glutamate and glutamine serve as the source of cellular nitrogen (Magasanik & Kaiser, 2002). Certain nitrogenous compounds like, ammonia, glutamine and glutamate are preferably used by the fungi and are considered as primary nitrogen sources, others like nitrate, nitrite, purines, amides, most amino acids and proteins are utilized only when primary nitrogen sources become growth limiting. These primary and alternative nitrogen sources are also referred to as preferred and non preferred/secondary nitrogen sources, respectively.

A conserved feature, by which selective use of available nitrogen sources is achieved in fungi, is the central role played by GATA factors. Members of this transcription factor family contain a DNA-binding zinc finger domain that recognizes the nucleotide sequences 5'-GATA-3' in the regulatory regions of their target genes, hence are called GATA factors (Marzluf, 1997). They affect a response commonly called Nitrogen Catabolite Repression (NCR) or simply nitrogen regulation. Under this control mechanism, expression of genes needed for uptake and catabolism of secondary nitrogen sources is prevented in presence of preferred nitrogen sources such as ammonium or glutamine. In the absence or limitation of preferred nitrogen sources, GATA factors activate transcription of these secondary, nitrogen sensitive, metabolic genes either alone or in conjugation with pathway specific factors. Nitrogen regulation in fungi is typically controlled by multiple GATA factors; some activate transcription while others negatively regulate expression (Magasanik & Kaiser, 2002; Marzluf, 1997). The filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* rely on a single highly conserved GATA factor, AreA and Nit2 respectively, to activate the expression of NCR sensitive genes. The homologs NmrA and Nmr1 antagonize AreA and Nit2 function, respectively, by interaction with zinc-finger domain (Marzluf, 1997). *S. cerevisiae* on the other hand, uses two, partially redundant, activating GATA factors encoded by *GLN3* and *GAT1/NIL1* (Marzluf, 1997). The relative contribution of Gln3p and Gat1p on expression of

target genes depends on the individual gene and the growth conditions (available nitrogen source). The activation of nitrogen sensitive genes by Gln3p and Gat1p is antagonized by two negatively acting GATA factors, Nil2p and Dal80p. These factors are proposed to antagonize Gln3p and Gat1p activation by competing for binding to GATA elements in the promoter of NCR regulated genes to fine tune expression. The configuration of the GATA sites and/or auxiliary elements in the promoters of nitrogen regulated genes is likely the key determinant of the binding affinities and specificities of these various GATA factors (Marzluf, 1997; Wong *et al.*, 2008). Additional control to Gln3p and Gat1p activity is provided by protein-protein interactions with Ure2p, which result in retention of GATA factors in the cytoplasm under nitrogen sufficient conditions, hence, these factors can not activate their target genes (Magasanik & Kaiser, 2002).

Several regulatory systems have been implicated in the response of *C. albicans* to nitrogen source availability. In analogy to *S. cerevisiae* the external amino acid sensing system in *C. albicans* is also called SPS sensor, consisting of homologs of Ssy1p, Ptr3p and Ssy5p. This plasma membrane associated sensor complex detects presence of amino acids and induces the expression of several permeases that facilitate uptake of amino acids from the medium (Brega *et al.*, 2004). Primary amino acid sensor component of the complex is encoded by *CSY1*, and the *csy1Δ* mutants fail to activate amino acid permease expression (Brega *et al.*, 2004). Enhanced expression of permeases is mediated by the transcription factors Stp1p and Stp2p, which are proteolytically processed by the activated SPS sensor complex. The processed form of Stp1p activates expression of genes required for protein utilization (e.g., *SAP2* and *OPT1*), whereas processed Stp2p induces expression of amino acid permease genes (e.g., *GAP1* and *GAP2*) (Martinez & Ljungdahl, 2005). The chaperone protein Csh3p also regulates the activation of Stp1p and Stp2p (Martinez & Ljungdahl, 2004). Responses to internal amino acid pools are regulated by the general amino acid control system which requires the transcription factor Gcn4p (Tripathi *et al.*, 2002). Starvation for a single amino acid induces multiple amino acid biosynthetic pathways and hyphal formation (Tripathi *et al.*, 2002). Observations that ammonium inhibits expression of peptide transporters (Basrai *et al.*, 1992; Payne *et al.*, 1991) and expression of *SAP2* (Banerjee *et al.*, 1991; Hube *et al.*, 1994; Ross *et al.*, 1990) indicate that nitrogen catabolite repression influences responses of *C. albicans* to the quality of available nitrogen source. Limjindaporn *et al.*, (2003) demonstrated that a GATA transcription factor, *GAT1* controls NCR in *C. albicans* and the gene is essential for its virulence. On the basis of preliminary experiments, lack of or reduction in secreted aspartic protease expression was hypothesized to be the reason for

attenuation of *gat1Δ* mutants. Molecular mechanisms by which *GAT1* regulate Sap expression were not demonstrated. Moreover, the full spectrum of *GAT1* influence on nitrogen metabolic capacity of *C. albicans* was not investigated.

2.2.2 Ammonium permease mediated filamentous growth in *C. albicans*

Ammonium is a preferred nitrogen source for microorganisms, and its transport across plasma membrane is mediated by an evolutionary conserved permease family, the Amt/Mep/Rh family (Andrade & Einsle, 2007). All members of this family are predicted to be membrane proteins with an extracytosolic amino terminus and a central hydrophobic core of 11 transmembrane helices (Thomas *et al.*, 2000). Structural and biochemical studies with the bacterial members of this family have proposed that these transporters form a channel that allows the passive diffusion of the ammonia gas (Khademi *et al.*, 2004; Knepper & Agre, 2004). Ammonium selectivity is attributed to the presence of a narrow hydrophobic pore that requires deprotonation of the translocating ammonium at the periplasmic side of the cell to form ammonia that can pass through the channel. Mutational analysis of the conserved residues which are involved in ammonium selectivity suggested that the mechanism of ammonium transport is evolutionarily conserved (Javelle *et al.*, 2006; Marini *et al.*, 2006).

In *S. cerevisiae* ammonium uptake is mediated by three members of the Amt/Mep/Rh family, Mep1p-Mep3p (Marini *et al.*, 1997). A mutant lacking all three *MEP* genes can not grow on media containing less than 5 mM ammonium as sole nitrogen source, however each permease by itself is sufficient to allow growth of the fungus under these conditions (Marini *et al.*, 1997). The *MEP* genes are subject to nitrogen control, i.e., their expression levels are highly induced when cells are grown under nitrogen limiting conditions (low concentrations of ammonium or presence of a non preferred nitrogen source in the growth medium).

Under nitrogen starvation conditions, the budding yeast *S. cerevisiae* switches to filamentous, pseudohyphal growth form, which is thought to enable these non-motile yeast cells to seek a preferable environment (Gimeno *et al.*, 1992). Ammonium permease Mep2p has been proposed to act as an ammonium sensor that regulates pseudohyphal growth of *S. cerevisiae* in response to low ammonium concentrations (Lorenz & Heitman, 1998). Under these conditions, the *mep2Δ* mutants do not switch to pseudohyphal growth, while *mep1Δ* mutants behave like the wild type. Some other fungal permeases are able to restore the pseudohyphal growth of *S. cerevisiae mep2Δ* mutants indicating that sensing and signaling function of Mep2p is preserved in specific members of the family across the fungal kingdom (Biswas & Morschhäuser, 2005; Javelle *et al.*, 2003a; 2003b; Smith *et al.*, 2003; Teichert *et*

al., 2008). Presumably, these permeases possess certain structural features which provide them with the signaling activity and these specific features are absent from other permeases that have only transport function (Smith *et al.*, 2003). However, it is not known how the ammonium transport activity of the ammonium permeases influences its signaling activity. In particular, if these permeases act as sensors of ammonium availability and couple ammonium transport with the regulation of a signal transduction pathway is not understood.

C. albicans also undergoes a transition from the budding yeast form to filamentous growth in response to nitrogen limitation. *C. albicans* expresses the *MEP1* and *MEP2* genes, which encode two ammonium transporters that enable growth of the fungus when limiting concentrations of ammonium are the only available nitrogen source. While deletion of either one of these two ammonium permeases does not affect the ability of *C. albicans* to grow at low ammonium concentrations, *mep1Δ mep2Δ* double mutants are unable to grow under these conditions. In addition to being an ammonium transporter, Mep2p, but not Mep1p, has a central function in the induction of filamentous growth on solid media under limiting nitrogen conditions (Biswas & Morschhäuser, 2005). However, Mep2p is not required for filamentous growth of *C. albicans* in response to other inducing signals, e.g. in the presence of serum. Therefore, the functions of the *C. albicans* ammonium permeases are similar to those of their counterparts in the model yeast *S. cerevisiae*. The C-terminal cytoplasmic domain of CaMep2p contains a signaling domain that is not required for ammonium transport but is essential for Mep2p-dependent morphogenesis (Biswas & Morschhäuser, 2005). CaMep2p activates both the cAMP-PKA and MAPK pathway in a Ras1p dependent manner via its cytoplasmic C-terminal tail to induce filamentous growth (Biswas & Morschhäuser, 2005). However, proteins that interact with Mep2p to activate the signal transduction pathways that induce hyphal development in response to nitrogen starvation are yet to be identified.

Beside structural features, the regulatory role of Mep2p in induction of filamentous growth is also due to its higher expression levels. Both ammonium permeases, Mep1p and Mep2p, are induced in response to nitrogen limitation but Mep2p is expressed at much higher levels than Mep1p. Promoter swapping experiments demonstrated that the differential expression levels are due to the stronger activity of the *MEP2* promoter as compared with the *MEP1* promoter and presumably also to differential transcript stability (Biswas & Morschhäuser, 2005). Expression of *MEP2* from the *MEP1* promoter resulted in reduced amounts of Mep2p, inefficient ammonium transport, and loss of its ability to induce filamentous growth. In contrast, expression of *MEP1* from the *MEP2* promoter resulted in increased Mep1p levels, which also conferred on it the ability to induce a weak filamentation.

These experimental evidences showed that Mep1p is a highly efficient ammonium transporter that needs to be expressed only at low levels to support growth on limiting ammonium concentrations. Contrastingly, Mep2p is a less efficient ammonium transporter that is expressed at high levels, which in turn is a prerequisite for the induction of filamentation. Since the control of *MEP2* expression is central to the regulation of nitrogen starvation induced filamentous growth in *C. albicans*, it is of paramount importance to identify the factors that regulate *MEP2* expression.

2.3 Aims of the study

C. albicans is the most common and arguably the most important causative agent of human fungal infections. *C. albicans* pathogenicity can be attributed to its ability to survive and thrive in multiple microenvironments within the host and to virulence factors that aid in proliferation of the fungus. The present study was aimed at understanding the control of virulence traits of *C. albicans*, dimorphic switching and production of secreted aspartic protease Sap2p, both of which are regulated by availability of nitrogen sources in the growth environment. Previous studies have illustrated that under nitrogen limiting conditions ammonium permease Mep2p, but not Mep1p has a regulatory role in inducing filamentous growth, and the C-terminal cytoplasmic tail of Mep2p has a specific signaling function. Moreover, higher expression levels of Mep2p in comparison to Mep1p contribute to the specific regulatory role of the permease (Biswas & Morschhäuser, 2005).

With this information, the present study set out to identify residues critical for the signaling function of Mep2p. An attempt was made to outline how signaling activity of Mep2p is regulated by ammonium availability and transport. Since appropriate levels of Mep2p are a prerequisite for regulation of filamentation, this research sought to identify the *cis*-acting sequences in the *MEP2* promoter as well as the *trans*-acting regulatory factors controlling *MEP2* expression and to elucidate their roles in morphogenesis. Possible roles, if any, of those transcription factors on other nitrogen regulated responses of *C. albicans* were also investigated.

3 Materials and Methods

3.1 Bacterial strain

Escherichia coli K12: *E. coli* strain DH5α (F⁻, *end*A1, *hsd*R17 [r$_k^-$, m$_k^-$], *sup*E44, *thi-1, rec*A1, *gyr*A96, *rel*A1, Δ[*arg*F-*lac*]U169, λ⁻, ɸ80d*lac*ZΔM15) (Bethesda Research Laboratories, 1986) was used for bacterial cloning experiments.

3.2 Plasmids

Following abbreviations are used in the description of plasmids: *caFLP*: *C. albicans*-adapted *FLP* gene encoding the site specific recombinase FLP; *cartTA*: *C. albicans*-adapted reverse tetracycline-dependent transactivator; *caSAT1*: *C. albicans*-adapted nourseothricin resistance marker (dominant selection marker); *FRT*: (FLP-recognition target) minimal recombination target sites of the FLP recombinase; *GFP*: Green fluorescent protein gene; P$_X$: Promoter of the given gene (*X*) ; P$_{tet}$: rtTA-dependent promoter; T$_{ACT1}$: Transcription termination sequence of the actin (*ACT1*) gene; *URA3*: Orotidine-5'-phosphate decarboxlylase gene, selection marker for uridine auxotrophic strains of *C. albicans*.

Table 1. Plasmids used in the study

Plasmids	Relevant insert	Reference
***MEP2* gene deletion cassette**		
pMEP2M2	[*5´MEP2-FRT*-P$_{SAP2}$-*caFLP*-T$_{ACT1}$-*URA3* -*FRT* -*3'MEP2*]-fragment	(Biswas & Morschhäuser, 2005)
pMEP2M5	[*5´MEP2-FRT*-P$_{SAP2}$-*caFLP*-T$_{ACT1}$-*caSAT1* -*FRT* -*3'MEP2*]-fragment	This study
Plasmid used for *URA3* reintegration		
pUR3	[*URA3*]-fragment	(Kelly *et al.*, 1987)
Plasmids containing wild-type or mutated *MEP2* alleles		
pMEP2Y122A	[*MEP2*^{Y122A}]-fragment	This study
pMEP2F126A	[*MEP2*^{F126A}]-fragment	This study
pMEP2W165A	[*MEP2*^{W165A}]-fragment	This study
pMEP2W167A	[*MEP2*^{W167A}]-fragment	This study

Plasmids	Relevant insert	Reference
pMEP2D180L	[$MEP2^{D180L}$]-fragment	(Biswas and Morschhäuser, unpublished)
pMEP2D180N	[$MEP2^{D180N}$]-fragment	This study
pMEP2H188A	[$MEP2^{H188A}$]-fragment	This study
pMEP2S243A	[$MEP2^{S243A}$]-fragment	This study
pMEP2H342A	[$MEP2^{H342A}$]-fragment	This study
pMEP2K1	[P_{MEP2}-*MEP2*-T_{ACT1}-*URA3-3´MEP2*]-fragment	(Biswas & Morschhäuser, 2005)
pMEP2K4	[P_{MEP2}-*MEP2*^{Y122A}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K5	[P_{MEP2}-*MEP2*^{F126A}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K6	[P_{MEP2}-*MEP2*^{W165A}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K7	[P_{MEP2}-*MEP2*^{W167A}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K8	[P_{MEP2}-*MEP2*^{D180L}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K9	[P_{MEP2}-*MEP2*^{D180N}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K10	[P_{MEP2}-*MEP2*^{H188A}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K11	[P_{MEP2}-*MEP2*^{S243A}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K12	[P_{MEP2}-*MEP2*^{H342A}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K13	[P_{MEP2}-*MEP2*-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K15	[P_{MEP2}-*MEP2*^{Y433F}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2K16	[P_{MEP2}-*MEP2*^{F239A}-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study

Plasmids containing GFP-tagged site specific *MEP2* mutant alleles

pMEP2G8	[P_{MEP2}-*MEP2*^{D180N}-*GFP*-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2G9	[P_{MEP2}-*MEP2*^{Y122A}-*GFP*-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2G10	[P_{MEP2}-*MEP2*^{F126A}-*GFP*-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2G11	[P_{MEP2}-*MEP2*^{W165A}-*GFP*-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2G12	[P_{MEP2}-*MEP2*^{W167A}-*GFP*-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2G13	[P_{MEP2}-*MEP2*^{H188A}-*GFP*-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2G14	[P_{MEP2}-*MEP2*^{S243A}-*GFP*-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study
pMEP2G15	[P_{MEP2}-*MEP2*^{H342A}-*GFP*-T_{ACT1}-*URA3-3´MEP2*]-fragment	This study

Plasmids	Relevant insert	Reference
pMEP2G16	[P$_{MEP2}$-MEP2^{F239A}-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
Plasmids used for analysing cytoplasmic tail of Mep2p		
pMEP2ΔC6	[P$_{MEP2}$-MEP2^{ΔC439}-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔC7	[P$_{MEP2}$-MEP2^{ΔC438}-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔC8	[P$_{MEP2}$-MEP2^{ΔC437}-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔC9	[P$_{MEP2}$-MEP2^{ΔC436}-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔC10	[$_{MEP2}$-MEP2^{ΔC435}-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔC11	[P$_{MEP2}$-MEP2^{ΔC434}-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔC12	[P$_{MEP2}$-MEP2^{ΔC433}-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔC12M1	[P$_{MEP2}$-MEP2^{ΔC433Y433F}-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔC13	[P$_{MEP2}$-MEP2^{ΔC432}-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
***MEP* hybrids**		
pMEP21H3	[P$_{MEP2}$-MEP2$^{1-418}$-MEP1$^{417-438}$-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP21H6	[P$_{MEP2}$-MEP2$^{1-418}$-MEP1$^{417-433}$-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP21H7	[P$_{MEP2}$-MEP2$^{1-418}$-MEP1$^{417-433F431Y}$-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
Plasmids used for *MEP2* promoter analysis		
pMEP2G2	[P$_{MEP2}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	(Biswas & Morschhäuser, 2005)
pMEP2G5	[P$_{MEP1}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP1]-fragment	(Biswas & Morschhäuser, 2005)
pMEP2G6	[P$_{MEP2}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔP1	[P$_{MEP2Δ-1014\ to\ -189}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔP2	[P$_{MEP2Δ-1014\ to\ -431}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔP3	[P$_{MEP2Δ-1014\ to\ -621}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔP4	[P$_{MEP2Δ-1014\ to\ -806}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔP5	[P$_{MEP2Δ-434\ to\ -189}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔP6	[P$_{MEP2Δ-1014\ to\ -288}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2ΔP7	[P$_{MEP2Δ-1014\ to\ -218}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study
pMEP2MP1	[P$_{MEP2M-206\ to\ -210}$-MEP2-GFP-T$_{ACT1}$-URA3-3´MEP2]-fragment	This study

Plasmids	Relevant insert	Reference
pMEP2MP2	[P$_{MEP2\text{M-264 to -268}}$-$MEP2$-$GFP$-T$_{ACT1}$-$URA3$-3´$MEP2$]-fragment	This study
pMEP2MP3	[P$_{MEP2\text{M-206 to -210, M-264 to -268}}$-$MEP2$-$GFP$-T$_{ACT1}$-$URA3$-3´$MEP2$]-fragment	This study
pMEP2K13	[P$_{MEP2}$-$MEP2$-T$_{ACT1}$-$URA3$-3´$MEP2$]-fragment	This study
pMEP2ΔP5A	[P$_{MEP2\Delta\text{-434 to -189}}$-$MEP2$-T$_{ACT1}$-$URA3$-3´$MEP2$]-fragment	This study
pMEP2ΔP6A	[P$_{MEP2\Delta\text{-1014 to -288}}$-$MEP2$-T$_{ACT1}$-$URA3$-3´$MEP2$]-fragment	This study
pMEP2MP1A	[P$_{MEP2\text{M-206 to -210}}$-$MEP2$-T$_{ACT1}$-$URA3$-3´$MEP2$]-fragment	This study
pMEP2MP2A	[P$_{MEP2\text{M-264 to -268}}$-$MEP2$-T$_{ACT1}$-$URA3$-3´$MEP2$]-fragment	This study
pMEP2MP3A	[P$_{MEP2\text{M-206 to -210, M-264 to -268}}$-$MEP2$-T$_{ACT1}$-$URA3$-3´$MEP2$]-fragment	This study

Plasmids containing *SAT1* flipper cassette for gene deletion and reintegration

Plasmids	Relevant insert	Reference
pGAT1M1	[5´$GAT1$-FRT-P$_{MAL2}$-$caFLP$-T$_{ACT1}$-$caSAT1$-FRT-3'$GLN3$]-fragment	This study
pGAT1M2	[5´$GAT1$-FRT-P$_{MAL2}$-$caFLP$-T$_{ACT1}$-$caSAT1$-FRT-3'$GAT1$]-fragment. *GAT1* gene deletion cassette.	This study
pGAT1K1	[$GAT1P$-$GAT1$-FRT-P$_{MAL2}$-$caFLP$-T$_{ACT1}$-$caSAT1$-FRT-3'$GAT1$]-fragment	This study
pGLN3M1	[5´$GLN3$-FRT-P$_{MAL2}$-$caFLP$-T$_{ACT1}$-$caSAT1$ -FRT -3´$OPT3$]-fragment	This study
pGLN3M2	[5´$GLN3$-FRT-P$_{MAL2}$-$caFLP$-T$_{ACT1}$-$caSAT1$-FRT-3'$GLN3$]-fragment. *GLN3* gene deletion cassette.	This study
pGLN3K1	[$GLN3P$-$GLN3$-FRT-P$_{MAL2}$-$caFLP$-T$_{ACT1}$-$caSAT1$ -FRT -3'$GLN3$]-fragment	This study
pSTP1M1	[5´$STP1$-FRT-P$_{MAL2}$-$caFLP$-T$_{ACT1}$-$caSAT1$-FRT -3´$GAT1$]-fragment	This study
PSTP1M2	[5´$STP1$-FRT-P$_{MAL2}$-$caFLP$-T$_{ACT1}$-$caSAT1$-FRT-3'$STP1$]-fragment. *STP1* gene deletion cassette.	This study

Plasmids containing *GFP* reporter constructs for analyzing *MEP1*, and *MEP2* expression

Plasmids	Relevant insert	Reference
pMEP1G4	[P$_{MEP1}$-$MEP1$-GFP-T$_{ACT1}$-$caSAT1$-3´$MEP2$]-fragment	This study
pMEP1PG1	[P$_{MEP1}$-GFP-T$_{ACT1}$-$caSAT1$-3´$MEP2$]-fragment	This study
pMEP2G7	[P$_{MEP2}$-$MEP2$-GFP-T$_{ACT1}$-$caSAT1$-3´$MEP2$]-fragment	This study
pMEP2PG1	[P$_{MEP2}$-GFP-T$_{ACT1}$-$caSAT1$-3´$MEP2$]-fragment	This study

Plasmids used for expressing *MEP2* and *MEP2*^{AC440} under the control of *ADH1* promoter

Plasmids	Relevant insert	Reference
pADH1E1	[P$_{ADH1}$-T$_{ACT1}$-$caSAT1$-3'$ADH1$ ´]-fragment	(Reuß & Morschhäuser, 2006)
pMEP2K2	[P$_{MEP1}$-$MEP2$-T$_{ACT1}$-$URA3$-3´$MEP1$]-fragment	(Biswas & Morschhäuser, 2005)

Plasmids	Relevant insert	Reference
pMEP2E4	[P$_{ADH1}$-*MEP2*-T$_{ACT1}$-*URA3-3′ADH1*]-fragment	This study
pMEP2ΔC2	[P$_{MEP2}$- *MEP2*^{ΔC440}-T$_{ACT1}$-*URA3-3′MEP2*]-fragment	(Biswas & Morschhäuser, 2005)
pMEP2ΔC2K2	[P$_{MEP1}$- *MEP2*^{ΔC440}-T$_{ACT1}$-*URA3-3′MEP1*]-fragment	This study
pMEP2ΔC2E2	[P$_{ADH1}$- *MEP2*^{ΔC440}-T$_{ACT1}$-*URA3-3′ADH1*]-fragment	This study

Plasmids containing genes under the control of tetracycline inducible (Tet) promoter

pNIM1	[P$_{ADH1}$-*cartTA* -T$_{ACT1}$- *caSAT1*- T$_{ACT1}$- *GFP*-P$_{tet}$ - *3′ADH1*]-fragment	(Park & Morschhäuser, 2005)
pTET1-GAT1-1	[P$_{ADH1}$-*cartTA* -T$_{ACT1}$- *caSAT1*- T$_{ACT1}$- *GAT1*^{2268}-P$_{tet}$ - *3′ADH1*]-fragment	This study
pTET1-GAT1-2	[P$_{ADH1}$- *cartTA* -T$_{ACT1}$- *caSAT1*- T$_{ACT1}$- *GAT1*^{2067}- P$_{tet}$ - *3′ADH1*]-fragment	This study
pTET1-GAT1-3	[P$_{ADH1}$- *cartTA* -T$_{ACT1}$- *caSAT1*- T$_{ACT1}$- *GAT1*^{2004}- P$_{tet}$ - *3′ADH1*]-fragment	This study
pTET1-GLN3	[P$_{ADH1}$- *cartTA* -T$_{ACT1}$-*caSAT1*-T$_{ACT1}$- *GLN3*- P$_{tet}$ - *3′ADH1*]-fragment	This study
pTET1-STP1	[P$_{ADH1}$- *cartTA* -T$_{ACT1}$- *caSAT1*-T$_{ACT1}$- *STP1*-P$_{tet}$ - *3′ADH1*]-fragment	This study
pTET1-STP1^{ΔN61}	[P$_{ADH1}$- *cartTA* -T$_{ACT1}$- *caSAT1*- T$_{ACT1}$- *STP1*^{ΔN61}-P$_{tet}$ - *3′ADH1*]-fragment	This study

Plasmids containing *GFP* reporter constructs for analyzing *OPT1*, *OPT3*, *SAP2*, and *STP1* expression

pOPT1G22	[P$_{OPT1}$-*GFP*-T$_{ACT1}$-*caSAT1-3′OPT1*]-fragment	(Reuß & Morschhäuser, 2006)
pOPT3G22	[P$_{OPT3}$-*GFP*-T$_{ACT1}$-*caSAT1-3′OPT3*]-fragment	(Reuß & Morschhäuser, 2006)
pSAP2G1	[P$_{SAP2-1}$ -*GFP*-T$_{ACT1}$-*caSAT1-3′SAP2*]-fragment	(Reuß & Morschhäuser, 2006)
pSTP1G1	[P$_{STP1}$-*GFP*-T$_{ACT1}$-*caSAT1-3′OPT1*]-fragment	This study
pSTP1G2	[P$_{STP1}$-*GFP*-T$_{ACT1}$-*caSAT1-3′STP1*]-fragment	This study

Plasmids used for expressing *SAP2* and *OPT1* from the *ADH1* promoter

pOPT1E1	[P$_{ADH1}$- *OPT1*-T$_{ACT1}$- *caSAT1-3′ADH1*]-fragment	(Reuß & Morschhäuser, 2006)
pSAP2ex7	[P$_{ADH1}$- *SAP2-1*-T$_{ACT1}$-*caSAT1-3′ADH1*]-fragment. Allele 1 of *SAP2* expressed from the *ADH1* promoter.	This study

3.3 *C. albicans* strains

Table 2. *C. albicans* strains used in this study

Strain	Parent	Relevant genotype[a]	Reference
SC5314		wild-type strain	(Gillum *et al.*, 1984)
CAI4	SC5314	*ura3Δ::imm434/ura3Δ::imm434*	(Fonzi & Irwin, 1993)
CAI4RU1A and B	CAI4	*ura3Δ::imm434/URA3*	This study
SAP2MS4A/B	SC5314	*sap2Δ::FRT /sap2Δ::FRT*	(Staib *et al.*, 2008)
mep2Δ single and mep1Δ mep2Δ double mutants			
SCMEP2M1A	SC5314	*mep2-1Δ::SAT1-FLIP [b]/MEP2-2*	This study
SCMEP2M1B	SC5314	*MEP2-1/mep2-2Δ::SAT1-FLIP*	This study
SCMEP2M2A	SCMEP2M1A	*mep2-1Δ::FRT/MEP2-2*	This study
SCMEP2M2B	SCMEP2M1B	*MEP2-1/mep2-2Δ::FRT*	This study
SCMEP2M3A	SCMEP2M2A	*mep2-1Δ::FRT/mep2-2Δ::SAT1-FLIP*	This study
SCMEP2M3B	SCMEP2M2B	*mep2-1Δ::SAT1-FLIP/mep2-2Δ::FRT*	This study
SCMEP2M4A	SCMEP2M3A	*mep2-1Δ::FRT/mep2-2Δ::FRT*	This study
SCMEP2M4B	SCMEP2M3B	*mep2-1Δ::FRT/mep2-2Δ::FRT*	This study
MEP2M4RU1A	MEP2M4A	*ura3Δ::imm434/URA3 mep2-1Δ::FRT/mep2-2Δ::FRT*	This study
MEP2M4RU1B	MEP2M4B	*ura3Δ::imm434/URA3 mep2-1Δ::FRT/mep2-2Δ::FRT*	This study
MEP2M4A/B	CAI4	*mep2-1Δ::FRT/mep2-2Δ::FRT*	(Biswas & Morschhäuser, 2005)
MEP2M5A	MEP2M4A	*mep2-1Δ::FRT/mep2-2Δ::URA3*	(Biswas & Morschhäuser, 2005)
MEP2M5B	MEP2M4B	*mep2-1Δ::URA3 /mep2-2Δ::FRT*	(Biswas & Morschhäuser, 2005)
MEP12M4A	MEP2M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/mep2-2Δ::FRT*	(Biswas & Morschhäuser, 2005)
MEP12M4B	MEP2M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/mep2-2Δ::FRT*	(Biswas & Morschhäuser, 2005)

Strain	Parent	Relevant genotype[a]	Reference
MEP12M6A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::URA3*	(Biswas & Morschhäuser, 2005)
MEP12M6B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::URA3 /mep2-2Δ::FRT*	(Biswas & Morschhäuser, 2005)

mep2Δ mutants expressing wild-type or mutated MEP2 alleles

Strain	Parent	Relevant genotype	Reference
MEP2MK1A	MEP2M4A	*mep2-1Δ::FRT/mep2-2Δ::MEP2-URA3*	(Biswas & Morschhäuser, 2005)
MEP2MK1B	MEP2M4B	*mep2-1Δ::MEP2-URA3/mep2-2Δ::FRT*	(Biswas & Morschhäuser, 2005)
MEP2MK6A	MEP2M4A	*mep2-1Δ::MEP2*[Y122A] *-URA3/mep2-2Δ::FRT*	This study
MEP2MK6B	MEP2M4B	*mep2-1Δ::FRT/mep2-2Δ::MEP2*[Y122A]*-URA3*	This study
MEP2MK7A	MEP2M4A	*mep2-1Δ::MEP2*[F126A] *-URA3/mep2-2Δ::FRT*	This study
MEP2MK7B	MEP2M4B	*mep2-1Δ::FRT/mep2-2Δ::MEP2*[F126A]*-URA3*	This study
MEP2MK9A	MEP2M4A	*mep2-1Δ::MEP2*[W167A] *-URA3/mep2-2Δ::FRT*	This study
MEP2MK9B	MEP2M4B	*mep2-1Δ::FRT/mep2-2Δ::MEP2*[W167A]*-URA3*	This study
MEP2MK17A/B	MEP2M4B	*mep2-1Δ::FRT/mep2-2Δ::MEP2*[F239A]*-URA3*	This study

mep1Δ mep2Δ double mutants expressing wild-type or mutated MEP2 alleles

Strain	Parent	Relevant genotype	Reference
MEP12MK2A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2-URA3*	(Biswas & Morschhäuser, 2005)
MEP12MK2B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2-URA3/mep2-2Δ::FRT*	(Biswas & Morschhäuser, 2005)
MEP12MK6A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*[Y122A]*-URA3*	This study
MEP12MK6B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*[Y122A] *-URA3/mep2-2Δ::FRT*	This study
MEP12MK7A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*[F126A]*-URA3*	This study
MEP12MK7B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*[F126A]*-URA3*	This study
MEP12MK8A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*[W165A]*-URA3*	This study
MEP12MK8B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*[W165A] *-URA3/mep2-2Δ::FRT*	This study

Strain	Parent	Relevant genotype[a]	Reference
MEP12MK9A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*^{W167A}*-URA3*	This study
MEP12MK9B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*^{W167A}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK10A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*^{D180L}*-URA3*	This study
MEP12MK10B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*^{D180L}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK11A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*^{D180N}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK11B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*^{D180N}*-URA3*	This study
MEP12MK12A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*^{H188A}*-URA3*	This study
MEP12MK12B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*^{H188A}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK13A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*^{S243A}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK13B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*^{S243A}*-URA3*	This study
MEP12MK14A	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*^{H342A}*-URA3*	This study
MEP12MK14B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*^{H342A}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK16A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*^{Y433F}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK16B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*^{Y433F}*-URA3*	This study
MEP12MK17A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*^{F239A}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK17B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2*^{F239A}*-URA3*	This study

mep1Δ mep2Δ double mutants expressing *GFP*-tagged *MEP2* alleles

Strain	Parent	Relevant genotype[a]	Reference
MEP12MG2A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2-GFP-URA3/mep2-2Δ::FRT*	(Biswas & Morschhäuser, 2005)
MEP12MG2B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/mep2-2Δ::MEP2-GFP-URA3*	(Biswas & Morschhäuser, 2005)
MEP12MG4A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2*^{D180L}*-GFP-URA3/* *mep2-2Δ::FRT*	(Biswas and Morschhäuser, unpublished)

Strain	Parent	Relevant genotype[a]	Reference
MEP12MG4B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*[D180L]*-GFP-URA3*	(Biswas and Morschhäuser, unpublished)
MEP12MG8A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*[D180N]*-GFP-URA3/ mep2-2Δ::FRT*	This study
MEP12MG8B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*[D180N]*-GFP-URA3*	This study
MEP12MG9A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*[Y122A]*-GFP-URA3*	This study
MEP12MG9B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*[Y122A]*-GFP-URA3/ mep2-2Δ::FRT*	This study
MEP12MG10A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*[F126A]*-GFP-URA3/ mep2-2Δ::FRT*	This study
MEP12MG10B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*[F126A]*-GFP-URA3*	This study
MEP12MG11A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*[W165A]*-GFP-URA3/ mep2-2Δ:: FRT*	This study
MEP12MG11B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*[W165A]*-GFP-URA3*	This study
MEP12MG12A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*[W167A]*-GFP-URA3/ mep2-2Δ:: FRT*	This study
MEP12MG12B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*[W167A]*-GFP-URA3*	This study
MEP12MG13A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*[H188A]*-GFP-URA3/ mep2-2Δ:: FRT*	This study
MEP12MG13B	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*[H188A]*-GFP-URA3*	This study
MEP12MG14A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*[S243A]*-GFP-URA3/ mep2-2Δ:: FRT*	This study
MEP12MG14B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*[S243A]*-GFP-URA3*	This study

Strain	Parent	Relevant genotype[a]	Reference
MEP12MG15A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*^{H342A}*-GFP-URA3/ mep2-2Δ:: FRT*	This study
MEP12MG15B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*^{H342A}*-GFP-URA3*	This study
MEP12MG16A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*^{F239A}*-GFP-URA3/ mep2-2Δ::FRT*	This study
MEP12MG16B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2Δ::MEP2*^{F239A}*-GFP-URA3*	This study

mep1Δ mep2Δ double mutants expressing truncated MEP2 alleles

Strain	Parent	Relevant genotype	Reference
MEP12MK2AΔC2	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*^{ΔC440}*-URA3/mep2-2Δ::FRT*	(Biswas & Morschhäuser, 2005)
MEP12MK2BΔC2	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT /mep2-2Δ::MEP2*^{ΔC440}*-URA3*	(Biswas & Morschhäuser, 2005)
MEP12MK2AΔC6	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT /mep2-2Δ::MEP2*^{ΔC439}*-URA3*	This study
MEP12MK2BΔC6	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*^{ΔC439}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK2AΔC7	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*^{ΔC438}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK2BΔC7	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT /mep2-2Δ::MEP2*^{ΔC438}*-URA3*	This study
MEP12MK2AΔC8	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT /mep2-2Δ::MEP2*^{ΔC437}*-URA3*	This study
MEP12MK2BΔC8	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*^{ΔC437}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK2AΔC9	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT /mep2-2Δ::MEP2*^{ΔC436}*-URA3*	This study
MEP12MK2BΔC9	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT /mep2-2Δ::MEP2*^{ΔC436}*-URA3*	This study
MEP12MK2AΔC10	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::MEP2*^{ΔC435}*-URA3/mep2-2Δ::FRT*	This study
MEP12MK2BΔC10	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT /mep2-2Δ::MEP2*^{ΔC435}*-URA3*	This study
MEP12MK2AΔC11	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT /mep2-2Δ::MEP2*^{ΔC434}*-URA3*	This study

Strain	Parent	Relevant genotype[a]	Reference
MEP12MK2BΔC11	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2^{ΔC434}-URA3/mep2-2Δ::FRT*	This study
MEP12MK2AΔC12	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT /mep2-2Δ::MEP2^{ΔC433}-URA3*	This study
MEP12MK2BΔC12	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2^{ΔC433}-URA3/mep2-2Δ::FRT*	This study
MEP12MK2AΔC12M1	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/* *mep2-2Δ::MEP2$^{ΔC433\ Y433F}$-URA3*	This study
MEP12MK2BΔC12M1	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2$^{ΔC433\ Y433F}$-URA3/* *mep2-2Δ::FRT*	This study
MEP12MK2AΔC13	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT /mep2-2Δ::MEP2^{ΔC432}-URA3*	This study
MEP12MK2BΔC13	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT /mep2-2Δ::MEP2^{ΔC432}-URA3*	This study
MEP12MK21H4A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/* *mep2-2Δ::MEP2$^{1-418}$-MEP1$^{417-438}$-URA3*	This study
MEP12MK21H4B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2$^{1-418}$-MEP1$^{417-438}$-URA3/* *mep2-2Δ:: FRT*	This study
MEP12MK21H6A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2$^{1-418}$-MEP1$^{417-433}$-URA3/* *mep2-2Δ::FRT*	This study
MEP12MK21H6B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/* *mep2-2Δ::MEP2$^{1-418}$-MEP1$^{417-433}$-URA3*	This study
MEP12MK21H7A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/* *mep2-2Δ::MEP2$^{1-418}$-MEP1$^{417-433\ F431Y}$-URA3*	This study
MEP12MK21H7B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::MEP2$^{1-418}$-MEP1$^{417-433\ F431Y}$-URA3/* *mep2-2Δ::FRT*	This study

Strains expressing a *MEP2-GFP* fusion under control of wild-type and mutated *MEP2* promoters in a *mep1Δ mep2Δ* double mutant background

Strain	Parent	Relevant genotype	Reference
MEP12MG6A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1::P$_{MEP2}$-MEP2-GFP-URA3/* *mep2-2Δ::FRT*	This study
MEP12MG6B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/* *mep2-2::P$_{MEP2}$-MEP2-GFP-URA3*	This study

34

Strain	Parent	Relevant genotype[a]	Reference
MEP12MG6ΔP1A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1::P$_{MEP2Δ1}$-MEP2-GFP-URA3/ mep2-2Δ::FRT*	This study
MEP12MG6ΔP1B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2::P$_{MEP2Δ1}$-MEP2-GFP-URA3*	This study
MEP12MG6ΔP2A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1::P$_{MEP2Δ2}$-MEP2-GFP-URA3/ mep2-2Δ:: FRT*	This study
MEP12MG6ΔP2B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2::P$_{MEP2Δ2}$-MEP2-GFP-URA3*	This study
MEP12MG6ΔP3A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2::P$_{MEP2Δ3}$-MEP2-GFP-URA3*	This study
MEP12MG6ΔP3B	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2::P$_{MEP2Δ3}$-MEP2-GFP-URA3*	This study
MEP12MG6ΔP4A	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1::P$_{MEP2Δ4}$-MEP2-GFP-URA3/ mep2-2Δ::FRT*	This study
MEP12MG6ΔP4B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2::P$_{MEP2Δ4}$-MEP2-GFP-URA3*	This study
MEP12MG6ΔP5A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1::P$_{MEP2Δ5}$-MEP2-GFP-URA3/ mep2-2Δ::FRT*	This study
MEP12MG6ΔP5B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2::P$_{MEP2Δ5}$-MEP2-GFP-URA3*	This study
MEP12MG6ΔP6A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2::P$_{MEP2Δ6}$-MEP2-GFP-URA3*	This study
MEP12MG6ΔP6B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1::P$_{MEP2Δ6}$-MEP2-GFP-URA3/ mep2-2Δ::FRT*	This study
MEP12MG6ΔP7A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1::P$_{MEP2Δ7}$-MEP2-GFP-URA3/ mep2-2Δ::FRT*	This study
MEP12MG6ΔP7B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1Δ::FRT/ mep2-2::P$_{MEP2Δ7}$-MEP2-GFP-URA3*	This study
MEP12MG6MP1A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT mep2-1::P$_{MEP2M1}$-MEP2-GFP-URA3/ mep2-2Δ:: FRT*	This study

Strain	Parent	Relevant genotype[a]	Reference
MEP12MG6MP1B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ::FRT/* *mep2-2*::P_{MEP2M1}-*MEP2-GFP-URA3*	This study
MEP12MG6MP2A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1*::P_{MEP2M2}-*MEP2-GFP-URA3/* *mep2-2Δ:: FRT*	This study
MEP12MG6MP2B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1*::P_{MEP2M2}-*MEP2-GFP-URA3/* *mep2-2Δ:: FRT*	This study
MEP12MG6MP3A	MEP12M4A	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1*::P_{MEP2M3}-*MEP2-GFP-URA3/* *mep2-2Δ:: FRT*	This study
MEP12MG6MP3B	MEP12M4B	*mep1-1Δ::FRT/mep1-2Δ::FRT* *mep2-1Δ:: FRT/* *mep2-2*::P_{MEP2M3}-*MEP2-GFP-URA3*	This study

Strains expressing *MEP2* under control of wild-type and mutated *MEP2* promoters in a *mep2Δ* background

Strain	Parent	Relevant genotype[a]	Reference
MEP2MK13A	MEP2M4A	*mep2-1*::P_{MEP2}-*MEP2-URA3/ mep2-2Δ::FRT*	This study
MEP2MK13B	MEP2M4B	*mep2-1Δ::FRT/mep2-2*::P_{MEP2}-*MEP2-URA3*	This study
MEP2MK13ΔP5A	MEP2M4A	*mep2-1*::$P_{MEP2Δ5}$-*MEP2-URA3/ mep2-2Δ::FRT*	This study
MEP2MK13ΔP5B	MEP2M4B	*mep2-1Δ::FRT/mep2-2*::$P_{MEP2Δ5}$-*MEP2-URA3*	This study
MEP2MK13ΔP6A	MEP2M4A	*mep2-1*::$P_{MEP2Δ6}$-*MEP2-URA3/mep2-2Δ::FRT*	This study
MEP2MK13ΔP6B	MEP2M4B	*mep2-1Δ::FRT/mep2-2*::$P_{MEP2Δ6}$-*MEP2-URA3*	This study
MEP2MK13MP1A	MEP2M4A	*mep2-1*::P_{MEP2M1}-*MEP2-URA3/* *mep2-2Δ::FRT*	This study
MEP2MK13MP1B	MEP2M4B	*mep2-1Δ::FRT/mep2-2*::P_{MEP2M1}-*MEP2-URA3*	This study
MEP2MK13MP2A	MEP2M4A	*mep2-1*::P_{MEP2M2}-*MEP2-URA3/* *mep2-2Δ::FRT*	This study
MEP2MK13MP2B	MEP2M4B	*mep2-1Δ::FRT/mep2-2*::P_{MEP2M2}-*MEP2-URA3*	This study
MEP2MK13MP3A	MEP2M4A	*mep2-1*::P_{MEP2M3}-*MEP2-URA3/* *mep2-2Δ::FRT*	This study
MEP2MK13MP3B	MEP2M4B	*mep2-1Δ::FRT/mep2-2*::P_{MEP2M3}-*MEP2-URA3*	This study

Strain	Parent	Relevant genotype[a]	Reference
gln3Δ mutants and complemented strains			
GLN3M1A	SC5314	_gln3-1Δ::SAT1-FLIP/GLN3-2_	This study
GLN3M1B	SC5314	_GLN3-1/gln3-2Δ::SAT1-FLIP_	This study
GLN3M2A	GLN3M1A	_gln3-1Δ::FRT/GLN3-2_	This study
GLN3M2B	GLN3M1B	_GLN3-1/gln3-2Δ::FRT_	This study
GLN3M3A	GLN3M2A	_gln3-1Δ::FRT/gln3-2Δ::SAT1-FLIP_	This study
GLN3M3B	GLN3M2B	_gln3-1Δ::SAT1-FLIP/gln3-2Δ::FRT_	This study
GLN3M4A	GLN3M3A	_gln3-1Δ::FRT/gln3-2Δ::FRT_	This study
GLN3M4B	GLN3M3B	_gln3-1Δ::FRT/gln3-2Δ::FRT_	This study
GLN3MK1A	GLN3M4A	_GLN3-SAT1-FLIP/gln3-2Δ::FRT_	This study
GLN3MK1B	GLN3M4B	_gln3-1Δ::FRT/GLN3-SAT1-FLIP_	This study
GLN3MK2A	GLN3MK1A	_GLN3-FRT/gln3-2Δ::FRT_	This study
GLN3MK2B	GLN3MK1B	_gln3-1Δ::FRT/GLN3-FRT_	This study
gat1Δ mutants and complemented strains			
GAT1M1A	SC5314	_gat1-1Δ::SAT1-FLIP/GAT1-2_	This study
GAT1M1B	SC5314	_GAT1-1/gat1-2Δ::SAT1-FLIP_	This study
GAT1M2A	GAT1M1A	_gat1-1Δ::FRT/GAT1-2_	This study
GAT1M2B	GAT1M1B	_GAT1-1/gat1-2Δ::FRT_	This study
GAT1M3A	GAT1M2A	_gat1-1Δ::FRT/gat1-2Δ::SAT1-FLIP_	This study
GAT1M3B	GAT1M2B	_gat1-1Δ::SAT1-FLIP/gat1-2Δ::FRT_	This study
GAT1M4A	GAT1M3A	_gat1-1Δ::FRT/gat1-2Δ::FRT_	This study
GAT1M4B	GAT1M3B	_gat1-1Δ::FRT/gat1-2Δ::FRT_	This study
GAT1MK1A	GAT1M4A	_GAT1-2-SAT1-FLIP [1]/gat1-2Δ::FRT_	This study
GAT1MK1B	GAT1M4B	_gat1-1Δ::FRT/GAT1-2-SAT1-FLIP_	This study
GAT1MK2A	GAT1MK1A	_GAT1-2-FRT/gat1-2Δ::FRT_	This study
GAT1MK2B	GAT1MK1B	_gat1-1Δ::FRT/GAT1-2-FRT_	This study

Strain	Parent	Relevant genotype[a]	Reference
gln3Δ gat1Δ double mutants and complemented strains			
Δgln3GAT1M1A	GLN3M4A	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::SAT1-FLIP/GAT1-2	This study
Δgln3GAT1M1B	GLN3M4B	gln3-1Δ::FRT/gln3-2Δ::FRT GAT1-1/gat1-2Δ::SAT1-FLIP	This study
Δgln3GAT1M2A	Δgln3GAT1M1A	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/GAT1-2	This study
Δgln3GAT1M2B	Δgln3GAT1M1B	gln3-1Δ::FRT/gln3-2Δ::FRT GAT1-1/gat1-2Δ::FRT	This study
Δgln3GAT1M3A	Δgln3GAT1M2A	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::SAT1-FLIP	This study
Δgln3GAT1M3B	Δgln3GAT1M2B	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::SAT1-FLIP/gat1-2Δ::FRT	This study
Δgln3GAT1M4A	Δgln3GAT1M3A	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT	This study
Δgln3GAT1M4B	Δgln3GAT1M3B	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT	This study
Δgln3GAT1MK1A	Δgln3GAT1M4A	GLN3-SAT1-FLIP/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT	This study
Δgln3GAT1MK1B	Δgln3GAT1M4B	gln3-1Δ::FRT/GLN3-SAT1-FLIP gat1-1Δ::FRT/gat1-2Δ::FRT	This study
Δgln3GAT1MK2A	Δgln3GAT1MK1 A	GLN3-FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT	This study
Δgln3GAT1MK2B	Δgln3GAT1MK1B	gln3-1Δ::FRT/GLN3-FRT gat1-1Δ::FRT/gat1-2Δ::FRT	This study
Δgln3GAT1MK3A	Δgln3GAT1M4A	gln3-1Δ::FRT/gln3-2Δ::FRT GAT1-2-SAT1-FLIP/gat1-2Δ::FRT	This study
Δgln3GAT1MK3B	Δgln3GAT1M4B	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/GAT1-2-SAT1-FLIP	This study
Δgln3GAT1MK4A	Δgln3GAT1MK3A	gln3-1Δ::FRT/gln3-2Δ::FRT GAT1-2-FRT/gat1-2Δ::FRT	This study
Δgln3GAT1MK4B	Δgln3GAT1MK3B	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/GAT1-2-FRT	This study
mep2Δ gat1Δ double mutants			
Δmep2GAT1M1A	SCMEP2M4A	mep2-1Δ::FRT/mep2-2Δ::FRT GAT1-1/gat1-2Δ::SAT1-FLIP	This study
Δmep2GAT1M1B	SCMEP2M4B	mep2-1Δ::FRT/mep2-2Δ::FRT gat1-1Δ::SAT1-FLIP/GAT1-2	This study
Δmep2GAT1M2A	Δmep2GAT1M1A	mep2-1Δ::FRT/mep2-2Δ::FRT GAT1-1/gat1-2Δ::FRT	This study
Δmep2GAT1M2B	Δmep2GAT1M1B	mep2-1Δ::FRT/mep2-2Δ::FRT gat1-1Δ::FRT/GAT1-2	This study

Strain	Parent	Relevant genotype[a]	Reference
Δ*mep2*GAT1M3A	Δ*mep2*GAT1M2A	*mep2-1Δ::FRT/mep2-2Δ::FRT* *gat1-1Δ::SAT1-FLIP/gat1-2Δ::FRT*	This study
Δ*mep2*GAT1M3B	Δ*mep2*GAT1M2B	*mep2-1Δ::FRT/mep2-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::SAT1-FLIP*	This study
Δ*mep2*GAT1M4A	Δ*mep2*GAT1M3A	*mep2-1Δ::FRT/mep2-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT*	This study
Δ*mep2*GAT1M4B	Δ*mep2*GAT1M3B	*mep2-1Δ::FRT/mep2-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT*	This study

Strains expressing *MEP2-GFP* and *MEP1-GFP* reporter gene fusions in wild-type, *gln3*Δ, *gat1*Δ and *gln3*Δ *gat1*Δ backgrounds

Strain	Parent	Relevant genotype[a]	Reference
SCMEP2G7A	SC5314	*mep2-1*::P$_{MEP2}$-*MEP2-GFP-caSAT1/MEP2-2*	This study
SCMEP2G7B	SC5314	*MEP2-1/mep2-2*::P$_{MEP2}$-*MEP2-GFP-caSAT1*	This study
SCMEP1G4A/B	SC5314	*MEP1/mep1*::P$_{MEP1}$-*MEP1-GFP-caSAT1*	This study
Δ*gln3*MEP2G7A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT* *mep2-1*::P$_{MEP2}$-*MEP2-GFP-caSAT1/MEP2-2*	This study
Δ*gln3*MEP2G7B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT* *MEP2-1/mep2-2*::P$_{MEP2}$-*MEP2-GFP-caSAT1*	This study
Δ*gln3*MEP1G4A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT* *MEP1/mep1*::P$_{MEP1}$-*MEP1-GFP-caSAT1*	This study
Δ*gln3*MEP1G4B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT* *MEP1/mep1*::P$_{MEP1}$-*MEP1-GFP-caSAT1*	This study
Δ*gat1*MEP2G7A	GAT1M4A	*gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP2-1/mep2-2*::P$_{MEP2}$-*MEP2-GFP-caSAT1*	This study
Δ*gat1*MEP2G7B	GAT1M4B	*gat1-1Δ::FRT/gat1-2Δ::FRT* *mep2-1*::P$_{MEP2}$-*MEP2-GFP-caSAT1/MEP2-2*	This study
Δ*gat1*MEP1G4A	GAT1M4A	*gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP1/mep1*::P$_{MEP1}$-*MEP1-GFP-caSAT1*	This study
Δ*gat1*MEP1G4B	GAT1M4B	*gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP1/mep1*::P$_{MEP1}$-*MEP1-GFP-caSAT1*	This study
Δ*gln3*Δ*gat1*MEP2G7A	Δ*gln3*GAT1M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP2-1/mep2-2*::P$_{MEP2}$-*MEP2-GFP-caSAT1*	This study
Δ*gln3*Δ*gat1*MEP2G7B	Δ*gln3*GAT1M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP2-1/mep2-2*::P$_{MEP2}$-*MEP2-GFP-caSAT1*	This study
Δ*gln3*Δ*gat1*MEP1G4A	Δ*gln3*GAT1M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP1/mep1*::P$_{MEP1}$-*MEP1-GFP-caSAT1*	This study
Δ*gln3*Δ*gat1*MEP1G4B	Δ*gln3*GAT1M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP1/mep1*::P$_{MEP1}$-*MEP1-GFP-caSAT1*	This study

Strains expressing P$_{MEP2}$-GFP and P$_{MEP1}$-GFP reporters in wild-type, gln3Δ, gat1Δ and gln3 gat1Δ backgrounds

Strain	Parent	Relevant genotype[a]	Reference
SCMEP2PG1A	SC5314	*mep2-1*::P$_{MEP2}$-*GFP-caSAT1/MEP2-2*	This study
SCMEP2PG1B	SC5314	*mep2-1*::P$_{MEP2}$-*GFP-caSAT1/MEP2-2*	This study
SCMEP1PG1A	SC5314	*mep1-1*::P$_{MEP1}$-*GFP-caSAT1/ MEP1-2*	This study
SCMEP1PG1B	SC5314	*MEP1-2/mep1-2*::P$_{MEP1}$-*GFP-caSAT1*	
Δ*gln3*MEP2PG1A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT* *MEP2-1/mep2-2*::P$_{MEP2}$-*GFP-caSAT1*	This study
Δ*gln3*MEP2PG1B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT* *mep2-1*::P$_{MEP2}$-*GFP-caSAT1/MEP2-2*	This study
Δ*gln3*MEP1PG1A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT* *mep1-1*::P$_{MEP1}$-*GFP-caSAT1/MEP1-2*	This study
Δ*gln3*MEP1PG1B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT* *MEP1-1/mep1-2*::P$_{MEP1}$-*GFP-caSAT1*	This study
Δ*gat1*MEP2PG1A	GAT1M4A	*gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP2-1/mep2-2*::P$_{MEP2}$-*GFP-caSAT1*	This study
Δ*gat1*MEP2PG1B	GAT1M4B	*gat1-1Δ::FRT/gat1-2Δ::FRT* *mep2-1*::P$_{MEP2}$-*GFP-caSAT1/MEP2-2*	This study
Δ*gat1*MEP1PG1A	GAT1M4A	*gat1-1Δ::FRT/gat1-2Δ::FRT* *mep1-1*::P$_{MEP1}$-*GFP-caSAT1/ MEP1-2*	This study
Δ*gat1*MEP1PG1B	GAT1M4B	*gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP1-1/mep1-2*::P$_{MEP1}$-*GFP-caSAT1*	This study
Δ*gln3*Δ*gat1*MEP2PG1A	Δ*gln3*GAT1M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP2-1/mep2-2*::P$_{MEP2}$-*GFP-caSAT1*	This study
Δ*gln3*Δ*gat1*MEP2PG1B	Δ*gln3*GAT1M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT* *mep2-1*::P$_{MEP2}$-*GFP-caSAT1/MEP2-2*	This study
Δ*gln3*Δ*gat1*MEP1PG1A	Δ*gln3*GAT1M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT* *mep1-1*::P$_{MEP1}$-*GFP-caSAT1/MEP1-2*	This study
Δ*gln3*Δ*gat1*MEP1PG1B	Δ*gln3*GAT1M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT* *gat1-1Δ::FRT/gat1-2Δ::FRT* *MEP1-1/mep1-2*::P$_{MEP1}$-*GFP-caSAT1*	This study

Strains expressing wild-type and hyperactive *MEP2* alleles from the *ADH1* promoter or carrying a control construct in wild-type and gln3Δ backgrounds

Strain	Parent	Relevant genotype[a]	Reference
SCADH1G4A/B	SC5314	*ADH1/adh1*::P$_{ADH1}$-*GFP-caSAT1*	This study
SCMEP2E4A/B	SC5314	*ADH1/adh1*::P$_{ADH1}$-*MEP2-caSAT1*	This study
SCMEP2ΔC2E2A/ B	SC5314	*ADH1/adh1*::P$_{ADH1}$-*MEP2^{ΔC440}-caSAT1*	This study

Strain	Parent	Relevant genotype[a]	Reference
Δ*gln3*ADH1G4A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT ADH1/adh1*::P*ADH1*-GFP-caSAT1	This study
Δ*gln3*ADH1G4B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT ADH1/adh1*::P*ADH1*-GFP-caSAT1	This study
Δ*gln3*MEP2E4A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT ADH1/adh1*::P*ADH1*-MEP2-caSAT1	This study
Δ*gln3*MEP2E4B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT ADH1/adh1*::P*ADH1*-MEP2-caSAT1	This study
Δ*gln3*MEP2ΔC2E2A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT ADH1/adh1*::P*ADH1*-MEP2^ΔC440-caSAT1	This study
Δ*gln3*MEP2ΔC2E2B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT ADH1/adh1*::P*ADH1*-MEP2^ΔC440-caSAT1	This study

Strains expressing P*SAP2*-GFP, P*OPT1*-GFP, P*OPT3*-GFP, and P*STP1*-GFP reporter gene fusions

Strain	Parent	Relevant genotype[a]	Reference
SCSAP2G1A/B	SC5314	*sap2-1*::P*SAP2-1*-GFP/SAP2-2	(Reuß & Morschhäuser, 2006)
SCOPT1G22A	SC5314	*OPT1-1/opt1-2*::P*OPT1*-GFP	(Reuß & Morschhäuser, 2006)
SCOPT1G22B	SC5314	*opt1-1*::P*OPT1*-GFP/OPT1-2	(Reuß & Morschhäuser, 2006)
SCOPT3G22A	SC5314	*opt3-1*::P*OPT3*-GFP/OPT3-2	(Reuß & Morschhäuser, 2006)
SCOPT3G22B	SC5314	*OPT3-1/opt3-2*::P*OPT3*-GFP	(Reuß & Morschhäuser, 2006)
SCSTP1G1A/B	SC5314	*STP1/stp1*::P*STP1*-GFP	This study
Δ*gln3*SAP2G1A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT sap2-1*::P*SAP2*-GFP/SAP2-2	This study
Δ*gln3*SAP2G1B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT sap2-1*::P*SAP2*-GFP/SAP2-2	This study
Δ*gln3*OPT1G22A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT OPT1-1/opt1-2*::P*OPT1*-GFP	This study
Δ*gln3*OPT1G22B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT OPT1-1/opt1-2*::P*OPT1*-GFP	This study
Δ*gln3*OPT3G22A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT opt3-1*::P*OPT1*-GFP/OPT3-2	This study
Δ*gln3*OPT3G22B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT OPT3-1/opt3-2*::P*OPT1*-GFP	This study
Δ*gln3*STP1G1A	GLN3M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT STP1/stp1*::P*STP1*-GFP	This study

Strain	Parent	Relevant genotype[a]	Reference
Δ*gln3*STP1G1B	GLN3M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT STP1/stp1*::P$_{STP1}$-*GFP*	This study
Δ*gat1*SAP2G1A	GAT1M4A	*gat1-1Δ::FRT/gat1-2Δ::FRT sap2-1*::P$_{SAP2}$-*GFP/SAP2-2*	This study
Δ*gat1*SAP2G1B	GAT1M4B	*gat1-1Δ::FRT/gat1-2Δ::FRT sap2-1*::P$_{SAP2}$-*GFP/SAP2-2*	This study
Δ*gat1*OPT1G22A	GAT1M4A	*gat1-1Δ::FRT/gat1-2Δ::FRT OPT1-1/opt1-2*::P$_{OPT1}$-*GFP*	This study
Δ*gat1*OPT1G22B	GAT1M4B	*gat1-1Δ::FRT/gat1-2Δ::FRT opt1-1*::P$_{OPT1}$-*GFP/OPT1-2*	This study
Δ*gat1*OPT3G22A	GAT1M4A	*gat1-1Δ::FRT/gat1-2Δ::FRT OPT3-1/opt3-2*::P$_{OPT1}$-*GFP*	This study
Δ*gat1*OPT3G22B	GAT1M4B	*gat1-1Δ::FRT/gat1-2Δ::FRT opt3-1*::P$_{OPT1}$-*GFP/OPT3-2*	This study
Δ*gat1*STP1G1A	GAT1M4A	*gat1-1Δ::FRT/gat1-2Δ::FRT STP1/stp1*::P$_{STP1}$-*GFP*	This study
Δ*gat1*STP1G1B	GAT1M4B	*gat1-1Δ::FRT/gat1-2Δ::FRT STP1/stp1*::P$_{STP1}$-*GFP*	This study
Δ*gln3*Δ*gat1*SAP2G1A	Δ*gln3*GAT1M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT sap2-1*::P$_{SAP2}$-*GFP/SAP2-2*	This study
Δ*gln3*Δ*gat1*SAP2G1B	Δ*gln3*GAT1M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT sap2-1*::P$_{SAP2}$-*GFP/SAP2-2*	This study
Δ*gln3*Δ*gat1*OPT1G22A	Δ*gln3*GAT1M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT OPT1-1/opt1-2*::P$_{OPT1}$-*GFP*	This study
Δ*gln3*Δ*gat1*OPT1G22B	Δ*gln3*GAT1M4B	*gat1-1Δ::FRT/gat1-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT opt1-1*::P$_{OPT1}$-*GFP/OPT1-2*	This study
Δ*gln3*Δ*gat1*OPT3G22A	Δ*gln3*GAT1M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT opt3-1*::P$_{OPT1}$-*GFP/OPT3-2*	This study
Δ*gln3*Δ*gat1*OPT3G22B	Δ*gln3*GAT1M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT OPT3-1/opt3-2*::P$_{OPT1}$-*GFP*	This study
Δ*gln3*Δ*gat1*STP1G1A	Δ*gln3*GAT1M4A	*gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT STP1/stp1*::P$_{STP1}$-*GFP*	This study
Δ*gln3*Δ*gat1*STP1G1B	Δ*gln3*GAT1M4B	*gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT STP1/stp1*::P$_{STP1}$-*GFP*	This study

Strain	Parent	Relevant genotype[a]	Reference
gln3Δ gat1Δ double mutants expressing _SAP2_ or _OPT1_ from the _ADH1_ promoter			
Δ_gln3_Δ_gat1_SAP2ex7A	Δ_gln3_GAT1M4A	_gln3-1_Δ::_FRT_/_gln3-2_Δ::_FRT_ _gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{ADH1}$-_SAP2_	This study
Δ_gln3_Δ_gat1_SAP2ex7B	Δ_gln3_GAT1M4B	_gln3-1_Δ::_FRT_/_gln3-2_Δ::_FRT_ _gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{ADH1}$-_SAP2_	This study
Δ_gln3_Δ_gat1_OPT1E1A	Δ_gln3_GAT1M4A	_gln3-1_Δ::_FRT_/_gln3-2_Δ::_FRT_ _gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{ADH1}$-_OPT1_	This study
Δ_gln3_Δ_gat1_OPT1E1B	Δ_gln3_GAT1M4B	_gln3-1_Δ::_FRT_/_gln3-2_Δ::_FRT_ _gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{ADH1}$-_OPT1_	This study
stp1Δ mutants			
STP1M1A/B	SC5314	_STP1_/_stp1_Δ::_SAT1-FLIP_	This study
STP1M2A	STP1M1A	_STP1_/_stp1_Δ::_FRT_	This study
STP1M2B	STP1M1B	_STP1_/_stp1_Δ::_FRT_	This study
STP1M3A	STP1M2A	_stp1_Δ::_FRT_/_stp1_Δ::_SAT1-FLIP_	This study
STP1M3B	STP1M2B	_stp1_Δ::_FRT_/_stp1_Δ::_SAT1-FLIP_	This study
STP1M4A	STP1M3A	_stp1_Δ::_FRT_/_stp1_Δ::_FRT_	This study
STP1M4B	STP1M3B	_stp1_Δ::_FRT_/_stp1_Δ::_FRT_	This study
gat1Δ mutants expressing different _GAT1_ versions from a tetracycline-inducible (Tet) promoter			
Δ_gat1_TET1-GAT1-1A	GAT1M4A	_gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{tet}$-_GAT1_[2268]	This study
Δ_gat1_TET1-GAT1-1B	GAT1M4B	_gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{tet}$-_GAT1_[2268]	This study
Δ_gat1_TET1-GAT1-2A	GAT1M4A	_gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{tet}$-_GAT1_[2067]	This study
Δ_gat1_TET1-GAT1-2B	GAT1M4B	_gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{tet}$-_GAT1_[2067]	This study
Δ_gat1_TET1-GAT1-3A	GAT1M4A	_gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{tet}$-_GAT1_[2004]	This study
Δ_gat1_TET1-GAT1-3B	GAT1M4B	_gat1-1_Δ::_FRT_/_gat1-2_Δ::_FRT_ _ADH1_/_adh1_::P$_{tet}$-_GAT1_[2004]	This study

Strain	Parent	Relevant genotype[a]	Reference
gln3Δ gat1Δ double mutants expressing STP1 or STP1 [ΔN61] from a tetracycline-inducible promoter			
Δgln3Δgat1TET1-STP1A	Δgln3GAT1M4A	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT ADH1/adh1::P_{tet}-STP1	This study
Δgln3Δgat1TET1-STP1B	Δgln3GAT1M4B	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT ADH1/adh1::P_{tet}-STP1	This study
Δgln3Δgat1TET1-STP1[ΔN61]A	Δgln3GAT1M4A	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT ADH1/adh1::P_{tet}-STP1 [ΔN61]	This study
Δgln3Δgat1TET1-STP1[ΔN61]B	Δgln3GAT1M4B	gln3-1Δ::FRT/gln3-2Δ::FRT gat1-1Δ::FRT/gat1-2Δ::FRT ADH1/adh1::P_{tet}-STP1 [ΔN61]	This study
stp1Δ mutants expressing STP1, GLN3, or GAT1 from a tetracycline-inducible promoter			
Δstp1TET1-STP1A	STP1M4A	stp1Δ::FRT/stp1Δ::FRT ADH1/adh1::P_{tet}-STP1	This study
Δstp1TET1-STP1B	STP1M4B	stp1Δ::FRT/stp1Δ::FRT ADH1/adh1::P_{tet}-STP1	This study
Δstp1TET1-GLN3A	STP1M4A	stp1Δ::FRT/stp1Δ::FRT ADH1/adh1::P_{tet}-GLN3	This study
Δstp1TET1-GLN3B	STP1M4B	stp1Δ::FRT/stp1Δ::FRT ADH1/adh1::P_{tet}-GLN3	This study
Δstp1TET1-GAT1-1A	STP1M4A	stp1-1Δ::FRT/stp1-2Δ::FRT ADH1/adh1::P_{tet}-GAT1[2268]	This study
Δstp1TET1-GAT1-1B	STP1M4B	stp1-1Δ::FRT/stp1-2Δ::FRT ADH1/adh1::P_{tet}-GAT1[2268]	This study
Δstp1TET1-GAT1-2A	STP1M4A	stp1-1Δ::FRT/stp1-2Δ::FRT ADH1/adh1::P_{tet}-GAT1[2067]	This study
Δstp1TET1-GAT1-2B	STP1M4B	stp1-1Δ::FRT/stp1-2Δ::FRT ADH1/adh1::P_{tet}-GAT1[2067]	This study
Δstp1TET1-GAT1-3A	STP1M4A	stp1-1Δ::FRT/stp1-2Δ::FRT ADH1/adh1::P_{tet}-GAT1[2004]	This study
Δstp1TET1-GAT1-3B	STP1M4B	stp1-1Δ::FRT/stp1-2Δ::FRT ADH1/adh1::P_{tet}-GAT1[2004]	This study

[a] Apart from the indicated features all strains have the genotype of their parental strain.

[b] SAT1-FLIP denotes the SAT1 flipper cassette.

3.4 Primers

Primers were obtained from MWG (Ebersberg, Germany). Restriction sites introduced into the primers are underlined; the substituted nucleotides either for introduction of restriction site or amino acid exchange are highlighted in bold; the start and stop codons are in italics. Primers with the suffix "p" are 5' phosphorylated.

Table 3. List of primers used in this study

Primer	No. of nucleotides	T_m (ºC)	Sequence (5' → 3')
GAT1	29	68.1	CCGATAACAATAA<u>**GGGCCC**</u>TCCCAATCAG
GAT2	28	68.0	TGTAGTGGCTGTG<u>**CTCGAG**</u>TTAAGCTGC
GAT3	30	65.4	GCTAATAATCAAG<u>**CCGCGG**</u>ATTGGTTAAAC
GAT4	29	63.9	TGCTATGTTCAGT<u>**GAGCTC**</u>ACATTTGAAG
GAT5	32	72.1	GAGGGTT<u>**CCGCGG**</u>CTAGTGGAGTCAATACATC
GAT6	32	69.5	TGACAGAG<u>**GAGCTC**</u>ATGATTGGGTTGGATCTG
GAT7	31	65.5	ATAGATGACA<u>**CTCGAG**</u>TTGATGATTGGGTTG
GAT8	21	52.0	AGATTGGTCAATTTAATCAGC
GAT9	18	51.4	ATTCTCAACGTCAGCATC
GAT10	21	55.9	AACTACTCCATTATGGAGACG
GAT11	30	66.8	ATGAATAGGTTGT<u>**GAGCTC**</u>GGTTGACTAGG
GAT12	33	63.3	ATATGGATCCATAATTCATGTTTAACCAATCCC
GAT13	29	66.7	TGTTGAAATCATGGTGCGGTTCAGGTTGG
GAT14	32	64.4	TTGTGATAATTGGGACATTGTATTAGTGGCAG
GAT15	31	68.2	AAGTCATACCACCACCTGAACTACCTCTGTC
GAT1-1	34	69.5	ATA**T**<u>**GTCGAC**</u>A*ATG*TACTACCGTGCTCGTCACTC
GAT1-2	33	60.8	ATAT<u>**GGATCC**</u>*TA*ATAATTCATGTTTAACCAATC
GAT1-3	37	63.9	A**T**A**T**<u>**GTCGAC**</u>A*ATG*ACAATGAATTTTAATCAAACAGG
GAT1-4	35	62.4	**ATAT**<u>**GTCGAC**</u>A*ATG*ATGTATATTAACAACAAATCG
GLN1	30	66.8	ATAAC<u>**GGGCCC**</u>TACCTAGAGGAATAAGTTC
GLN2	30	68.1	GACTGACTATTCG<u>**CTCGAG**</u>TCATTTGTCCC
GLN3	30	69.5	GGGGATTATAAGG<u>**CCGCGG**</u>ATTGGTTGAAG
GLN4	29	69.5	GTCGTTTAGGTCAC<u>**GAGCTC**</u>CACGAGATG
GLN5	31	64.2	ATATT<u>**GGATCC**</u>TAGAGTTTGCAAACACGTAC
GLN6	30	62.7	GTCATTA<u>**GGATCC**</u>AAAGTATATACTATGCC
GLN7	25	63.0	ACTACAACAACGACGTCGTATCACC
GLN8	22	56.5	CATCTTCCACTTCATTATCTGG
GLN9	22	56.5	CATACTAGAGAATGTGAACACC

Primer	No. of nucleotides	T$_m$ (ºC)	Sequence (5' → 3')
GLN10	28	63.7	TTTT<u>CT**CGA**G</u>GGACAAATGACTACATCG
GLN11	35	68.3	ACGT**GGA**<u>TCC</u>TCAAATGTCAAACTTCAACCAATCC
MEP3	34	62.1	TAAATAC**GG**<u>TACC</u>CAAACGATTGGCTTGAATGTC
MEP24	30	64.9	CCAGACA**CTCGA**GTTATTAACTATTCAGAG
MEP32	29	68.4	AAAGAACT**G**<u>GATCC</u>ATTTTTAGCTTCTCC
MEP39p	27	77.9	TGGCGGTCCAGTTCACGAAAACTCTGG
MEP44	31	65.5	CAGCTATCTT<u>GGTACC</u>TCATCAATCAATTGC
MEP51	30	61.3	GAAAAA<u>TCTAGA</u>AATCCCTATTGTGATTGG
MEP52	26	63.2	AACCAC**TCTAGA**TTTACCCCACTTCG
MEP53	32	64.4	GTGATGC<u>TCTAGA</u>TAAATACAATACCCAAACG
MEP54	34	63.4	ATGATTT<u>TCTAGA</u>ATATACCATGAAGTACCAAGC
MEP55	30	64.0	TCCTCGT<u>TCTAGA</u>TACTAATGGTTGATACG
MEP60	32	65.6	GGGGTAAA<u>TCTAGA</u>GTGGTTAAAAGGATATCC
MEP61	31	60.2	TCTCAAT<u>TCTAGA</u>ATTATTCCTGTATATTGC
MEP62	35	60.1	TTTT<u>TCTAGA</u>AAATTGTTTATCAGTGTGAAAAATC
MEP63	30	62.7	CTGGGAAATTGT<u>**TCTAG**A</u>GTGTGAAAAATC
MEP64	30	62.7	GATTTTTCACAC<u>**TCTAG**A</u>ACAATTTCCCAG
MEP65	29	58.2	CCTGTATATTGC<u>**AGATC**T</u>ATATTTTTTC
MEP66	29	58.2	GAAAAAAATAT<u>AGATCT</u>GCAATATACAGG
MEP67p	25	64.6	CCGGCGAA**ATT**CAATGCACCTAAGC
MEP68p	32	70.8	CAGCAGCAAACATCCCTTGG**GC**AAGACAGAAC
MEP69p	32	70.8	CAGCAGCA**GC**CATCCCTTGGTAAAGACAGAAC
MEP70p	25	63.0	TCACTGCTATTTTGATGGCTGGTGC
MEP71p	25	66.3	CCGGCGAAATCCAATGCACCTAAGC
MEP72p	27	69.5	TGGCGGTCCAGTT**GCC**GAAAACTCTGG
MEP73p	22	56.5	ACAGTAAACAACAGTCAACCAG
MEP74p	26	68.0	CCTATTGCCTAT**GCC**ACATGGGGTGG
MEP75p	23	67.8	GG**C**CCCACCGTTGAAACCGTACC
MEP76p	23	60.6	ACTGGTAACTCTTCCATGCGTTC
MEP77p	22	58.4	GTCCATACCATCATCAATCTGC
MEP78p	26	71.1	GTGTGGGCACTC**GC**TGGTGTTGGTGG
MEP79	29	59.6	AAAGAAC**AGAT**<u>*CT*</u>*A*ATTTTTAGCTTCTCC
MEP80p	32	70.8	CCTATTGCCTATTGGACA**GCC**GGTGGAAATGG
MEP81	32	64.4	TTGGTTTC<u>AGAT**CT**</u>*A*GTCATCAGCATAATAGG
MEP82	30	61.3	TTGGTTTCA<u>AGAT*CT*</u>*A*ATCAGCATAATAGG
MEP83	41	68.4	TTGGTTTCAGGAT<u>AGAT**CT**</u>*A*AGCATAATAGGCATATTCACC
MEP84	36	66.1	TTCAGGATCGT<u>**AGAT***CT*</u>*A*ATAATAGGCATATTCACC
MEP85	37	68.4	TTCAGGATCGTCATC<u>**AGAT***CT*</u>*A*ATAGGCATATTCACC
MEP86	38	69.5	ATCGTCATCAGC**AGAT**<u>*CT*</u>*A*GGCATATTCACCAATTTGG

Primer	No. of nucleotides	T$_m$ (°C)	Sequence (5' → 3')
MEP87	38	67.3	ATCGTCATCAGCAT**AGAT*CTA*ATATTCACCAATTTGGG
MEP88	38	67.3	ATCGTCATCAGCAT**AGAT*CTA*AA**ATTCACCAATTTGGG
MEP89p	22	64.0	TTGGGCCAAGTCGGTTCCCAAC
MEP90p	28	60.7	ATTGGTGAAT**T**TGCCTATTATGCTGATG
MEP91	37	63.9	TCACTTCAAAGATCTAATAAGCAAATTCACCAATTT
MEP92	37	63.9	TCACTTCAAAGATCTAATAAGCATATTCACCAATTT
MEP94p	27	72.6	GGACCCACCGTT**GGC**ACCGTACCAACC
STP1-1	38	65.1	**ATATA**GTCGAC**AAA*ATG*CCACCAATACAAAGATTAAG
STP1-2	30	58.6	AAACT**AGAT**C**TTAATCTAGTAATAGATTGC
STP1-3	28	62.2	TTACGTACCACAACTTATACCATCAAGC
STP1-4	37	67.2	AACCAAT**GTCGAC**AACTATGTTGATACTTTCCATAGG
STP1-5	28	65.1	ATGAAAAGATAA**GGGCCC**ATGGAAAGCC
STP1-6	26	56.9	AGTTATA**GTCGAC**GTTCTTTAATATG
STP1-7	23	58.9	ATATATC**CTGCA**GGTGTAAAGGC
STP1-8	24	61.0	TAATGAA**GAGCTC**GAACCTGAACG
STP1-9	24	64.4	AATGGAAGAT**CCGCGG**CTGTTTCC

3.5   Materials

Sources of chemicals, enzymes and equipments used in this study were:

Amersham, Applichem, Boehringer, Difco, Eppendorf, Gibco, Gilson, Greiner, Merck, New England Biolabs (NEB), MWG-Biotech, Oxoid, Peqlab, Pharmacia, Roth, Serva and Sigma.

Table 4. Equipments used in the study

Equipments used	Source
Centrifuge	
Tabletop centrifuge (Biofuge Pico)	Heraeus
4° centrifuge (Megafuge 2.0R)	
Confocal Laser Scanning Microscope	Zeiss
(Zeiss LSM 510 + Zeiss Axiovert 100)	
Digital camera Coolpix 4500	Nikon

Equipments used	Source
Electrophoresis apparatus	
Agarose gel (DNA Sub Cell GT/Mini Sub Cell GT)	Bio-Rad
Polyacrylamide gel (Mini Protein 3)	Bio-Rad
Agarose-Formadehyde-Gel	Fröbel
Electroporation apparatus (Easyjet prima)	Equibio
FACSCalibur cytometry system	Becton Dickinson
Geldocumentation system	Bio-Rad
Hybridization oven Shake `n´ Stack	Hybaid
Incubators	
for *E. coli* (37°C) (Type B6200)	Heraeus
for *C. albicans* (30°C) (Model 400)	Memmert
Shaking incubators	
for *E. coli* (37°C) Innova 4300	New Brunswick Scientific
for *C. albicans* (30°C) Certomat BS-1	B. Braun Biotech
Spectrophotometer (Ultrospec 3000)	Pharmacia Biotech
Stereozoom microscope SMZ800	Nikon
Thermocycler Cyclone 25	Peqlab
UV-Crosslinker Sratalinker 1800	Stratagene
Vacuum-Blotter	Pharmacia Biotech

3.6   Methods

For all microbiology and molecular biology procedures standard protocols were followed from Sambrook *et al.*, (1989) or Ausubel *et al.*, (1989).

    All solutions and media were made in double distilled and distilled water, respectively. All solutions and media were sterilized by autoclaving or filter sterilized by passing through a 0.22 micron Millipore filter.

3.6.1   Growth and maintenance of *E. coli* strains

Recombinant *E. coli* strains were routinely grown in LB liquid medium (1% Peptone, 0.5% Yeast extract, 0.5% NaCl) under the selection pressure (either 100 µg/ml ampicillin or 30 µg/ml chloramphenicol). For growth on plates, 1.5% agar [Difco™ agar granulated, BD] was added to the media. The cells in liquid culture were grown at 37°C for 16-18 hours with shaking at 200 rpm and the plates were incubated at 37°C until the colonies appeared. While liquid cultures were processed to isolate plasmid DNA, the plates with streaked colonies were stored at 4°C.

3.6.2   Growth and maintenance of *C. albicans* strains

C. albicans strains were routinely grown in YPD liquid medium (2% Peptone, 1% Yeast extract, 2% Glucose) at 30°C shaker with rpm of 250. For growth on plates 1.5% agar was added to the YPD medium.

3.6.3   Selection media for *C. albicans* transformants

Uridine prototrophic *C. albicans* strains were selected and propagated on SD agar plates (0.67% yeast nitrogen base without amino acids [BIO 101, Vista, Calif.], 2% glucose, 1x CSM-URA [BIO 101, Vista, Calif.] and 1.5% agar). To support growth of uridine auxotrophic strains, 100 µg/ml uridine was added to the media. For the selection of nourseothricin-resistant transformants, 200 µg/ml nourseothricin (Werner Bioagents, Jena, Germany) was added to YPD agar plates. Nourseothricin-sensitive derivatives in which the *SAT1* flipper was excised by FLP-mediated recombination, were obtained by growing transformants for 6 h in YPM medium (2% Peptone, 1% Yeast extract, 2% Maltose) without selective pressure. On plates containing 20 µg/ml of nourseothricin, Nou^S clones were identified by their small colony size in comparison to the Nou^R parental strain. These Nou^S clones were confirmed by restreaking on YPD plates containing 100 µg/ml of nourseothricin.

3.6.4   Phenotypic assays

To observe *MEP2* expression, strains were grown overnight in liquid minimal proline medium (0.17% yeast nitrogen base without amino acids without ammonium sulphate [BIO 101, Vista, Calif.], 2% glucose, 0.1% proline) diluted 50 fold, and grown for six hours at 30°C in SD medium in which the standard concentration of 76 mM ammonium was replaced

by 100 µM ammonium or other nitrogen sources as indicated in the text. To study filamentation, and Mep2p expression on solid media, overnight cultures of the strains in YPD medium were appropriately diluted and plated on SD agar plates (washed agar 2%) containing 100 µM ammonium or other nitrogen sources. Individual colonies were photographed after 6 days of growth at 37°C. Growth of the strains was assayed by incubating strains for 4 days at 30°C on SD agar plates containing 1 mM ammonium. Filamentation was also tested on agar plates containing 10% fetal calf serum (PAA Laboratories GmbH).

To test for growth on BSA as the sole nitrogen source, strains were grown at 30°C in YCB-BSA medium (2.34 % yeast carbon base [YCB, Difco, Heidelberg, Germany], 0.4% bovine serum albumin [BSA, Sigma, Deisenhofen, Germany], pH 4.0). Experiments in which normal growth of *gat1Δ* and *gln3Δ gat1Δ* mutants was required, 0.2% yeast extract was added to YCB-BSA medium (YCB-BSA-YE). For induction of tet-promoter, 50 µg/ml doxcycycline was added to the experimental medium.

3.6.5   Small scale plasmid DNA isolation (Miniprep)

Miniprep was carried out by modified alkaline lysis method (Sambrook *et al.*, 1989). Cells were harvested at 13,000 rpm for 1 minute at RT. Medium was removed by aspiration and bacterial pellets were suspended completely in 100 µl Solution I (25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 50 mM Glucose). 200 µl freshly prepared Solution II (1% SDS, 0.2 N NaOH) was added and mixed by inverting tubes 3-4 times. 150 µl ice cold Solution III (3 M sodium acetate pH 5.2) was added to the cell lysate, mixed thoroughly by inversion and then incubated on ice for 10 minutes. To remove cell debris, bacterial cell lysate was centrifuged at 13,000 rpm for 5 minutes at RT and the supernatant was collected and extracted once with 200 µl phenol/chloroform/isoamyl alcohol (25:24:1). Plasmid DNA was precipitated, by adding 1 ml 96% ethanol to the supernatant and then mixed by inversion. The plasmid pellet was recovered by centrifugation at 13,000 rpm for 5 minutes at RT, the supernatant was discarded, the pellet washed once with 70% ethanol and air-dried. The pellet was dissolved in 50 µl double distilled water (100-200 ng/l DNA), and 1 µl RNase A (50 mg/ml) was added to each sample.

3.6.6   Polymerase Chain Reaction (PCR)

As high fidelity DNA polymerase either Phusion (Finnzymes) or Elongase (Invitrogen) was used for all PCR applications. Usually the PCR reaction volume was 50 µl which contain 1-2 ng plasmid DNA or 0.1-0.5 µg genomic DNA as template, 0.2 mM of each dNTP, 0.5 µM of each primer, 1 unit of enzyme and polymerase specific buffer. The PCR reaction was performed on Thermocycler Cyclone 25 (Peqlab) following the programme recommended by the enzyme manufacturers.

Following are the cycling conditions when using Phusion enzyme:

Initially the DNA was denatured at 98°C for 30 s followed by 30 cycles of amplifications.

Denaturation at 98°C for 10 s

Annealing temperature (optimized) 45-55°C for 30 s } 30 cycles

Extension at 72°C for 30 s /1 kb product

Final extension was done at 72°C for 10 minutes in order to fill the incomplete extension products. The annealing temperature was adjusted on the basis of the T_m of the primers (mentioned in Table 3) used for amplification. The template amount, number of cycles and cycling conditions were varied to maximize the product yield. Successful amplification was confirmed by agarose gel electrophoresis and the PCR product was purified using Qiagen kit (Qiagen GmbH, Hilden, Germany).

3.6.7   DNA digestion with restriction enzymes

DNA digestions were done using 10x 'one-for all buffer' (330 mM Tris-acetate pH 7.9, 660 mM K-acetate, 100 mM Mg-acetate, 5 mM DTT, 0.1% BSA) with final concentration of 1x in the digestion reaction. Amount of enzyme per µg of DNA used was according to manufacturer's instructions. All preparative digestion reactions were generally done in a 50 µl reaction volume, at 37ºC for 6 h, containing 30-35 µl of plasmid DNA (inserts) or 10 µl of plasmid DNA (vectors). The digests were then mixed with 10x DNA stop buffer (0.2% bromophenol blue, 0.2 M EDTA, 50% glycerol) to a final concentration of 1x, and loaded on a 1% agarose gel along with 1 kb DNA ladder (Invitrogen).

3.6.8   Gel electrophoresis and gel elution of DNA fragments

Agarose gel electrophoresis of DNA was routinely carried out in 1x TAE (1 liter 50x TAE stock solution : 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA pH 8.0 in distilled water). After electrophoresis, the gels were stained in ethidium bromide (10

mg/ml in water) and photographed using a gel documentation system. The sizes of the fragments were estimated by measuring the relative mobility of the bands in comparison to markers of known molecular size (1 kb DNA ladder, Invitrogen), run in a lane alongside.

3.6.9   Elution of inserts from agarose gel

To release insert of interest, digested DNA sample was loaded on the agarose gel and run overnight at low voltage till the band of interest was well separated from the vector backbone. Required band was cut out with a scalpel. The DNA was eluted from the gel slice using the 'Gene Clean Kit' (QBiogene, Heidelberg, Germany). 400 µl NaI was added to the gel slice and incubated at 50°C till the agarose melted (10-15 minutes). 7 µl glass-milk was added and thoroughly mixed by vortexing; it was then incubated on ice for 5 minutes. Washed 3 times with 200 µl 'New Wash' solution 1 (13,000 rpm, 1 min). 11 µl distilled water was added to the pellet, mixed well, and incubated at 50°C for 5 minutes. After centrifugation at 13,000 rpm for 2 minutes, the supernatant was collected which contains the fragment of interest. Digested PCR products were eluted from the gel similarly. Linear DNA fragments needed for *C. albicans* transformation were eluted in 6 µl distilled water, instead of 11 µl.

3.6.10   Cloning gene of interest in vectors

Ligation: The vector most commonly used in these studies was pBluescript KS II (Stratagene, Heidelberg, Germany). Ligation reactions were set up at 1:5 ratio of vector and insert (in general) in a 20 µl reaction volume containing 10 µl of 2x Quick ligase buffer (NEB) and 1 unit of Quick ligase enzyme (NEB). A vector-only control ligation reaction was also set up during each ligation experiment. Ligation reactions were carried out at RT for 15-30 min.

Transformation of E. coli

Preparation of E. coli DH5α competent cells: *E. coli* competent cells were prepared using calcium chloride method. Single colony of DH5α was inoculated in 10 ml LB medium and grown at 37°C with shaking at 200 rpm for 16-18 hours. The overnight grown culture was diluted 1:100 in 50 ml fresh LB medium and incubated at 37°C shaker for 3-4 hours (till OD_{600}= 0.7-0.9). The culture was then transferred to chilled 50 ml tubes and centrifuged at 3,000 rpm for 10 min at 4°C in a chilled rotor. The supernatant was discarded and the cell pellet was resuspended in 20 ml of ice cold 100 mM calcium chloride The cell mix was

incubated on ice for 30 minutes and centrifuged at 4,000 rpm for 10 minutes at 4°C. The pellet was then resuspended gently in 2.5 ml of 100 mM calcium chloride and 86% glycerol was added to a final concentration of 15% (v/v). Aliquotes of 200 µl competent cells were made in the eppendorf tubes and stored at -80°C for later use.

Transformation of competent cells:

For each transformation a frozen aliquot of competent cells was thawed on ice. The ligation reaction mixture (20 µl) was added to 200 µl competent *E. coli* cells and incubated for 30 minutes on ice. The cells were subjected to heat shock at 42°C (water-bath) for 90 seconds and then chilled on ice for 2 minutes. After adding 1 ml LB medium the cells were allowed to grow for 1h at 37°C. Finally, the cells were spread on LB selection plate (containing ampicillin or chloramphenicol). These plates were incubated at 37°C until the colonies appeared (~16 hours).

3.6.11   Screening of recombinants

3 µl of miniprep plasmid DNA of clones was digested with appropriate restriction enzymes at 37°C in a 20 µl reaction mix for 1-2 h and separated on a 1% agarose gel. After staining in ethidium bromide the gel was observed in gel documentation system and correct recombinants were selected based on their expected band pattern on gel. Sequences of these clones were also verified, in order to avoid selecting such recombinants which may contain undesired nucleotide substitutions that can arise in PCR.

DNA-Sequencing

For sequencing of the cloned fragments, plasmid DNA was isolated as described earlier. The DNA sample was purified in following manner: Volume of the sample was made up to 200 µl by adding distilled water. After extracting once with phenol/chloroform/IAA (25:24:1) and once with chloroform/IAA (24:1), the DNA was precipitated by addition of 0.1 volume 3 M sodium acetate pH 5.2 and 2.5 volumes 100% ethanol and incubation at -70°C for 1 h. The DNA pellet was recovered by centrifugation at 13,000 rpm for 5 minutes at RT. After washing with 70% ethanol the DNA pellet was dissolved in 20 µl distilled water. Reaction mixture containing 1 µl of the plasmid DNA, 1 µl primer (100 pmole/µl) chosen as per the requirement of cloned fragment to be sequenced, and 5 µl distilled water was sent to Sequence Laboratories Göttingen GmbH (Göttingen, Germany).

3.6.12 *C. albicans* **transformation**

C. albicans strains were transformed by electroporation as described by Köhler *et al.*, (1997), with slight modifications. A single colony of the strain to be transformed was inoculated in 10 ml YPD medium (supplemented with 100 μg/ml uridine if strain is *ura3* negative), grown overnight at 30ºC shaker (250 rpm). Cells from the preculture were diluted 10^{-4} in 50 ml fresh YPD medium and grown at 30°C shaker till the culture reached mid log phase (OD_{600}= 1.6-2.2). Cells were collected by centrifugation at 4,000 rpm at 4°C for 5 minutes. The cell pellet was then suspended in 8 ml of sterile distilled water. After addition of 1 ml of 10x TE (100 mM Tris-HCl pH 7.5, 10 mM EDTA, pH 7.5) and 1 ml of 1M lithium acetate (pH 7.5), the suspension was incubated in rotary shaker for 60 minutes at 30°C. 250 μl of 1 M dithiothreitol (DTT) was then added, and the cells were incubated again for 30 min at 30°C with shaking. After addition of 40 ml of water the cells were centrifuged (4,000 rpm, 4°C, 5 minutes), cell pellet was then washed sequentially in 25 ml of ice-cold water and 5 ml of ice-cold 1 M sorbitol. The supernatant was discarded and the washed cell pellet was resuspended in 50 μl of 1 M sorbitol and kept on ice.

Five μl of the linear DNA fragments was mixed with 40 μl of electrocompetent cells in a 0.2 cm cuvette (PeqLab, Erlangen, Germany). For control, 5 μl of sterile distilled water was used instead of DNA. Electroporation was carried out at 1.8 kV using an electroporation apparatus (Equibio, Kent, UK). After electroporation, the cells were transferred to eppendorf tubes using 1 ml of 1 M sorbitol. When *URA3* gene was used as the selection marker, the electroporated cells were collected by centrifugation (4,000 rpm, 4°C, 2 minutes) resuspended in 100 μl of residual sorbitol and plated on the minimal media selection plate directly. When the transforming cassette contained *caSAT1* (dominant nourseothricin resistance marker), the electroporated cells after resuspension in 1 ml YPD medium were divided into two parts so that two independent first round transformants could be obtained. The volume of each part was adjusted to 1 ml by adding 500 μl YPD and the samples were then incubated for 4-6 h at 30°C. After incubation, 100 μl from the sample was spread on YPD plates containing 200 μg/ml of nourseothricin. The plates were incubated at 30°C until colonies appeared. Individual colonies were streaked on respective selection media plates for further analysis.

3.6.13 **Genomic DNA isolation from** *C. albicans*

Strains were grown overnight in 10 ml YPD medium at 30°C. The cells were collected by centrifugation at 4,000 rpm for 5 minutes, resuspended in 1 ml 1M Sorbitol and transferred to

2 ml micro centrifuge tubes. The cells were washed by centrifugation at 4,000 rpm for 5 minutes, the supernatant was discarded and the cell pellet was resuspended in 1 ml SCEM buffer (1M sorbitol, 100 mM sodium citrate pH 5.8, 50 mM EDTA, 2% β-mercaptoethanol, 500 U/ml lyticase [Sigma]) and incubated at 37°C for 45 minutes. The protoplasts thus obtained were centrifuged (13,000 rpm, 5 minutes), suspended in 800 µl proteinase-buffer (100 mM Tric-Cl pH 7.5, 50 mM EDTA pH 7.5, 0.5% SDS, 1 mg/ml proteinase K [Sigma]) and incubated at 60°C for 30 minutes. After 2 times extraction with phenol/chloroform/IAA (25:24:1), the nucleic acids were precipitated by adding 600 µl isopropyl alcohol to each sample. After centrifugation at 13,000 rpm for 5 minutes the pellet was washed with 70% ethanol, air-dried and dissolved in 200 µl distilled water containing 2 µl RNaseA (10 mg/ml). After 30 minutes incubation at 37°C, the DNA was extracted once with phenol/chloroform/ IAA (25:24:1) and once with chloroform/IAA (24:1) and precipitated with 200 µl isopropanol. It was then centrifuged at 13,000 rpm for 5 minutes, washed in 70% ethanol, air-dried and dissolved in 100 µl distilled water.

3.6.14   Southern hybridization

10 µg of genomic DNA from *C. albicans* transformants was digested overnight with appropriate restriction enzymes in a 30 µl reaction volume at 37°C. The reactions were stopped by adding DNA stop buffer to 1x final concentration. Digested samples, along with 1 kb DNA ladder (Invitrogen) as marker, were loaded on a 1% agarose gel. The gel was run overnight at 36-40 V in 1x TAE buffer. After electrophoresis the gel was stained with ethidium bromide, and photographed.

DNA transfer

A nylon membrane (15 cm x 14 cm, Schleicher & Schuell, Dassel, Germany) was pre-wet for few seconds in distilled water and equilibrated in 20x SSC (3 M NaCl, 0.3 M tri-sodium citrate dihydrate) for 5 minutes. The gel was rinsed with distilled water and aligned on top of the membrane in vacuum blot apparatus (Phamacia Biotech). In a sequential order, the gel was treated with different solutions (15 min each) under vacuum, Solution A (0.25 N HCl) for depurination of the DNA, Solution B (1.5 M NaCl, 0.5 N NaOH) for denaturing DNA and then with Solution C (1.5 M NaCl, 0.5 M Tris-Cl pH 7.5) for neutralization. Transfer was done with 20x SSC for 90 minutes. After the transfer was complete, the membrane was first soaked in 0.4N NaOH for 1 minute to denature the DNA and then treated with 0.2M Tris-HCl (pH 7.5) for 1 minute for neutralization. The wet membrane was placed on Whatmann

filter paper and DNA was fixed to membrane by UV-cross linking using Stratalinker (Stratagene).

Hybridization

Hybridization was performed with the 'ECL labelling and detection kit' (GE Healthcare, Braunschweig, Germany) as per instructions of the manufacturer. The membrane was pre-wet in 2x SSC for 1 minute and then placed in a hybridization bottle. For prehybridization, the membrane was incubated with 15 ml ECL hybridization buffer for 1-2 h at 42°C in a hybridization oven. The probe was prepared as per manufacturer's instruction using 100 ng DNA of interest (gel eluted) and 2 ng 1 kb DNA ladder (Invitrogen) and then was added to the prehybridization buffer. Hybridization was carried out at 42°C for 16-18 hours. After hybridization, the membrane was washed at 42°C, one time with 5x SSC for 15 minutes, 2 times with Wash Buffer I (6 M Urea, 0.4% SDS, 0.5x SSC) for 10 minutes and finally in 2x SSC at RT for 15 minutes. For signal detection, the washed membrane was incubated in a mixture of detection solution 1 and 2 (1:1) for 1 min, then wrapped securely in saran wrap, and finally exposed to Amersham hyperfilm ECL in a film cassette for 5-90 minutes. For rehybridizing with a 2nd probe the blot was washed in 2x SSC for 10 min and then steps for hybridization, as described above, were followed.

3.6.15 RNA isolation from *C. albicans*

All solutions used for RNA isolation were made either in DEPC treated water or treated with DEPC after preparation and RNase-free plastic ware was used in all procedures of RNA handling. For DEPC treatment 0.1% DEPC (v/v) was added and the solutions were incubated at 37°C overnight and autoclaved.

Hot acidic phenol method

Total RNA was isolated from log-phase cultures of the *C. albicans* strains in liquid Synthetic Low Ammonium Dextrose (SLAD) medium by the standard hot acidic phenol method (Ausubel *et al.*, 1989). Cells grown under this *MEP2* inducing condition were collected by centrifugation and the pellet was resuspended in 600 µl TES buffer (10 mM Tris-Cl pH 7.5, 10 mM EDTA, 0.5% SDS prepared in DEPC water). 600 µl water saturated phenol was added and mixed thoroughly by vortexing. The mixture was heated at 65°C for 1 h with intermittent mixing. The hot mixture was then chilled in an ethanol-dry ice bath for 5 seconds and then centrifuged at 12,000 rpm for 10 minutes at 4°C. Without disturbing the interphase, the upper aqueous phase was carefully transferred to another tube and reextracted once again with water saturated phenol and twice with TE saturated phenol/chloroform/IAA (25:24:1).

The RNA was precipitated by adding 0.1 volume 3 M sodium acetate pH 5.2 and twice the volume chilled 96% ethanol. The RNA precipitate was collected by centrifugation (12,000 rpm, 10 min, 4ºC). The RNA pellet was washed with 70% ethanol in DEPC water, dried and dissolved in DEPC treated water. Concentration of RNA was estimated by measuring the absorbance at 260 nm (OD260= 1.0 equals 40 μg/ml RNA).

3.6.16   Northern hybridization

A formaldehyde denatured RNA gel, which contains 1.2% agarose in 1x MOPS buffer and 2.2 M formaldehyde (Applichem, pH 3-3.5), was prepared. To prepare the RNA sample, 30 μg RNA in 11.25 μl DEPC water was mixed with 5 μl 10x MOPS buffer (0.2 M MOPS pH 7.0, 20 mM sodium acetate, 10 mM EDTA), 25 μl deionised formamide and 8.75 μl 37% formaldehyde. After incubation at 55°C for 15 minutes, 10 μl RNA loading buffer (50% glycerol, 1 mM EDTA, 0.002% bromophenol blue and 0.002% xylene cyanol) was added to the mix and the samples were loaded in the wells. Electrophoresis was done in 1x MOPS buffer at 150 V for 3-4 h (bromophenol blue had migrated 2/3 length of the gel).

Afterwards, the gel was washed at RT in DEPC treated water twice (30 min each) on a rotator, followed by equilibration in 10x SSC for 45 minutes. The RNA was transferred to nylon membrane by overnight capillary transfer using 20x SSC as transfer buffer. RNA was cross linked to the membrane by using a UV-cross linker and the amount of RNA loaded was verified by methylene blue staining (0.02% methylene blue in 0.3 M sodium acetate) of the blot.

Probe labeling and hybridization

The membrane was pre-wet in 6x SSC for 1 minute and then placed in hybridization bottle. For prehybridization, the membrane was incubated with 15 ml of pre warmed (65°C) rapid hybridization buffer (Rapid-hyb buffer, GE Healthcare) for 1 h at 65°C in a hybridization oven. Labeling of DNA for probe was done using the rediprime II random prime labelling system (GE Healthcare, Braunschweig, Germany). For this, 25 ng of the template DNA fragment (gel eluted) was taken in 45 μl 1x TE buffer. The DNA was denatured by heating to 100°C for 5 min in a boiling water bath, and then chilled quickly by placing on ice for 5 minutes. The tube was centrifuged briefly to bring down the contents to bottom and the denatured DNA was then added to the reaction tube which is provided with the kit. This reaction tube contains buffered solution of dATP, dGTP, dTTP, exonuclease free Klenow enzyme and random primers in a dried, stabilized form. 5 μl of [α^{32}P]-dCTP (50 μCi from 3,000 Ci/mmol specific activity) was added to the reaction tube and the contents of tube were

mixed thoroughly by pipetting. The reaction mix was incubated at 37°C for 30 min and the reaction was stopped by adding 5 µl of 0.2 M EDTA. The labeled DNA was denatured by heating at 100°C for 5 min, chilled on ice and then added to the hybridizing sample.

Hybridization was carried out at 65°C for 3-4 hours in a hybridization oven. Afterwards, the membrane was washed with Wash solution I (2x SSC, 0.1% SDS) for 20 minutes at 42°C and 2 times for 15 minutes in Wash solution II (0.1x SSC and 0.1% SDS) at 65°C. Washing time was varied according the radioactivity present in the blot. After each wash the background count was monitored with a Hand Monitor to avoid washing off the specifically bound signal. The washed membrane was then wrapped securely in a saran wrap and autoradiographed, by exposing to Kodak X-Omat™ film at -80°C for 6 hours to 2 days, depending on the signal strength.

3.6.17   5'-Rapid amplification of cDNA ends (5' RACE) assay

C. albicans strain SC5314 was grown to log-phase in liquid SLAD medium and total RNA was isolated using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). 5' RACE analysis was performed using the 5'/3' RACE kit, 2nd Generation (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's specifications. First-strand cDNA was synthesized in 20 µl reaction mix containing 2 µg of the template RNA which was reverse transcribed using the antisense primer GAT13 (see Table 3 for sequence of the primer). A homopolymeric dA-tail was added to the purified cDNA using recombinant terminal transferase and dATP provided with the kit. With the help of DNA polymerase enzyme (Phusion), the dA-tailed cDNA was amplified using the 5' RACE oligo dT-anchor primer (5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTV-3'; V=A, C or G) and the *GAT1*-specific antisense primer GAT14. The product was further amplified using the 5' RACE PCR anchor primer (5'-GACCACGCGTATCGATGTCGAC-3') and the *GAT1*-specific antisense primer GAT15. The PCR products of both reactions were purified and sent to sequencing with the primer GAT15, using the protocol for DNA sequencing as described in section 3.6.1.8.

3.6.18   SDS polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting

To investigate Sap2p secretion or its proteolytic activity (monitored by BSA degradation), supernatants of *C. albicans* strains grown at 30°C in YCB-BSA-YE (pH 4.0) were analyzed on polyacrylamide gel. While BSA in medium appears as an intense signal between 50 and

80 kDa; the thin Sap2p band at 43 kDa is detectable only after complete degradation of BSA and is otherwise masked by BSA degradation products. Culture supernatants were collected at different time points as mentioned in text. 4 µl of protein sample buffer (5x Sample buffer stock: Solution I (1.1 g SDS, 0.41 g EDTA, 0.17 g NaH_2PO_4 x $2H_2O$, 1.1 ml β-mercaptoethanol, distilled H_2O was added to make up the volume to 10 ml) + Solution II (20 mg bromophenolblue in 10 ml of 50% glycerin) was added to 15 µl of the supernatants to be analyzed. These samples were then analyzed on 12% polyacrylamide gel; Resolving gel: 2.5 ml Solution B (1.5 M Tris-Cl pH 8.8, 0.4% SDS), 4.0 ml gelstock (30% acrylamide solution containing 0.8% bisacrylamide [Roth]), 50 µl 10% ammonium persulphate (APS), 5 µl TEMED, dH_2O was added for making volume to 10 ml; Stacking gel: 1.25 ml Solution C (0.5 M Tris-Cl pH 6.8, 0.4% SDS), 0.65 ml gelstock, 25 µl 10% APS, 5 µl TEMED, dH_2O was added to make the volume to 5 ml. 10 µl of Precision Plus Protein Standards all blue size marker (Bio-Rad, München, Germany) was loaded in a lane along with the samples for determining the molecular weight of the proteins. Electrophoresis was performed on a minigel apparatus (Bio-Rad) at 35 mA for 1 h in 1x SDS running buffer (10x Stock solution: 30.2 g Tris, 188 g glycine, 1% SDS in 1litre dH_2O).

Protein bands were visualized by overnight staining of gel with Colloidal coomassie dye (40 ml Stock solution (0.1% Coomassie-brilliant-blue R250 [Serva], 85% phosphoric acid, 0.75 M ammonium sulphate) + 10 ml ethanol). Proteins were transferred onto the nitrocellulose membrane (Schleicher & Schuell) in Towdin buffer (25 mM Tris pH8.1-8.5, 192 mM glycine and 20% methanol), using a Semi-Dry-Trans-Blot SD blot apparatus (Bio Rad) at 15 V for 1 h. After blocking of the membrane which was done for 1 hour at RT in 10 ml of 1x TBS (Solution: 8 g NaCl, 0.2 g KCl, 3.0 g Tris, pH was adjusted to 7.4 with HCl) + 5% non fat milk powder (Applichem), the membrane was given primary antibody treatment for 1 hour at RT i.e., antibodies raised against Sap2p (provided by Michel Monod, University of Lausanne, Switzerland) at dilution 1:1000 in 10 ml TBS+ 5% non fat milk powder. Afterwards, the membrane was given secondary antibody treatment for 30 min at RT, with an anti-rabbit (1:10000) antibody. Washing of the membrane was done thrice, 5 min each time at RT, in 1x TBS and signals were detected with the ECL labelling and detection kit (GE Healthcare, Braunschweig, Germany).

3.6.19 *GFP* expression analysis

Fluorescence microscopy

Localizations of GFP fusion proteins were determined using a Zeiss LSM 510 inverted confocal laser scanning microscope equipped with a ZEISS Axiovert 100 microscope. Imaging scans were acquired with an Argon laser of 488 nm wavelength and corresponding filter settings for GFP and parallel transmission images. The cells were observed with with 63× immersion oil objective.

Flow cytometry

Cells were grown for the indicated times in the various experimental media, washed and suspended in 1x PBS (phosphate buffered saline) to an OD_{600} of 0.1 and kept on ice. Fluorescence-activated cell sorter (FACS) analysis was performed with a FACSCalibur cytometry system (Becton Dickinson, Heidelberg, Germany) equipped with an argon laser emitting at 488 nm. Fluorescence was measured on the FL1 fluorescence channel equipped with a 530-nm band-pass filter. Twenty thousand cells were analyzed per sample and were counted at low flow rate. Fluorescence data was collected by using logarithmic amplifiers. The mean of analyzed events was calculated using the Histogram Stats in the CellQuest Pro software. Unless mentioned otherwise the strain SC5314, which does not carry *GFP*, was included as a negative control in all experiments.

3.6.20 Ammonium uptake assays

Ammonium uptake was assayed as described previously (Biswas & Morschhäuser, 2005). Briefly, strains were grown to late log phase in minimal proline medium (0.17% YNB without amino acids and $[NH_4]_2SO_4$ [BIO 101 Vista Calif.], 0.1% proline, 2% glucose) and the cultures were diluted to an OD_{600} of 1.0-1.2 in the same medium plus 250 µM ammonium sulfate. At the indicated times 1 ml of the culture was taken, the cells removed by centrifugation, and the ammonium concentration in the culture supernatant determined using a glutamate dehydrogenase-linked assay. Reaction mixture contains 50 mM imidazole pH 7.3, 20 mM α-ketoglutarate, 0.1 mg/ml NADH, 0.01 mg/ml EDTA and 10 units of glutamate dehydrogenase from bovine liver (Sigma). Absorbance at 340 nm was measured one minute after the addition of NADH to the reaction mixture. In this reaction $NH_4^+ + NADH + H^+ + $ α-ketoglutarate is converted to glutamate $ + NAD^+ + H_2O$ and a higher A340 value due to increased NADH concentration indicates ammonium removal by the cells.

4 Results

4.1 Mutational analysis of ammonium permease Mep2p of *C. albicans*

In response to nitrogen starvation, *C. albicans* switches from yeast to filamentous growth form. This morphogenetic switch is controlled by the ammonium permease Mep2p. It is not yet clear whether the ammonium transport by Mep2p is required for its signaling activity or not. The C-terminal cytoplasmic domain of Mep2p contains a signaling domain that is not required for ammonium transport but is essential for Mep2p-dependent morphogenesis (Biswas & Morschhäuser, 2005). Mutational analysis of Mep2p was done to identify the residues of the C-terminal cytoplasmic domain that are critical for the signaling function of the permease and to investigate how the ammonium transport activity of the Mep2p influences its ability to induce filamentous growth under limiting nitrogen conditions.

4.1.1 Identification of the minimal region in the C-terminal cytoplasmic tail of Mep2p that is required for the induction of filamentous growth

Previously, it was reported that deletion of the last 40 amino acids of the C-terminal cytoplasmic domain of Mep2p did not impair its ability to induce filamentous growth of *C. albicans* in response to nitrogen limitation. In fact, cells expressing the *MEP2*^{AC440} allele had a hyperfilamentous phenotype, presumably due to increased mRNA levels of the truncated allele in comparison to those of the wild-type allele (Biswas & Morschhäuser, 2005). However, deletion of 17 additional amino acids abolished the capacity of *MEP2* to induce filamentous growth, although the *MEP2*^{AC423} allele was expressed at equally high levels and ammonium uptake by the truncated Mep2p was not affected. This suggested that at least some residues in the region between amino acids 424 and 440 of Mep2p (shown in Fig. 3A) are part of a signaling domain that is specifically required for morphogenesis but not for ammonium transport. In order to define the minimal region that is essential for the induction of filamentous growth, progressive C-terminal truncations of Mep2p starting from amino acid 440 were generated.

Construction of plasmids containing truncated MEP2 alleles

Plasmid pMEP2K1 (see Fig. 2) (Biswas & Morschhäuser, 2005) which contains the full length *MEP2* allele served as the basis for generation of C-terminally truncated *MEP2* alleles. The truncated *MEP2* alleles were generated by amplifying the *MEP2* gene from pMEP2K1

with primer MEP3 and one of the primers MEP81 to MEP87 or MEP59, which introduce stop codons at the desired positions (primers are listed in Table 3). The SalI-BglII fragment from each PCR (truncated *MEP2* allele) was cloned together with a KpnI-SalI fragment from pMEP2K1 into KpnI/BglII-digested pMEP2K1, resulting in replacement of the full-length *MEP2* in pMEP2K1 by a truncated *MEP2* allele. The plasmids thus obtained are pMEP2ΔC6 to pMEP2ΔC13, which contain the $MEP2^{\Delta C439}$ to $MEP2^{\Delta C432}$ alleles, respectively.
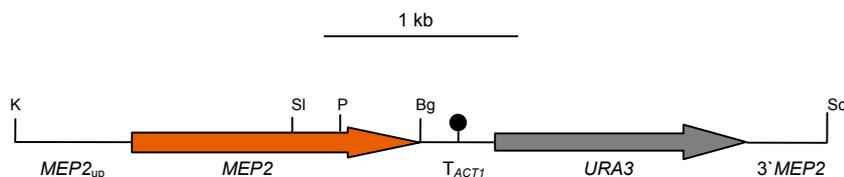
Fig. 2. Structure of the insert of plasmid pMEP2K1, which contains full-length *MEP2* allele, is shown. The *MEP2* coding region is represented by the red arrow, the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the filled circle, and the *URA3* selection marker by the grey arrow. *MEP2* upstream and downstream regions are represented by the solid lines. Only relevant restriction sites are indicated: Bg, BglII; K, KpnI; P, PstI; ScI, SacI; Sl, SalI.

In order to express the truncated alleles of *MEP2* in *mep1Δ mep2Δ* double mutants, the mutants (strains MEP12M4A/B) were transformed with the KpnI-SacI fragment from the plasmids pMEP2ΔC6 to pMEP2ΔC13. Single-copy integration of the constructs into one of the genomic *MEP2* alleles, which can be distinguished by an *Eco*RI restriction site polymorphism (Biswas & Morschhäuser, 2005), was confirmed by Southern hybridization (data not shown) and two independent transformants were used for further analysis in each case (strain descriptions are provided in Table 2).

The ability of the transformants to filament on SLAD plates was evaluated. As expected, all tested truncated alleles rescued the growth defect of the double mutants under limiting ammonium conditions and strains expressing the $MEP2^{\Delta C439}$ to $MEP2^{\Delta C433}$ alleles also exhibited the hyperfilamentous phenotype (Fig. 3B, panels a-c, and data not shown). In contrast, strains expressing the $MEP2^{\Delta C432}$ allele were unable to filament (Fig, 3B, panel d), indicating that the tyrosine at position 433 is the last residue that is essential for signaling by Mep2p. Interestingly, Mep2p is highly similar in this region to Mep1p, which does not normally induce filamentation in *C. albicans* (see Fig. 3A). Previous studies have shown that substituting the C-terminal tail of Mep1p for the C-terminus of Mep2p resulted in abolishment of filamentation (Biswas & Morschhäuser, 2005). To exclude the possibility that

the C-terminus of Mep1p, which is longer than that of Mep2p, contains an inhibitory domain, new hybrid alleles were generated in which the last 22 codons of the hyperactive $MEP2^{\Delta C440}$ and last 17 codons of hyperactive $MEP2^{\Delta C435}$ alleles were replaced by the corresponding sequence of $MEP1$. For construction of these new plasmids, pMEP21H2, which contains a hybrid $MEP2^{1-418}$-$MEP1^{417-534}$ allele, was used (Biswas & Morschhäuser, 2005). Truncated derivatives in which a stop codon was inserted behind codons 438 or 433 of $MEP1$ were generated by amplification of a part of the hybrid gene with the primer pairs MEP39p/MEP23 or MEP39p/MEP91, which was then substituted for the corresponding region in pMEP21H2, resulting in plasmids pMEP21H4 and pMEP21H6, respectively. Relevant inserts from these plasmids were integrated at the original $MEP2$ locus and correct integrants confirmed by Southern hybridization (data not shown) were selected for further studies.

Strains expressing the hybrid $MEP2^{1-418}$-$MEP1^{417-438}$ and $MEP2^{1-418}$-$MEP1^{417-433}$ alleles did not filament (Fig. 3B, panels e and f), demonstrating that residues in the region between amino acids 419 and 433 of Mep2p in which it differs from Mep1p must be essential for signaling. As highlighted in Fig. 3A, the important Y433 of Mep2p is replaced by phenylalanine at the corresponding position (F431) in Mep1p. Importance of the presence of a tyrosine at this position in signaling was investigated by introducing a Y433F mutation in the $MEP2^{\Delta C433}$ allele and, also by substituting the phenylalanine to tyrosine in the hybrid $MEP2^{1-418}$-$MEP1^{417-433}$ allele. For this, plasmids pMEP2ΔC12M1 and pMEP21H7, which are identical to pMEP2ΔC12 and pMEP21H6, respectively, but contain the Y433F mutation, were constructed. Using primer pair MEP3/MEP88, plasmid pMEP2ΔC12M1 was created in the same way as the other plasmids that contain truncated $MEP2$ alleles (see page 61-62). In a manner analogous to construction of plasmid pMEP21H6 (described above), pMEP21H7 was generated using primers MEP39p and MEP92. The substitution of tyrosine by phenylalanine resulted in reduced filamentation of cells expressing the $MEP2^{\Delta C433\ Y433F}$ allele (Fig. 3B, compare panels c and h), suggesting that phenylalanine can only partially substitute for the function of Y433 in signaling. For expression of $MEP2^{Y433F}$ allele, in which tyrosine is replaced by phenylalanine in full-length Mep2p, plasmid pMEP2K15 was generated by amplification of the N- terminal and C-terminal parts of $MEP2$ with the primer pairs MEP3/MEP89p and MEP90p/MEP9, followed by ligation of the KpnI- and BglII-digested PCR products into the same sites of pMEP2K1. However, transformants carrying the Y433F mutant allele of Mep2p exhibited normal filamentation (Fig. 3B, compare panels i and k). Conversely, replacement of phenylalanine by tyrosine in the hybrid protein did not confer the

ability to filament upon cells expressing the *MEP2*¹⁻⁴¹⁸-*MEP1*^{417-433 F431Y} allele (Fig. 3B, panel g), demonstrating that other amino acids in which Mep2p differs from Mep1p are critical for the signaling activity of Mep2p.

(A)

```
                                        433
                                         ▽
        Mep2p   419 DEEMLGTDLAQIGEYAYYADDD 440


        Mep1p   417 NGEEAGVDEDQIGEFAYDYVEV 438
                        *   *  *   ****  **
```

(B)
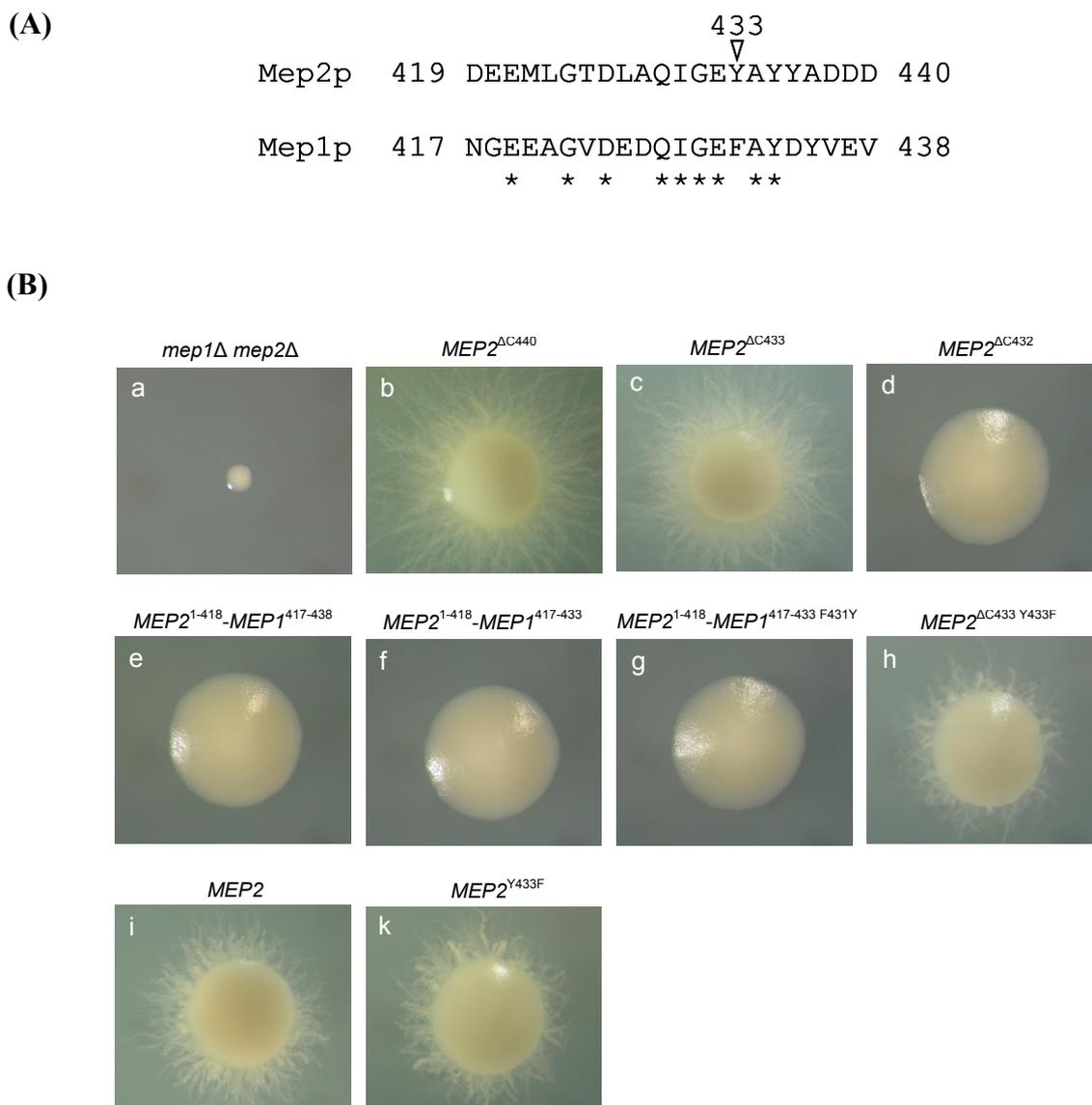
Fig. 3. **(A)** Alignment of the region between amino acids 419 and 440 of Mep2p with the corresponding region in Mep1p. Identical residues are marked by the stars below the alignment. The tyrosine at position 433 of Mep2p is labeled by an arrowhead. **(B)** Colony phenotype of *mep1Δ mep2Δ* double mutants expressing the indicated *MEP2* and *MEP2-MEP1* hybrid alleles. Individual colonies were photographed after 6 days of growth on SLAD plates at 37°C. Following strains were used: MEP12M6A/B, in which only the *URA3* gene, but not *MEP2*, was reintegrated, were used as controls (*mep1Δ mep2Δ*), MEP12MK2A/BΔC2 (*MEP2*^{ΔC440}), MEP12MK2A/BΔC12 (*MEP2*^{ΔC433}), MEP12MK2A/BΔC13 (*MEP2*^{ΔC432}), MEP12MK21H4A/B (*MEP2*¹⁻⁴¹⁸-*MEP1*⁴¹⁷⁻⁴³⁸), MEP12MK21H6A/B (*MEP2*¹⁻⁴¹⁸-*MEP1*⁴¹⁷⁻⁴³³), MEP12MK21H7A/B (*MEP2*¹⁻⁴¹⁸-*MEP1*^{417-433F431Y}), MEP12MK2A/BΔC12M1 (*MEP2*^{ΔC433Y433F}), MEP12MK2A/B (*MEP2*) and MEP12MK16A/B (*MEP2*^{Y433F}). The two independently constructed series of strains behaved identically and only one of them is shown in each case.

4.1.2 Expression pattern and ammonium uptake capacity of mutated Mep2p proteins

In *S. cerevisiae* and *C. albicans*, the signaling activity of the Mep2p is thought to be influenced by ammonium binding or transport by Mep2p (Biswas & Morschhäuser, 2005; Lorenz & Heitman, 1998). Experimental data indicate that ammonium transport by Mep2p is required for its ability to induce pseudohyphal growth in *S. cerevisiae* (Boeckstaens *et al.*, 2007; Marini *et al.*, 2006). However, an alternative possibility was suggested for *C. albicans* (Biswas & Morschhäuser, 2005) in which Mep2p can induce filamentous growth in the absence of its ammonium transport activity (i.e., when ammonium is absent or present only at low concentrations), and signaling is inhibited when Mep2p is engaged in ammonium transport. This hypothesis for CaMep2p was investigated by generating transport-deficient Mep2p derivatives. Mutations were carried out on those conserved residues of CaMep2p which, based on structural and biochemical studies of ammonium permeases of other organisms, were proposed to be required for ammonium transport (Khademi *et al.*, 2004; Marini *et al.*, 2006).

The conserved aspartate at position 160 of *E. coli* AmtB (in the mature protein after removal of the signal sequence) has been proposed to be an initial ammonium binding site (Thomas *et al.*, 2000). Therefore, D180 of Mep2p, which corresponds to D160 of AmtB, was mutated to leucine (Biswas & Morschhäuser, unpublished) i.e., to an amino acid that has a similar backbone structure but lacks the negatively charged group of aspartate. The D180N mutation was also generated because a mutation of the equivalent residue in *S. cerevisiae* Mep2p, D186 to asparagine, is reported to allow normal expression of the protein and abolishment of ammonium transport and Mep2p-dependent pseudohyphal growth (Marini *et al.*, 2006). Resolution of the AmtB crystal structure has revealed additional highly conserved residues that might stabilize ammonium in an outer vestibule of the protein (F103, F107, W148, S219) as well as two pore-lining histidines (H168, H318) that are predicted to make contacts with ammonium during transport (Khademi *et al.*, 2004; Knepper & Agre, 2004). As mutation of these residues might abolish ammonium transport, the corresponding amino acids in Mep2p (Y122, F126, W167, H188, S243, H342) as well as W165, which is located near W167, were changed to alanine.

Construction of plasmids containing MEP2 alleles with single amino acid substitutions
For pMEP2D180N, the N-terminal part of *MEP2* plus upstream sequences was amplified from pMEP2K1 with the primer pair MEP3/MEP67p and the remaining part of the *MEP2*

coding region was amplified with primer pair MEP39p/MEP32. The two fragments were digested with KpnI and BamHI, respectively, and ligated together into the vector pBluescript. Plasmids pMEP2Y122A, pMEP2F126A, pMEP2W165A, pMEP2W167A, pMEP2H188A, pMEP2S243A, and pMEP2H342A were generated in an analogous fashion using the primer pairs MEP3/MEP68p and MEP70p/MEP32, MEP3/MEP69p and MEP70p/MEP32, MEP3/MEP73p and MEP74p/MEP32, MEP3/MEP68p and MEP80p/MEP32, MEP3/MEP71p and MEP72p/MEP32, MEP3/MEP75p and MEP76p/MEP32, and MEP3/MEP77p and MEP78p/MEP32, respectively. A PstI-SacI [C-terminal part of *MEP2*-T_{ACT1} - *URA3-3' MEP2*] fragment from pMEP2K1 (Fig. 2) was then ligated between the same sites of the plasmids listed above and also of plasmid pMEP2D180L (Biswas & Morschhäuser, unpublished) to generate plasmids pMEP2K4 to pMEP2K12 containing the $MEP2^{Y122A}$, $MEP2^{F126A}$, $MEP2^{W165A}$, $MEP2^{W167A}$, $MEP2^{D180L}$, $MEP2^{D180N}$, $MEP2^{H188A}$, $MEP2^{S243A}$, and $MEP2^{H342A}$ alleles, respectively (summarized in Table 5). Plasmids containing GFP-tagged versions of the mutated *MEP2* alleles were created by substituting the KpnI-BamHI fragments from plasmids pMEP2D180N, pMEP2Y122A, pMEP2F126A, pMEP2W165A, pMEP2W167A, pMEP2H188A, pMEP2S243A, and pMEP2H342A for the corresponding fragment in the pMEP2G2 (Biswas & Morschhäuser, 2005), resulting in plasmids pMEP2G8 to pMEP2G15, respectively (see Table 5). Using relevant inserts from the plasmids enlisted in Table 5, the mutated *MEP2* alleles were targeted at the original *MEP2* locus and two independent transformants were used for further analysis in each case (strain descriptions are provided in Table 2).

Table 5. Overview of the single amino acid substitutions in *MEP2* alleles.

Primer pairs	Substitution in *MEP2* alleles	Plasmids containing mutated *MEP2* alleles	Plasmids with GFP-tagged mutated *MEP2* alleles
MEP3/MEP68p and MEP70p/MEP32	MEP2Y122A	pMEP2K4	pMEP2G9
MEP3/MEP69p and MEP70p/MEP32	MEP2F126A	pMEP2K5	pMEP2G10
MEP3/MEP73p and MEP74p/MEP32	MEP2W165A	pMEP2K6	pMEP2G11
MEP3/MEP68p and MEP80p/MEP32	MEP2W167A	pMEP2K7	pMEP2G12
MEP3/ MEP38p and MEP39p/ MEP32	MEP2D180L	pMEP2K8	pMEP2G3
MEP3/MEP67p and MEP39p/MEP32	MEP2D180N	pMEP2K9	pMEP2G8
MEP3/MEP71p and MEP72p/MEP32	MEP2H188A	pMEP2K10	pMEP2G13
MEP3/MEP75p and MEP76p/MEP32	MEP2S243A	pMEP2K11	pMEP2G14
MEP3/MEP77p and MEP78p/MEP32	MEP2H342A	pMEP2K12	pMEP2G15
MEP3/MEP94p and MEP76p/MEP32	MEP2F239A	pMEP2K16	pMEP2G16

The capacity of the mutated *MEP2* alleles to restore growth of the *mep1Δ mep2Δ* double mutants on plates containing limiting ammonium concentrations was assessed. Strains expressing the *MEP2*^{W165A}, *MEP2*^{W167A}, and *MEP2*^{S243A} alleles grew as well as the strains containing a wild-type *MEP2* allele (Fig. 4), indicating that the mutated proteins were still able to transport ammonium, and a weaker growth was restored by the *MEP2*^{Y122A} and *MEP2*^{F126A} alleles. In contrast, no growth was observed for transformants carrying the D180L, D180N, H188A, and H342A mutant alleles of Mep2p that behaved like *mep1Δ mep2Δ* double mutants, indicating that these mutations rendered the ammonium permease nonfunctional.
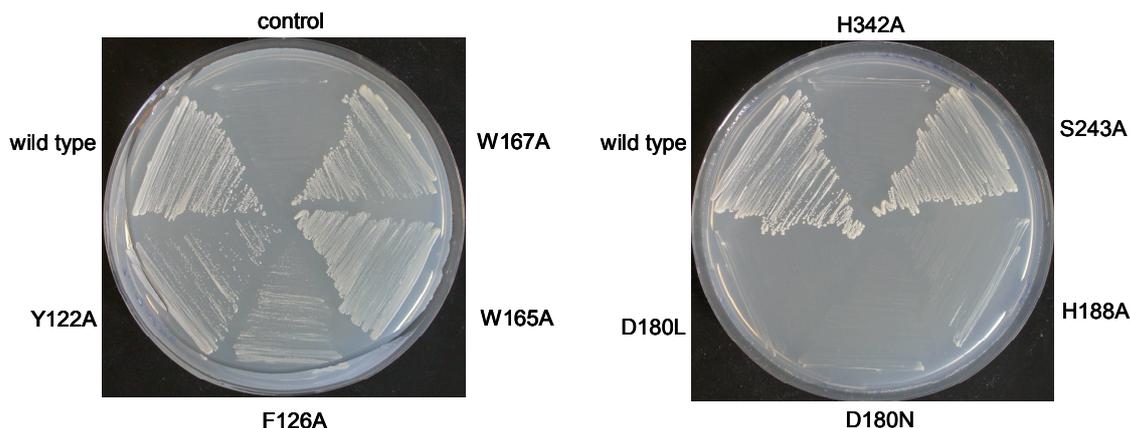
Fig. 4. Growth of *mep1Δ mep2Δ* double mutants expressing wild-type or mutated *MEP2* alleles, as indicated, on plates containing limiting concentrations (1 mM) of ammonium. The plates were incubated for 4 days at 30°C. Following strains were used: MEP12M6A/B, in which only the *URA3* gene, but not *MEP2*, was reintegrated, were used as controls, MEP12MK2A/B (wild type), MEP12MK6A/B (Y122A), MEP12MK7A/B (F126A), MEP12MK8A/B (W165A), MEP12MK9A/B (W167A), MEP12MK10A/B (D180L), MEP12MK11A/B (D180N), MEP12MK12A/B (H188A), MEP12MK13A/B (S243A), and MEP12MK14A/B (H342A). The two independently constructed series of strains behaved identically and only one of them is shown in each case.

To test whether the mutated Mep2p proteins were correctly expressed in the cell membrane, C-terminally *GFP*-tagged versions of all alleles were introduced into *mep1Δ mep2Δ* double mutants. Expression of these fusion proteins was observed by fluorescence microscopy. Mep2p-GFP fusion proteins containing the Y122A, F126A, W165A, W167A, and S243A mutations were all correctly localized at the cell periphery (Fig. 5A). Cells expressing *GFP*-tagged *MEP2*^{W165A} and *MEP2*^{S243A} alleles exhibited similar fluorescence as those expressing a *GFP*-tagged wild-type *MEP2*, but the Y122A, F126A, and W167A mutations resulted in reduced fluorescence of the corresponding transformants. Fluorescence was not detected in strains expressing GFP-tagged Mep2p variants containing the D180L, D180N, H188A, and H342A mutations (data not shown). Therefore, these latter mutations, which abolished growth at low ammonium concentrations, did not specifically affect ammonium transport but rather impaired Mep2p expression or protein stability. With the help of strains carrying the *GFP*-tagged mutated Mep2p alleles, the expression levels of the Mep2p variants were analyzed by flow cytometry. Cells containing the *MEP2*^{D180L}, *MEP2*^{D180N}, and *MEP2*^{H342A} alleles showed only background fluorescence values (Fig. 5B), hence, confirming that these proteins were not expressed at detectable levels. Fluorescence of cells containing the *MEP2*^{H188A} allele was marginally higher (3.5-fold) than that of control cells without a *GFP*-tagged *MEP2* gene. The W165A and S243A mutations resulted in only slightly reduced

fluorescence as compared with the wild-type control (53% and 62% of wild-type levels). In line with the microscopic observations, fluorescence of the cells expressing the $MEP2^{Y122A}$, $MEP2^{F126A}$, and $MEP2^{W167A}$ alleles was more strongly reduced (20%, 23%, and 21%, respectively, of wild-type levels).

(A)

(B)
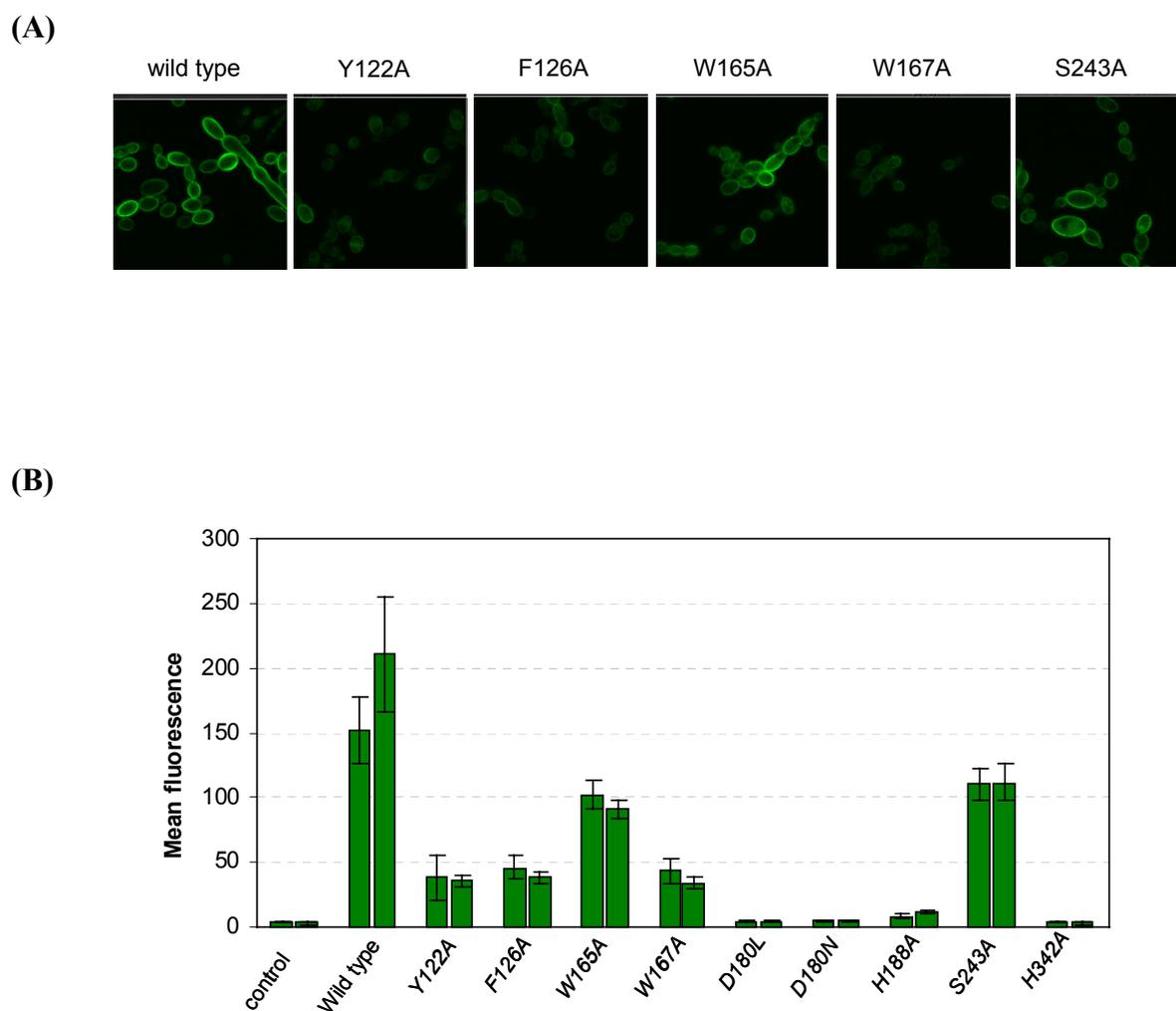
Fig. 5. Expression of GFP-tagged Mep2p proteins. Overnight cultures of the strains, *mep1Δ mep2Δ* double mutants carrying wild-type or mutated *MEP2* alleles, in SD-Pro medium were diluted 50-fold in SLAD medium and were grown for 6 hours at 30°C. **(A)** Localization of GFP-tagged Mep2p confirmed by fluorescence microscopy. **(B)** Fluorescence of the strains was quantified by flow cytometry. The bars represent the means and standard deviations from five (control, wild type, Y122A, F126A, W165A, W167A, S243A) or two (D180L, D180N, H188A, and H342A) experiments performed with each of two independently constructed series of strains. The following strains were used: MEP12M6A/B (control), MEP12MG2A/B (wild type) MEP12MG9A/B (Y122A), MEP12MG10A/B (F126A), MEP12MG11A/B (W165A), MEP12MG12A/B (W167A), MEP12MG4A/B (D180L), MEP12MG8A/B (D180N), MEP12MG13A/B (H188A), MEP12MG14 A/B (S243A), and MEP12MG15A/B (H342A).

4.1.3 Signaling activity of mutated Mep2p proteins

The effect of the various amino acid substitutions on the signaling activity of Mep2p was determined by observing the ability of strains expressing the corresponding *MEP2* alleles to filament under nitrogen limiting conditions. As expected, *MEP2* alleles containing the D180L, D180N, H188A, and H342A mutations, whose encoded proteins were not detectably expressed in the cell membrane, did not restore filamentous growth of *mep1Δ mep2Δ* double mutants on plates containing limiting concentrations of different nitrogen sources (data not shown). In contrast, strains expressing the *MEP2*^{W165A} and *MEP2*^{S243A} alleles exhibited wild-type filamentation on SLAD plates (Fig. 6). Filamentous growth was slightly reduced in strains expressing the *MEP2*^{Y122A} allele as compared with the wild-type control, which could be explained by the 5-fold reduced expression levels of the mutated Mep2p protein. However, filamentation on SLAD plates was completely abolished in strains containing the *MEP2*^{F126A} and *MEP2*^{W167A} alleles (Fig. 6), although the corresponding Mep2p proteins were expressed at similar levels as Mep2p containing the Y122A substitution (Fig. 5), indicating that the F126A and W167A mutations impaired the signaling activity of Mep2p.
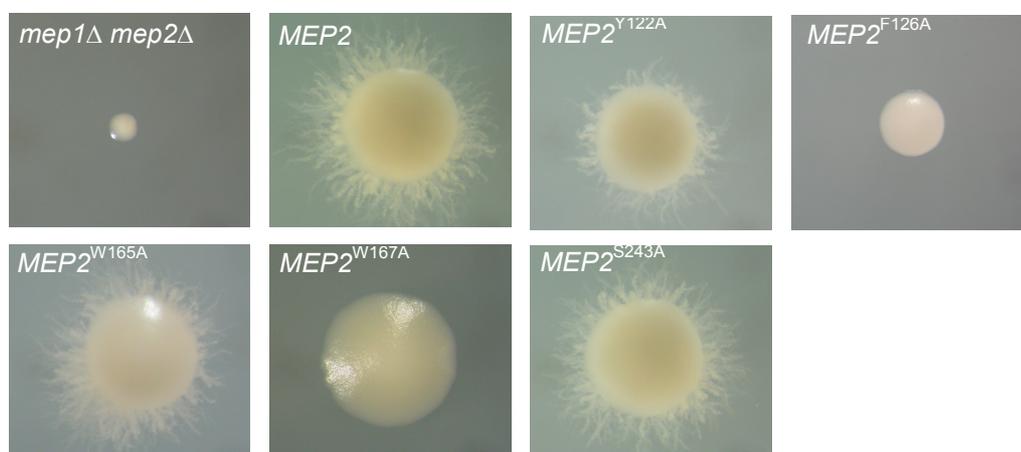
Fig. 6. Filamentation of strains expressing the indicated *MEP2* alleles in *mep1Δ mep2Δ* background. Individual colonies were photographed after 6 days of growth at 37°C on SLAD plates. The two independently constructed series of strains behaved identically and only one of them is shown. Strains MEP12M6A/B (*mep1Δ mep2Δ*) were used as controls.

In order to understand if the signaling activity of mutated Mep2p proteins correlates with their ammonium transport ability, ammonium uptake capacities of strains expressing

wild-type, $MEP2^{Y122A}$, $MEP2^{F126A}$, $MEP2^{W167A}$, and $MEP2^{S243A}$ alleles were compared in an ammonium removal assay (described in section 3.6.20). The S243A mutation, which did not impair growth and filamentation on SLAD plates, had no detectable effect on ammonium uptake (Fig. 7A). In contrast, ammonium uptake in cells expressing the $MEP2^{Y122A}$, $MEP2^{F126A}$, and $MEP2^{W167A}$ alleles was reduced to different levels in comparison to cells expressing a wild-type $MEP2$ allele. Interestingly, the W167A mutation, which abolished nitrogen starvation-induced filamentation, had a weaker effect on ammonium uptake than the Y122A substitution, which still allowed filamentous growth. Therefore, the signaling activity of the mutated Mep2p proteins was not directly correlated with their ammonium transport activity.

The ammonium uptake capacity of cells expressing the mutated $MEP2$ alleles corresponded well with their ability to grow in liquid medium containing limiting ammonium concentrations (Fig. 7B). The F126A mutation, which had the severest effect on ammonium uptake, also reduced growth of the cells most strongly. The Y122A mutation, which had an intermediate effect on ammonium transport, also resulted in a significant growth reduction. However, the minor effect of the W167A mutation on ammonium uptake did not translate into a detectable growth defect of the cells.

Filamentation capacity of cells expressing mutated $MEP2$ alleles was also analyzed on plates containing limiting concentrations of other nitrogen sources, such as urea or the amino acid proline. Under these conditions, ammonium permeases are not required for growth, but Mep2p is still necessary for the induction of filamentation (Biswas & Morschhäuser, 2005). The effect of the various mutations on filamentous growth on plates containing 100 µM urea corresponded to that observed on SLAD plates, i.e., the Y122A substitution resulted in a slight reduction of filamentation, whereas the F126A and W167A mutations abolished filamentous growth (Fig. 8). Interestingly, on plates containing 100 µM proline, strains expressing the $MEP2^{F126A}$ allele produced filamentous colonies, whereas the W167A mutation abolished filamentation also under these conditions. Proline is known to induce filamentation in *C. albicans* (Land *et al.*, 1975), although at the limiting concentrations used here, Mep2p is nevertheless required for filamentous growth. It seems that Mep2p containing the F126A mutation can still support filamentation when additional signaling pathways are activated.

(A)

(B)
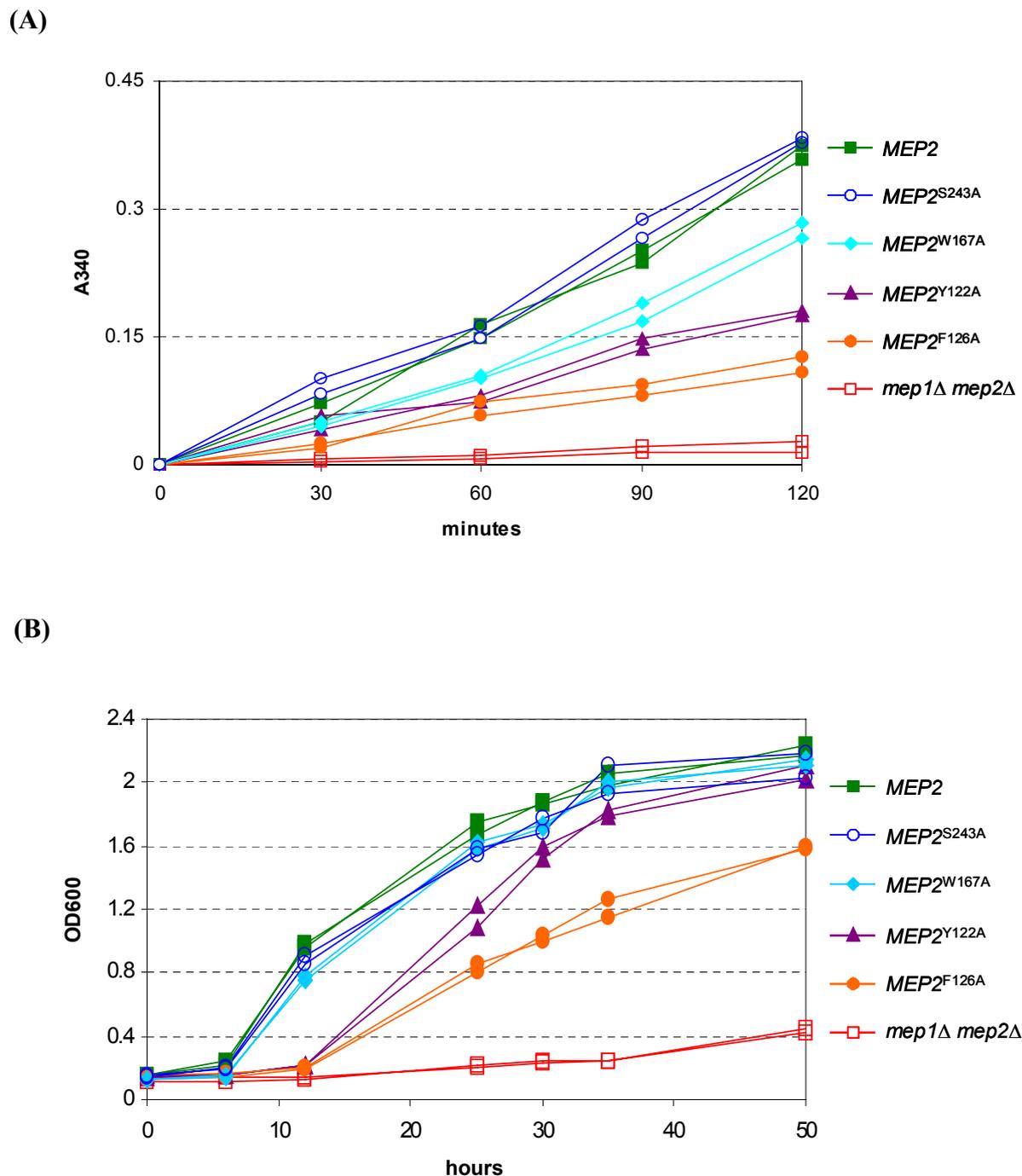
Fig. 7. **(A)** Ammonium uptake by *mep1Δ mep2Δ* double mutants expressing wild-type or mutated *MEP2* alleles. Two independently constructed strains were used in each case. Following strains were used: MEP12M6A/B (*mep1Δ mep2Δ*), MEP12MK2A/B (*MEP2*), MEP12MK6A/B (*MEP2*[Y122A]), MEP12MK7A/B (*MEP2*[F126A]), MEP12MK9A/B (*MEP2*[W167A]), and MEP12MK13A/B (*MEP2*[S243A]). **(B)** Growth of the same strains in liquid medium under limiting ammonium conditions. Precultures of the strains grown in SD-Pro medium were diluted 10^{-2} in SD medium containing 1 mM ammonium as the sole nitrogen source and growth was monitored by measuring the optical densities of the cultures over time.
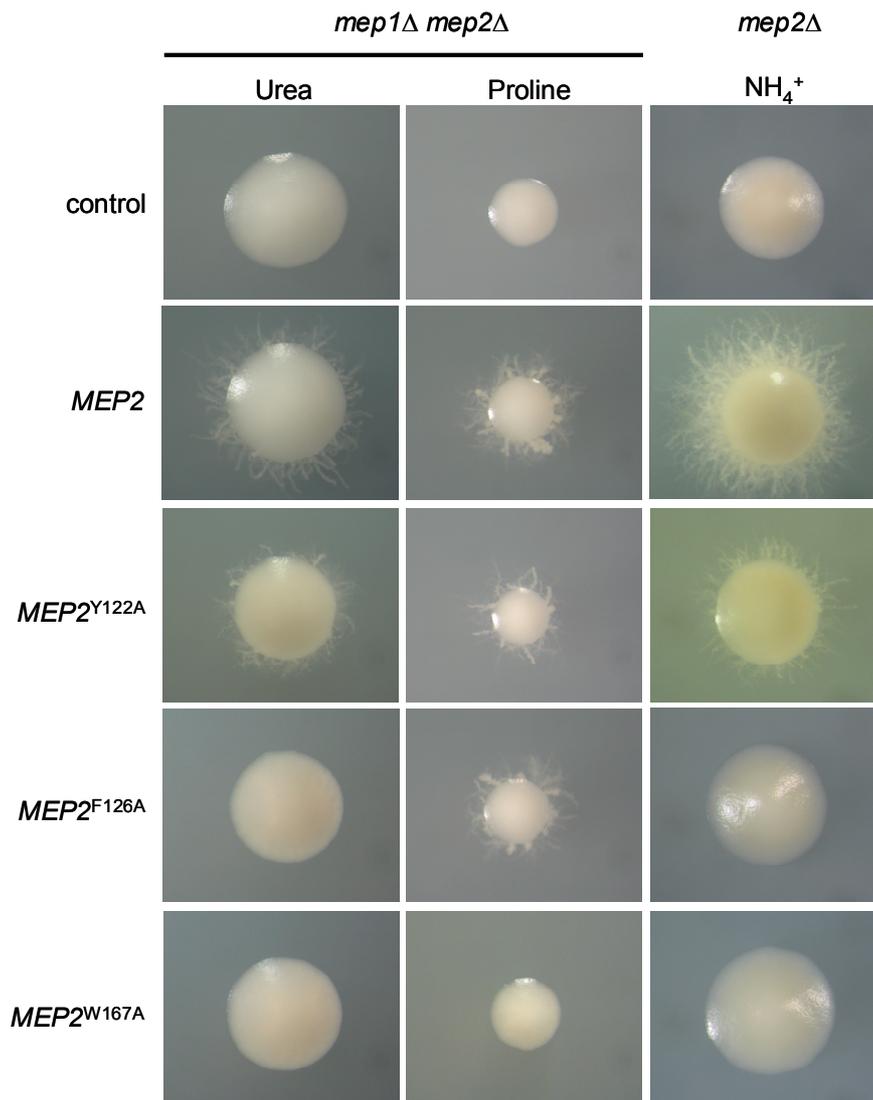
Fig. 8. Filamentation of *mep1Δ mep2Δ* double mutants (left two panels) and *mep2Δ* single mutants (right panels) expressing wild-type or mutated *MEP2* alleles on agar plates containing 100 µM of the indicated nitrogen sources. Individual colonies were photographed after 6 days of growth at 37°C. The *mep1Δ mep2Δ* double mutants (strains MEP12M6A/B) and the *mep2Δ* single mutants (strains MEP2M5A/B) were used as controls. The two independently constructed series of strains behaved identically and only one of them is shown.

In a complementary approach to study the effect of *MEP2* mutations on signaling independent of their effect on growth of the cells, the *MEP2*[Y122A], *MEP2*[F126A], and *MEP2*[W167A] alleles were introduced into *mep2Δ* single mutants, which can grow normally on SLAD plates because they express Mep1p. The filamentation behavior of these strains mirrored that of the *mep1Δ mep2Δ* double mutants expressing the same alleles (Fig. 8, right panels), i.e., the Y122A mutation resulted in reduced filamentous growth and the F126A and W167A mutations abolished filamentation.

4.1.4   An F239A mutation abolishes ammonium uptake and induction of filamentous growth by Mep2p

Very recently, Javelle *et al*. reported that mutation of the conserved phenylalanine at amino acid position 215 in AmtB of *E. coli* resulted in abolishment of ammonium transport (Javelle *et al*., 2008). F215, together with F107, is thought to form an extracytosolic gate to the conductance channnel of AmtB, and F215 was proposed to be essential for the deprotonation of ammonium to allow transport of ammonia into the cell. To obtain a similarly transport-deficient Mep2p protein in *C. albicans*, the corresponding residue, F239, was changed to alanine. Plasmid pMEP2K16 containing the F239A mutation was generated by ligating the PstI-SacI fragment from plasmid pMEP2K1 into the PstI/SacI-digested pMEP2G16 (described below). Indeed, expression of the *MEP2*^F239A allele did not rescue the growth defect of *mep1Δ mep2Δ* double mutants on SLAD plates (Fig. 9A), indicating that the F239A mutation abolished ammonium uptake by Mep2p. In order to investigate if the mutated protein is correctly expressed in the cell membrane a plasmid pMEP2G16, which contains *GFP*-tagged *MEP2*^F239A allele, was generated in following manner: the N- terminal and C-terminal parts of *MEP2* were amplified from pMEP2K1 with the primer pairs MEP3/MEP94p and MEP76p/MEP32. The PCR products were digested at an internal NsiI site and at the introduced BamHI site, respectively, and ligated together into the NsiI/BamHI-digested pMEP2G2 (Biswas & Morschhäuser, 2005).

(A)   **(B)**
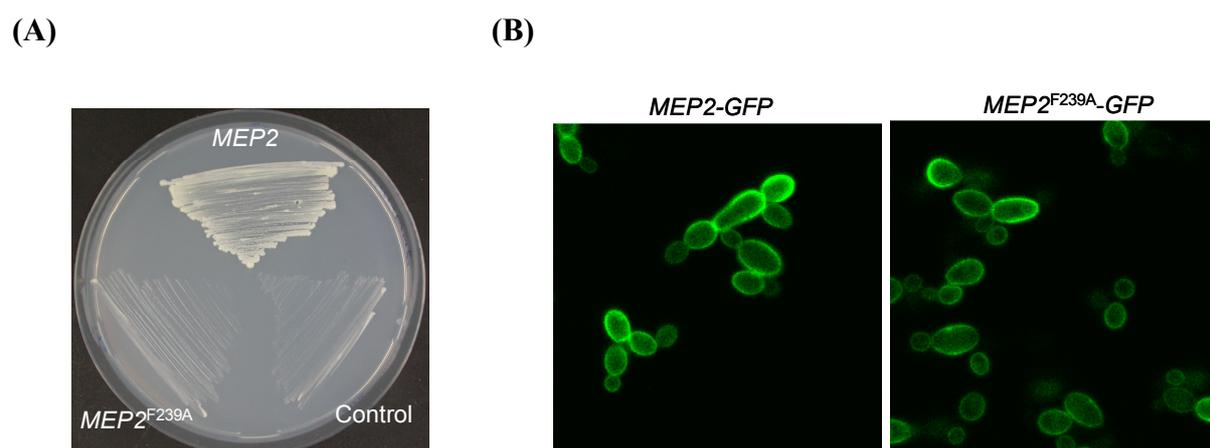
Fig. 9. (A) Growth of *mep1Δ mep2Δ* double mutants expressing wild-type *MEP2* or the *MEP2*^F239A allele. The strains were grown for 4 days at 30°C on SD plates containing 2 mM ammonium. The strains used were as follows: MEP12M6A/B (control), MEP12MK2A/B (*MEP2*), and MEP12MK17A/B (*MEP2*^F239A)   **(B)** Localization of *GFP*-tagged wild-type or *MEP2*^F239A alleles. Strains MEP12MG2A/B (*MEP2-GFP*) and MEP12MG16A/B (*MEP2*^F239A-*GFP)* were grown for 6 hours at 30°C in liquid SLAD medium and observed by fluorescence microscopy. The two independently constructed series of strains behaved identically in both experiments and only one of them is shown.

The mutated protein was properly localized on the cell surface as demonstrated by fluorescence microscopy of the cells expressing a GFP-tagged Mep2p[F239A] (Fig. 9B), and quantification of the cellular fluorescence by flow cytometry showed that the expression levels of the mutated protein reached approximately 35% of those of wild-type Mep2p (data not shown). The transport-deficient Mep2p was then investigated for its ability to induce filamentation. *mep1Δ mep2Δ* double mutants expressing the *MEP2*[F239A] allele were unable to filament on agar plates containing limiting concentrations of different nitrogen sources (Fig. 10, left three panels), and the mutated allele did also not restore filamentous growth of *mep2Δ* single mutants on SLAD plates (Fig. 10, right panel). Therefore, F239 is essential for both ammonium transport and induction of filamentous growth by Mep2p in *C. albicans*.
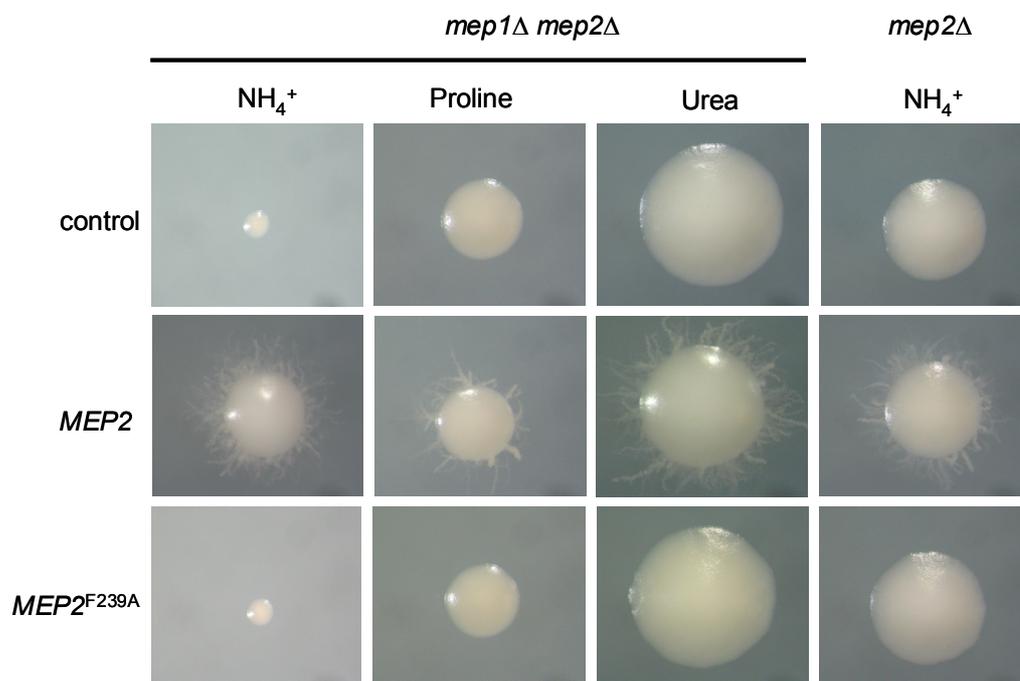
Fig. 10. Filamentation of *mep1Δ mep2Δ* double mutants (left three panels) and *mep2Δ* single mutants (right panels) expressing wild-type *MEP2* or the *MEP2*[F239A] alleles on agar plates containing 100 µM of the indicated nitrogen sources. Individual representative colonies were photographed after 6 days of growth at 37°C. The *mep1Δ mep2Δ* double mutants (strains MEP12M6A/B) and the *mep2Δ* single mutants (strains MEP2M5A/B) were used as controls. The two independently constructed series of strains behaved identically and only one of them is shown.

4.2 Control of ammonium permease expression and nitrogen starvation-induced filamentous growth in *C. albicans*

Under nitrogen limiting conditions the morphogenetic regulatory role of Mep2p is attributed, at least in part, to its high expression levels. Since the control of *MEP2* expression is central to the regulation of nitrogen starvation-induced filamentous growth in *C. albicans* (Biswas & Morschhäuser, 2005), factors involved in the induction of its expression were investigated.

4.2.1 Two putative GATA factor binding sites in the *MEP2* promoter are essential for the upregulation of *MEP2* expression under limiting nitrogen conditions

In order to understand how expression of Mep2p is regulated, the regulatory elements in the *MEP2* promoter which mediate its induction in response to nitrogen limitation were identified. For this purpose, a *GFP*-tagged *MEP2* gene was used as reporter and expressed under the control of wild-type and mutated *MEP2* promoters in *mep1Δ mep2Δ* double mutants. By this approach Mep2p expression could be monitored by observing both the fluorescence of the cells and the capacity of the tagged ammonium permease to restore growth of the double mutants at low ammonium concentrations. The reporter fusions were constructed in such a manner so that they could be integrated at the original *MEP2* locus (Fig. 11), since it has been reported that integration of *C. albicans* genes at a different genomic site may affect their expression (Lay *et al.*, 1998).

Plasmid constructions for MEP2 promoter analysis

Plasmid pMEP2G6, which contains a *GFP*-tagged *MEP2* gene under the control of the wild-type *MEP2* promoter and served as the basis for the introduction of deletions and mutations in the *MEP2* regulatory region (Fig. 11), was generated in the following way: A KpnI-XhoI fragment containing *MEP2* upstream sequences from positions -1478 to -5 with respect to the start codon was amplified from genomic DNA of *C. albicans* strain SC5314 with the primer pair MEP44/MEP24. The *MEP2* promoter fragment was then cloned together with an XhoI-BamHI [*MEP2* coding region] fragment from plasmid pMEP2G5 (Biswas & Morschhäuser, 2005) into the KpnI/BamHI-digested pMEP2G2 (Biswas & Morschhäuser, 2005). The cloned *MEP2* promoter fragment contained the polymorphic EcoRI site, which is present only in the *MEP2-1* allele (Biswas & Morschhäuser, 2005). To construct pMEP2ΔP1, a distal *MEP2* promoter fragment (positions -1478 to -1015) was amplified with the primers MEP44/MEP55 and a proximal *MEP2* promoter fragment (positions -188 to -5) was amplified with the primers MEP51/MEP24. The PCR products were digested with KpnI/XbaI and XbaI/XhoI,

respectively, and cloned together into the KpnI/XhoI-digested pMEP2G6. To create pMEP2ΔP2, a proximal *MEP2* promoter fragment (positions -431 to -5) was amplified with the primers MEP52/MEP24 and substituted for the XbaI-XhoI fragment in pMEP2ΔP1. Additional deletion constructs were made in an analogous fashion: Proximal *MEP2* promoter fragments from positions -620 to -5, -805 to -5, -287 to -5, and -217 to -5 were amplified with the primers MEP53/MEP24, MEP54/MEP24, MEP61/MEP24, and MEP62/MEP24, respectively, and substituted for the XbaI-XhoI fragment in pMEP2ΔP1 to generate pMEP2ΔP3, pMEP2ΔP4, pMEP2ΔP6, and pMEP2ΔP7. To construct pMEP2ΔP5, a distal *MEP2* promoter fragment (positions -1478 to -435) was amplified with the primer pair MEP44/MEP60 and used to replace the distal *MEP2* promoter fragment in pMEP2ΔP1. For pMEP2MP1, in which the GATA sequence centered at position -208 is replaced by an XbaI site, a distal *MEP2* promoter fragment (positions -1478 to -210), amplified with the primers MEP44/MEP64 and a proximal *MEP2* promoter fragment (positions -205 to -5), amplified with the primers MEP63/MEP24, were fused at the introduced XbaI site and substituted for the wild-type *MEP2* promoter in pMEP2G6. For pMEP2MP2, in which the GATA sequence centered at position -266 is replaced by a BglII site, a distal *MEP2* promoter fragment (positions -1478 to -269), amplified with the primers MEP44/MEP66, and a proximal *MEP2* promoter fragment (positions -266 to -5), amplified with the primers MEP65/MEP24, were fused at the introduced BglII site and substituted for the wild-type *MEP2* promoter in pMEP2G6. To introduce both mutations into the *MEP2* promoter, the same proximal *MEP2* promoter fragment was amplified from pMEP2MP1, fused with the distal *MEP2* promoter fragment, and substituted for the wild-type *MEP2* promoter in pMEP2G6 to create pMEP2MP3. KpnI-SacI fragment from each of these plasmids (Fig.11) was used to transform *mep1Δ mep2Δ* double mutants. Replacement of the resident wild-type promoter by mutated *MEP2* promoters was verified by Southern hybridization analysis (data not shown) and two independent transformants were used for further analysis in each case (see Table 2 for strain descriptions).

In accord with the earlier results (Biswas & Morschhäuser, 2005), expression of the *MEP2-GFP* fusion from the wild-type *MEP2* promoter resulted in strong fluorescence of the cells in SLAD medium and restoration of the growth to wild-type levels on plates containing limiting ammonium concentrations (Figs. 11 and 12A).
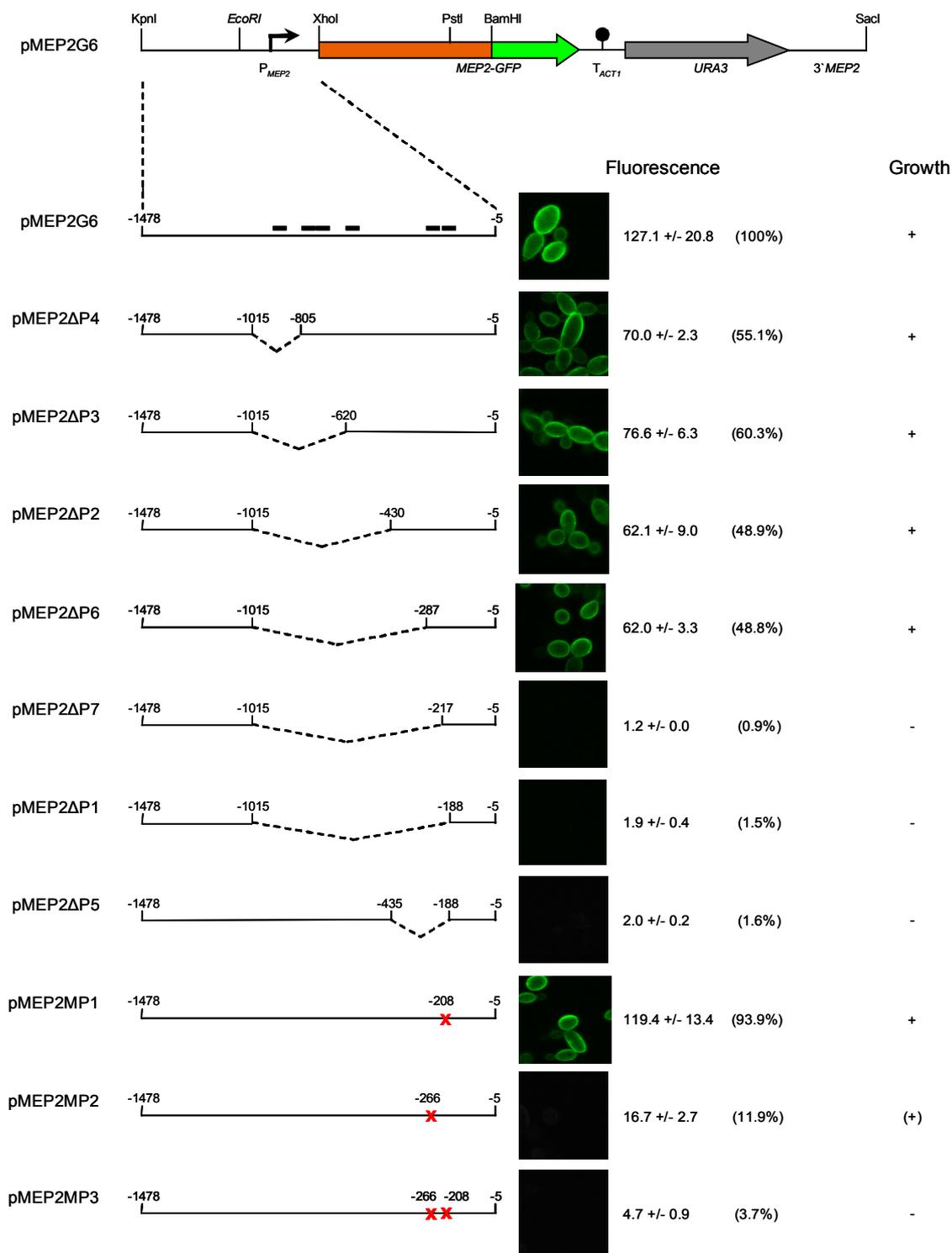
Fig. 11. *MEP2* promoter analysis. The structure of the insert of plasmid pMEP2G6, which contains a *GFP*-tagged *MEP2* gene under control of the wild-type *MEP2* promoter, is shown on top. The *MEP2* and *GFP* coding regions are represented by the colored box and the green arrow, respectively, the transcription termination sequence of the *ACT1* gene (T$_{ACT1}$) by the filled circle, and the *URA3* selection marker by the grey arrow. *MEP2* upstream and downstream regions are represented by the solid lines, the *MEP2* promoter (P$_{MEP2}$) is symbolized by the bent arrow. Relevant restriction sites are shown, the polymorphic EcoRI site, which is present only in the *MEP2-1* allele, is highlighted in italics. Enlarged representations of the *MEP2* regulatory region with the introduced deletions and mutations are shown below and the names of the corresponding plasmids are indicated to the left. The extent of *MEP2* promoter sequences contained in the various plasmids is given. Internal deletions are

indicated by the dashed lines. The locations of GATAA sequences within 1 kb upstream of the *MEP2* start codon are indicated by the short black bars in the wild-type promoter. The mutations of the GATAA sequences centered at positions -266 and -208 are marked by an X. The phenotypes conferred by the various constructs upon integration into *mep1Δ mep2Δ* double mutants are shown to the right. Fluorescence of the cells was observed after 6 hours of growth at 30°C in liquid SLAD medium. The fluorescence micrographs show representative cells and the mean fluorescence of the two independently constructed reporter strains measured by flow cytometry is given. The percentage values in parentheses are with respect to the wild-type *MEP2* promoter, which was set to 100%. Strain SC5314, which does not carry *GFP*, was used as a negative control. Background fluorescence value of this strain (1.6) was subtracted from those of the reporter strains. Growth of the strains at limiting ammonium concentrations was as follows: +, wild-type growth; (+), weak growth; -, no growth.

Deletion of *MEP2* upstream sequences ranging from positions -1014 up to -288 with respect to the start codon (ΔP2, ΔP3, ΔP4, ΔP6) reduced *MEP2* expression only by about 50% and did not detectably affect growth at limiting ammonium concentrations, but deletion of additional sequences to positions -218 or -189 (ΔP1, ΔP7) abolished *MEP2* expression and transformants carrying these fusions behaved like *mep1Δ mep2Δ* double mutants. A shorter deletion ranging from positions -434 to -189 (ΔP5) produced the same phenotype, suggesting that sequences within this region control *MEP2* induction in response to nitrogen limitation. Inspection of the DNA sequence of this region revealed that it contained two putative GATA transcription factor binding sequences (GATAA) (Magasanik & Kaiser, 2002) located at positions -264 to -268 and -206 to -210 on the antisense strand. In order to investigate the involvement of these GATAA sequences in the regulation of *MEP2* expression, these sequences were mutated in the full-length *MEP2* promoter. The GATAA sequence centered at -266 was changed to GATCT, and the other centered at -208 was changed to CTAGA. Whereas mutation of the GATAA sequence at -208 alone (MP1) had no effect, mutation of the GATAA sequence at -266 (MP2) resulted in strongly reduced *MEP2* expression and only weak restoration of growth of its transformants at limiting ammonium concentrations. Mutation of both GATAA sequences (MP3) reduced *MEP2* expression almost to background levels and the corresponding transformants were unable to grow at low ammonium concentrations, like *mep1Δ mep2Δ* double mutants. Taken together these results demonstrated that two proximal GATAA sequences are important for *MEP2* expression, and indicate that GATA transcription factors may be involved in the induction of *MEP2* under nitrogen starvation conditions. Though there are a number of additional GATAA sequences located at more distal sites in the *MEP2* upstream region (Fig. 11), the results obtained with promoter deletion and mutation analysis indicate that these sequences are neither required nor sufficient for *MEP2* expression.

4.2.2   Filamentous growth is correlated with *MEP2* expression levels

Under limiting nitrogen conditions, the higher expression levels of *MEP2* than those of *MEP1* are reported to be a prerequisite for normal filamentation that is mediated by Mep2p (Biswas & Morschhäuser, 2005). The effect of reduced Mep2p expression from mutated *MEP2* promoters on filamentous growth was analyzed. This was accomplished by expressing *MEP2* from various mutated *MEP2* promoters displaying different activities in a *mep2Δ* background, thus allowing normal ammonium uptake due to the presence of the *MEP1* gene. To express *MEP2* gene from wild-type and mutated *MEP2* promoters, a PstI-SacI fragment from pMEP2K1 (Fig. 2) (Biswas & Morschhäuser, 2005) was substituted for the PstI-SacI fragment in plasmids pMEP2G6, pMEP2ΔP5, pMEP2ΔP6, pMEP2MP1, pMEP2MP2, and pMEP2MP3 (see Fig. 11), thereby generating pMEP2K13, pMEP2ΔP5A, pMEP2ΔP6A, pMEP2MP1A, pMEP2MP2A, and pMEP2MP3A, respectively, in which the *GFP*-tagged *MEP2* is replaced by wild-type *MEP2*. A KpnI-SacI fragment from each of these plasmids was used to transform *mep2Δ* single mutants and correct integrants were verified by Southern analysis (data not shown).

The normal Mep2p expression levels, as from the MP1 promoter, resulted in wild-type filamentation (Fig.12B). A slight reduction of Mep2p expression by about 50%, as from the ΔP6 promoter, also did not detectably affect filamentous growth. However, the strongly (ca. 8-fold) reduced Mep2p expression levels obtained from the MP2 promoter severely affected the ability of the strains to produce filamentous colonies in response to nitrogen starvation. Most colonies (70-80%) were non-filamentous and a minority (20-30%) showed only weak filamentation. As expected, reduction of Mep2p expression to nearly background levels, as from the ΔP5 or MP3 promoters, completely abolished filamentous growth. Hence, nitrogen starvation-induced filamentous growth directly correlated with Mep2p expression levels.

(A)

(B)
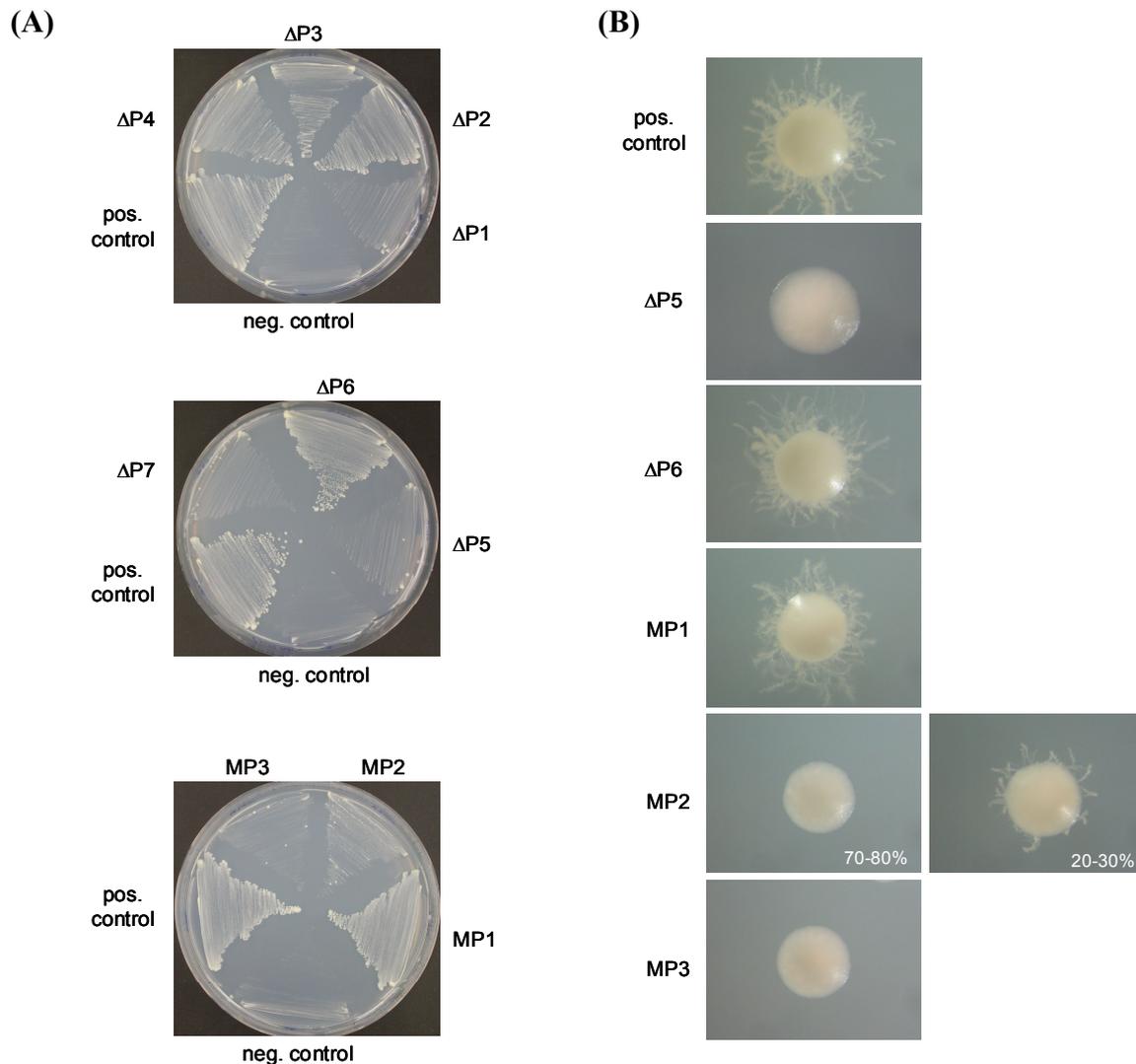
Fig. 12. (A) Growth of *mep1Δ mep2Δ* double mutants expressing a *GFP*-tagged *MEP2* gene from the wild-type *MEP2* promoter and derivatives containing the indicated deletions or mutations. The strains were grown for 4 days at 30°C on SD plates containing 1 mM ammonium. The following strains were used: MEP12M6A/B (neg. control), MEP12MG6A/B (pos.control), MEP12MG6ΔP1A/B (ΔP1), MEP12MG6ΔP2A/B (ΔP2), MEP12MG6 ΔP3A/B (ΔP3), MEP12MG6ΔP4A/B (ΔP4), MEP12MG6ΔP5A/B (ΔP5), MEP12MG6ΔP6A/B (ΔP6), MEP12M G6ΔP7A/B (ΔP7), MEP12MG6MP1A/B (MP1), MEP12MG6MP2A/B (MP2), MEP12MG6MP3A/B (MP3). **(B)** Filamentation of *mep2Δ* mutants expressing *MEP2* from the wild-type *MEP2* promoter and derivatives containing the indicated deletions or mutations. The strains were grown for 6 days at 37°C on SLAD plates and individual representative colonies were photographed. The following strains were used: MEP2MK13A/B (pos. control), MEP2MK13ΔP5A/B (ΔP5), MEP2MK13ΔP6A/B (ΔP6), MEP2MK13MP1A/B (MP1), MEP2MK13 MP2A/B (MP2), MEP2MK13MP3A/B (MP3). The two independently constructed strains used in A and B experiments behaved identically and only one of them is shown in each case.

4.2.3   GATA factors *GLN3* and *GAT1* in *C. albicans*

In *S. cerevisiae*, *MEP2* expression requires at least one of the two GATA transcription factors Gln3p and Gat1p/Nil1p, which are involved in the transcriptional activation of many nitrogen-regulated genes (Lorenz & Heitman, 1998; Marini *et al.*, 1997). *C. albicans*

possesses homologs of *GLN3* and *GAT1* (Braun *et al.*, 2005; Limjindaporn *et al.*, 2003) and their roles in regulating *MEP2* expression were explored. For this purpose, deletion mutants were generated from the *C. albicans* wild-type strain SC5314, lacking either *GLN3*, *GAT1* or both, using the *SAT1*-flipping strategy (Reuß *et al.*, 2004).

4.2.4   Construction of *GLN3* and *GAT1* deletion mutants

Knock-out mutant strains in the diploid *C. albicans* were constructed using a dominant selection marker based method, the *SAT1* flipper system (Reuß *et al.*, 2004). The *SAT1*-flipping cassette is depicted in Fig. 13A. It consists of *C. albicans*-adapted nourseothricin resistance gene *caSAT1* as selection marker; *C. albicans*-adapted recombinase gene *caFLP* under the control of the inducible *MAL2* promoter; and is flanked by direct repeats of 34 bp minimal target sequences of the *FLP* recombinase (*FLP* recognition target, *FRT*). The strategy for sequential gene disruption using the *SAT1* flipper is outlined in Fig. 13B. Gene disruption plasmids were created by cloning sequences upstream and downstream of the target gene onto either side of the *SAT1* cassette. *C. albicans* is transformed with the linearized insert (obtained after digesting the deletion plasmid with appropriate enzymes) and transformants were selected on medium containing nourseothricin. The flanking sequences of the gene direct integration of the cassette into one allele of the target gene by homologous recombination. Transformants containing the correct insertion are nourseothricin resistant (Nou^R) and are grown in medium containing maltose or glucose (since *MAL2* promoter is leaky) without selection pressure. In this medium *MAL2* promoter is induced, resulting in expression of the *FLP* gene and excision of the *SAT1* flipper by FLP-mediated site specific recombination. The heterozygous nourseothricin sensitive (Nou^S) derivatives are easily identified by their smaller colony size on YPD plates containing 20 µg/ml nourseothricin as compared with their Nou^R parental strains. A second round of integration and subsequent excision of the *SAT1* cassette in the heterozygous mutants generates the desired homozygous knock-out mutants, which differ from the wild-type strain by the two disrupted alleles of the target gene, both of which contain one copy of the *FRT* site.

(A)

(B)
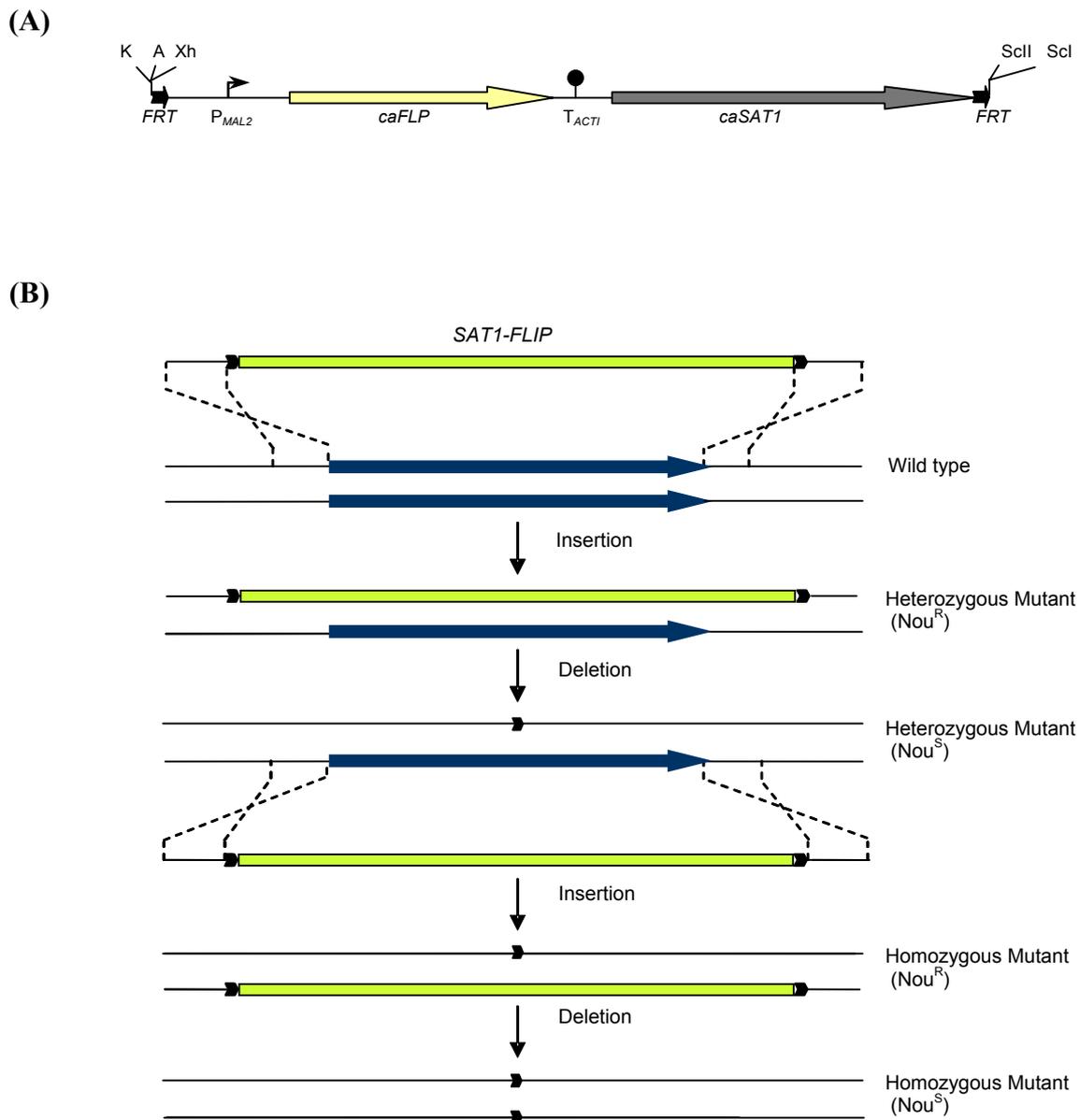
Fig. 13. (A) Structure of the *SAT1* flipper cassette (Reuß *et al.*, 2004). The *C. albicans*-adapted recombinase gene (*caFLP*) is represented by the yellow arrow; the nourseothricin resistance marker (c*aSAT1)* by the grey arrow; the inducible *MAL2* promoter (P*MAL2*) by the bent arrow; and the transcription termination of the *C.albicans ACT1* gene (T*ACT1*) by the filled circle. The FLP recombination target sequences (*FRT*) are indicated by the black arrows. Only relevant restriction sites are given: A, ApaI; K, KpnI; ScI, SacI; ScII, SacII; Xh, XhoI. **(B)** Schematic representation of gene disruption in *C. albicans* using *SAT1* flipper.

GLN3 mutant series

To obtain *gln3∆* mutants, a *GLN3* deletion cassette pGLN3M2 was constructed. For generating this plasmid, an ApaI-XhoI *GLN3* upstream fragment (positions -204 to +6) was amplified from the genomic DNA of *C. albicans* strain SC5314 with the primer pair GLN1/ GLN2. A SacII-SacI *GLN3* downstream fragment (positions +2025 to +2293) was amplified

with the primer pair GLN3/GLN4. The *GLN3* upstream and downstream fragments were substituted for the *OPT1* flanking sequences in pOPT1M3 (Reuß *et al.*, 2004) to result in pGLN3M2, in which the *GLN3* coding region from position +7 to +2024 (23 bp before stop codon) is replaced by *SAT1* flipper (Fig. 14A). Nucleotide positions are with respect to the *GLN3* start codon.

(A)
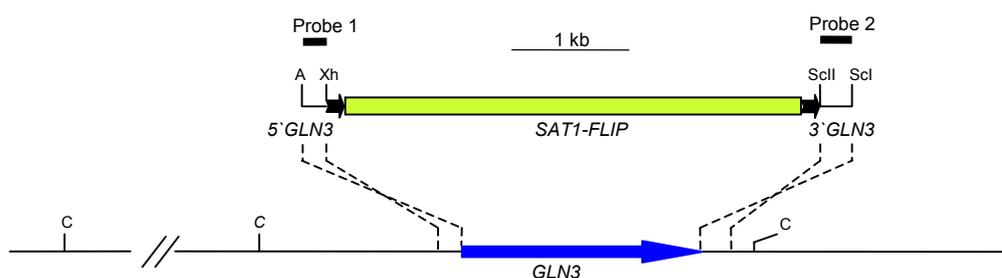
(B)
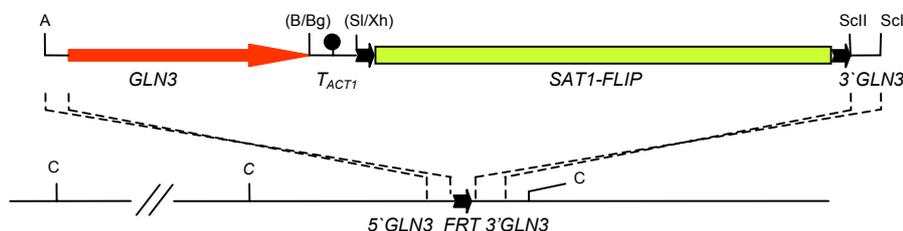
Fig. 14. (A) Structure of the deletion cassette from plasmid pGLN3M2 (top), which was used to delete the *GLN3* ORF, and genomic structure of the *GLN3* locus in strain SC5314 (bottom). The upstream and downstream regions of *GLN3* are represented by the solid lines. Details of the *SAT1* flipper are presented in Fig.14A. The probes used for Southern hybridization analysis of the mutants are indicated by the black bars. **(B)** Structure of the DNA fragment from pGLN3K1 (top), which was used for reintegration of an intact *GLN3* copy (represented by red arrow) into one of the disrupted *GLN3* loci in the *gln3Δ* single and *gln3Δ gat1Δ* double mutants (bottom). Only relevant restriction sites are given: A, ApaI; B, BamHI; Bg, BglII; C, ClaI; ScI, SacI; ScII, SacII; Sl, SalI; Xh, XhoI. Sites shown in parenthesis were destroyed by the cloning procedure. The ClaI site marked in italics is present only in the *GLN3-1* allele.

The two *GLN3* alleles in strain SC5314 could be distinguished by a ClaI restriction site polymorphism (Fig. 14A, Fig. 15 lane1) and were arbitrarily designated as allele 1 (*GLN3-1,* located on the smaller ClaI fragment) and allele 2 (*GLN3-2,* larger ClaI fragment). Steps involved in construction of *GLN3* mutant series are documented in Fig. 15. The wild-type strain SC5314 was transformed with ApaI-SacI fragment of pGLN3M2. Two

independent transformants in which the *SAT1* flipper had integrated into either the *GLN3-1* allele (strain GLN3M1A) or into the *GLN3-2* allele (strain GLN3M1B) of the parental strain were selected. These Nou[R] strains were inoculated in YPM medium for 6 h allowing FLP-mediated excision of *SAT1* flipper cassette resulting in the Nou[S] heterozygous mutants GLN3M2A and GLN3M2B (Fig. 15, lanes 2 and 3). In order to inactivate the remaining intact allele of *GLN3*, transformation of the heterozygous mutants was done with the same deletion cassette (strain GLN3M3A/B) and subsequent excision of the *SAT1* flipper generated Nou[S] homozygous *gln3Δ* mutants GLN3M4A and GLN3M4B (Fig. 15, lanes 4 and 5).
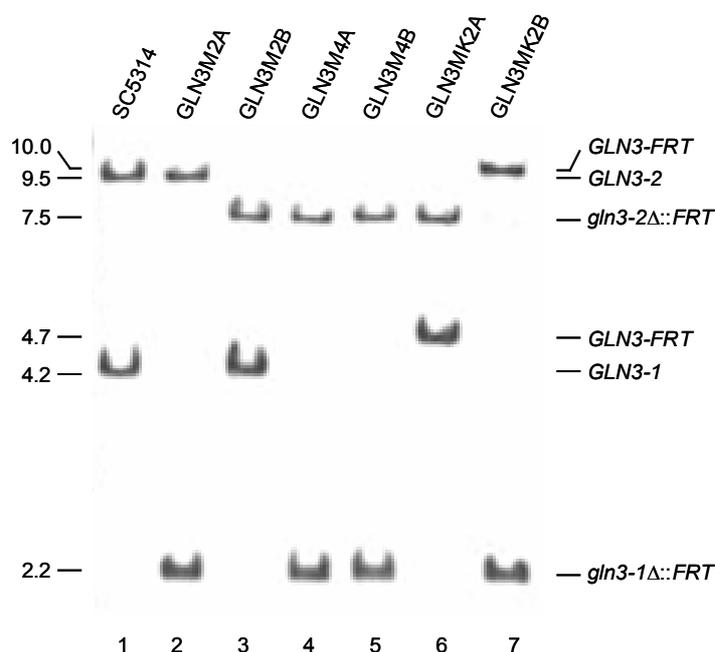
Fig. 15. Southern hybridization of ClaI-digested genomic DNA of the wild-type strain SC5314 and *GLN3* mutant derivatives with the *GLN3*-specific probe 1 shown in Fig. 14A. The sizes of the hybridizing fragments (in kb) are given on the left side of the blot and their identities are indicated on the right.

Reintegration of an intact *GLN3* copy into its original locus was done with the help of the *SAT1* flipper using plasmid pGLN3K1. For generating this plasmid an ApaI-BamHI fragment containing the complete *GLN3* ORF and upstream sequences (from position -204 to +2074) was amplified with the primers GLN1 and GLN5 from genomic DNA of *C. albicans* strain SC5314. This fragment was cloned together with a BglII-SalI fragment from pCBF1M4 containing the *ACT1* transcription termination sequence (Biswas *et al.*, 2003) into the ApaI/XhoI-digested pGLN3M2 to create pGLN3K1 (Fig. 14B). The *gln3Δ* null mutants (strains GLN3M4A/B) were transformed with ApaI-SacI fragment of pGLN3K1. Nou[R] transformants GLN3MK1A and GLN3MK1B were derived from GLN3M4A and GLN3M4B respectively. The *SAT1* flipper was then deleted from these transformants resulting in the complemented strains GLN3MK2A/B (Fig. 15, lanes 6 and 7).

GAT1 mutant series

GAT1 gene was deleted in analogous manner in SC5314 and *gln3*Δ strains to generate *gat1*Δ single as well as *gln3*Δ *gat1*Δ double mutants. To create a *GAT1* deletion construct, an ApaI-XhoI fragment containing *GAT1* upstream sequences (from position -429 to -69) was amplified from the genomic DNA of *C. albicans* strain SC5314 with the primer pair GAT1/ GAT2. A SacII-SacI *GAT1* downstream fragment (from position +2003 to +2559) was amplified with the primers GAT5 and GAT6. The *GAT1* upstream and downstream fragments were substituted for the *GLN3* flanking sequences in pGLN3M2 to result in pGAT1M2, in which the *GAT1* coding region from position -68 to +2002 (63 bp before the stop codon) is replaced by the *SAT1* flipper (Fig. 16A).
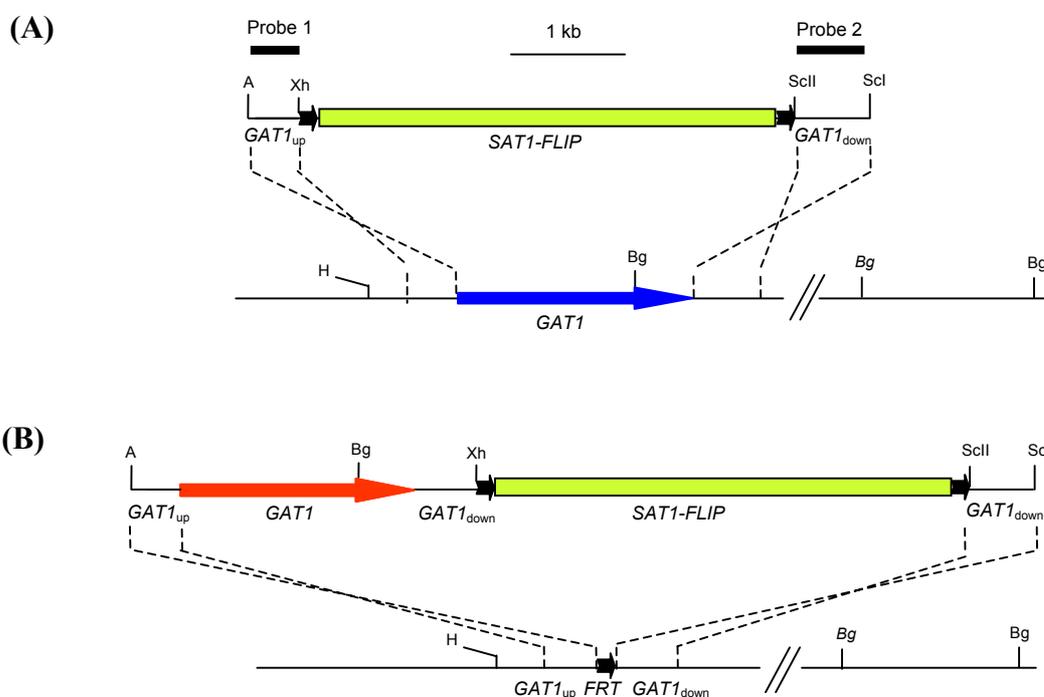
Fig. 16. (A) Structure of the deletion cassette from plasmid pGAT1M2 (top), which was used to delete the *GAT1* ORF, and genomic structure of the *GAT1* locus in strain SC5314 (bottom).The probes used for Southern hybridization analysis of the mutants are indicated by the black bars. **(B)** Structure of the DNA fragment from pGAT1K1 (top), which was used for reintegration of an intact *GAT1* copy into one of the disrupted *GAT1* loci in the *gat1*Δ single and *gln3*Δ *gat1*Δ double mutants (bottom). The probes used for Southern hybridization analysis of the mutants are indicated by the black bars. The *GAT1* coding region is represented by the red arrow and the upstream and downstream regions by the solid lines. Only relevant restriction sites are given: A, ApaI; Bg, BglII; H, HindIII; ScI, SacI; ScII, SacII; Xh, XhoI. The BglII site marked in italics is present only in the GAT1-1 allele.

The *GAT1* alleles of strain SC5314 can be distinguished by a downstream BglII restriction site polymorphism (Fig. 16A, Fig. 17 lane1) and were arbitrarily designated as allele 1 (*GAT1-1,* located on the smaller BglII fragment) and allele 2 (*GAT1-2,* larger BglII fragment). Insertion of the *SAT1* flipper (ApaI-SacI fragment from pGAT1M2) into either the

GAT1-1 allele (strain GAT1M1A) or into the *GAT1-2* allele (strain GAT1M1B) of the parental strain and subsequent FLP-mediated excision of the cassette produced the heterozygous mutants GAT1M2A and GAT1M2B (Fig. 17, lanes 2 and 3). Using the same deletion cassette, the remaining wild-type *GAT1* alleles of heterozygous mutants were inactivated. Recycling of the *SAT1* flipper from these independently constructed Nou^R mutants generated Nou^S homozygous *gat1Δ* mutants GAT1M4A and GAT1M4B (Fig. 17, lanes 4 and 5).
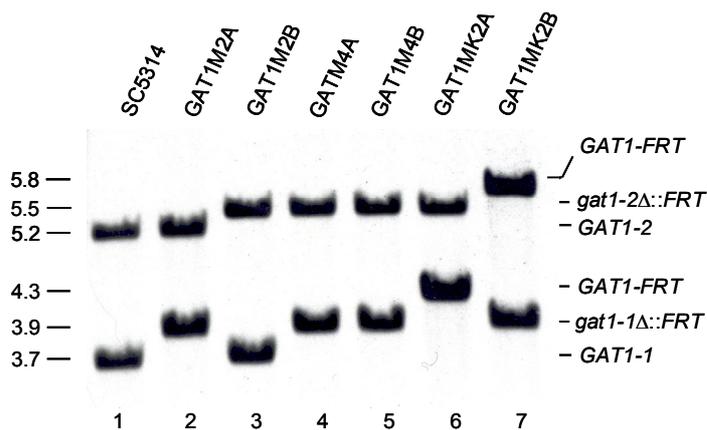
Fig. 17. Southern hybridization of Hind III-BglII digested genomic DNA of the wild-type strain SC5314 and *GAT1* mutant derivatives with the *GAT1*-specific probe 2 shown in Fig.16A. The sizes of the hybridizing fragments (in kb) are given on the left side of the blot and their identities are indicated on the right.

Reintroduction of an intact *GAT1* allele in the mutant background was done using plasmid pGAT1K1. For generating this plasmid, an ApaI-XhoI fragment containing the *GAT1* coding region and flanking sequences (from positions -429 to +2558) was amplified from genomic DNA of strain SC5314 with the primers GAT1 and GAT7. This PCR fragment was substituted for the *GAT1* upstream region in the plasmid pGAT1M2 (Fig. 16B). GAT1M4A and GAT1M4B strains were transformed with ApaI-SacI fragment of pGAT1K1. Transformants in which the cassette was targeted to either of the two inactivated *gat1Δ* alleles were selected and recycling of the *SAT1* flipper from these strains give rise to complemented strains GAT1MK2A and GAT1MK2B (Fig. 17, lanes 6 and 7).

To construct *gln3Δ gat1Δ* double mutants, the *gln3Δ* mutants (strains GLN3M4A and GLN3M4B) were used as parental strains and were transformed with the *GAT1* deletion cassette (pGAT1M2). One transformant from each parental strain, in which the *SAT1* flipper cassette had integrated either into the *GAT1-1* allele (strain Δ*gln3*GAT1M1A) or into the *GAT1-2* allele (strain Δ*gln3*GAT1M1B) was selected. Excision of the *SAT1* flipper from these strains by FLP-mediated recombination resulted in Nou^S derivatives Δ*gln3*GAT1M2A/B (Fig. 18, lanes 4 and 5). Second round of integration and subsequent excision of *GAT1* deletion cassette in the heterozygous Δ*gln3*GAT1M2A/B mutants resulted in inactivation of the

remaining intact allele of *GAT1,* generating *gln3*GAT1M4A/B (Fig. 18, lanes 6 and 7). Re-integration of an intact *GLN3* copy (using pGLN3K1 [Fig. 14B]) into either of its inactivated loci in the homozygous *gln3*Δ *gat1*Δ double mutants, followed by excision of the *SAT1* flipper cassette produced complemented strains Δ*gln3*GAT1MK2A/B (Fig. 18, lanes 8 and 9). Similarly an intact *GAT1* copy (using pGAT1K1 [Fig.16B]) was reintroduced into *gln3*Δ *gat1*Δ mutants and subsequent excision of *SAT1* flipper yielded the complemented strains Δ*gln3*GAT1K4A/B (Fig. 18, lanes 10 and 11). Integrity of the *GLN3* locus in the mutants was confirmed by probing the blot that is used for Fig. 18 with the *GLN3*- specific probe 1 (data not shown).
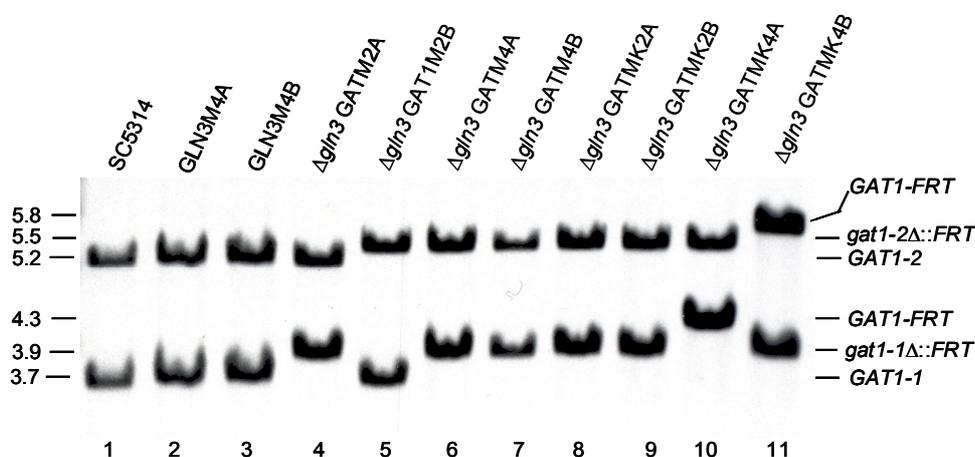
Fig. 18. Southern hybridization of HindIII-BglII digested genomic of wild-type strain SC5314, the *gln3*Δ parental strins GLN3M4A and B, and *GAT1* mutant derivatives with the *GAT1*-specific probe 2 shown in Fig. 16A. The sizes of the hybridizing fragments (in kb) are given on the left side of the blot and their identities are indicated on the right.

4.2.5 GATA factors Gln3p and Gat1p control *MEP2* expression

To assess whether *GLN3* and *GAT1* participate in the induction of *MEP2* expression in response to nitrogen limitation, expression levels of the GFP-tagged Mep2p in the wild-type strain SC5314, the *gln3*Δ and *gat1*Δ single mutants, and the *gln3*Δ *gat1*Δ double mutants were compared. For integration of *MEP2-GFP* fusion (Fig. 19) into either of the two *MEP2* alleles, plasmid pMEP2G7 was used. This plasmid was generated in following manner: A BamHI-PstI [*GFP*-T$_{ACT1}$-*caSAT1*] fragment from pADH1G3 (Park and Morschhäuser, unpublished) was cloned together with a PstI-SacI [3'*MEP2*] fragment from pMEP2G2 into the BamHI/SacI-digested pMEP2G2.
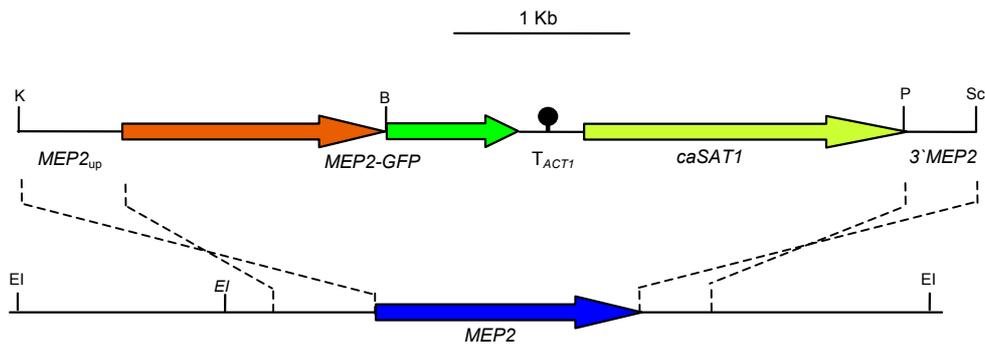
Fig. 19. Structure of the DNA fragment from pMEP2G7 (top), which was used to introduce *MEP2-GFP* fusion into the original *MEP2* locus (bottom). The *MEP2* and *GFP* coding regions are represented by the red arrow and the dark green arrow, respectively, the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the filled circle, and the *caSAT1*selection marker by the light green arrow. *MEP2* upstream and downstream regions are represented by the solid lines. Only relevant restriction sites are given: B, BamHI; EI, EcoRI; K, KpnI; P, PstI; ScI, SacI. The polymorphic EcoRI site, which is present only in the *MEP2-1* allele, is highlighted in italics.

The Mep2p-GFP reporter strains in different genetic backgrounds were grown in liquid media containing limiting amounts (100 µM) of ammonium, glutamine, proline, or urea and fluorescence of the cells was measured by flow cytometry. Irrespective of the nitrogen source, Mep2p expression was strongly reduced in the *gln3Δ* and *gat1Δ* single mutants and was below the detection limit in the *gln3Δ gat1Δ* double mutants under all tested conditions (Fig. 21A).

Additionally, with the help of a P_{MEP2}-*GFP* fusion (Fig. 20), the effect of the absence of GATA factors on *MEP2* promoter activity was also compared. For integration of the P_{MEP2}-*GFP* fusion at the original *MEP2* locus, plasmid pMEP2PG1 was constructed. For this, SalI-PstI [*GFP*-T_{ACT1}-*caSAT1*] fragment from pOPT1G22 (Reuß & Morschhäuser, 2006) was cloned into XhoI/PstI-digested pMEP2G6. In accord with the results obtained with C-terminal fusion of GFP to Mep2p; *MEP2* promoter activity was greatly decreased in the mutants lacking either *GLN3* or *GAT1*, and abolished in the double mutants (Fig. 21B).
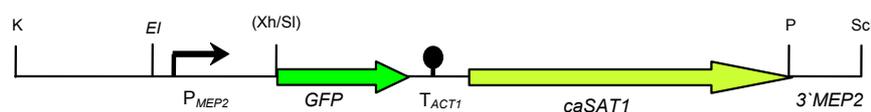

Fig. 20. Structure of the DNA fragment from plasmid pMEP2PG1 that was used to introduce P_{MEP2}-*GFP* fusion into the original *MEP2* locus. The *GFP* coding region is represented by the dark green arrow, T_{ACT1} by the filled circle, and the *caSAT1* selection marker by the light green arrow. *MEP2* upstream and downstream regions are represented by the solid lines, the *MEP2* promoter (P_{MEP2}) is symbolized by the bent arrow. Only relevant restriction sites are given: EI, EcoRI; K, KpnI; P, PstI; Sl, SalI;ScI, SacI; Xh,XhoI. Sites shown in parentheses were destroyed by the cloning procedure. The polymorphic EcoRI site is highlighted in italics.

(A)

(B)
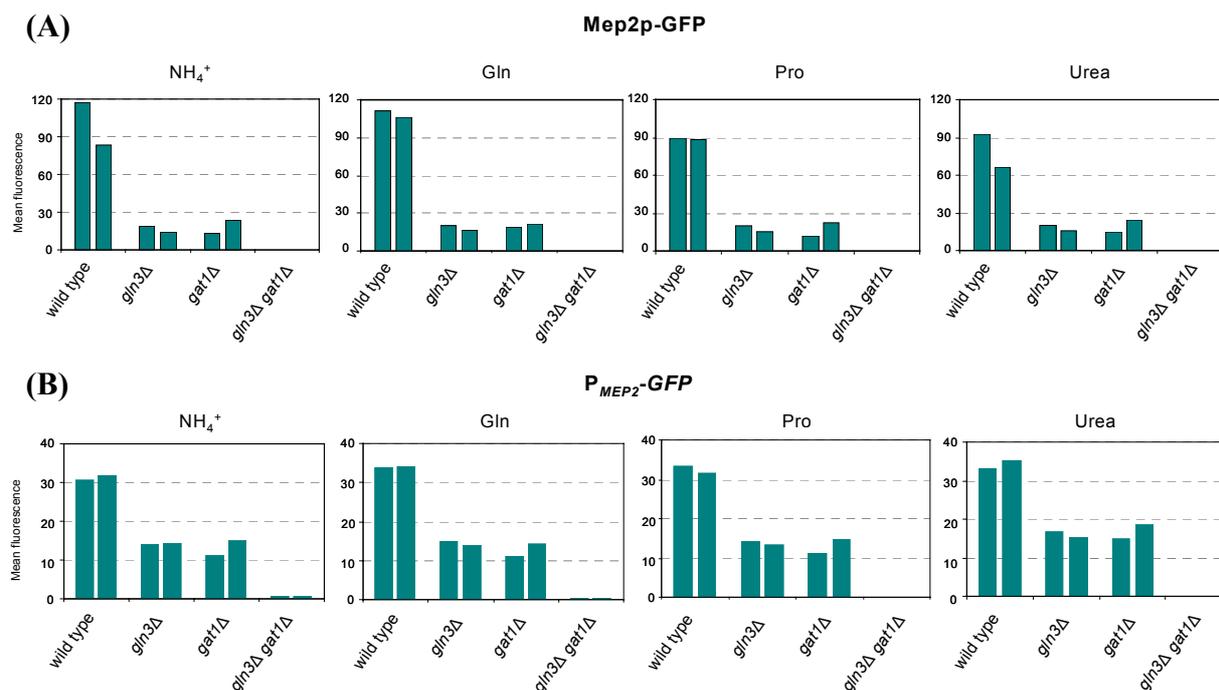
Fig. 21. (A) Expression of GFP-tagged Mep2p and **(B)** P_{MEP2}-*GFP* in the wild type, *gln3Δ* and *gat1Δ* single mutants, and *gln3Δ gat1Δ* double mutants in liquid media containing limiting concentrations (100 μM) of the indicated nitrogen sources. Overnight cultures of the reporter strains in SD-Pro medium were diluted 50-fold in the test media and grown for 6 h at 30°C. Fluorescence of the cells was measured by flow cytometry Strain SC5314, which does not carry *GFP*, was used as a negative control and the background fluorescence values of this strain (between 1.6 and 2.45 in the various experimental media) were subtracted from those of the reporter strains. The first columns show the results obtained with the A series and the second columns show the results obtained with the B series of the reporter strains (descriptions of strains are provided in Table 2). Note that the scale of the *y*-axis is different for Mep2p-GFP and P_{MEP2}-GFP.

To confirm that GATA factors mediate *MEP2* induction under nitrogen limitation, *MEP2* mRNA levels were compared in the wild-type and GATA factor mutant backgrounds by Northern hybridization. As can be seen in Fig. 22, the result is identical to those obtained with GFP quantification experiments. The *MEP2* transcript is absent from the *gln3Δ gat1Δ* double mutants, the faint band of slightly higher molecular weight seen in these and all other strains represents a cross-hybridizing transcript. In summary, it can be stated that analogous to *S. cerevisiae*, expression of *MEP2* in *C. albicans* is controlled by both GATA transcription factors Gln3p and Gat1p.
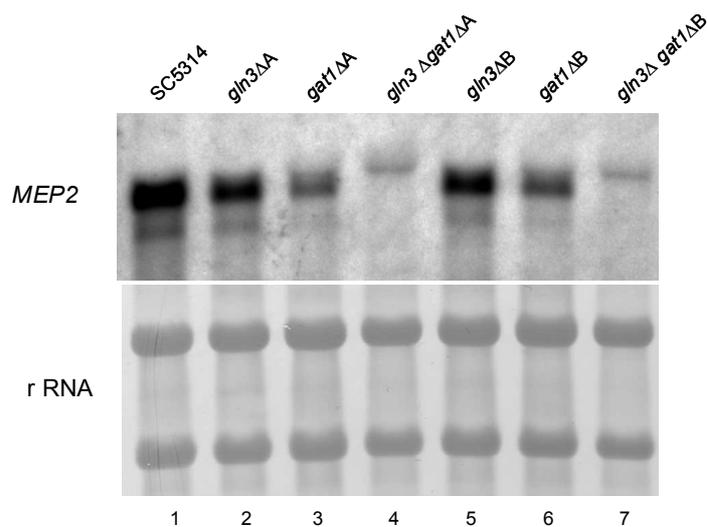
Fig. 22. Detection of *MEP2* mRNA by Northern hybridization with a *MEP2*-specific probe. Overnight cultures of the wild-type strain SC5314 (lane 1), the *gln3*Δ mutants GLN3M4A (lane 2) and GLN3M4B (lane 5), the *gat1*Δ single mutants GAT1M4A (lane 3) and GAT1M4B (lane 6), and the *gln3*Δ *gat1*Δ double mutants Δ*gln3*GAT1M4A (lane 4) and Δ*gln3*GAT1M4B (lane 7) in SD-Pro medium were diluted 50-fold in liquid SLAD medium and RNA was isolated after 6 h of growth at 30°C.

4.2.6 Ammonium permease *MEP1* expression is also regulated by GATA factors

To test whether the GATA transcription factors also regulate expression of the ammonium permease *MEP1*, reporter strains were generated in wild-type and GATA factor mutant backgrounds in a manner analogous to the Mep2p reporter strain constructions. Using the fragments from plasmids pMEP1G4 or pMEP1PG1, the GFP-tagged Mep1p or P_{MEP1}-GFP fusions were introduced into either of the two *MEP1* alleles. For monitoring expression levels of Mep1p-GFP as well as promoter activity of *MEP1*, the reporter strains were grown in liquid media containing limiting amounts (100 μM) of ammonium, glutamine, proline, or urea and fluorescence of the cells was measured by flow cytometry. In agreement with results from previous study (Biswas & Morschhäuser, 2005), Mep1p was found to be expressed at much lower levels than Mep2p in the wild-type background (compare mean fluorescence values of Fig. 21 with those of Fig. 23). As shown in Fig. 23, Mep1p expression was slightly reduced in the *gln3*Δ mutants in comparison to wild-type. In contrast, *MEP1* expression was elevated in the *gat1*Δ mutants. Only very low levels of Mep1p expression were observed in the *gln3*Δ *gat1*Δ double mutants. Therefore, both Gln3p and Gat1p also regulate *MEP1* expression.

Mep1p-GFP
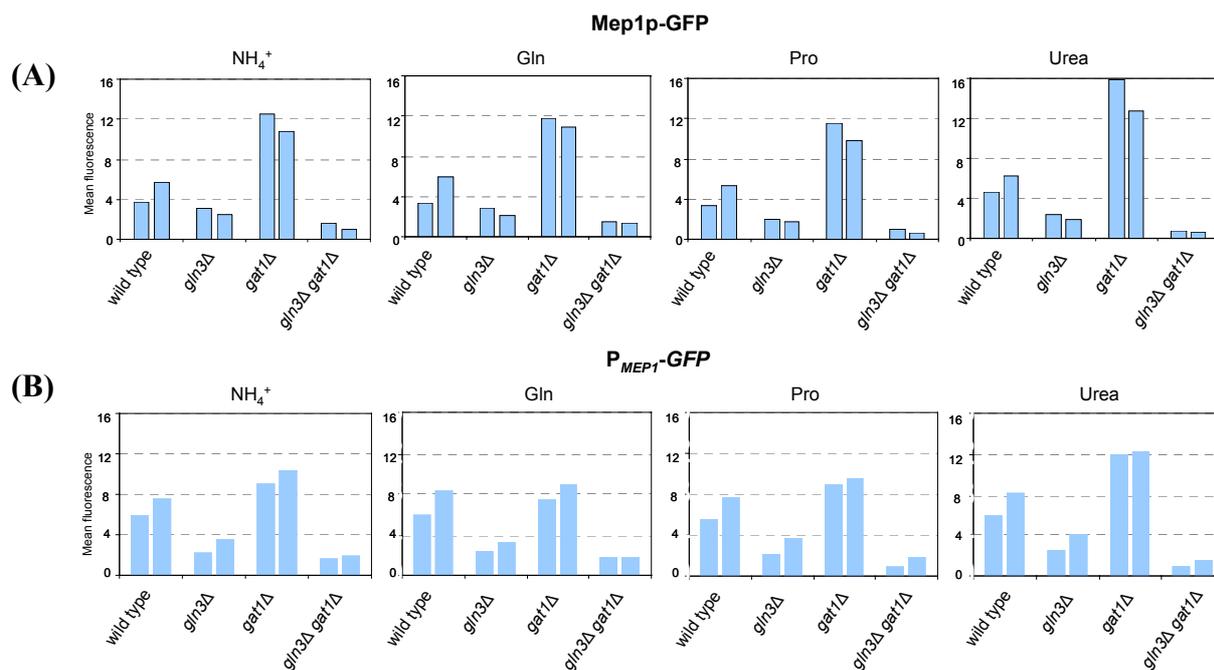
P_{MEP1}-GFP

Fig. 23. (A) Expression of GFP-tagged Mep1p and **(B)** P*MEP1*-*GFP* in the wild type, *gln3Δ* and *gat1Δ* single mutants, and *gln3Δ gat1Δ* double mutants in liquid media containing limiting concentrations (100 μM) of the indicated nitrogen sources. Overnight cultures of the reporter strains in SD-Pro medium were diluted 50-fold in the test media and grown for 6 h at 30°C. Fluorescence of the cells was measured by flow cytometry. Strain SC5314, which does not carry *GFP*, was used as a negative control and the background fluorescence values of this strain (between 1.6 and 3.72 in the various experimental media) were subtracted from those of the reporter strains. The first columns show the results obtained with the A series and the second columns show the results obtained with the B series of the reporter strains (descriptions of strains are provided in Table 2).

4.2.7   Gln3p regulates nitrogen starvation-induced filamentous growth in *C. albicans*

Growth of the GATA factor mutant strains was tested on agar plates containing limiting concentrations of different nitrogen sources (Fig. 24). All mutants grew as well as the wild-type strain SC5314 on SLAD plates, indicating that the reduced Mep1p expression levels in the *gln3Δ gat1Δ* double mutants still allowed sufficient ammonium uptake for normal growth. However, the *gln3Δ* mutants showed delayed and strongly reduced filamentation on SLAD plates as well as on plates containing 100 μM urea, proline, glutamate or glutamine as the sole nitrogen source, conditions in which *MEP2* is required for filamentous growth (Biswas & Morschhäuser, 2005). While wild-type colonies started to produce filaments after about 3 days of incubation, no filamentous colonies of *gln3Δ* mutants were seen at day 4, and fewer and shorter filaments than in the wild type were observed after 6 days (Fig. 24 and data not shown). On the other hand, deletion of *GAT1* did not affect filamentation on these plates and *gln3Δ gat1Δ* double mutants behaved like the *gln3Δ* single mutants, except on urea plates on which no filamentation was observed in the double mutants. Reintroduction of *GLN3* into the

gln3Δ single and the *gln3Δ gat1Δ* double mutants restored filamentation to wild-type levels, confirming that the filamentous growth defect of the mutants was due to *GLN3* inactivation. No filamentation defect of the mutants was observed on plates containing serum, indicating that *GLN3* is specifically required for normal filamentous growth in response to nitrogen limitation.
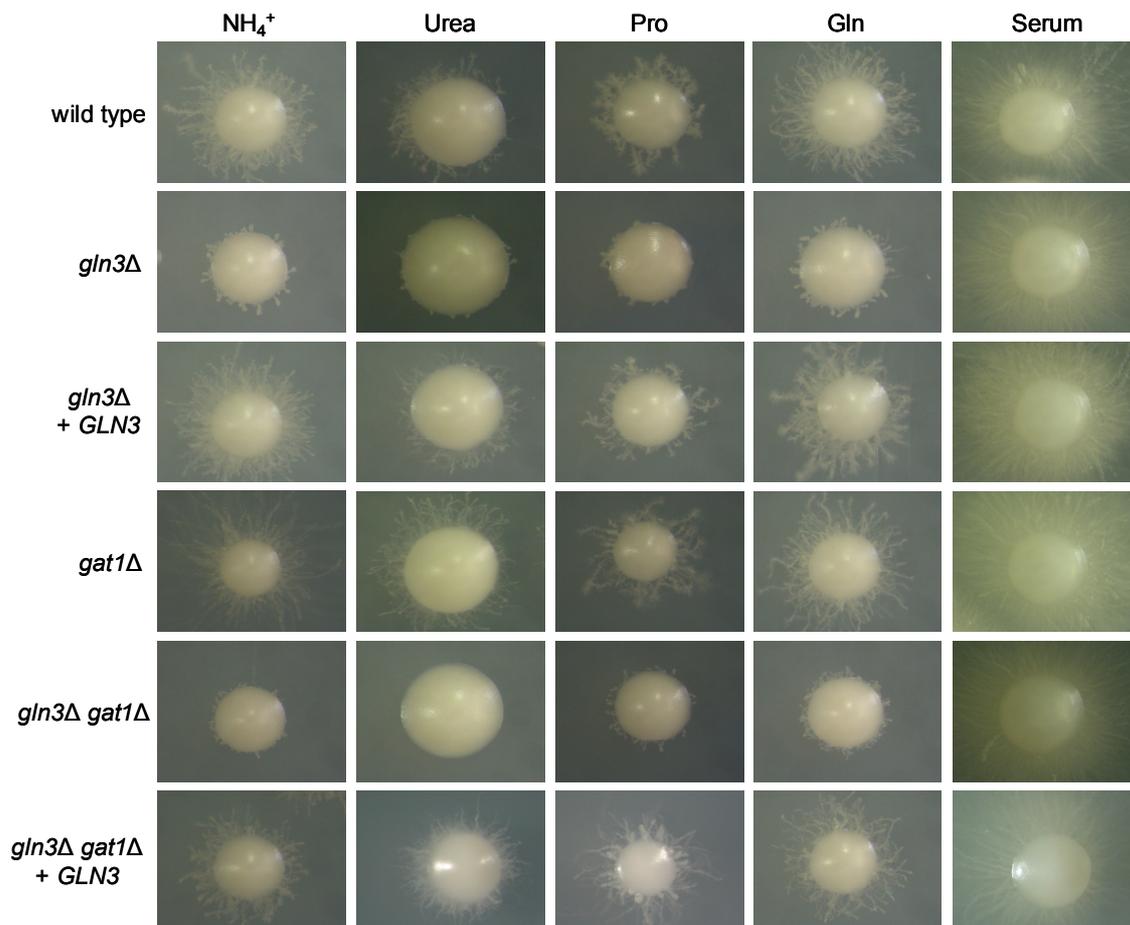

Fig. 24. YPD precultures of the strains were appropriately diluted and spread on SD agar plates containing the indicated nitrogen sources at a concentration of 100 μM or on agar plates containing serum as an inducer of filamentous growth. Individual colonies were photographed after 6 days of incubation at 37°C. The following strains were used: SC5314 (wild type), GLN3M4A/B (*gln3Δ*), GLN3MK2A/B (*gln3Δ + GLN3*), GAT1M4A/B (*gat1Δ*), Δgln3GAT1M4A/B (*gln3Δ gat1Δ*), Δgln3GAT1MK2A/B (*gln3Δ gat1Δ + GLN3*). Independently constructed mutants and complemented strains behaved identically and only one of them is shown in each case.

To investigate whether the filamentation phenotype of the mutants correlated with Mep2p expression on solid media, expression of the GFP-tagged Mep2p in the corresponding reporter strains on SLAD plates was inspected microscopically. As in liquid SLAD medium, Mep2p expression was strongly reduced in both *gln3Δ* and *gat1Δ* single mutants and was

undetectable in the *gln3Δ gat1Δ* double mutants also on filamentation inducing solid medium (Fig. 25). Therefore, the reduced *MEP2* expression in strains lacking *GLN3* correlated with their filamentation defect. In contrast, deletion of *GAT1* did not affect filamentous growth under these conditions, despite the fact that *MEP2* expression was similarly reduced in *gln3Δ* and *gat1Δ* mutants.
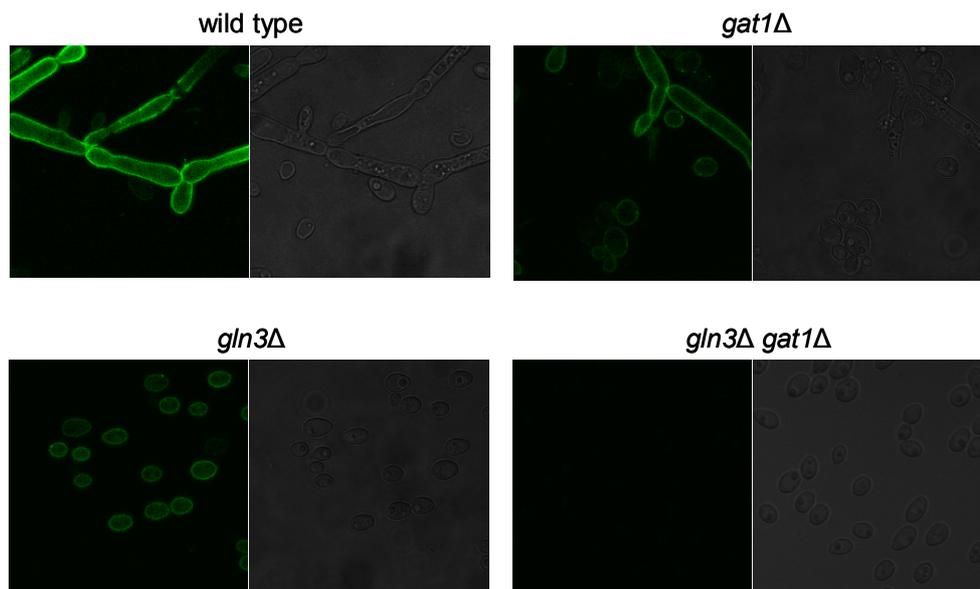

Fig. 25. Expression of the GFP-tagged Mep2p in wild-type (strains SCMEP2G7A/B), *gat1Δ* (strains *gat1Δ*MEP2G7A/B), *gln3Δ* (strains *gln3Δ*MEP2G7A/B), and *gln3Δgat1Δ* mutant (strains *gln3Δ gat1Δ* MEP2 G7A/B) backgrounds on SLAD plates. The pictures show fluorescence and corresponding phase contrast micrographs of cells taken from colonies of the reporter strains grown for 6 days at 37°C.

4.2.8 Inactivation of *GAT1* activates *MEP2*-independent filamentation pathways

The ability of the *gat1Δ* mutants to filament under limiting nitrogen conditions despite strongly reduced Mep2p expression levels suggested that nitrogen starvation-induced filamentous growth may not depend on the Mep2p in the prototrophic wild-type strain SC5314, in contrast to its auxotrophic derivative CAI4, which was the parent of *mep2Δ* mutants in the previous study (Biswas & Morschhäuser, 2005). To exclude the possibility that inappropriate *URA3* expression levels at the *MEP2* locus were responsible for the filamentation defect of the *mep2Δ* mutants, revertant strains were constructed, in which the *URA3* gene was inserted back at its original locus in the two independently constructed *mep2Δ* mutants (strains MEP2M4A/B) as well as in the parental strain CAI4, using a 4.8 kb

BglII-PstI fragment from pUR3 (Kelly *et al.*, 1987). The resulting revertant strains of *mep2*Δ mutants, MEP2M4R1A and B exhibited the same filamentation defect as the previously constructed *mep2*Δ mutants, whereas the wild-type control strains CAI4R1A and B showed normal filamentation (Fig. 26A). A further test for the requirement of Mep2p for nitrogen starvation-induced filamentous growth in *C. albicans* was done using strains in which *MEP2* was deleted in the prototrophic wild-type strain SC5314 with the help of the *SAT1* flipping strategy. For this purpose, plasmid pMEP2M5 was generated in which the *caSAT1* selection marker was substituted for the *URA3* marker in the pMEP2M2 (Biswas & Morschhäuser, 2005). To obtain pMEP2M5, an EcoRI-PstI [3'*caFLP*-T$_{ACT1}$-*caSAT1*] fragment from pGLN3M2 was ligated between the same sites of plasmid pMEP2M2 (Biswas & Morschhäuser, 2005). Two independently generated *mep2*Δ mutants (data not shown) exhibited the same filamentous growth defect on SLAD plates as the *mep2*Δ mutants which were used in the earlier studies (Fig. 26B, top panels). These results confirmed that Mep2p controls nitrogen starvation-induced filamentous growth in *C. albicans*.


(A) wild type   *mep2*Δ

(B) wild type   *mep2*Δ
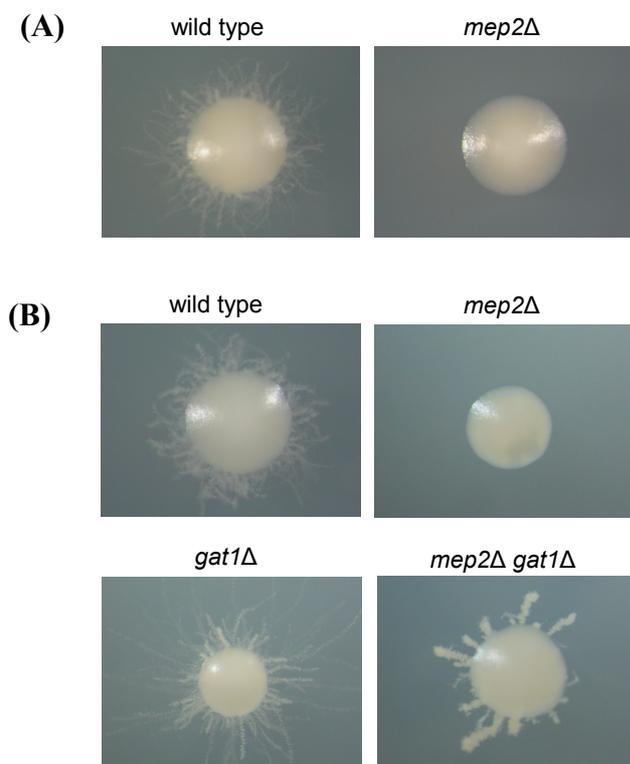
gat1Δ   *mep2*Δ *gat1*Δ

Fig. 26. (A) Filamentation of *URA3* revertant strains. The following strains were used: CAI4RU1A/B (wild type), MEP2M4RU1A/B (*mep2*Δ). **(B)** Filamentation phenotype of *mep2*Δ *gat1*Δ double mutants was compared to that of *mep2*Δ and *gat1*Δ single mutants. The following strains were used: SC5314 (wild type), SCMEP2M4A/B (*mep2*Δ), GAT1M4 A/B (*gat1*Δ), and Δ*mep2*GAT1M4A/B (*mep2*Δ *gat1*Δ). In both experiments A and B, YPD precultures of the strains were appropriately diluted and spread on SLAD agar plates. Individual colonies were photographed after 6 days of incubation at 37°C. Independently constructed mutants behaved identically and only one of them is shown in each case.

It was hypothesized that the absence of a functional Gat1p might result in the activation of filamentation inducing signaling pathways that do not require high *MEP2* expression levels or are even independent of Mep2p. To explore this possibility, *GAT1* was

deleted in the two *mep2Δ* mutants constructed from strain SC5314. Strikingly, both independently generated *mep2Δ gat1Δ* double mutants regained the ability to filament under nitrogen starvation conditions, albeit not to wild-type levels (Fig. 26B, lower panels). Therefore, the ability of the *gat1Δ* mutants to filament normally despite strongly reduced Mep2p expression levels can be explained by the activation of additional signaling pathways, which can induce filamentation in response to nitrogen starvation even in the absence of Mep2p (see discussion).

4.2.9   Forced *MEP2* expression bypasses the requirement of *GLN3* for filamentous growth

If the failure to express *MEP2* at appropriate levels was the cause of the filamentous growth defect of the *gln3Δ* mutants, then forced expression of *MEP2* from a different promoter should restore normal filamentation. To test this hypothesis, *MEP2* under control of the *ADH1* promoter was expressed in the wild-type strain SC5314 and in the *gln3Δ* mutants using the relevant insert from pMEP2E4 (Fig. 27). For generation of pMEP2E4, an XhoI-BglII [*MEP2* coding region] fragment from pMEP2K2 (Biswas & Morschhäuser, 2005) was ligated between the same sites of plasmid pADH1E1 (Reuß & Morschhäuser, 2006). In addition, the hyperactive $MEP2^{\Delta C440}$ allele, which is strongly overexpressed presumably due to enhanced transcript stability (Biswas & Morschhäuser, 2005), was also expressed from the *ADH1* promoter. For this, plasmid pMEP2ΔC2E2 was generated in analogous fashion, an XhoI-BglII [$MEP2^{\Delta C440}$ allele] fragment from pMEP2ΔC2K2 was cloned into XhoI/BglII-digested pADH1E1. As a control, the *GFP* gene was integrated at the *ADH1* locus instead of *MEP2* in an identical fashion by using the plasmid pADH1G4 (Park and Morschhäuser, unpublished).
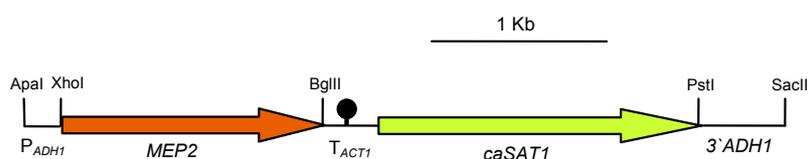

Fig. 27. Structure of the DNA fragment from plasmid pMEP2E4 which was used to integrate *MEP2* allele at the *ADH1* locus. The *MEP2* coding region is represented by the red arrow, T_{ACT1} by the filled circle, and the *caSAT1* selection marker by the light green arrow. *ADH1* upstream and downstream regions are represented by the solid lines. Relevant restriction sites are indicated.

Expression of an additional *MEP2* copy from the *ADH1* promoter had no detectable effect on filamentation of the wild-type strain SC5314 (Fig. 28), but expression of the hyperactive *MEP2*$^{\Delta C440}$ allele from the *ADH1* promoter in the same strain resulted in a hyperfilamentous phenotype, as was previously reported for strain CAI4 expressing the *MEP2*$^{\Delta C440}$ allele from the native *MEP2* promoter (Biswas & Morschhäuser, 2005). Forced expression of *MEP2* from the *ADH1* promoter partially rescued the filamentation defect of the *gln3*Δ mutants and expression of the hyperactive *MEP2*$^{\Delta C440}$ allele resulted in the same hyperfilamentous phenotype as in the wild-type background (Fig. 28). Therefore, forced expression of *MEP2* overcomes the filamentous growth defect caused by *GLN3* inactivation.
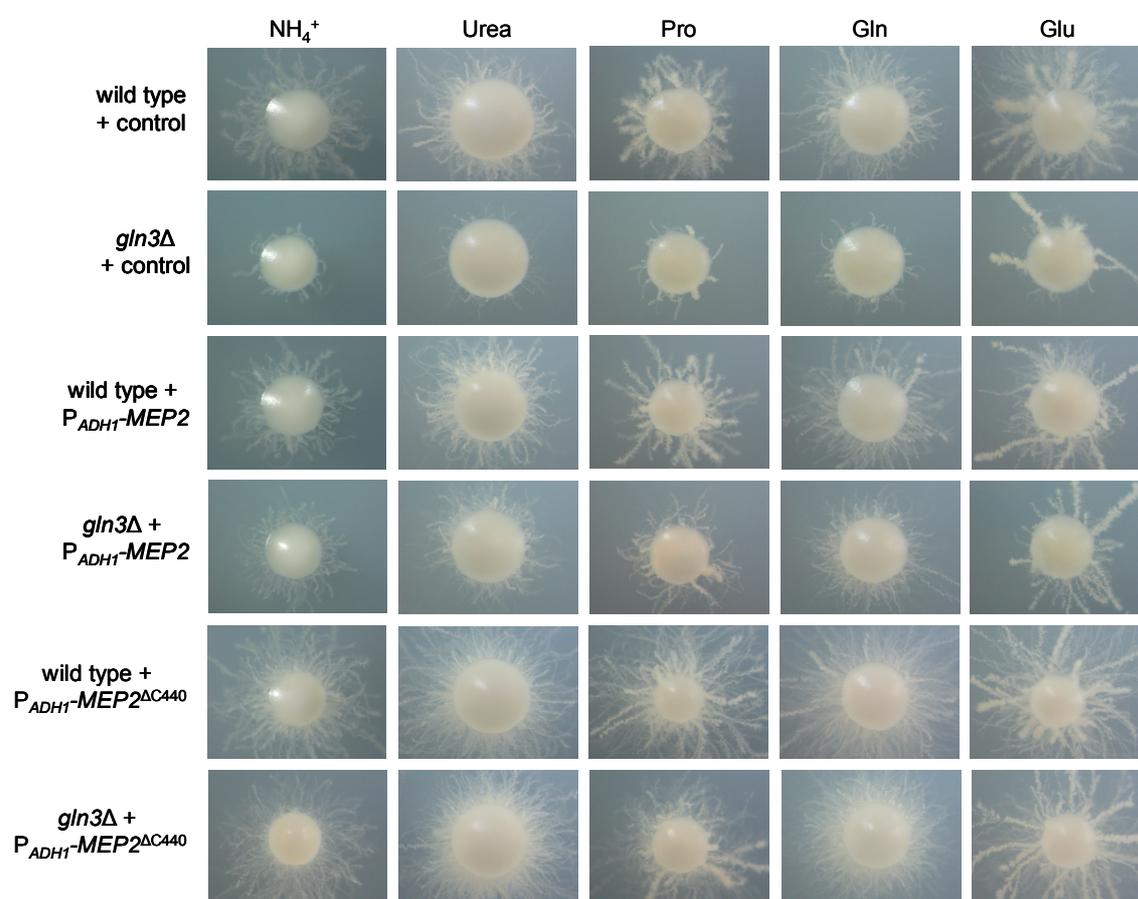

Fig. 28. Forced expression of *MEP2* overcomes the filamentous growth defect of *gln3*Δ mutants. YPD precultures of the strains were appropriately diluted and spread on SD agar plates containing the indicated nitrogen sources at a concentration of 100 μM. Individual colonies were photographed after 6 days of incubation at 37°C. The following strains were used: SCADH1G4A/B (wild type + control), Δ*gln3*ADH1G4A/B (*gln3*Δ + control), SCMEP2E4A/B (wild type + P$_{ADH1}$-*MEP2*), Δ*gln3*MEP2E4A/B (*gln3*Δ + P$_{ADH1}$-*MEP2*), SCMEP2ΔC2E2A/B (wild type + P$_{ADH1}$-*MEP2*$^{\Delta C440}$), Δ*gln3*MEP2ΔC2E2A/B (*gln3*Δ + P$_{ADH1}$-*MEP2*$^{\Delta C440}$). Independently constructed mutants and complemented strains behaved identically and only one of them is shown in each case.

4.3 Secreted aspartic protease expression in *C. albicans* is controlled by a transcription factor regulatory cascade

C. albicans can use proteins as the sole source of nitrogen for growth. The secreted aspartic protease Sap2p degrades proteins mainly to oligopeptides, which are then taken up into the cell by oligopeptide transporters encoded by the *OPT* gene family (Reuß & Morschhäuser, 2006). Deletion of *SAP2* or several members of the *OPT* gene family renders *C. albicans* unable to grow in YCB-BSA medium, in which a protein, bovine serum albumin (BSA), is the only available nitrogen source (Hube *et al.*, 1997; Reuß & Morschhäuser, 2006; Staib *et al.*, 2002; 2008).

4.3.1 The GATA transcription factors Gln3p and Gat1p are required for growth of *C. albicans* on proteins

Proteins can be considered as secondary nitrogen source for *C. albicans*, as the expression of genes required for their utilization, i.e., *SAP2* and the oligopeptide transporters *OPT1* and *OPT3*, is induced in the presence of proteins and *SAP2* is repressed even in the presence of proteins when preferred nitrogen sources, like ammonium or amino acids, are available (Banerjee *et al.*, 1991; Hube *et al.*, 1994; Reuß & Morschhäuser, 2006; Ross *et al.*, 1990). In fungi, GATA transcription factors are known to regulate the expression of genes required to utilize alternative or secondary nitrogen sources. Hence, the growth behaviors of *C. albicans* *gln3Δ*, *gat1Δ*, and *gln3Δ gat1Δ* mutant strains were analyzed in YCB-BSA medium which contains BSA as the only available nitrogen source. Growth of the *gln3Δ* mutants was slightly reduced as compared with the wild-type parental strain SC5314 (Fig. 29A). In contrast, the *gat1Δ* mutants had a severe growth defect (Fig. 29B) and growth of the *gln3Δ gat1Δ* double mutants was virtually abolished (Fig. 29C). Complementation of the *gln3Δ* and *gat1Δ* single mutants with the respective functional alleles restored wild-type growth (Fig. 29 A, B). The double mutants complemented with *GLN3* behaved like *gat1Δ* single mutants (compare Fig. 29 B and C). Reintroduction of *GAT1* into the double mutants also restored growth (Fig. 29C). However, a *GAT1* copy number effect was observed in the absence of *GLN3*, because the double mutants in which one *GAT1* allele was reintroduced grew less well than the *gln3Δ* single mutants, which contained both *GAT1* alleles (compare Fig. 29 A and C). The same effect was observed in the *gln3Δ* mutants in which one of the *GAT1* wild-type alleles had been deleted (data not shown). These results demonstrated that both GATA factors, but especially *GAT1*, are required to efficiently utilize proteins as a nitrogen source.
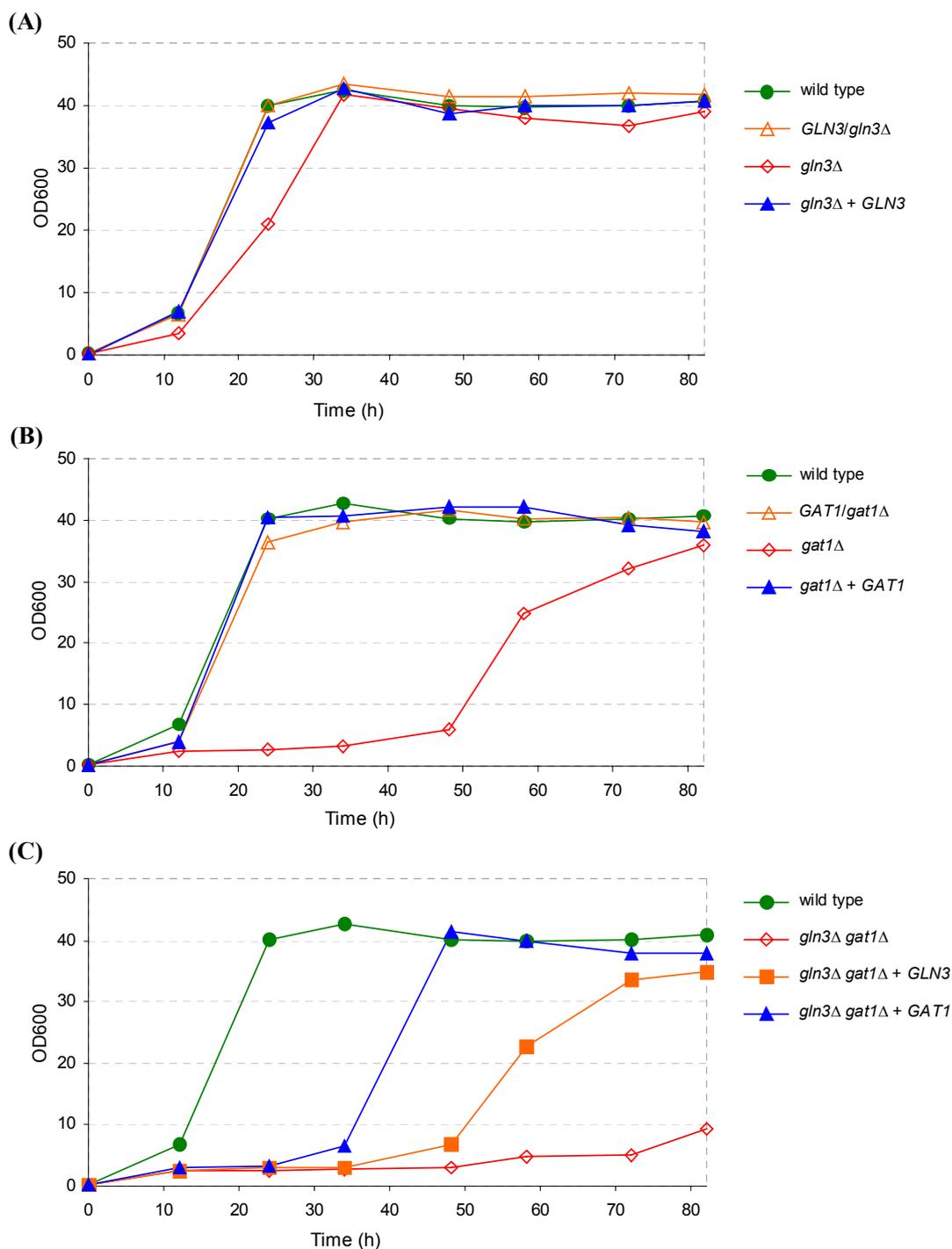
Fig. 29. *GAT1* and *GLN3* are required for utilization of proteins as a nitrogen source. Overnight cultures of the strains in YPD medium were diluted 10^{-2} in YCB-BSA medium and incubated at 30°C. Growth was monitored by measuring the OD600 (optical density at 600 nm) of the cultures at the indicated times. All strains were tested in parallel, but the results for the *gln3*Δ mutants, *gat1*Δ mutants, and *gln3*Δ *gat1*Δ double mutants are shown separately in panels A-C for better illustration, together with the results for the corresponding complemented strains. The results for the wild-type strain SC5314 are shown in all three panels. Besides wild type following strains were used: **(A)** GLN3M2A/B (*GLN3/gln3*Δ), GLN3M4A/B (*gln3*Δ), GLN3MK2A/B (*gln3*Δ+*GLN3*). **(B)** GAT1M2A/B (*GAT1/gat1*Δ), GAT1M4A/B (*gat1*Δ), GAT1M K2A/B (*gat1*Δ+*GAT1*). **(C)** Δ*gln3*GAT1M4/B (Δ*gln3*Δ*gat1*), Δ*gln3*GAT1MK2A/B (Δ*gln3*Δ*gat1*+ *GLN3*), Δ*gln3*GAT1MK4A/B (Δ*gln3*Δ*gat1*+*GAT1*). The two independently constructed series of mutants and complemented strains behaved identically and only one of them is shown for clarity.

99

4.3.2   Functional analysis of the *GAT1* gene

In assembly 19 of the *C. albicans* genome sequence, the open reading frame defined as *GAT1* (orf19.1275) is 2067 bp in length and encodes a predicted protein of 688 amino acids. However, Limjindaporn *et al*. obtained a plasmid clone from a genomic library of strain SC5314, the strain used for genome sequencing, in which an upstream stop codon was changed to a sense codon, extending the N-terminal part of the *GAT1* ORF by 201 bp to encode a predicted protein of 755 amino acids (Limjindaporn *et al.*, 2003). When *GAT1* was amplified in this work from strain SC5314 for reintroduction into the *gat*Δ single and *gln3*Δ *gat1*Δ double mutants, it was found that the cloned *GAT1* gene in the resulting plasmid pGAT1K1 (Fig. 16B) contained the stop codon reported in the genome sequence. Since the *GAT1* alleles of strain SC5314 can be distinguished by a downstream BglII restriction site polymorphism, the remaining intact *GAT1* allele was amplified from the heterozygous *GAT1*/*gat1*Δ mutants GAT1M2A and GAT1M2B, in which either of the two alleles was inactivated (Fig. 17, lanes 2 and 3). Sequencing of the resulting clones confirmed that strain SC5314 contained two polymorphic *GAT1* alleles. The allele located on the smaller BglII fragment, which was arbitrarily designated as allele 1, was identical to the one reported by Limjindaporn *et al*. (2003), whereas allele 2 was identical to orf19.1275 of the *C. albicans* genome sequence. As both heterozygous *gat1* mutants grew as well as the wild-type strain in YCB-BSA medium and the growth defect of the homozygous *gat1*Δ mutants was fully complemented by the *GAT1-2* allele containing the upstream stop codon (Fig. 29B), both *GAT1* alleles must be functional.

Recently, a different start codon located 63 bp further downstream has been assigned to orf19.1275 in assemblies 20 and 21 of the *C. albicans* genome sequence. Now this *GAT1* ORF would be only 2004 bp and would encode a predicted protein of 667 amino acids. In order to investigate which of the start codons are included in the *GAT1* transcript 5' RACE was performed. This analysis revealed that the *GAT1* mRNA starts at an adenine that is located 60 nucleotides upstream of the first start codon and therefore includes all three potential *GAT1* ORFs. To explore if all three potential *GAT1* ORFs, which are referred from here onwards as $GAT1^{2268}$, $GAT1^{2067}$, and $GAT1^{2004}$ according to their length, encode functional proteins, these different ORFs were expressed from a tetracycline-inducible (Tet) promoter in the *gat1*Δ mutants. Construction of plasmids is documented in Table 6 and structure of the relevant DNA fragments used for transformation of *gat1*Δ mutants is depicted in Fig. 30. Growth of the corresponding transformants was monitored in YCB-BSA medium.

Table 6 Construction of plasmids in which different *GAT1* ORFs are expressed from the tetracycline-inducible promoter. Introduced restriction sites and their positions are mentioned. These nucleotide positions are with respect to that start codon of *GAT* as reported by Limjindaporn *et al.*, (2003).

| Primers and template DNA	Restriction sites	Plasmid construction	Plasmid	Transformants
GAT1-3 GAT1-2 (GAT1M2B)	SalI at –7 BamHI at +2268	SalI/SmaI and SmaI/BamHI digested PCR fragments cloned into SalI /BglII digested pNIM1	pTET-GAT1-1	Δ*gat1*TET1 GAT1-1A/B
GAT1-1 GAT1-2 (GAT1M2A)	SalI at +195 BamHI at +2268	SalI/SmaI and SmaI/BamHI digested PCR fragments cloned into SalI /BglII digested pNIM1	pTET-GAT1-2	Δ*gat1*TET1 GAT1-2A/B
GAT1-4 GAT1-2 (GAT1M2A)	SalI at +258 BamHI at +2268	SalI/SmaI and SmaI/BamHI digested PCR fragments cloned into SalI /Bgl II digested pNIM1	pTET-GAT1-3	Δ*gat1*TET1 GAT1-3A/B
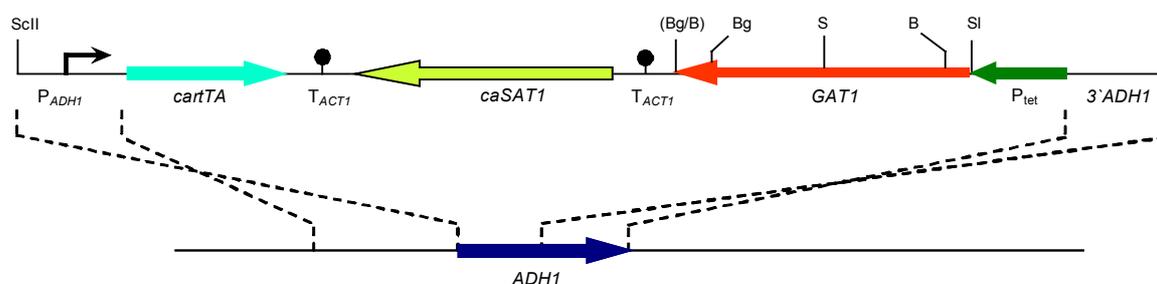
Fig. 30. Structure of the DNA cassettes that were used for expression of the *GAT1*^{2268}, *GAT1*^{2067}, and *GAT1*^{2004} alleles under control of a tetracycline-inducible promoter (P$_{tet}$) after integration into the *ADH1* locus of the *gat1*Δ mutants. Bent arrow symbolize promoter *ADH1* (P$_{ADH1}$), P$_{tet}$ is represented by the green arrow, the filled circles represent T$_{ACT1}$, which serves for proper transcription termination of the *Candida*-adapted, reverse tetracycline-dependent transactivator (*cartTA*) and the target genes in this cassette (Park & Morschhäuser, 2005). Only relevant restriction sites explaining plasmid construction or used to excise the whole cassette from the vector backbone are shown: A, ApaI; B, BamHI; Bg, BglII; S, SmaI; Sl, SalI; ScII, SacII.

As shown in Fig. 31, all three *GAT1* ORFs fully restored growth of the *gat1*Δ mutants. Therefore, even the short *GAT1*^{2004} allele seems to encode a functional protein. Recently, comparison of Gat1p orthologs among different fungal species revealed presence of a highly conserved sequence motif in the N-terminal of the protein (Wong *et al.*, 2008), and in *C. albicans* this motif would be encoded by 27 nucleotides upstream of the third start codon. Therefore, it is unlikely that the third start codon (*GAT1*^{2004}) is utilized during translation of Gat1p, which if used, would not include this conserved sequence in the protein. The N-terminal motif has been shown to be dispensable for function of AreA in *Aspergillus nidulans* (Caddick & Arst, 1998), which also seems to be the case for Gat1p (AreA homolog in *C. albicans*) and would explain restoration of growth of *gat1*Δ mutants by the *GAT1*^{2004} allele.
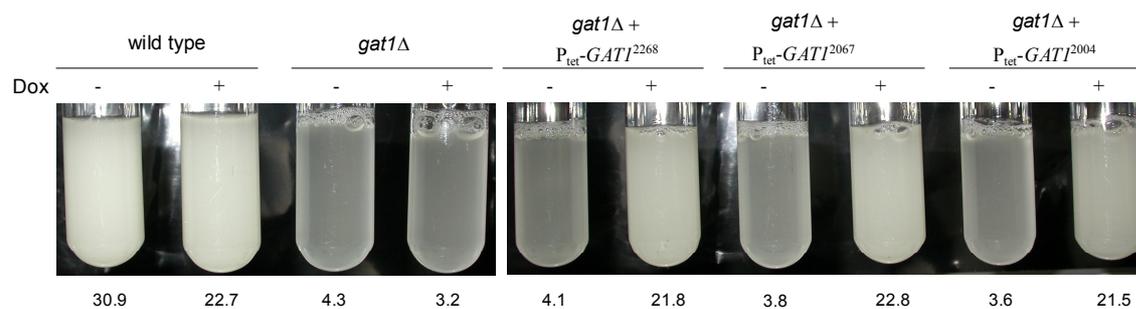
Fig. 31. Complementation of the growth defect of *gat1Δ* mutants by different *GAT1* alleles. Growth of the parental strain SC5314 (wild type), *gat1Δ* mutants, and transformants containing the indicated P$_{tet}$-*GAT1* fusions in YCB-BSA medium in the absence (-) or presence (+) of 50 μg/ml doxycycline. The cultures were photographed after 20 h of growth at 30ºC and their optical densities are given below the tubes. Note that doxycycline slightly reduced growth of the wild-type strain under these conditions.

4.3.3 *GLN3* and *GAT1* control expression of the secreted aspartic protease *SAP2* and oligopeptide transporters *OPT1* and *OPT3*

Secreted aspartic protease Sap2p and oligopeptide transporters are required by *C. albicans* for its ability to utilize proteins as a nitrogen source and expression of the *SAP2*, *OPT1*, and *OPT3* genes is induced in YCB-BSA medium (Hube *et al.*, 1994; Reuß & Morschhäuser, 2006; Staib *et al.*, 2002). To investigate whether expression of these genes depends on *GLN3* and *GAT1*, reporter gene fusions in which *GFP* was placed under the control of the respective promoters were introduced into the *gln3Δ, gat1Δ,* and *gln3Δ gat1Δ* mutants. These strains were grown in YCB-BSA-YE medium, which also induces *SAP2* and *OPT* gene expression but allows growth of *sap2Δ* and *optΔ* mutants (Reuß & Morschhäuser, 2006; Staib *et al.*, 2002), and the *gln3Δ gat1Δ* mutants also had no growth defect in this medium. The activity of all three tested promoters was reduced in the *gln3Δ* and *gatΔ* single mutants, with a pronounced effect in the *gat1Δ* mutants. Very low or undetectable promoter activity was observed in mutants lacking both transcription factors (Fig. 32).

The failure of the *gln3Δ gat1Δ* mutants to express *SAP2* was also confirmed by ana-lyzing culture supernatants of the wild type and mutants grown in YCB- BSA-YE on SDS-polyacrylamide gels (Fig. 33). The wild-type strain SC5314 had completely degraded the BSA within 8 h of growth in this medium and Sap2p expression was readily detected by Western immunoblotting with an anti-Sap2p antibody (Fig. 33, lanes 2). BSA degradation was delayed in the *gln3Δ* (Fig. 33, lanes 3 and 7) and *gat1Δ* (Fig. 33, lanes 4 and 8) mutants and reduced amounts of Sap2p were detected in the culture supernatant of the latter. However, the strong differences in *SAP2* promoter activity in the wild-type and *gln3Δ* and *gat1Δ*

8 h **15 h**
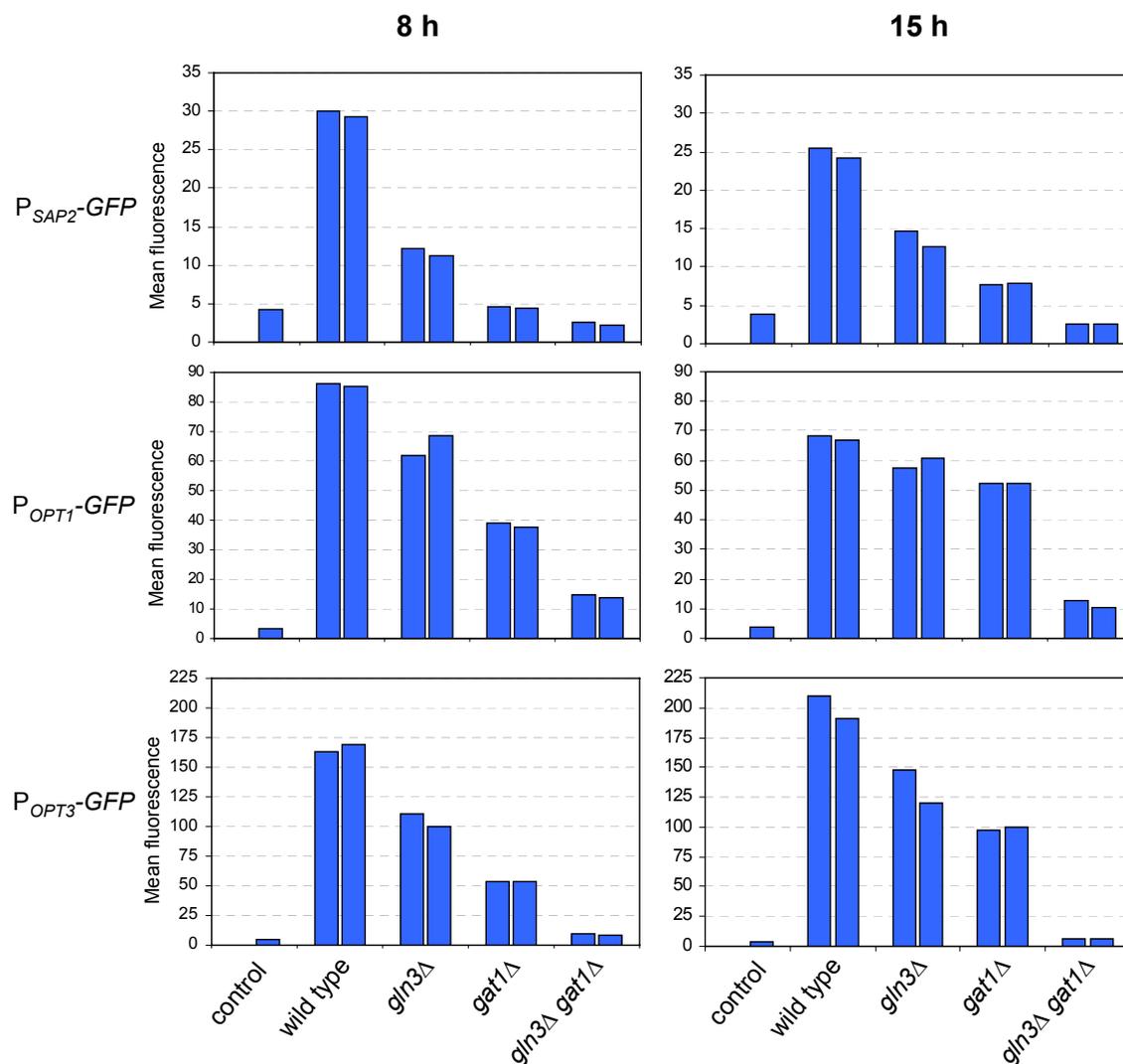
Fig. 32. The GATA transcription factors Gat1p and Gln3p control expression of *SAP2* and the oligopeptide transporters *OPT1* and *OPT3*. Overnight cultures of strains (description provided in Table 2) expressing *GFP* under control of the indicated promoter in a wild-type, *gln3Δ*, *gat1Δ*, or *gln3Δ gat1Δ* background in YPD medium were diluted 10^{-2} in YCB-BSA-YE medium, grown for 8 h (left panels) or 15 h (right panels) at 30°C and analyzed by flow cytometry. The mean fluorescence of each cell population is given (arbitrary units). The first columns show the results obtained with the A series and the second columns show the results obtained with the B series of the reporter strains. The parental strain SC5314, which does not contain *GFP*, was used as a negative control.

mutants (Fig. 32) were not reflected in Sap2p levels produced by these strains. It could be due to the fact that in all reporter strains the promoter activity of the *SAP2-1* allele was determined and most of the secreted Sap2p was probably produced from the more strongly expressed *SAP2-2* allele, which is essential for growth in YCB-BSA (Staib *et al.*, 2002). Only very limited BSA degradation was detected in the culture supernatants of the *gln3Δ gat1Δ* double mutants and Sap2p was not detectably expressed in these strains, which behaved similar to a *sap2Δ* mutant (compare lanes 5 and 9 with lane 6 in Fig. 33). These results demonstrate that the GATA transcription factors Gat1p and Gln3p control the expression of genes that are known to be required for growth of *C. albicans* on proteins.

103
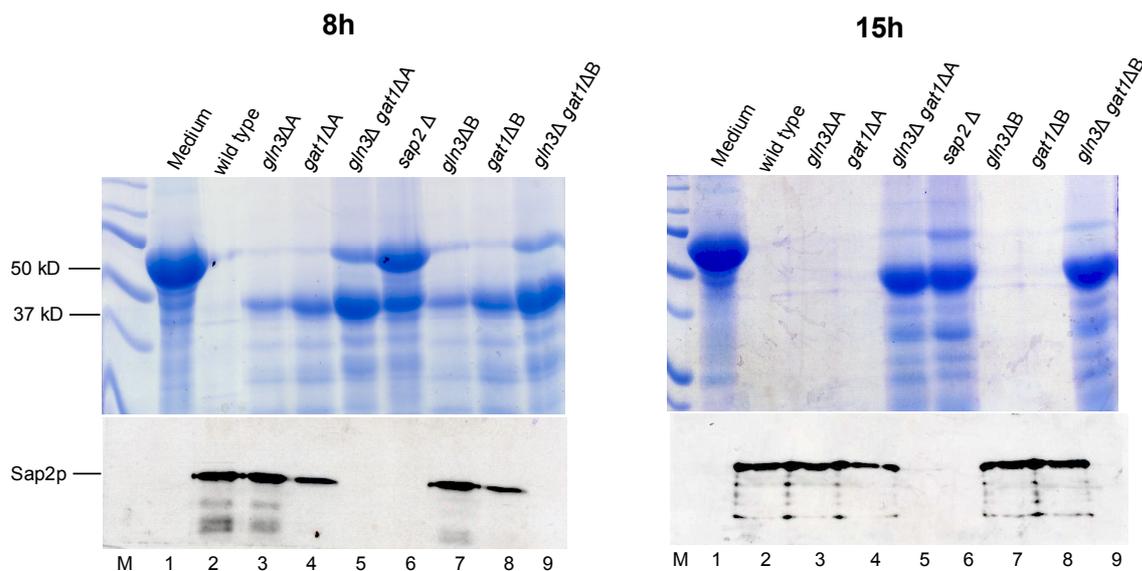
Fig. 33. Sap2p expression and BSA degradation by the wild-type strain SC5314 (lane 2) and the two independently constructed A and B series of *gln3*Δ (lanes 3 and 7), *gat1*Δ (lanes 4 and 8), and *gln3*Δ *gat1*Δ mutants (lanes 5 and 9). Strains were grown for 8 h (left) or 15 h (right) in YCB-BSA-YE medium at 30°C and the culture supernatants were analysed by SDS-PAGE (top) and by Western immunoblotting with an anti Sap2p-antibody (bottom). A *sap2*Δ mutant (lane 6) as well as uninoculated medium (lane1) were included as controls. M, molecular size marker.

4.3.4 Forced expression of *SAP2* overcomes the growth defect of *gln3*Δ *gat1*Δ mutants

To investigate if the growth defect of mutants lacking the GATA transcription factors Gln3p and Gat1p was caused by their inability to adequately express *SAP2* and oligopeptide trans-porters, *SAP2* and *OPT1* were expressed from the strong *ADH1* promoter in the *gln3*Δ *gat1*Δ mutants. Integration of *OPT1* and *SAP2* at the *ADH1* locus was achieved using relevant insert from plasmid pOPT1E1 (Reuß & Morschhäuser, 2006) and pSAP2ex7, respectively. For creating plasmid pSAP2ex7, a SalI-PstI [N-terminal part of *SAP2*] fragment and a PstI-BamHI [C-terminal part of *SAP2*] fragment from pSAP2ex1 (Staib & Morschhäuser, unpublished) were cloned into XhoI-BglII-digested pOPT4E1 (Reuß & Morschhäuser, 2006). Previous studies have shown that expression of *OPT1* from the *ADH1* promoter can rescue the growth defect in YCB-BSA of mutants lacking multiple oligopeptide transporters (Reuß & Morschhäuser, 2006). Growth of the *gln3*Δ *gat1*Δ double mutants expressing *SAP2* from the *ADH1* promoter was restored, though not to wild-type levels, contrastingly, expression of *OPT1* alone had no effect (Fig. 34). In summary, these results indicate that the failure to induce *SAP2* expression is the main reason for the inability of the *gln3*Δ *gat1*Δ mutants to utilize proteins as a nitrogen source.
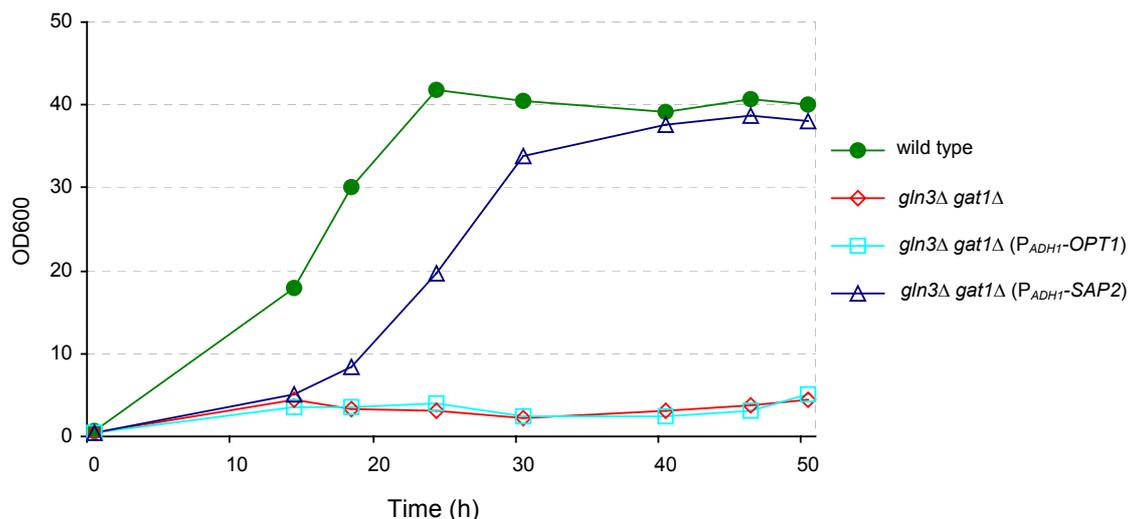
Fig. 34. Forced *SAP2* expression restores growth of *gln3Δ gat1Δ* double mutants in YCB-BSA. YPD overnight cultures of the wild-type strain SC5314, *gln3Δ gat1Δ* double mutants (strains Δ*gln3*GAT1M4A/B)*,* and transformants expressing *OPT1* (strains Δ*gln3*Δ*gat1*OPT1E1A/B) or *SAP2* (strains Δ*gln3*Δ*gat1*SAP2ex7A/B) from the *ADH1* promoter were diluted 10^{-2} in YCB-BSA and incubated at 30°C. Growth was monitored by measuring the optical density of the cultures at the indicated times. The two independently constructed series of mutants and transformants behaved identically and only one of them is shown.

4.3.5 Forced expression of the transcription factor *STP1* bypasses the requirement of GATA transcription factors for growth on proteins

The transcription factor Stp1p is known to regulate expression of *SAP2* and *OPT1* in response to the presence of micromolar concentrations of extracellular amino acids (Martinez & Ljungdahl, 2005). Under these conditions, Stp1p is proteolytically processed to its activated form and localizes to the nucleus to induce expression of its target genes. Mutants lacking *STP1* do not express *SAP2* and *OPT1* and can not utilize proteins as a nitrogen source. In order to investigate the relationship between Stp1p and the GATA transcription factors Gln3p and Gat1p, the ability of a constitutively active Stp1p that lacks the N-terminal inhibitory domain to restore growth of the *gln3Δ gat1Δ* mutants in YCB-BSA was tested. For this purpose, an *STP1*$^{\Delta N61}$ allele was integrated into the genome of the *gln3Δ gat1Δ* mutants under the control of the Tet promoter using the relevant insert from plasmid pTET1-STP1$^{\Delta N61}$. This plasmid was generated by amplifying the *STP1* ORF lacking codons 2-61 with the primers STP1-1 and STP1-2 and ligating the SalI/BglII-digested PCR product between the same sites in pNIM1 (see Fig. 30 for analogy). Doxycycline-induced expression of the *STP1*$^{\Delta N61}$ allele fully restored growth of the mutants (Fig. 35). In fact, the transformants expressing this allele started to grow even earlier than the wild-type strain SC5314 after transfer from YPD to

YCB-BSA medium. This could be due to the fact that expression of the activated transcription factor allowed a faster adaptation of the cells to the switch of the nitrogen source even in the absence of the GATA transcription factors. This result indicated that Gln3p and Gat1p might be regulating either *STP1* expression or Stp1p activation. These possibilities were investigated by monitoring growth phenotypes of *gln3Δ gat1Δ* transformants expressing wild-type *STP1* allele from the Tet promoter. For this, the *STP1* ORF was PCR-amplified with the primers STP1-4 and STP1-2, digested at the SalI site introduced before the start codon and at an internal SphI site, and cloned into the SalI/SphI-digested pTET1-STP1^{ΔN61} to generate pTET1-STP1, and the SacII-ApaI fragment from this plasmid was used to transform *gln3Δ gat1Δ* mutants. As shown in Fig. 35, doxycycline-induced expression of wild-type *STP1* also rescued the growth defect of the *gln3Δ gat1Δ* mutants, although growth of the strains was somewhat delayed in comparison with that of the wild-type strain SC5314. Therefore, forced expression of *STP1* bypasses the requirement of *GLN3* and *GAT1* for growth of *C. albicans* on proteins.
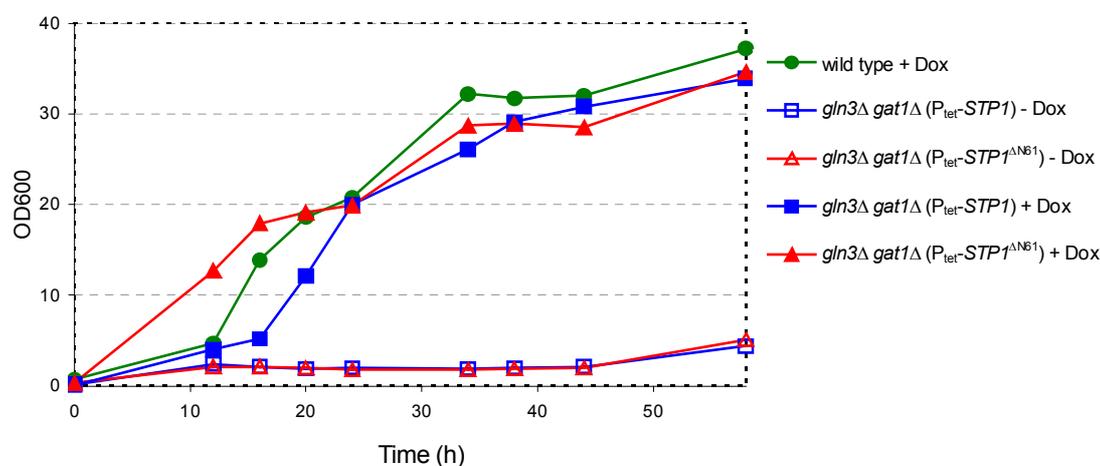

Fig. 35. Forced expression of *STP1* overcomes the growth defect of *gln3Δ gat1Δ* double mutant in YCB-BSA. YPD overnight cultures, of the wild-type strain SC5314 and transformants of the *gln3Δ gat1Δ* double mutants expressing full-length *STP1* (strains Δ*gln3*Δ*gat1*TET1-STP1A/B) or the constitutively active *STP1*^{ΔN61} allele (strains Δ*gln3*Δ*gat1*TET1-STP1^{ΔN61}A/B) from the Tet promoter, were diluted 10^{-2} in YCB-BSA in the absence or presence of 50 µg/ml doxycycline and incubated at 30°C. Growth was monitored by measuring the optical density of the cultures at the indicated times. The two independently constructed series of transformants behaved identically and only one of them is shown.

In order to examine if, vice versa, forced expression of *GLN3* or *GAT1* would also allow cells lacking Stp1p to grow on proteins, *STP1* null mutants were constructed from the wild-type strain SC5314 using the *SAT1* flipper strategy. To create an *STP1* deletion cassette, an ApaI-SalI fragment from pSTP1PG1 (described in next section) containing *STP1* upstream

sequences (from position -489 to -13) was substituted for the *GAT1* upstream fragment in the ApaI/XhoI-digested pGAT1M2 (Fig.16A) to result in pSTP1M1. A SacII-SacI *STP1* down-stream fragment (from position +1194 to +1585) was then amplified with the primers STP1-9 and STP1-8 and cloned between the same sites in pSTP1M1 to generate pSTP1M2. Using the ApaI-SacI fragment from pSTP1M2, sequential disruption of *STP1* alleles in the strain SC5314 was done (verified by Southern analysis, data not shown). *GLN3*, *GAT1*^{2268}, *GAT1*^{2067}, and *GAT1*^{2004} alleles were expressed from the Tet promoter (Table 6 and Fig. 30) in the *stp1Δ* mutants. For expression of *GLN3* from the Tet promoter plasmid pTET1-GLN3 was constructed in following manner: the *GLN3* coding region was amplified with the primers GLN10 and GLN11, and the PCR product digested at the introduced XhoI and BamHI sites and cloned in pBluescript to generate pGLN3. The XhoI-BamHI fragment from pGLN3 was then ligated between the SalI and BglII sites of pNIM1 to produce pTET1-GLN3.

In agreement with a previous report (Martinez & Ljungdahl, 2005), the *stp1Δ* mutants exhibited a growth defect in YCB-BSA medium, which was complemented by expression of *STP1* from the Tet promoter (Fig. 36). In contrast, tetracycline-induced expression of *GLN3* and *GAT1* did not rescue the growth defect of the *stp1Δ* mutants, indicating that the GATA transcription factors can not efficiently induce *SAP2* expression in the absence of Stp1p.
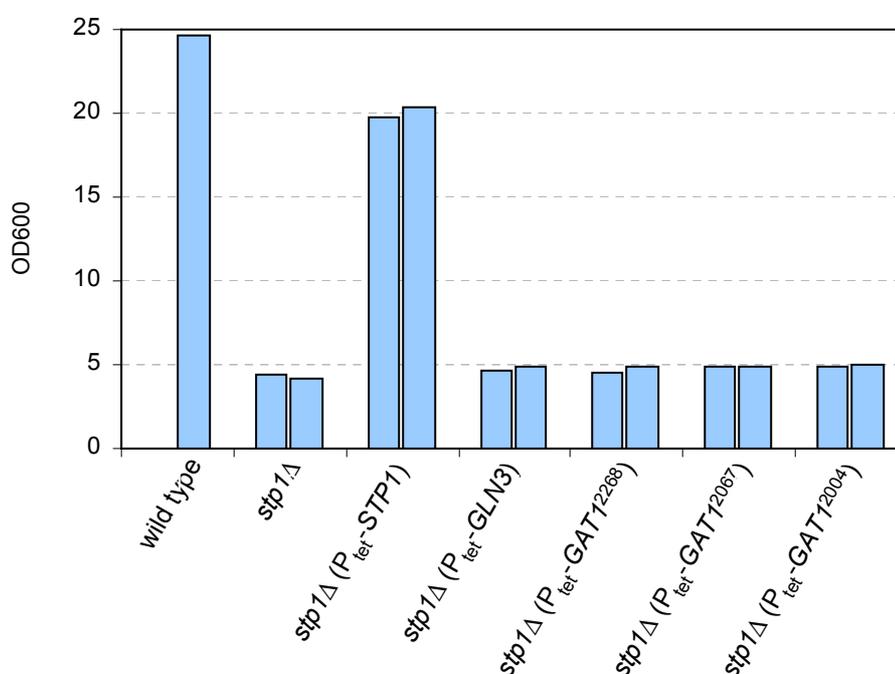
Fig. 36. Forced expression of *GLN3* and *GAT1* does not rescue the growth defect of *stp1Δ* mutants. YPD overnight cultures, of the wild-type strain SC5314, two independently generated *stp1Δ* mutants (strains STP1M4A/B), and transformants expressing *STP1* (strains Δ*stp1*TET-STP1A/B), *GLN3* (strains Δ*stp1*TET-GLN3A/B), ORF *GAT1*^{2268} (strains Δ*stp1*TET-GAT1-1A/B), ORF *GAT1*^{2067} (strains Δ*stp1*TET-GAT1-2A/B) or ORF *GAT1*^{2004} (strains Δ*stp1*TET-GAT1-3A/B), from the Tet promoter were diluted 10^{-2} in YCB-BSA containing 50 µg/ml doxycycline. The OD of the cultures was measured after 20 h of growth at 30°C.

4.3.6 Expression of the transcription factor *STP1* is controlled by Gln3p and Gat1p

The foregoing results suggest that Gln3p and Gat1p are required for the utilization of proteins because they regulate *STP1* expression, which in turn induces *SAP2*. To investigate whether *STP1* expression is regulated by the GATA transcription factors, *STP1* reporter fusion (P*STP1*-*GFP*) was introduced at the original *STP1* locus in the wild-type strain SC5314 and the *gln3Δ*, *gat1Δ*, and *gln3Δ gat1Δ* mutants using relevant insert from the plasmid pSTP1PG2. For generating this plasmid, the *STP1* upstream region was amplified with the primer pair STP1-5/STP1-6 and the PCR product was digested at the introduced ApaI/SalI sites and substituted for the *OPT1* upstream region in the ApaI/SalI-digested plasmid pOPT1G22 (Reuß & Morschhäuser, 2006) to produce pSTP1PG1. A *STP1* downstream fragment was then amplified with the primer pair STP1-7/STP1-8, digested at the introduced PstI and SacI sites, and ligated between the same sites in pSTP1PG1 to generate pSTP1PG2 (see Fig. 20 for analogy).

    STP1 promoter activity in the reporter strains was compared during growth of the strains in YCB-BSA-YE medium. As can be seen in Fig. 37, both GATA transcription factors were required for normal *STP1* expression levels. However, contribution of Gat1p in regulating *STP1* expression seems to be higher than that of Gln3p. Nevertheless, basal *STP1* expression was observed in the absence of both transcription factors, as the fluorescence of *gln3Δ gat1Δ* double mutants containing the reporter fusion was significantly above the background.
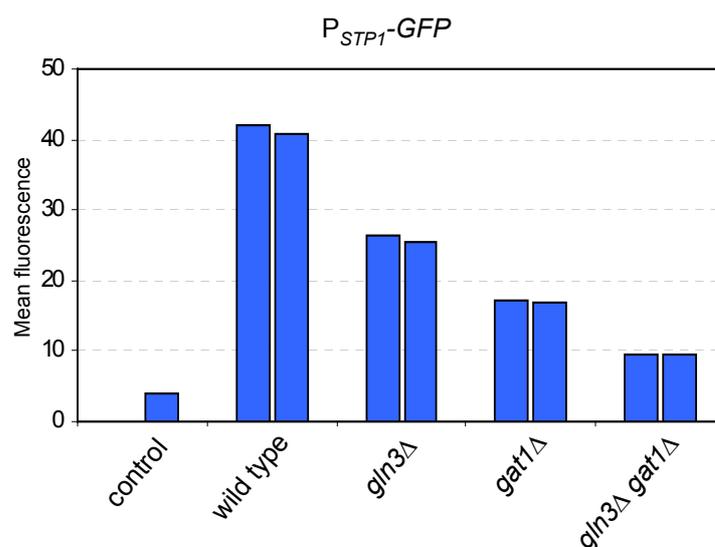
Fig. 37. *STP1* expression is controlled by GATA transcription factors. YPD overnight cultures of reporter strains expressing *GFP* under control of the *STP1* promoter in a wild-type (strains SCSTP1G1A/B), *gln3Δ* (strains Δ*gln3*STP1G1A/B), *gat1Δ* (strains Δ*gat1*STP1G1A/B) or *gln3Δgat1Δ* (strains Δ*gln3*Δ*gat1* STP1G1A/B) along with control strain (SC5314) were diluted 10^{-2} in YCB-BSA-YE medium, grown for 8 h at 30°C, and analyzed by flow cytometry.

4.3.7 Nitrogen catabolite repression of *SAP2* is mediated by regulation of *STP1* expression

The fact that GATA factors Gln3p and Gat1p control *STP1* expression levels indicated that *STP1*, like *SAP2*, is regulated by nitrogen catabolite repression. This hypothesis was validated by observing the influence of nitrogen source availability on *STP1* expression levels. Using *STP1* reporter fusion, the promoter activity was determined in the wild-type strain SC5314 in minimal SD medium containing high (100 mM) and low (100 µM) concentrations of the preferred nitrogen source ammonium as well as in YCB-BSA-YE medium in the absence and presence of ammonium. For comparison, the activity of the *SAP2* promoter was monitored under the same conditions. As shown in Fig. 38, high ammonium concentrations repressed *STP1* expression approximately by twofold, both in minimal SD medium and in YCB-BSA-YE, demonstrating that *STP1* is indeed under nitrogen catabolite repression. *SAP2* promoter activity was detected only in the inducing medium YCB-BSA-YE and reduced to background
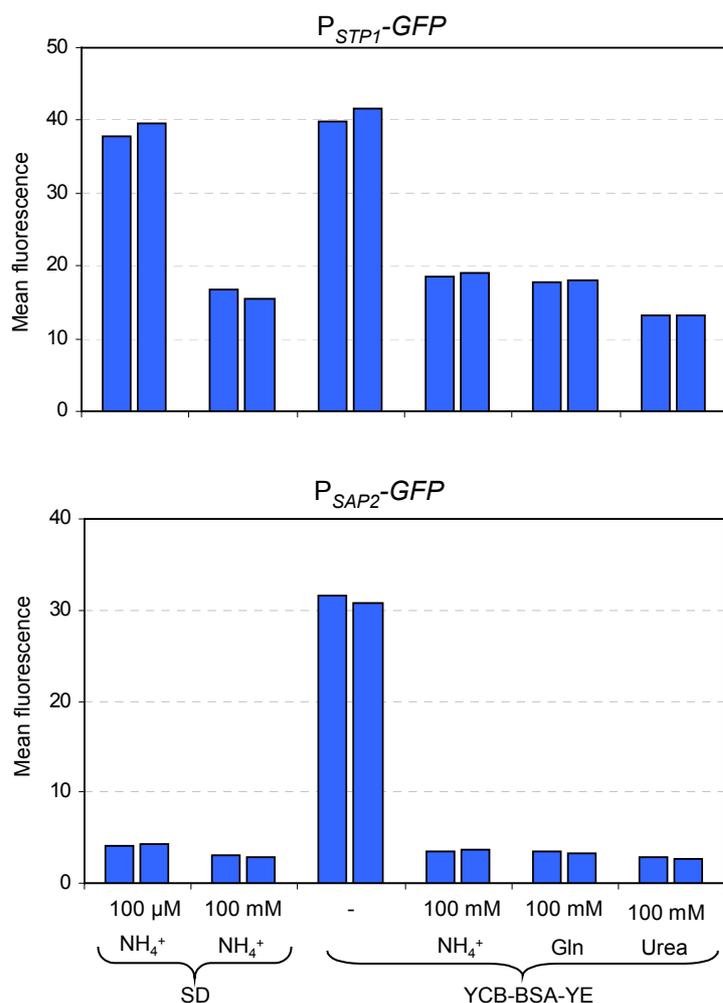
Fig. 38. *STP1* expression is subject to nitrogen catabolite repression. YPD overnight cultures of reporter strains containing a P$_{STP1}$-*GFP* (top) or a P$_{SAP2}$-*GFP* fusion (bottom) in a wild-type background i.e, strains SCSTP1G1A/B and SCSAP2G1A/B, were diluted 10^{-2} in SD medium containing 100 µM or 100 mM ammonium and in YCB-BSA-YE medium without or with 100 mM ammonium, glutamine (Gln) or urea, grown for 8 h at 30°C, and analyzed by flow cytometry. The mean fluorescence of each cell population is shown. The first columns show the results obtained with the A series and the second columns show the results obtained with the B series of the reporter strains. The parental strain SC5314, which does not contain *GFP*, was used as a negative control. Background fluorescence values of this strain were between 2.6 and 3.8 in the various media used in the experiment.

levels by the addition of ammonium. High concentration of an amino acid (glutamine) or urea in both SD and YCB-BSA-YE medium had similar repressing effect on *STP1* and *SAP2* expression (results obtained with SD medium are not shown). These findings suggested that nitrogen catabolite repression of *SAP2* might be mediated at least in part through the regulation of *STP1* expression by the GATA transcription factors Gln3p and Gat1p. In such a scenario, forced expression of *STP1* should overcome *SAP2* repression by preferred nitrogen sources. This possibility was investigated by monitoring BSA degradation and Sap2p expression during growth of the wild-type strain SC5314 and *gln3Δ gat1Δ* strains expressing wild-type or constitutively active forms of *STP1* in YCB-BSA-YE in the absence or presence of high ammonium levels. As can be seen in Fig. 39, ammonium suppressed Sap2p expression and BSA degradation by the wild-type strain SC5314, regardless of the presence or absence of doxycycline (Fig. 39, lanes 1 to 4). In contrast, *gln3Δ gat1Δ* strains expressing
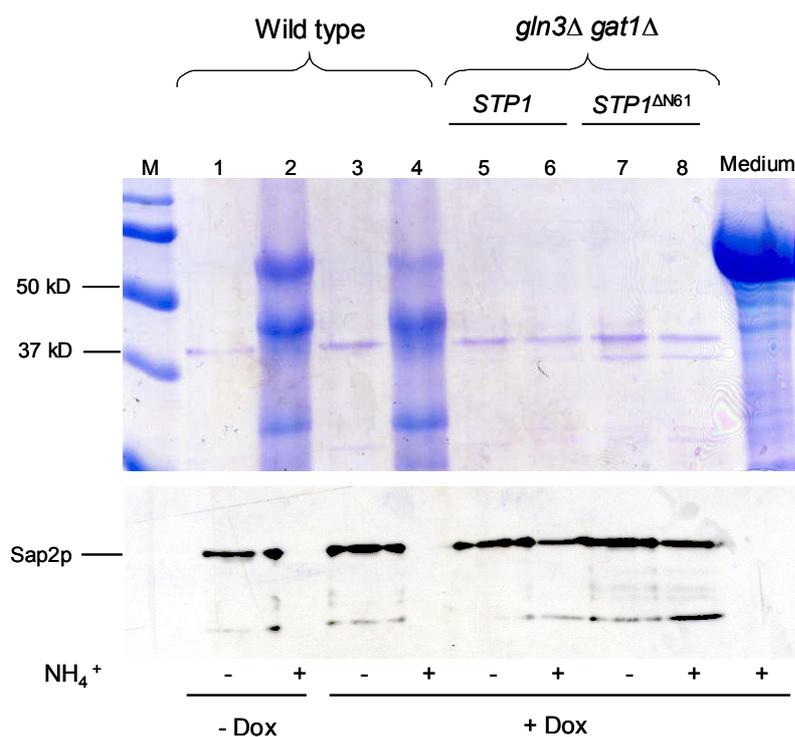
Fig. 39. Nitrogen catabolite repression of *SAP2* is mediated by Gln3p and Gat1p-dependent control of *STP1* expression. The wild-type strain (SC5314), and transformants of the *gln3Δ gat1Δ* double mutants expressing full-length *STP1* (strains Δ*gln3*Δ*gat1*TET1-STP1A/B) or the constitutively active *STP1*$^{\Delta N61}$ allele (strains Δ*gln3*Δ*gat1*TET1-STP1$^{\Delta N61}$A/B) from the Tet promoter were grown for 27 h at 30°C in YCB-BSA-YE in the presence (+) or absence (-) of doxycycline (50 μg/ml) and ammonium (100 mM) as indicated. The culture supernatants were analysed by SDS-PAGE (top) and by Western immunoblotting with an anti Sap2p-antibody (bottom). Uninoculated growth medium was used as a control. M, molecular size marker. The two independently constructed series of transformants behaved identically and only one of them is shown.

110

STP1 or *STP*Δ^{N61} from the Tet-inducible promoter degraded the BSA and expressed Sap2p even in the presence of ammonium (Fig. 39, lanes 5 to 8). This result demonstrated that forced *STP1* expression overcomes the repressive effect of ammonium on Sap2p secretion, and thus *STP1* expression levels play a decisive role in nitrogen catabolite repression of *SAP2*.

5 Discussion

5.1 Mutational analysis of Mep2p

Microorganisms sense the availability of nutrients in the environment to induce the expression of genes whose encoded products are required for uptake and utilization of these nutrients (Forsberg & Ljungdahl, 2001; Holsbeeks *et al.*, 2004). In many cases, membrane proteins that are related to transporters which mediate the uptake of the nutrient into the cell, but have lost their transport function, serve as extracellular nutrient sensors and activate signaling pathways to induce a cellular response e.g. glucose sensors Rgt2p and Snf3p, amino acid sensor Ssy1p in *S. cerevisiae* (Didion *et al.*, 1998; Iraqui *et al.*, 1999; Klasson *et al.*, 1999; Özcan *et al.*, 1996). However, there are few examples in which the transporters themselves also have a sensing and signaling function such as the general amino acid permease Gap1p and the phosphate permeases Pho84p and Pho87p in *S. cerevisiae* (Donaton *et al.*, 2003; Giots *et al.*, 2003; Lorenz & Heitman, 1998). The yeast ammonium permease Mep2p and functionally related proteins from other fungi, which mediate uptake of ammonium into the cell, are also believed to be signaling proteins that sense ammonium in the environment and, as a response, activate signal transduction pathways to induce developmental processes in these organisms (Biswas & Morschhäuser, 2005; Javelle *et al.*, 2003b; Lorenz & Heitman, 1998; Rutherford *et al.*, 2008b; Smith *et al.*, 2003; Teichert *et al.*, 2008). It is presently unknown how exactly ammonium controls signaling activity of the ammonium permeases, and what are the important structural features that link signaling of ammonium permeases to ammonium availability. An N-terminal domain has been implicated in the signaling function of Mep2p in *S. cerevisiae* (Lorenz & Heitman, 1998), and the C-terminal cytoplasmic tail of *C. albicans* Mep2p was shown to contain a specific signaling domain that is required for the induction of filamentation, but dispensable for ammonium uptake (Biswas & Morschhäuser, 2005). A signaling function of the C-terminal cytoplasmic tail has also been shown for bacterial ammonium permeases. AmtB of *E. coli* and *Rhodobacter capsulatus* bind via their C-terminal tail to the PII protein GlnK, which controls ammonium uptake and, in *R. capsulatus* nitrogenase activity (Javelle & Merrick, 2005; Tremblay & Hallenbeck, 2008).

In the present work, the C-terminal signaling domain of *C. albicans* Mep2p was delimited by generating progressive truncated versions of the protein and assessing the ability of the mutated Mep2p to induce filamentous growth. This analysis demonstrated that the C-terminal 47 amino acids of CaMep2p are dispensable for nitrogen starvation-induced

filamentation and that Y433 is the last amino acid that is required for signaling. A hybrid protein in which the region between amino acid 419 to 435 of Mep2p was replaced by the corresponding region from Mep1p was unable to induce filamentation, demonstrating that one or more of the eight amino acids in which the two proteins differ in this region are essential for the signaling function of Mep2p. It is possible that other residues in this region which are present in both Mep1p and Mep2p are also part of the signaling domain. Previously, Mep1p was shown to induce a low level of filamentation when overexpressed from the *MEP2* promoter, suggesting that specific contacts of the C-terminal tail with the remainder of the protein also govern the signaling capacity of the ammonium permeases (Biswas & Morschhäuser, 2005). This was further supported by the observation that ammonium permeases from other fungi can also restore pseudohyphal growth in *S. cerevisiae mep2Δ* mutants (Javelle *et al.*, 2003a; 2003b; Smith *et al.*, 2003; Teichert *et al.*, 2008). Therefore, it is likely that signaling competence of an ammonium permease does not depend only on universally conserved amino acid residues but also on specific interactions within each individual protein. It is likely that all these ammonium permeases act similarly on the same signal transduction pathway to induce pseudohyphal growth when expressed in *S. cerevisiae*. However, an understanding of how signaling is achieved by ammonium permeases requires identification of the interaction partners of these proteins, which are currently not known in any of these organisms. Recently, a different model was proposed to explain how Mep2p regulates filamentous growth in *S. cerevisiae* (Boeckstaens *et al.*, 2008). It was shown that ScMep1p and ScMep2p have different pH optima and therefore the two proteins may differ in the mechanism of ammonium transport. Transport by Mep2p would involve a deprotonation step, whereas transport by Mep1p would not, resulting in opposite effects on internal pH variations, and it was suggested that Mep2p regulates filamentous growth indirectly by influencing pH. In *C. albicans* such a model of indirect control of filamentation by Mep2p via pH regulation is difficult to reconcile with the finding that the C-terminal cytoplasmic tail of Mep2p contains a domain that is dispensable for ammonium transport but essential for signaling, as it seems unlikely that removal of the cytoplasmic tail would affect the deprotonation step at the extracellular gate and alter the mechanism of ammonium transport through the channel.

In an attempt to elucidate how exactly ammonium availability and transport controls the signaling activity of Mep2p, mutational analysis of the protein was done. Signaling is hypothesized to occur either when Mep2p is engaged in ammonium transport or in absence of ammonium transport. In *S. cerevisiae*, there are several lines of evidence that suggest that the

signaling activity of Mep2p depends on its ammonium transport activity. Amino acid substitutions that blocked the transport activity of Mep2p abolished pseudohyphal growth and a mutation that result in increased transport activity also enhanced pseudohyphae formation (Boeckstaens *et al.*, 2007; Marini *et al.*, 2006; Rutherford *et al.*, 2008a). Therefore, the model proposed was that ammonium transport by ScMep2p is required for its sensor role in the induction of pseudohyphal growth. Since filamentous growth of *C. albicans* is repressed at higher ammonium concentrations, even while Mep2p is still expressed, an alternative model was suggested to explain how signaling activity of Mep2p might be regulated. In this model, Mep2p would induce filamentous growth as long as ammonium is absent or present at low concentrations, i.e., when most Mep2p proteins in the cell membrane are not engaged in ammonium transport. At higher ammonium concentrations the transport activity of Mep2p would be increased, which in turn would inhibit its signaling activity and explain why *C. albicans* continues to grow in the budding yeast form instead of switching to filamentous growth under these conditions. Therefore, ammonium would not induce but inhibit the signaling activity of the ammonium sensor Mep2p (Biswas & Morschhäuser, 2005). Such a model would be supported by the identification of transport-deficient Mep2p proteins that are still able to induce filamentous growth. Therefore, several conserved amino acid residues in CaMep2p were mutated in this study, which from structural and functional analyses of ammonium permeases of other organisms were supposed to be required for ammonium transport and ability of the mutated proteins to mediate ammonium uptake and filamentous growth in *C. albicans* was tested.

Mutation of several amino acid residues (D180, H188, and H342) abolished expression of Mep2p, indicating that these amino acids are important for the stability of the protein. The negatively charged aspartate at D160 of AmtB of *E. coli* had been proposed to function as an initial binding site for the positively charged ammonium ion (Thomas *et al.*, 2000) and its mutation to alanine resulted in complete loss of transport activity, despite wild-type expression levels (Javelle *et al.*, 2004). However, substitution of equivalent residue D180 to leucine in CaMep2p resulted in abolishment of expression of the mutated Mep2p. A similar mutation in Mep2p of *S. cerevisiae* (D186A) resulted in stacking of the protein in the endoplasmic reticulum, but a D186N substitution allowed normal expression of the protein and abolished ammonium transport and Mep2p-dependent pseudohyphal growth (Marini *et al.*, 2006). Therefore, D180 was also changed to asparagine in Mep2p of *C. albicans* in order to explore if this substitution would similarly affect ammonium transport and signaling. In this case as well, no expression of the mutated protein could be detected. Therefore, no

specific function could be assigned to D180 apart from its importance in protein stability. Similarly, mutated Mep2p proteins in which the highly conserved histidines H188 and H342 were changed to alanine could also not be detected in the cell membrane. In contrast, mutation of the analogous residues in AmtB of *E. coli* (H168 and H318) or *R. capsulatus* (H193 and H342) allowed normal expression of the proteins, but abolished ammonium transport (Javelle *et al.*, 2006; Tremblay & Hallenbeck, 2008). In the same way, alanine substituted variants of corresponding residues (H194 and H348) in Mep2p of *S. cerevisiae* were normally expressed and localized at cell membrane, but while H194A mutant protein was ammonium transport deficient (i.e., did not restore growth of *mep1Δ mep2Δ mep3Δ* mutants at low ammonium concentrations), Mep2^{H342A} variant was transport proficient (Rutherford *et al.*, 2008a). Therefore, Mep2p of *C. albicans* seems to be more sensitive to mutations in these highly conserved amino acid residues than ammonium permeases from other organisms, which are still normally expressed.

Mutation of four other residues (Y122, F126, W167, S243), which have been predicted to participate in the recruitment and coordination of the ammonium ion (Khademi *et al.*, 2004; Knepper & Agre, 2004), allowed functional expression of Mep2p. An S243A substitution did not affect localization and functions of Mep2p i.e., ammonium transport and signaling by the Mep2^{S243A} protein is comparable to that of wild type Mep2p. However, mutation of equivalent residue in AmtB of *E. coli* (S219A) resulted in increased methylammonium transport (four times) without much influence on the expression levels of the protein (Javelle *et al.*, 2008). Substitution of alanine for Y122, F126, and W167 also allowed expression of the mutated ammonium permeases in the cytoplasmic membrane, although at strongly reduced levels, and it affected ammonium transport to different degrees. Mutational studies on these three residues have been done in other organisms. A Y133I mutation in Amt1;1 from *Lycopersicon esculentum* resulted in decreased methylammonium transport despite normal expression levels and cellular localization (Mayer *et al.*, 2006). We found that mutation of the corresponding residue Y122 in CaMep2p also decreased ammonium uptake but expression levels of the mutated protein were also reduced (20% of wild-type levels). An F131A mutation in AmtB of *R. capsulatus* completely abolished ammonium transport, although the protein was normally expressed and localized to the membrane (Tremblay & Hallenbeck, 2008). In contrast, an analogous F126A substitution still allowed some ammonium transport by CaMep2p. A W178L substitution in LeAmt1;1 (corresponding to W167 of CaMep2p) also decreased ammonium transport despite normal expression levels and localization of the protein (Mayer *et al.*, 2006). On the contrary, an analogous W148L mutation in AmtB of *E. coli* even

increased ammonium flux (Fong *et al.*, 2007). Minor effect of the W167A mutation on ammonium transport was observed for CaMep2p, which may be explained by the reduced expression levels of the mutated protein. A F239A mutation in CaMep2p resulted in a transport deficient protein which was localized to the membrane, this effect is similar to what was observed for mutation of its equivalent residue (F215) in *E. coli* AmtB (Javelle *et al.*, 2008). Collectively, these results illustrate that the relative significance of different amino acid residues for the function of ammonium permeases may vary between different organisms. As shown in the present study, several residues that can be mutated in other ammonium permeases without affecting their expression and localization seem to be indispensable for the stability of Mep2p in *C. albicans*.

It was previously demonstrated that the transport and signaling functions of *C. albicans* Mep2p can be separated (Biswas & Morschhäuser, 2005). However, so far this conclusion was based on mutated proteins lacking the C-terminal cytoplasmic tail, which contains (part of) the signaling domain. Contrastingly, W167 is located in an extracytoplasmic loop of Mep2p and its mutation abolished its ability to induce filamentous growth, although it had only a minor effect on ammonium transport. This finding demonstrates that alterations in the extracellular portion of Mep2p can affect intracellular signaling without influencing the transport activity of the protein. In *S. cerevisiae* experimental evidence suggest that ammonium transport through Mep2p is required for its signaling activity. D186N and H194A mutations in ScMep2p abolished both ammonium transport and pseudohyphal growth, despite normal localization of the proteins (Marini *et al.*, 2006; Rutherford *et al.*, 2008a). Conversely, a G349C mutation in ScMep2p resulted in increased ammonium transport and concomitantly enhanced pseudohyphal growth (Boeckstaens *et al.*, 2007). On the other hand, a H194E mutation in ScMep2p abolished pseudohyphal growth despite the fact that ammonium transport by the mutated protein was increased (Boeckstaens *et al.*, 2008), and a H348A mutation despite being transport proficient abolished pseudohyphal growth (Rutherford *et al.*, 2008a). In the present work a mutated CaMep2p protein was generated that was defective for ammonium transport despite normal localization. Mutation of F239 abolished both ammonium uptake and induction of filamentous growth by Mep2p in *C. albicans*, supporting the model that signaling by Mep2p depends on ammonium transport. However, results of the present study indicate that the effect of mutations on signaling may also be caused by an alteration of protein structure and not necessarily be due to altered ammonium transport, as exemplified by the W167A mutation which abolished signaling without having a strong effect on ammonium transport. Contrastingly, the Y122A mutation had a stronger impact on

ammonium transport than the W167A mutation, but nevertheless, hardly influenced Mep2p-dependent filamentous growth. Therefore, a definite conclusion about how ammonium transport affects signaling by Mep2p can not be drawn from mutational analyses alone.

Apart from the effect of ammonium on the signaling activity of Mep2p, there are differences in the regulation of filamentous growth by ammonium availability in *C. albicans* and *S. cerevisiae*. Dominant-active *RAS1* or *GPA2* alleles, which activate the cAMP-PKA signaling pathway, or the addition of exogenous cAMP can bypass the requirement of *MEP2* for filamentous growth in response to limiting nitrogen concentrations in both *C. albicans* and *S. cerevisiae*. However, increased ammonium concentrations suppress morphogenesis in *C. albicans* strains expressing dominant-active *RAS1* or *GPA2* alleles or in the presence of exogenous cAMP, indicating that ammonium or its metabolic products inhibit filamentation downstream of these regulators (Biswas & Morschhäuser, 2005). In contrast, dominant-active *RAS1* or *GPA2* alleles or exogenous cAMP stimulate pseudohyphal growth even at high ammonium concentrations in *S. cerevisiae* (Lorenz & Heitman, 1998). Additionally, forced overexpression of *MEP2* from a galactose-inducible promoter induced pseudohyphal growth also under nitrogen-replete conditions, demonstrating that ammonium limitation *per se* is not required for the induction of the ammonium-responsive dimorphic switch in *S. cerevisiae* (Rutherford *et al.*, 2008a). On the contrary, filamentous growth was suppressed in *C. albicans* cells containing the hyperactive *MEP2*$^{\Delta C440}$ allele at increased ammonium concentrations (10 mM) at which *MEP2* is still expressed (Biswas & Morschhäuser, 2005), and the same was found when wild-type or the hyperactive *MEP2*$^{\Delta C440}$ allele was expressed from the constitutively active *ADH1* promoter (data not shown). Therefore, the control of morphogenesis by nitrogen availability differs between *C. albicans* and *S. cerevisiae*, and these species-specific differences may well extend to the control of Mep2p signaling activity itself.

5.2 The GATA transcription factors Gln3p and Gat1p control *MEP2* expression and filamentous growth

The specific role of Mep2p in nitrogen starvation-induced filamentous growth is due, at least in part, to its higher expression levels in comparison to Mep1p. Lowering *MEP2* expression levels, when expressed from the *MEP1* promoter, resulted in loss of ability to induce filamentous growth (Biswas & Morschhäuser, 2005). The filamentous growth defect of

strains expressing *MEP2* from the *MEP1* promoter in a *mep1Δ mep2Δ* background was not caused simply by the slower growth of these strains on SLAD plates due to inefficient ammonium uptake, since the defect was also observed when the P*MEP1*-*MEP2* fusion was expressed in *mep2Δ* single mutants, which can grow at wild-type rates because of the presence of an intact *MEP1* allele (K. Biswas and J. Morschhäuser, unpublished results). A correlation between filamentous growth and Mep2p expression levels was also found in the present study when *MEP2* was expressed from mutated *MEP2* promoters with different levels of activity. Strains in which Mep2p expression levels were reduced by 50% showed normal filamentous growth, whereas those with strongly reduced Mep2p expression levels (circa 8 fold) were severely affected in the ability to produce filaments under nitrogen limiting conditions. Obviously, expressing Mep2p at appropriate levels is a prerequisite for *C. albicans* to be able to induce the switch from yeast to filamentous growth in response to nitrogen starvation. Hence, knowledge of regulators that control *MEP2* expression is necessary. In *C. albicans MEP2* expression, like its counterpart in *S. cerevisiae* is subject to nitrogen control i.e., enhanced *MEP2* expression levels under limiting nitrogen conditions (Biswas & Morschhäuser, 2005; Marini *et al.*, 1997). Typical for a nitrogen regulated gene the upstream regulatory region of *MEP2* possesses several 5'-GATA-3' sequences. In this study two putative GATA factor binding sites in the *MEP2* promoter were found to be essential for upregulation of *MEP2* under limiting nitrogen conditions. While mutation of the GATAA sequence centered at position -208 alone had no significant effect, the same mutation almost completely abolished *MEP2* expression when combined with a mutation of the GATAA sequence centered at position -266, which by itself already reduced *MEP2* expression very strongly. Since a single GATAA sequence is essential, but not adequate for nitrogen regulation in *S. cerevisiae* (Magasanik & Kaiser, 2002), it is possible that in the absence of the GATAA sequence at -208 (as in pMEP2MP1), one of the (dispensable) GATAA sequences located further upstream or other regulatory sequences can act in concert with the more important GATAA sequence at -266. Importance of the two GATAA sites in the induction of *MEP2* expression suggested that members of GATA transcription factor family might be involved in the regulation of *MEP2* expression under limiting nitrogen conditions.

In *S. cerevisiae*, the GATA transcription factors Gln3p and Gat1p promote high-level expression of *MEP2*, but the contribution of each factor depends on the available nitrogen source (Marini *et al.*, 1997). Under nitrogen limiting conditions, when nitrogen sources were used at concentrations of 100 μM, *MEP2* expression was induced in *C. albicans* regardless of

the nature of the nitrogen source (Biswas & Morschhäuser, 2005). In the present work *MEP2* expression levels were quantified using the same concentrations as stated above and it was found that Gln3p and Gat1p both are required for full *MEP2* expression in *C. albicans*. Strains lacking either *GLN3* or *GAT1* had strongly reduced *MEP2* expression under all conditions tested and no *MEP2* expression was detected in the absence of both transcription factors. Liao *et al.,* (2008) reported that when using nitrogen source at a concentration of 10 mM (in contrast to 100 μM used in this study), *MEP2* expression become dependent on the quality of nitrogen source, low expression in presence of glutamine and enhanced expression levels in proline medium. The authors also suggested that Gln3p and Gat1p can regulate *MEP2* expression independent of the nature of nitrogen source, which is in agreement with present results, and added that alternate factors may control *MEP2* expression in regard to nitrogen quality. In the case of regulation of *MEP1* expression, Gln3p was required for activation of transcription; unexpectedly Gat1p was found to have a negative effect on Mep1 expression levels under all tested conditions. These results demonstrated that relative contribution of Gln3p and Gat1p on transcription depends on their target gene.

C. albicans strains deleted for *GLN3* were also severely impaired for filamentous growth under nitrogen starvation conditions, a phenotype that correlated well with the reduced *MEP2* expression levels in these mutants. Therefore, the inability of the *gln3Δ* mutants to induce *MEP2* expression at appropriate levels may be responsible for their filamentation defect. In fact, when a *MEP2* copy was expressed from the *ADH1* promoter in the *gln3Δ* mutants, filamentation was partially restored, and forced overexpression of a hyperactive *MEP2* allele completely rescued the filamentation defect of the *gln3Δ* mutants and resulted in a hyperfilamentous phenotype, as in a wild-type background. *S. cerevisiae gln3Δ* mutants are also defective in pseudohyphal differentiation, but expression of *MEP2* from a heterologous, inducible promoter did not restore filamentation, indicating that Gln3p has additional targets that are critical for regulation of pseudohyphal growth (Lorenz & Heitman, 1998). Although results of the present study do not exclude the possibility that in *C. albicans* Gln3p also has other targets in addition to *MEP2* that are normally required for the induction of filamentous growth in response to nitrogen starvation, the expression of the hyperactive *MEP2* allele bypasses the need to activate these pathways. A similar observation was made in previous study which demonstrated that expression of the hyperactive *MEP2* allele from its own promoter overcame the filamentation defect of *cph1Δ* and *efg1Δ* single mutants, but not that of *cph1Δ efg1Δ* double mutants, although both transcription factors, which are at the end of a MAP kinase cascade and a cAMP-dependent signaling pathway, respectively, are normally

required for nitrogen starvation-induced filamentous growth of *C. albicans* (Biswas & Morschhäuser, 2005).

Surprisingly, the *gat1Δ* mutants, despite having similarly reduced *MEP2* expression as the *gln3Δ* mutants, do not show a filamentation defect. It is possible that the reduced *MEP2* expression levels seen in both mutants are still sufficient to induce filamentation, but other target genes, which are also required for normal filamentous growth, are affected by inactivation of *GLN3* but not *GAT1*. In fact, the expression levels of GFP-tagged Mep2p in the *gln3Δ* and *gat1Δ* mutants were still higher (roughly 2-fold) than those in strains expressing the *MEP2-GFP* fusion from the *MEP1* promoter, which were not sufficient for filamentation (data not shown). Another possibility that could account for normal filamentation observed in *gat1Δ* mutants is that absence of the Gat1p transcription factor in *C. albicans* may activate filamentation inducing signaling pathways that do not require higher *MEP2* expression levels or are even independent Mep2p. It was found that in contrast to the wild-type, filamentation in *gat1Δ* mutants has become partially independent of the presence of functional *MEP2* gene as the *mep2Δ gat1Δ* mutants can filament on SLAD plates. Wild-type filamentation in the *gat1Δ* mutants still depends on Gln3p activity as *gln3Δ gat1Δ* double mutants exhibited the same strong filamentation defect as *gln3Δ* single mutants. Increase in expression levels of *MEP1,* when expressed from the *MEP2* promoter is reported to confer Mep1p the ability to induce a weak filamentation (Biswas & Morschhäuser, 2005). However, the increased *MEP1* expression levels observed in *gat1Δ* mutants (ca. 2.5 fold) and *gat1Δ mep2Δ* mutants could not account for the activation of Mep2p independent pathways, since the *gat1Δ mep2Δ mep1Δ* triple mutant exhibit filaments under nitrogen limitation (Somisetty and Morschhäuser, personal communication). Future challenge is to elucidate the molecular mechanisms which induce filamentation under nitrogen limiting conditions in a Mep2p and Gat1p-independent manner.

Results of this study show similarities and differences with those reported by Liao *et al.,* (2008). In contrast to the findings presented here, they reported that filamentation phenotype of *gln3Δ* and *gat1Δ* null mutants differed in relation to nitrogen source. They found that filamentation of *gln3Δ* single and *gln3Δ gat1Δ* double mutants was only marginally reduced in medium containing glutamine and proline as nitrogen source, and *gat1Δ* mutants were impaired in filamentation on ammonium and urea. These differences were attributed to the variation of the nitrogen limiting conditions, nitrogen sources were used at a concentration of 1mM in those studies versus 100 μM in the present study, differences in experimental procedures or possibly to the genetic backgrounds of the strains (Liao *et al.*, 2008). They

proposed that in addition to reduced Mep2p expression levels, the hyphal defect of *gln3Δ* mutants may also be due to defect in ammonium assimilation since non-filamentous phenotype of *gln3Δ* mutants was observed only on ammonium and urea (which is degraded to ammonium and then assimilated in similar manner), and expression of *GDH3*, encoding NADP-dependent glutamate dehydrogenase, a key enzyme of ammonium assimilation, was greatly reduced in *GLN3* null mutants. However, this line of reasoning failed to explain the notable reduction in filamentation of *gat1Δ* mutants observed by them on ammonium and urea since *GDH3* expression was unaltered in these strains (Liao *et al.*, 2008).

The present work demonstrated that by placing *MEP1* and *MEP2* under the control of the GATA transcription factors Gln3p and Gat1p, *C. albicans* ensures that these ammonium permeases are expressed only when the preferred nitrogen source ammonium is present at low concentrations or absent. The signaling activity of Mep2p then also induces morphogenesis, allowing the fungus to fine tune its growth mode according to environmental conditions.

5.3 A transcription factor regulatory cascade controls secreted aspartic protease expression in *C. albicans*

The ability to utilize proteins as a nitrogen source, which is conferred by the secreted aspartic protease Sap2p, is important for growth of *C. albicans* under both *in vitro* and *in vivo* conditions. Besides being growth defective in YCB-BSA medium, which contains the protein bovine serum albumin (BSA) as the sole nitrogen source, the *sap2Δ* mutants also exhibit reduced virulence in animal model of candidiasis (De Bernardis *et al.*, 1999; Hube *et al.*, 1997; Staib *et al.*, 2002). However, the molecular mechanisms involved in regulating the expression of *SAP2* are poorly understood. The present study illustrated that GATA transcription factors Gln3p and Gat1p control expression of *SAP2*. Limjindaporn *et al.*, (2003) already reported that in preliminary experiments a *gat1Δ* mutant was deficient in the utilization of BSA as a nitrogen source. This observation was confirmed and extended further by the findings in the present work that growth of *gat1Δ* mutants in YCB-BSA medium was strongly delayed, although the mutants eventually reached the same optical density as the wild-type after several days of growth, while mutants lacking both *GLN3* and *GAT1* failed to grow in this medium. The limiting factor responsible for the growth defect of *gln3Δ gat1Δ* double mutants was the abolishment of Sap2p expression, whereas Sap2p production was still

observed in either of the single mutants. Therefore, both Gln3p and Gat1p contribute to *SAP2* expression, although Gat1p clearly plays a more prominent role than Gln3p.

The induction of *SAP2* expression in the presence of proteins and its repression by sufficient amounts of a preferred nitrogen source, like ammonium or amino acids in high concentrations, has been known for many years (Banerjee *et al.*, 1991; Hube *et al.*, 1994; Ross *et al.*, 1990). However, insights into molecular basis of *SAP2* regulation were missing. Initial reports have proposed that peptides which are produced from proteins by basal extracellular proteolytic activity serve as the inducers of *SAP2* expression (Hube *et al.*, 1994; Lerner & Goldman, 1993). However, recently it was suggested that micromolar concentrations of amino acids, which may also be produced during the degradation of extracellular proteins and signal the availability of proteins, are the actual inducers of *SAP2* (Martinez & Ljungdahl, 2005). Extracellular amino acids are sensed at the cell surface by the SPS sensor, which then induces the proteolytic activation of two latent transcription factors Stp1p and Stp2p. The activated transcription factors are targeted to the nucleus where each of these factors has a specific subset of target genes. While Stp2p induces the expression of genes involved in amino acid uptake, Stp1p activates genes that are required for the utilization of proteins as a nitrogen source, the secreted aspartic protease *SAP2* and the oligopeptide transporters *OPT1* and *OPT3*. Stp1p is essential for *SAP2* and *OPT1* expression (*OPT3* is also induced by Stp2p) and *stp1Δ* mutants can not grow on protein as the sole nitrogen source. Conversely, cells expressing a truncated, constitutively active *STP1* allele, which lacks the N terminal inhibitory domain, do not need an inducer and express *SAP2* and *OPT1* even in the absence of proteins. In the presence of high (millimolar) concentrations of amino acids, Stp1p, but not Stp2p levels are downregulated, thereby ensuring that amino acid permeases are adequately expressed while the expression of enzymes and transporters required for the utilization of proteins is shut off. As Stp1p was readily detected in the presence of high concentrations of ammonium, Martinez and Ljungdahl concluded that steady-state levels of Stp1p are affected by amino acid availability, but not by the overall nitrogen status of the cell (Martinez & Ljungdahl, 2005). However, Stp1p levels in media containing high and low ammonium levels were not compared by them. Results from the present study clearly demonstrated that *STP1* expression is downregulated at high concentrations of ammonium or other nitrogen sources, like amino acids and urea, which may at least partially explain the repression of *SAP2* expression when sufficient amounts of preferred nitrogen sources are available.

In this work a link between the two types of transcription factors that are essential for *SAP2* expression and growth of *C. albicans* on proteins, the general regulators Gln3p and Gat1p and the specific regulator Stp1p, is provided. In Fig. 40, the schematic representation describes how both the positive and negative control of *SAP2* expression is achieved. When preferred nitrogen sources like ammonium become limiting, Gln3p and Gat1p increase expression of *STP1*, which seems to be a prerequisite for *SAP2* expression. However, nitrogen starvation alone is not sufficient and *SAP2* induction still needs a positive signal, which is provided by the presence of low concentrations of amino acids that result in the proteolytic activation of Stp1p. Conversely, even in the presence of proteins (inducing signal) *SAP2* expression is repressed when sufficient amounts of preferred nitrogen sources are available, because under these conditions *STP1* expression is down-regulated to levels that may not be adequate for *SAP2* expression. It is not clear whether the observed twofold down-regulation of *STP1* is sufficient to abolish *SAP2* expression, and it is well possible that Gln3p and Gat1p also directly activate *SAP2* or control other genes that contribute to *SAP2* expression. However, such an additional effect of Gln3p and Gat1p on *SAP2* expression would be insufficient, as *stp1*Δ mutants do not detectably express *SAP2* and are unable to grow in YCB-BSA medium. In contrast, forced expression of *STP1* from the Tet-inducible promoter relieved *SAP2* expression from its dependence on Gln3p and Gat1p, demonstrating that the control of *STP1* expression levels is a central aspect in the regulation of *SAP2* and in the decision on whether to use available proteins as a nitrogen source.
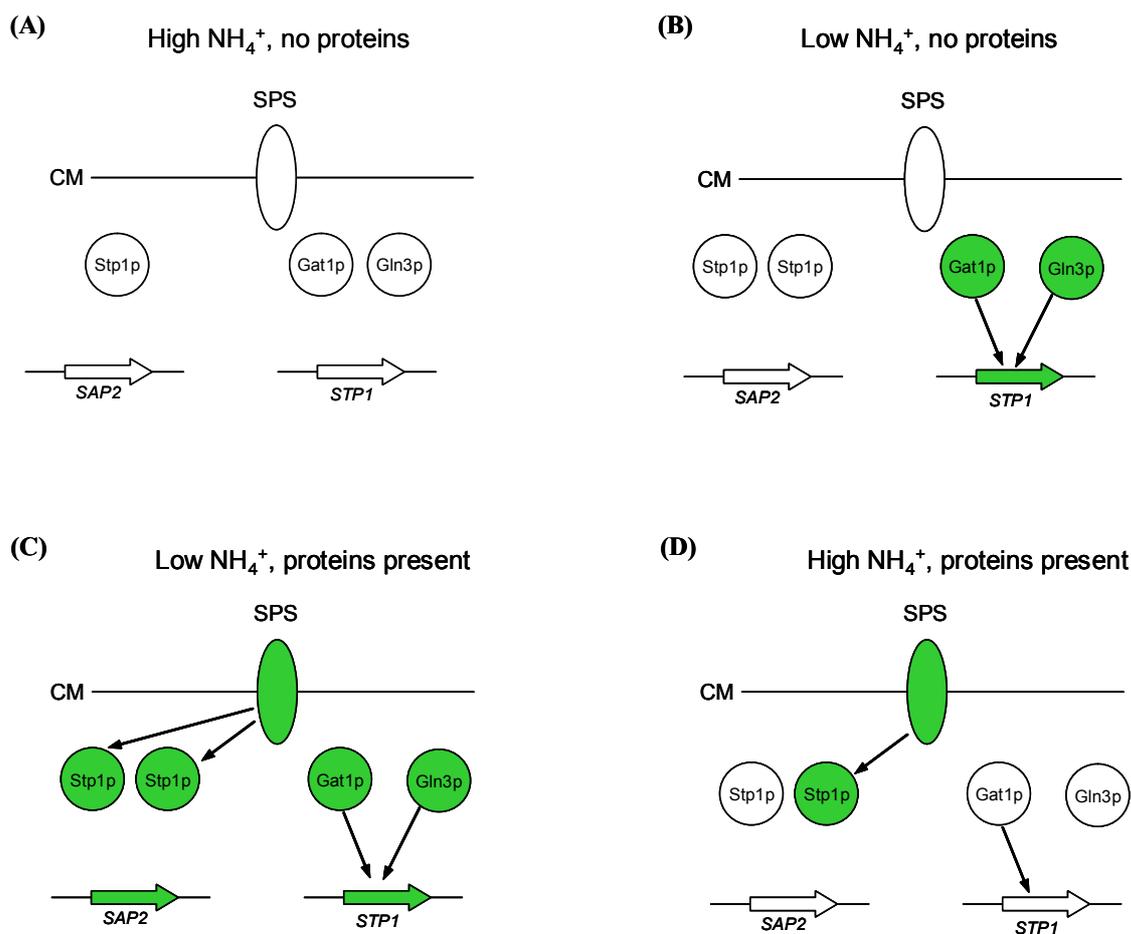
Fig. 40. Schematic explaining the regulation of *SAP2* expression by the transcription factors Stp1p, Gln3p, and Gat1p. Unshaded block arrows and spheres symbolize genes and proteins, respectively, in their inactive state. The activated state is indicated by the green shading. An increase in Stp1p levels is indicated by two corresponding spheres instead of only one.

(A) Under nitrogen replete conditions (in this model represented by high NH_4^+), the GATA factors Gln3p and Gat1p have only basal activity and *STP1* is expressed at low levels. In the absence of proteins, Stp1p is not activated by the SPS sensor and *SAP2* is not expressed.

(B) Under nitrogen limiting conditions, Gln3p and Gat1p are activated and induce expression of their target genes, including *STP1*. However, the absence of proteins prevents activation of Stp1p by the SPS sensor and *SAP2* is not expressed.

(C) When proteins are the only available nitrogen source, Gln3p and Gat1p ensure high *STP1* expression levels. Micromolar concentrations of amino acids generated by basal proteolytic activity induce the SPS sensor to activate Stp1p, which in turn induces *SAP2* expression.

(D) When both proteins and sufficient concentrations of a preferred nitrogen source, like ammonium, are available, Gln3p and Gat1p are not activated and *STP1* expression levels remain low. Stp1p may still be activated by the SPS sensor under these conditions, but this is not sufficient to allow *SAP2* expression.

Additional possible regulatory mechanisms, e.g., direct regulation of *SAP2* by Gln3p and Gat1p or the contribution of other regulators, are not depicted in this model**.**

As discussed in section 5.2, the GATA transcription factor, Gln3p also controls nitrogen starvation-induced filamentous growth of *C. albicans* by regulating expression of *MEP2*. The filamentous growth defect of *gln3Δ* mutants can be overcome by forced overexpression of *MEP2* and the requirement of *MEP2* can be bypassed by dominant-active *RAS1*[G13V] or *GPA2*[Q354L] alleles (Biswas & Morschhäuser, 2005). However, high concentrations of ammonium still suppress filamentous growth in cells expressing hyperactive *RAS1* or *GPA2*, demonstrating that ammonium can also act downstream of these regulators. In contrast, ammonium could not inhibit *SAP2* expression in strains expressing *STP1* from a tetracycline-inducible promoter, lending further support to the idea that the regulation of *STP1* expression is a decisive factor in the control of *SAP2* expression. Therefore, by using a regulatory cascade in which the general regulators Gln3p and Gat1p control the expression of the specific transcription factor Stp1p, which in turn mediates *SAP2* expression, *C. albicans* places *SAP2* expression under nitrogen control and ensures proper expression of this long-known virulence determinant.

Direct experimental evidences exhibiting the requirement of GATA factors in virulence of *C. albicans* were provided by Limjindaporn *et al.*, (2003) and Liao *et al.*, (2007). In a mouse model of disseminated candidiasis, the *gln3Δ* mutant showed significantly reduced virulence, but a *gat1Δ* mutant was avirulent. Differences in virulence phenotypes of mutants indicate that each factor makes distinct contributions to the ability of *C. albicans* to survive in its host. It is likely that Gln3p and Gat1p may each independently or redundantly activate a subset of genes involved in virulence, and Gat1p regulated genes have a more critical role. Thus nitrogen regulation by GATA factors is central to the pathogenicity of *C. albicans*.

Conclusions and future directions

The present study provides valuable insights into how the GATA transcription factors, Gln3p and Gat1p, control nitrogen regulated virulence traits of *C. albicans*. These factors exhibit partially overlapping, but distinct roles in regulating the expression of those genes that help *C. albicans* to deal with different nitrogen environmental conditions. This is supported by the findings that under nitrogen limiting conditions both GATA factors control Mep2p expression levels and Gln3p is also an important regulator of this filamentous growth. Gln3p and Gat1p also enable growth of *C. albicans* on proteins by being part of a regulatory cascade that governs expression of *SAP2* and Gat1p has a prominent role in regulating Sap2p expression levels. Hence, the relative contributions of Gln3p and Gat1p vary with their target genes and

the availability of nitrogen source. A full appreciation of their role awaits a complete analysis of the gene targets and processes regulated by them.

Further, mutational analysis of the morphogenetic regulatory ammonium permease Mep2p reveals residues that are required for ammonium transport and signaling. The molecular mechanisms by which Mep2p links ammonium availability to the induction of filamentous growth are yet to be identified. The identity of signaling partners that interact with Mep2p to activate the signal transduction pathways which induce hyphal development in response to nitrogen starvation is yet to be revealed.

6 References

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7 Appendix

7.1 Publications and Presentations

Publications:

Dabas, N., and Morschhäuser, J. (2007). Control of ammonium permease expression and filamentous growth by the GATA transcription factors *GLN3* and *GAT1* in *Candida albicans.* Eukaryotic Cell: 5: 875-88

Dabas, N., and Morschhäuser, J. (2008). A transcription factor regulatory cascade controls secreted aspartic protease expression in *Candida albicans.* Molecular Microbiology: 69(3): 586-602

Presentations:

Dabas, N., and Morschhäuser, J. Control of ammonium permease expression and filamentous growth by the transcription factors *GLN3* and *GAT1* in *Candida albicans,* 58 Jahrestagung der DGHM, 1-4.10.2006, Würzburg, Germany

Dabas, N., and Morschhäuser, J. Control of ammonium permease expression and filamentous growth by the GATA transcription factors *GLN3* and *GAT1* in *Candida albicans.* FEBS Advanced Lecture Course on Human Fungal Pathogens, 11-17.5.2007, La Colle sur Loup, France. Poster.

Dabas, N., and Morschhäuser, J. Regulation of virulence traits by the GATA transcription factors *GLN3* and *GAT1* in *Candida albicans.* New Trends in Infectious Disease Research, 3rd Joint Ph.D. Students Meeting of the Collaborative Research Centers SFB 630 & SFB 544, 28-29.6.2007, Retzbach, Germany.

Dabas, N., and Morschhäuser, J. Regulation of nitrogen-regulated virulence traits of *Candida albicans* by GATA transcription factors. Colonisation and Infection by Human-Pathogenic Fungi, Colloquium of the DFG Priority Programme 1160, 8-9.11.2007, Jena, Germany.

Dabas, N., and Morschhäuser, J. A transcription factor regulatory cascade controls secreted aspartic protease expression in *Candida albicans.* 9th ASM Conference, Candida and Candidiasis, 24.-28.3.2008, New Jersey, USA. Poster.

7.2 Curriculum Vitae

Name	Neelam Dabas
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Date of Birth	15.02.1981
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Academic Qualifications

1987-1999   All India Senior School Certificate Examination
Deen Bandhu Public School, Ghevra, Delhi, India

1999-2002   Bachelor of Science (General group Biology)
Miranda House College, University of Delhi, India

2002-2004   Master of Science (Life Sciences)
Jawaharlal Nehru University (JNU), New Delhi, India
Title of M.Sc. Dissertation:
"Functional characterization of Cdr1p, a Multidrug
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Research Experience

2004-2008   Ph.D. thesis work in the group of Prof. Dr. J. Morschhäuser
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