Trehalose pathway regulates filamentation response in Saccharomyces cerevisiae

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Title:
Trehalose pathway regulates filamentation response in Saccharomyces cerevisiae

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Abstract:
In Saccharomyces cerevisiae, the diploid cells undergo either pseudohyphal differentiation or sporulation in response to carbon and nitrogen source depletion. Distinct pathways are known to regulate the processes of filamentation and sporulation in response to nutritional stress. Here, we report the novel finding that the trehalose pathway which is essential for sporulation, is involved in pseudohyphae formation both via GPR1 as well as RAS2 mediated signaling. Our observations indicate that GPR1 is epistatic over TPS1 in signaling for filamentation. Further, we have demonstrated that the pseudohyphal defect of the ras2 mutant is overcome upon disruption of TPS2. Thus, our results indicate that TPS1 and TPS2 may be involved in cell fate decision between meiosis and filamentation response under nutrient depleting conditions. Further, monitoring pseudohyphae formation under limiting glucose condition unravelled the possibility that TPS1 and TPS2 exert opposing effects to trigger filamentation response.

Key Words: TPS1, TPS2, RAS2, GPR1, Pseudohyphae, trehalose

Statements and Declarations:
The authors have no competing interests to declare that are relevant to the content of this article.

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INTRODUCTION

Organisms have evolved a plethora of developmental and differentiation mechanisms to overcome nutritional deprivation. Yeast, *Saccharomyces cerevisiae*, undergoes both metabolic as well as morphologic differentiation in order to overcome nutrient deprivation (Gagiano et al. 2002, Gancedo 2008). Diploid cells of *S. cerevisiae* have the potential to either sporulate or achieve pseudohyphal differentiation in response to carbon and/or nitrogen depletion (Gimeno et al. 1992, Iyer et al. 2008). Sporulation occurs when carbon and nitrogen are depleted (Honigberg and Purnapatre 2003). Although pseudohyphal differentiation was originally thought to occur only in response to low ammonium and high glucose (Lengeler et al. 2000, Lorenz 1997), Iyer et al. (2008) demonstrated that in addition to signaling from low ammonium, low glucose signaling was also necessary for filamentation response. Despite intense investigations, it is still unclear as to how these two developmental processes of pseudohyphae formation and sporulation emerge in response to low nitrogen and low glucose (Cullen and Sprague 2012, Iyer et al. 2008). While distinct low glucose and low ammonium signaling pathways implement pseudohyphal differentiation by regulating the levels of cAMP, the nature of the crosstalk between glucose and ammonium signaling has remained largely elusive (Cullen and Sprague 2012, Gagiano et al. 2002).

Signaling for pseudohyphal transition in response to low ammonium occurs via MEP2, an ammonium transporter, (Lorenz and Heitman 1998). It was demonstrated that MEP2 is a transceptor i.e. in addition to signaling, the transport function of Mep2 was necessary for filamentation response (Rutherford et al. 2008). *NPR1*, a TORC1 effector kinase, positively regulates MEP2 under conditions of poor nitrogen availability to trigger pseudohyphae formation (Boeckstaens et al. 2007, Boeckstaens et al. 2014). Although it has been demonstrated that MEP2 signals via cAMP (Lorenz and Heitman 1998), the underlying mechanisms have not yet been fully elucidated. (Rutherford et al. 2008).

In contrast to the above, how the glucose responsive, *GPR1-GPA2* signaling impinges on the cAMP-PKA pathway (Cullen and Sprague 2012, Xue and Hirsch 1998) to induce filamentation response is relatively well understood. It was previously reported *KRH1/2* interfere with *GPR1-GPA2* coupling thereby inhibiting downstream signaling via cAMP (Harashima and Heitman 2005). Although it was tacitly assumed that *KRH1* and *KRH2* are functionally redundant, Iyer and Bhat demonstrated that they are non-redundant and uncovered distinct roles for these two kelch proteins in inducing pseudohyphae in low glucose (Iyer and Bhat 2017). Thus, this study highlighted the significance of low glucose in filamentation. This was corroborated by the observation that *FLO11*, a key gene regulating pseudohyphal differentiation (Rupp et al. 1999), is glucose repressed (Kuchin et al. 2002). Further, *SNF1*, a gene under carbon catabolite repression, is required for formation of pseudohyphae (Kuchin et al. 2003). Thus, it is evident that glucose is a key component of pseudohyphal differentiation process.

While cAMP mediated signaling via the *GPR1-GPA2* axis is known to function in a *RAS2* dependent fashion (Lorenz et al. 2000), more recently it was demonstrated that *RAS* signaling is indirectly regulated through the glycolytic flux. Accumulation of trehalose-6-phosphate (T6P) results in elevated levels of Fructose 1,6-bis-phosphate which in turn caused activated *RAS* signaling (Peeters et al. 2017). This was mediated by the absence of suppression of *HXK2* by T6P. Further, it was demonstrated that apoptosis induced by
activated RAS signaling probably caused the growth defect exhibited by the *tps1* mutant in high glucose (Peeters et al. 2017), invoking the possibility of a role for trehalose metabolism in pseudohyphal differentiation. This idea does not seem to be farfetched given the observation that the components of the trehalose biosynthetic pathway are essential for sporulation (De Silva-Udawatta and Cannon 2001), which also is a response to nutrient depletion. In yeast, trehalose performs a variety of functions ranging from serving as a carbon source to a stress protectant, by conferring resistance to adverse environmental conditions (Bonini et al. 2000, Miao et al. 2017). The trehalose pathway involves two enzymes namely *TPS1* (trehalose phosphate synthase) and *TPS2* (trehalose phosphate phosphatase). *TPS1* catalyzes the formation of T6P from UDP-Glucose and glucose-6-phosphate (Thevelein and Hohmann, 1995). T6P appears to regulate glucose mediated signaling (Deroover et al. 2016).

Interestingly, observations in *Candida albicans* suggested a possible link between trehalose synthesis and virulence. Evidence in *C. albicans* indicated that disruption of *TPS1* results in a decrease in virulence of the strain (Zaragoza et al. 1998). Disruption of *TPS2* caused a reduction in virulence without affecting hyphae formation (Van Dijck et al. 2002). Further, it was demonstrated that *TPS2* and *GPR1* functioned synergistically in trehalose metabolism as well as virulence (Maidan et al. 2008). In contrast to *S. cerevisiae*, *TPS1* disruption in *C. albicans* did not affect growth on glucose (Zaragoza et al. 1998, Van Dijck et al. 2002). The *TPS* genes have also been shown not only to regulate differentiation in *C. albicans* but also to regulate several processes in plants ranging from cell morphology to architecture of inflorescence and other developmental processes (Chary et al. 2008).

Based on the above and the observation that low glucose is pivotal in pseudohyphae formation as well as in restoring the glycolytic imbalance in a *tps1* mutant, we hypothesized that the trehalose biosynthetic pathway may be involved in filamentation response. This hypothesis is further supported by the correlation observed between low trehalose levels and pseudohyphae formation (Iyer et al. 2008). Despite the availability of a vast body of literature on the components of trehalose pathway, it’s role in pseudohyphal differentiation has hitherto been unexplored in *S. cerevisiae*. Here, we show that the *tps1* but not *tps2* mutant is defective in pseudohyphae formation. This is the first report on the involvement of the trehalose biosynthetic pathway in pseudohyphal differentiation in *S. cerevisiae*. The use of SLALD (Synthetic low ammonia low dextrose) medium enabled the dissection of the independent roles of *TPS1* and *TPS2*. Our results demonstrate that *TPS1* and *TPS2* may regulate RAS2 differentially depending upon the availability of nutrients to signal filamentation via cAMP.
MATERIALS AND METHODS

**Media, Strains and Plasmids:** The strains used in this study are isogenic derivatives of Σ1278b strain. The strains were constructed using standard methods (Adams et al. 1997, Wach et al. 1994) and are listed in Table 1. Genes were disrupted using either the KanMX or Hygromycin cassette. The double disruptants were generated by mating the individual mutants followed by sporulation and segregation of haploids.

**Pseudohyphal growth assay:** Synthetic low ammonia dextrose (SLAD) medium with 2% glucose or Synthetic low ammonia low dextrose (SLALD) medium with 0.05% glucose were used to score pseudohyphal growth (Gimeno et al.1992, Lorenz and Heitman 1997, Iyer et al. 2008). Cells were spread for single colonies and incubated for 6 days at 30°C unless mentioned otherwise. Images of colonies were captured at 10X magnification using a Nikon Coolpix 8400 attached to a Nikon TS 100 microscope. The images are representative of at least three repetitions of each experiment.

**Spotting Assay:** Cells were grown to 0.5 OD, washed twice and five-fold serial dilutions were spotted on yeast extract peptone dextrose (YPD) or yeast extract peptone galactose (YPGal) agar. Images were captured after 2 days of incubation.

**Western Blot Analysis:** Crude cell extracts were prepared as described by (Peeters et al. 2017). Briefly, cells at OD of 3–4 in YPD medium were collected and washed with ice-cold water. For analysis in SLALD medium, cells at OD 3-4 were collected by centrifugation, washed twice, transferred to SLALD (one-fifth volume) and incubated for an additional 8 hrs. 200mg cells were lysed with 0.2g of glass beads after adding lysis buffer as described (Peeters et al. 2017). Centrifugation at 8000 rpm for 5 min yielded the crude protein extract used for western blot analysis. The blots were developed with antibodies against yeast Ras2p from Santacruz Biotechnology Inc., according to the manufacturer’s recommendations.
RESULTS:

Glucose growth defect of the \textit{tps1} mutant is both strain as well as ploidy dependent

It is a long standing observation that mutation in \textit{TPS1} causes a growth defect on fermentable carbon sources (Van Heerden et al. 2014, Peeters et al. 2017). It is pertinent to note here that this phenotype was observed in a haploid of the W303 strain background (Peeters et al. 2017). In the study by van Herdeen et al (2014), the diploid strain of S288c was used (personal communication). Our analysis showed that although the \textit{tps1Δ} haploid strain in the \textit{∑1278} background had a growth defect (Fig. 1), the diploid strain was able to grow well on glucose (Fig. 2). Thus, the glucose growth phenotype of the \textit{tps1} mutant strains varies with different lineages as well as the ploidy status of the cell.

\textit{TPS1} is required for filamentation response

Trehalose synthesis in yeast occurs in response to adverse environmental conditions, including nutritional stress. Since filamentation occurs in response to nutrient limitation, we hypothesized that \textit{TPS1} and/or \textit{TPS2} may have a role in pseudohyphal differentiation. Therefore, independent mutants of \textit{tps1} and \textit{tps2} as well as the \textit{tps1tps2} double mutant were analyzed on SLAD as well as SLALD media (Fig. 3). As expected, \textit{tps1} was defective in pseudohyphae formation. In contrast, however, the \textit{tps2} mutant had no filamentation defect. Further, pseudohyphae formation was slightly enhanced in the \textit{tps2} mutant as compared to the wild type indicating that \textit{TPS2} may be a negative regulator. Surprisingly the \textit{tps1tps2} double mutant formed pseudohyphae on SLALD but not SLAD medium. In high glucose the double mutant phenotype was the same as that of the \textit{tps1} mutant whereas in low glucose it was the same as that of \textit{tps2} mutant, indicating that \textit{TPS1} and \textit{TPS2} may signal differently depending on the availability of glucose. Further, heterodiploids in the \textit{TPS1} as well as the \textit{TPS2} loci exhibit different phenotypes as compared to the homodiploids (Fig. 4), indicating that the effective concentrations of intermediates of the trehalose synthesis pathway may have a role in signaling.

The next question was to determine the pathway through which \textit{TPS1} acted. Studies in \textit{C. albicans} have demonstrated that \textit{TPS} enzymatic activity was higher in the \textit{gpr1Δ} strain. Further a synergistic action between \textit{TPS2} and \textit{GPR1} had been proposed in virulence (Maidan et al. 2008). To determine if there was any interaction between \textit{GPR1} and \textit{TPS1} or \textit{TPS2}, in \textit{S. cerevisiae}, pseudohyphal growth of \textit{tps1gpr1} and \textit{tps2gpr1} mutants was monitored. Both the haploid as well as the diploid strains of the \textit{tps1gpr1} double mutant exhibited a growth defect on high glucose (data not shown). However, in SLALD medium, the filamentation defect of \textit{tps1} mutant was overcome upon disruption of \textit{GPR1} (Fig. 5 and Fig. 6), indicating that \textit{GPR1} was epistatic over \textit{TPS1} in signaling for pseudohyphae via the cAMP-PKA pathway. This is corroborated by the observation that extraneous addition of cAMP overcomes the filamentation defect of the \textit{tps1} mutant (Fig. 7). It was demonstrated earlier that \textit{GPR1} suppressed filamentation under low glucose conditions (Iyer et al. 2008). The current observations indicate that \textit{GPR1} is epistatic over \textit{TPS1} in signaling for filamentation when glucose is limiting. Although, the individual effects of \textit{tps2} or \textit{gpr1} mutation is enhanced pseudohyphae, the combined effect results in reduction of filamentation (see \textit{tps2/tps2} in Fig. 3, \textit{gpr1/gpr1} and \textit{gpr1/gpr1 tps/tps2} in Fig. 5). This is as opposed to that
observed in C. albicans where mutation in TPS2 and GPR1 results in a synergistic effect in suppression of filamentation (Maidan et al. 2008).

**TPS1 and TPS2 have a differential role in regulating RAS2 depending upon nutrient availability**

It has been observed that *tps1* mutation results in activation of *RAS2*. However, the effect of *tps2* mutation on *RAS2* is not known. In order to determine whether the effect on *RAS2* is limited only to *TPS1* or does *TPS2* also have a role, *RAS2* was disrupted in the *tps1* as well as the *tps2* mutant. Interestingly, disruption of *tps2* but not *tps1* restored pseudohyphae formation in the *ras2* mutant (Fig. 8). This phenotype was enhanced in SLALD as compared to SLAD medium (compare left and right panels of Fig. 8). Therefore, expression of Ras2p was monitored under nutrient complete (YPD medium) as well as nutrient depleted (SLALD medium) conditions in the diploid strains of *tps1Δ* and *tps2Δ*. Contrary to earlier reports in the haploid *tps1* mutant where *RAS2* is activated in glucose causing the growth defect (Peeters et al., 2017), we observed that Ras2p expression was suppressed in the diploid *tps1* mutant in YPD medium (Fig. 9). This meant that regulation of Ras2p by *TPS1* resulted in opposite effects in the haploid versus the diploid cell. Our observations indicated that this mutant was unable to form pseudohyphae. Since, Ras2p is suppressed in high glucose in a diploid *tps1* mutant, the defect in pseudohyphae formation is probably due to repression of Ras2p. In SLALD medium, however, Ras2p expression was suppressed in *tps2* but not *tps1* mutant (Fig. 10). Thus indicating that *TPS1* and *TPS2* regulate *RAS2* differentially under conditions of nutrient abundance or depletion. Given that the *ras2tps2* double mutant puts forth pseudohyphae in SLALD medium, it is possible that *TPS1* and *TPS2* regulate *RAS2* to appropriate levels for filamentation by exerting opposing effects.
DISCUSSION

Trehalose has been shown to perform a range of functions in the cell (Perfect et al. 2017), eventually leading to stress protection. Although there is a large body of data available on the deleterious effects of TPS1 or TPS2 mutations in S. cerevisiae, the exact mechanism of action of trehalose is unclear. Gibney et al. (2015) demonstrated that phenotypes of the tps1 mutant could not be reversed by simply increasing intracellular concentration of trehalose. This meant that the phenotypes were not due to the depletion of intracellular trehalose concentration per se. It is possible that the trehalose pathway exerts a more complex metabolic effect on the physiology of the cell.

It has been demonstrated that both TPS1 and TPS2 are essential for sporulation (De Silva-Udawatta and Cannon 2001). It is possible that these enzymes of the trehalose pathway have a signaling function in addition to the metabolic effect. Our observations unravel a hitherto unknown role for the trehalose pathway in regulation of pseudohyphal differentiation in yeast. We hypothesize that TPS1 and TPS2 coordinate to regulate the expression of RAS2 based on glucose availability and thereby affect the downstream concentration of cAMP. It is thus possible that components of the trehalose biosynthetic pathway determine whether the cell goes into pseudohyphal differentiation or sporulation in response to nutritional stress. This is in accordance with an earlier observation that intracellular concentration of trehalose is inversely proportional to pseudohyphae formation (Iyer et al. 2008).

Available literature indicates that TPS1 and its orthologues occur in a wide range of organisms and play an important role in development and differentiation. TPS1/2 regulate dimorphic transition and thereby virulence in Candida albicans (Van Dijck et al. 2002) as well as Magnaporthe grisea (Wilson et al. 2007). TPS1 has also been implicated in the virulence of other pathogens such as Aspergillus and Cryptococcus (Perfect et al. 2017). There is evidence to show that TPS genes regulate multiple processes involved in growth and development in plants as well (Satoh-Nagasawa et al. 2006, Chary et al. 2008). It is significant to note that this pathway, which is important for virulence and stress resistance of several pathogenic fungi, does not exist in mammals. Thus, understanding the nuances of the pathway have far reaching consequences in developing novel antifungal targets. Since it has been observed that trehalose increases protein stability under stressful conditions including hypoxia, it also has a potential to be applied in therapeutics for mammalian cell injury due to hypoxia or anoxia.
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**Table 1**: Strains generated in this study: Haploids are listed below. The corresponding diploids were used for pseudohyphal growth assay.

| Genotype                                | Reference                        |
|-----------------------------------------|----------------------------------|
| `ura3-52/ura3-52 MATa/α` *(Wild type)*  | Lorenz and Heitman (1997)        |
| `tps1::G418 ura3-52 MATa`               | This study                       |
| `tps2::G418 ura3-52 MATa`               | This study                       |
| `tps1::G418  tps2::G418 ura3-52 MATa`   | This study                       |
| `tps1::G418  gpr1::G418 ura3-52 MATa`   | This study                       |
| `tps2::G418  gpr1::G418 ura3-52 MATa`   | This study                       |
| `tps1::G418  ras2::hph ura3-52 MATa`    | This study                       |
| `tps2::G418  ras2::hph ura3-52 MATa`    | This study                       |
FIGURE LEGENDS:

**Fig. 1** Glucose growth phenotype of the haploid tps mutants. Three dilutions (five-fold) were spotted.

**Fig. 2** Glucose growth phenotype of the diploid tps mutants. Three dilutions (five-fold) were spotted.

**Fig. 3** Pseudohyphal growth phenotype of the tps mutants on SLAD and SLALD media as indicated. Three independent colonies are shown.

**Fig. 4** Pseudohyphal growth phenotype of the heterodiploid *tps* mutants on SLAD as well as SLAD medium as indicated. The three images represent independent colonies.

**Fig. 5** Filamentation of the *tps* mutants in the background of *gpr1* disruption. Three independent colonies are shown.

**Fig. 6** Pseudohyphae formed by the *tps1gpr1* double mutant is enhanced on prolonged incubation of 8 days. Three independent colonies are shown.

**Fig. 7** Effect of extraneous addition of cAMP on filamentation of the *tps1* mutant. The three images represent independent colonies.

**Fig. 8** Pseudohyphal growth phenotype of the *tps* mutants in the background of *ras2* mutation. Three independent colonies are shown.

**Fig. 9** Expression of Ras2p in the *tps* mutants grown in YPD medium.

**Fig. 10** Expression of Ras2p in the *tps* mutants grown in SLALD medium.
Figures

**Figure 1**
Glucose growth phenotype of the haploid tps mutants. Three dilutions (five-fold) were spotted.

**Figure 2**
Glucose growth phenotype of the diploid tps mutants. Three dilutions (five-fold) were spotted.
Figure 3

Pseudohyphal growth phenotype of the tps mutants on SLAD and SLALD media as indicated. Three independent colonies are shown.

Figure 4

Pseudohyphal growth phenotype of the heterodiploid tps mutants on SLAD as well as SLAD medium as indicated. The three images represent independent colonies.
Figure 5

Filamentation of the tps mutants in the background of gpr1 disruption. Three independent colonies are shown.
Figure 6

Pseudohyphae formed by the tps1gpr1 double mutant is enhanced on prolonged incubation of 8 days. Three independent colonies are shown.
**Figure 7**

Effect of extraneous addition of cAMP on filamentation of the tps1 mutant. The three images represent independent colonies.
Figure 8

Pseudohyphal growth phenotype of the tps mutants in the background of ras2 mutation. Three independent colonies are shown

|        | WT     | ras2Δ   | tps1Δ   | tps2Δ   |
|--------|--------|---------|---------|---------|
|        | /ras2Δ | /tps1Δ  | /tps2Δ  |         |

![Image showing WT, ras2Δ, tps1Δ, and tps2Δ growth phenotypes](image)

Ras2p

G6PD

Cells grown in YPD

Figure 9

Expression of Ras2p in the tps mutants grown in YPD medium
Expression of Ras2p in the tps mutants grown in SLALD medium

Figure 10

Expression of Ras2p in the tps mutants grown in SLALD medium