Specificity of MAPK signaling towards *FLO11* expression is established by crosstalk from cAMP pathway

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Abstract In budding yeast, elements of a single MAP Kinase cascade are shared to regulate a wide range of functions such as mating, differentiation and osmotic stress. However, cells have programmed to execute correct event in response to a given input signal without cross activating other responses. Studies have observed that magnitude and duration of MAPK activation encodes specificity. Similarly, the differential regulation of Tec1p, a transcriptional activator of invasive growth gene, *FLO11* by MAP kinases has been observed to bring specificity in mating and invasive growth signaling. However, the understanding of interactions between the shared components and other signaling pathways related to the phenotypic response in contributing towards specificity remains unclear. We specifically address the crosstalk of cAMP pathway with MAPK pathway in haploid invasive growth and show the contribution and importance of cAMP pathway towards invasive growth irrespective of the activation status of MAPK pathway. Our analysis shows that crosstalk from cAMP pathway in haploids might offer an advantage in terms of amplifying the observed weak signaling through MAPK pathway. Further, we show that such a crosstalk in haploids leads to higher *FLO11* expression than diploids. We also demonstrate the positive and

negative role of Tpk1 and Tpk3 in haploid invasive growth. Finally, we observe that a cross-inhibition at gene level brought about by cAMP pathway controlled inhibitor, Sfl1, perhaps help in deamplifying the MAPK signal and also in preventing *FLO11* expression in the absence of cAMP pathway activation.

Keywords Crosstalk · Invasive growth · Pheromone · Signaling pathway · Steady state modeling · Systemic properties

Introduction

Biological networks involving multiple signaling pathways often crosstalk resulting in complexity. Crosstalk between signaling pathways is critical in deciding the outcome of the final phenotypic response. Crosstalk helps in providing systemic properties such as integrating multiple signals and signal amplification. These interactions also help in controlling the gene expression. In *Saccharomyces cerevisiae*, filamentous growth network comprising of cAMP and MAPK pathway is shown to control a flocculin gene, *FLO11* expression (Liu et al. 1993; Pan and Heitman 1999; Rupp et al. 1999). *FLO11* is shown to mediate pseudohyphae growth in diploids under nitrogen starvation and a related phenomenon called haploid invasive growth under glucose starvation. (Roberts and Fink 1994; Lo and Dranginis 1998; Rupp et al. 1999; Cullen and Sprague 2000). Simulations have shown that crosstalk between these two pathways is a key regulatory design necessary to control *FLO11* expression (Neelajan et al. 2007). However, the significance of the crosstalk in haploid invasive growth remains unclear. We address this issue using a steady state model.

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Invasive and pseudohyphal growth is characterized by unipolar budding pattern and by cells that remain attached after budding to form filaments (Gimeno et al. 1992; Roberts and Fink 1994; Cullen and Sprague 2000). Haploids cells display stronger adhesiveness than diploid cells and filaments of haploids penetrate solid agar more efficiently (Roberts and Fink 1994). This difference in haploid and diploids is attributed to the transcript levels of *FLO11* under different activating conditions (Rupp et al. 1999; Braus et al. 2003). Activation of cAMP and MAPK pathways direct the cells to activate two different transcriptional activators Flo8p and Ste12p/Tec1p, respectively, which binds to the *FLO11* promoter (Madhani and Fink 1997; Lo and Dranginis 1998; Rupp et al. 1999). Both Ras2p and Gpa2p act as upstream activator of cAMP pathway to activate adenylate cyclase, which in turn elevates intracellular cAMP concentration (Fig. 1). cAMP

activates the yeast PKAs, each of which consists of a catalytic subunit Tpk1/2/3p, and a cAMP-binding regulatory subunit, Bcy1p (reviewed in Gagiano et al. 2002; Palecek et al. 2002). Tpk2p is shown to have a dual function in *FLO11* expression. It regulates the activities of the transcriptional activator Flo8p and a transcriptional repressor, Sfl1 (Robertson and Fink 1998; Pan and Heitman 1999; Conlan and Tzamarias 2001). Tpk1/3p is shown to inhibit both pseudohyphae and invasive growth, where *Δtpk1/3* mutant strain was shown to enhance the phenotype (Robertson and Fink 1998; Pan and Heitman 1999). Hydrolysis of cAMP by cAMP-phosphodiesterases Pde1p and Pde2p restores PKA to the inactive state. Studies have shown that PKA mediated phosphorylation of phosphodiesterase, Pde1p brings about feedback inhibition of cAMP synthesis (Pingsheng et al. 1999). Further, Ras2p is also shown to activate MAPK cascade which comprises of protein

Fig. 1 A simplified network involved in the invasive growth in haploids. Nutrient signal activates Ras2p and Gpa2p, which binds to adenylate cyclase to signal through cAMP-PKA cascade. The signaling through cAMP-PKA cascade includes cAMP binding to PKA complex to release its catalytic subunits Tpk1/2/3p from regulatory subunit Bcy1p. Tpk2p phosphorylates Flo8 and an inhibitor Sfl1 to activate *FLO11*, a flocculin gene involved in invasive growth. Pde1 and Pde2 are cAMP—phosphodiesterases which hydrolysis cAMP to AMP. Tpk1/3 exerts a negative feedback by activating Pde1. Activated Ras2p

can also signal through MAPK cascade to activate Kss1p (MAPK). The signaling through MAPK cascade includes Ste20 (MAPKKKK), Ste11 (MAPKKK) and Ste7 (MAPKK). The activated Kss1p activates Ste12p/Tec1p, another transcriptional activator of *FLO11*. Tpk1/2/3 of cAMP pathway cross-activates Ste12p/Tec1p. The pheromone signal activates GPCR system, which signals through elements of the same MAPK cascade to activate Fus3, which brings about degradation of Tec1 and activation of Ste12p. The Ste12p binds to pheromone response element (PRE), thereby helping in mating specific functions

kinases Ste20p (MAPKKKK), Ste11p (MAPKKK) and Ste7p (MAPKK) (Mosch et al. 1996) (Fig. 1). Ste7p mediated phosphorylation of Kss1 (MAPK) is required for efficient invasive growth. Kss1p activates Ste12 by phosphorylating it, which in turn activates the transcription of *TEC1* (Bardwell et al. 1994). Transcriptional activators Ste12p and Tec1p bind cooperatively to filamentous growth response elements (FREs) identified in the *FLO11* promoter (Madhani and Fink 1997; Rupp et al. 1999).

Under non-inducing conditions, *FLO11* promoter region is embedded in the tightly packed chromatin structure with a region for binding of an inhibitor, Sfl1 (Fischer 2005). Inhibitor serves as an antagonist to chromatin remodeling and thereby inhibits *FLO11* transcription (Fleming and Pennings 2001; Fischer 2005). Desilencing of *FLO11* promoter takes place with removal of the inhibitor by cAMP pathway and then followed by chromatin remodeling activity under inducing conditions (Robertson and Fink 1998; Fleming and Pennings 2001; Fischer 2005). A particular intriguing aspect of this network is the involvement of the transcriptional activator Ste12p in both mating and invasive growth in haploids (Fields and Herskowitz 1987; Roberts and Fink 1994). These two developmental programs are activated by the same three-step MAPK Cascade (Liu et al. 1993; Mosch et al. 1996). The specificity in MAPK signaling has been studied extensively with regard to pheromone response and invasion pathway (Madhani and Fink 1997; Schwartz and Madhani 2004; Elion et al. 2005; Flatauer et al. 2005). A well established fact that brings specificity in these phenotypic states is the differential regulation of Tec1p, a transcriptional activator that bind cooperatively with Ste12 to filamentous growth response elements (FREs) by two MAPKs, Fus3p and Kss1p confers signaling specificity to mating and invasive growth, respectively (Bao et al. 2004; Bruckner et al. 2004).

Our previous steady state modeling of the regulatory network involved in *FLO11* expression demonstrated that *FLO11* expression is highly sensitive to cAMP pathway compared to MAPK pathway and further, the activation level of cAMP pathway primarily controls *FLO11* expression compared to MAPK pathway (Neelajan et al. 2007). In addition, we also demonstrated that a minimum activation level of MAPK pathway is required for *FLO11* expression. However, the significance of our earlier results with regard to haploid invasive growth was not analyzed. It is also interesting to note that a low concentration of pheromone induces invasive growth in haploids whereas higher concentration does not, indicating that the magnitude of MAPK signaling activation brings about difference in these phenotypic states (Erdman and Snyder 2001). Surprisingly, in haploids, a cross-activation of MAPK pathway by cAMP pathway has been reported (Mosch et al. 1999). A hyperactive PKA in the absence of Ras2p is

shown to stimulate expression of filamentous response element (FRE) reporter gene to levels comparable to strains with an activated MAPK pathway (Mosch et al. 1999). Also, overexpression of any of the Tpk's were also shown to stimulate FRE and as well as invasive growth. This is interesting considering the fact that Tpk1/3 is shown to have negative effect on invasive growth (Robertson and Fink 1998). Further, different experiments in diploids have failed to observe the presence of cross activation of MAPK pathway by cAMP pathway (Mosch et al. 1996, Lorenz and Heitman 1997). Therefore, we directed our analysis in understanding the significance and role of this crosstalk in haploids with regard to MAPK pathway specificity towards *FLO11* expression.

Our analysis shows that crosstalk from cAMP pathway amplify the basal level of signaling through MAPK pathway in haploid invasive growth. We show that the cross-activation of MAPK pathway in haploids helps in increasing the *FLO11* expression by overcoming the weak signaling through MAPK pathway. Our analysis indicates that such a cross-activation in diploids is limited due to observed low total Ste12p concentration, which results in weak *FLO11* expression. We demonstrate that overexpression of Tpk1/3 in haploids and diploids bring about different phenotypic responses. Further, we observe that cross-inhibition at the gene level brought about by the inhibitor controlled by cAMP pathway deamplify the signal to regulate the *FLO11* expression.

Methods

Signaling pathways involved in pseudohyphae growth also take part in the regulation of haploid invasive growth and is not cell type specific. Therefore, the simulations of the regulatory network involved in the haploid invasive growth were performed with reduced number of components including all the key biomolecular interactions of the network (Fig. 1). For a detailed steady state model of yeast filamentous growth network see Neelajan et al. 2007. In order to characterize the steady state behavior of cAMP pathway, the components of cAMP-PKA activation module involving activation of Tpk1/2/3 by activated adenylate cyclase along with negative feedback inhibition of cAMP synthesis by PKA were considered (Fig. 1).

Further, in order to characterize the steady-state behavior of the complete MAP kinase cascade, we fit the signal response with a Hill curve for Kss1p activation by activated Ras2p (i.e. Ras2GTP) through MAPK cascade.

$$Kss1p = \frac{Kss1_{max} Ras2GTP^{nH}}{K_{0.5}^{nH} + Ras2GTP^{nH}} \quad (1)$$

where Hill's Coefficient, $nH = 2$ and half saturation constant, $K_{0.5} = 5$ nM. These parameters were estimated from Neelajan et al. 2007. Kss1p then activates the Ste12Tec1 transcription complex by phosphorylating it to Ste12p-Tec1p. It is assumed that Ste12p and Tec1p are pre-associated in a complex to activate *FLO11* expression. In haploids, MAPK cascade also activates the transcriptional activator Ste12 for regulating mating specific functions in response to pheromone binding to G-protein coupled receptor (Bardwell et al. 1994; Bardwell 2005) (Fig. 1).

In case of haploid invasive growth, Tpk1/2/3 of cAMP pathway cross activates the MAPK pathway controlled transcriptional activator Ste12Tec1 to express *FLO11* (Mosch et al. 1999). Therefore, the rate equation for Ste12Tec1 activation by Tpk1/2/3 and Kss1p at steady state is given by

$$k5 \times [Ste12Tec1] \times \left[\frac{Tpk1}{Km5} + \frac{Tpk2}{Km6} + \frac{Tpk3}{Km7} + \frac{Kss1p}{Km8} \right] = k6 \times [Ste12pTec1p] \times \frac{E6}{Km9} \quad (2)$$

'k5' and 'k6' are the rate constant for phosphorylation and dephosphorylation reaction, respectively. 'Km5/6/7' and 'Km9' are their respective Michaelis–Menten constants. 'E6' represents phosphatase. It is assumed that all the Tpk have equal affinity towards Ste12Tec1.

The fractional transcriptional expression of *FLO11* was quantified by Eq. 3 (Neelajan et al. 2007).

$$f_{FLO11} = \frac{FLO11_Ste12pTec1p_Flo8p}{FLO11t} \quad (3)$$

FLO11_Flo8p_Ste12p_Tec1p represents *FLO11* with both Flo8p and Ste12pTec1p complex bound to it and *FLO11_t* is the total *FLO11* concentration.

The fractional activation of Flo8 and Ste12Tec1 complex will give the measure of fractional activation of these pathways and could be varied to study the effect of these pathways on *FLO11* expression. The fractional activation of Flo8 is given by Eq. 4

$$f_{Flo8p} = \frac{Flo8p}{Flo8t} \quad (4)$$

where *Flo8p* is the fraction of the total Flo8 concentration (*Flo8_t*) in the phosphorylated state. Similarly, the fraction of the total Ste12Tec1 complex concentration in the activated (phosphorylated) state is given by Eq. 5.

$$f_{Ste12pTec1p} = \frac{Ste12pTec1p}{Ste12pTec1t} \quad (5)$$

The framework reported by Goldbeter and Koshland was used to model the network at steady state and

accordingly an equivalent rate constant and Michaelis–Menten constant nomenclature scheme was applied (Koshland et al. 1982). The steady state equations for covalent modification cycles, equilibrium relationships for allosteric interactions, and mass balance equations for total species are listed in the supplementary material. These equations were solved numerically using Fsolve program of MATLAB (The Math-Works Inc.). The accuracy of the simulation was verified by numerically checking the mass balance of all species. All component enzyme concentrations are represented with respect to the whole cell volume. Most of the kinetic/equilibrium constants were taken from the literature. The reactants like ATP and PPi concentrations were assumed to be constant. The total component concentrations, rate constants, the Michaelis–Menten constant and dissociation constants are listed in the supplementary material along with references.

Results

Cross-activation of MAPK pathway by cAMP pathway

The steady state model was used to study the effect of cross-activation of MAPK pathway by cAMP pathway on *FLO11* expression in haploids. The fractional activation of the most downstream activators of cAMP and MAPK pathway were given as input to the system and the dose responses were evaluated with and without the cross-activation of MAPK pathway by cAMP pathway. The cross-activation of MAPK pathway by Tpk occurs at the level of activation of Ste12Tec1 by Kss1p, a downstream kinase of MAPK pathway. Figure 2a shows the predicted dose response curves with respect to Kss1p. The cross activation of Ste12Tec1 complex by PKA of cAMP pathway was varied by varying the 'Km' value of Tpk for the transcriptional activator Ste12Tec1 complex (see Eq. 2). The *FLO11* expression increased with activated Kss1p concentration, however the dose response varied with the strength of cross-activation. Figure 2a, curve 'a' shows the response curve without the cross-activation of MAPK pathway. For this case, the Hill's coefficient was 1.8 and the Kss1p concentration required for 90% expression of *FLO11* was 15 nM. For Km = 450 nM, the Kss1p concentration required for 90% expression of *FLO11* was 5 nM with a Hill's coefficient of 1.8 (Fig. 2a, curve d). Hence, 3-fold amplification was observed with the inclusion of cross-activation of MAPK pathway by cAMP pathway, while the sensitivity remained unchanged. The dose response with Km = 300 nM became independent of Kss1p indicating that MAPK pathway is dispensable to *FLO11* expression at this 'Km' value (Fig. 2a, curve g). Figure 2b shows the *FLO11* expression with respect to

Fig. 2 Effect of cross-activation of MAPK pathway by cAMP pathway. (a) Fractional dose response is shown with respect to Kss1p of MAPK pathway, with cross-activation of MAPK pathway by Tpks of cAMP pathway included. Curve 'a' shows the case without cross-activation. The strength of cross activation was varied by varying the 'Km' value of Tpks for the transcriptional activator Ste12Tec1 complex, b (1500 nM), c (600 nM), d (450 nM), e (375 nM), f (330 nM) and g (300 nM). (b) *FLO11* expression with respect to upstream activator Ras2GTP. Curve 'a' represents the case without cross-activation of MAPK pathway, whereas curve 'b'

represents the case with cross-activation ($K_m = 450$ nM). The requirement of Ras2GTP decreases with cross-activation. (c) Fractional dose response with respect to upstream activator of cAMP pathway (Gpa2p) and MAPK pathway (Ras2GTP), when the fractional activation of either pathway was fixed at 20% while the other was varied in presence of cross-activation. Curve 'a' represents dose response when fractional activation of cAMP pathway was restricted to 20%. Curve 'b' represents dose response when fractional activation of MAPK pathway was restricted to 20%

Ras2GTP, upstream activator of MAPK pathway with $K_m = 450$ nM and without cross-activation. In the absence of cross-activation (curve a), the activated Ras2p concentration required for more than 90% *FLO11* expression was 8 nM, whereas in the presence of cross-activation it was 4 nM (curve b), indicating two fold amplification with respect to upstream activator. However, the sensitivity of the response did not vary significantly in the presence and absence of the crosstalk (Hill's coefficient of 3.5 and 3.1, respectively). Figure 2c shows the dose response curves with respect to upstream activators Gpa2p and Ras2GTP of cAMP and MAPK pathway, respectively, when fractional activation of either of the pathways was fixed equal to 20%, while the other was varied in the presence of cross-activation from cAMP pathway. We observe that *FLO11* expression gets restricted to about 30 % when the fractional activation of cAMP pathway was restricted to 20% (Fig. 2c, curve a), while complete expression was observed when MAPK pathway was restricted to 20% (Fig. 2c,

curve b). We observe that a high MAPK activity is dependent on the activation level of cAMP pathway for *FLO11* expression demonstrating that cAMP pathway has a bigger role to play in haploid invasive growth.

Cross-inhibition of *FLO11* expression at the transcriptional level

cAMP and MAPK pathways are also known to crosstalk at the gene level. cAMP pathway controlled inhibitor represses the transcription of the *FLO11* expression. *FLO11* promoter in the silenced state is bound by Sfl1, hence the removal of inhibitor becomes pre-requisite for *FLO11* expression under inducing conditions. Figure 3 shows the dose response curve with respect to Ste12pTec1p complex concentrations. In the presence of inhibitor in the system, the Ste12pTec1p concentration required for 90% *FLO11* expression was 60 nM (Fig. 3, curve a) with a

Fig. 3 Effect of cross-inhibition at transcriptional level. Fractional dose response is shown with respect to transcriptional activator Ste12pTec1p of MAPK pathway. Curve 'a' shows the case when the inhibitor, Sfl1 is present in the system and curve 'b' is the case when inhibitor is absent

Hill's coefficient of 2.5, whereas in the absence of inhibitor the Hill's coefficient was 1.9 and the Ste12pTec1p concentration required was 30 nM (Fig. 3, curve b). This indicated that inhibitor plays a role in deamplifying the signal by two fold without much change in the sensitivity. In the absence of activation of cAMP pathway, activation of MAPK pathway becomes immaterial as inhibitor continues to repress. The role of inhibitor in eukaryotic gene expression becomes important in terms of bringing deamplification in the signal, which helps the cells to respond only if the activator concentration exceeds certain threshold value to bring about gene expression.

Differences in *FLO11* expression in haploids and diploids

Both haploid and diploids use cAMP and MAPK signaling pathways to regulate *FLO11* expression. However, the *FLO11* expression differs significantly in both the strain (Rupp et al. 1999; Braus et al. 2003). Haploids are shown to have higher transcript levels of *FLO11* compared to diploids. Further, the cross-activation of MAPK pathway by cAMP pathway has not been observed in diploids (Mosch et al. 1996; Lorenz and Heitman 1997). It has been reported that the total concentration of Ste12 in diploids is 5–10 fold less than that of haploids (Fields and Herskowitz 1987; Nelson et al. 2003). Therefore, we studied the impact of the crosstalk and the concentration of Ste12 on *FLO11* expression in diploids. Figure 4 shows the dose response curve for diploids (curve a) and haploids (curve c) with respect to an upstream activator Ras2GTP. The response

curve for haploids demonstrated a Hill's coefficient of 3.5 and a half saturation constant of 1.8 nM. At a Ras2GTP concentration of 4 nM, more than 90% of expression was observed. At this concentration of Ras2GTP, the diploids demonstrated only about 25% expression. However, on introducing the crosstalk of Tpk, the *FLO11* expression was enhanced to 50% as seen in the curve 'b' of Fig. 4. This implies that both the factors i.e. crosstalk and lower total Ste12 concentration had a role to play in regulating *FLO11* expression. Considering the fact that diploids depend on *FLO11* expression for pseudohyphae growth as $\Delta flo11$ strain fail to produce pseudohyphae (Lo and Dranginis 1998), it is quite possible that fractional expression observed in our simulation is sufficient for inducing weak *FLO11* expression and hence pseudohyphae growth in diploids. However, the difference between 25 and 50% of *FLO11* expression in terms of pseudohyphae growth might be significant. We also find that the increase in activation levels of MAPK pathway does not increase the *FLO11* expression in diploids significantly because of the limiting amounts of the total Ste12 concentration. Thus, the cross-activation requirement is also negated due to low availability of total Ste12 concentration.

Effect of overexpressing Tpk1/3 on *FLO11* expression

Overexpression of Tpk1/3 has shown to inhibit filamentous growth only in diploids and not in haploids (Mosch et al. 1999; Pan and Heitman 1999). However, $\Delta tpk1/3$ mutation

Fig. 4 *FLO11* expression in haploids and diploids. Fractional dose response is shown with respect to upstream activator Ras2GTP. Curve 'a' represents the *FLO11* expression in diploids, where the total concentration of Ste12 is 5–10 fold less than haploids and cross-activation of MAPK pathway by cAMP pathway is not observed. Curve 'b' shows the case when the cross-activation is included in diploids. Curve 'c' represents the case for haploids, which shows complete expression

has shown to enhance filamentous growth both in haploids and diploids (Robertson and Fink 1998; Pan and Heitman 1999). Therefore, we studied the positive and negative effect of Tpk1/3 on *FLO11* expression in our analysis. Overexpressing Tpk1/3 by 3 fold in our current model drastically reduced the activated Tpk2p and Flo8p concentration through complete loss of PKA activation (similar to Flo8p mutant) due to strong negative feedback on cAMP synthesis. Also, overexpression brought about drastic increase in the activated Ste12pTec1p concentration through cross-activation of MAPK pathway in haploids. However, in both haploids and diploids it resulted in complete loss of *FLO11* expression in our model as we have quantified fractional *FLO11* expression with both Flo8p and Ste12pTec1p bound to it. This difference in haploids was therefore analyzed further by considering a positive role for Tpk1/3 in controlling Flo8p and Sfl1. Studies in diploids have shown that Tpk1/3 may activate *FLO11* expression in $\Delta sfl1tpk2$ mutants by promoting Flo8p binding to *FLO11* promoter, however significant fraction of Flo8p remains unbound (Pan and Heitman 2002). Further, $\Delta tpk2$ mutant completely suppressed *FLO11* expression indicating that Tpk1/3 has lesser effect on Sfl1 deactivation. Therefore, simulations were carried out including Tpk1/3 in the rate equation of Flo8p and Sfl1 (Supplementary information). The parameters were chosen in such way that Tpk1/3 only activates a smaller fraction (10%) of Flo8p and have lesser effect on Sfl1 under wild type conditions. Simulations shows an enhanced *FLO11* expression in diploid $\Delta sfl1tpk2$ mutant condition, which is in accordance with experimental observation (Robertson and Fink 1998; Pan and Heitman 2002). Figure 5 shows the effect of overexpressing Tpk1 in wild type haploids and diploids. The fractional *FLO11* expression observed in diploids (50%) decreases with increasing Tpk1 concentration. The *FLO11* expression is restricted below 10% for Tpk1 concentration between 100 and 400 nM (Curve a). However, the expression starts to increase steadily with further increase in Tpk1. In haploids, the observed *FLO11* expression (90%) decreases below 70 % for Tpk1 concentration between 80 and 400 nM beyond which it starts to increase (Fig. 5 curve b). For an overexpressed Tpk1 concentration of 1000 nM, the haploids show invasive growth (80%), whereas diploids show a restricted pseudohyphal growth (18%) relative to wild type conditions. Haploids and diploids show wild type invasive and pseudohyphae growth, respectively on overexpressing the Tpk1 concentration by 30 fold.

Discussion

In the current analysis, we use steady modeling to quantify a simplified network involving crosstalk with reference to

Fig. 5 Effect of overexpressing Tpk1 in haploids and diploids. Fractional dose response with increasing concentration of Tpk1. Curve 'a' represents the effect of increasing Tpk1 concentration on observed *FLO11* expression in diploids with cross-activation (50 %). Curve 'b' represents the effect of increasing Tpk1 concentration on observed *FLO11* expression in haploids (90%)

haploid invasive growth. Cross-activation of MAPK pathway by cAMP pathway amplifies the signaling through MAPK pathway and further reduces the requirement of MAPK pathway for *FLO11* expression. We observed that an additional input to MAPK pathway by way of cross-activation has a role to play in achieving MAPK specificity towards *FLO11* expression. We also show that the cross-inhibition by cAMP pathway controlled inhibitor at the gene level deamplifies the MAPK signal and has a role in ensuring the repression of *FLO11* expression during strong mating signal through pheromone pathway.

Elements of single MAP-Kinase-Cascade are shared in haploids to regulate a wide range of functions such as mating, differentiation and osmotic stress. In spite of this haploids have programmed to execute correct event in response to a given input signal without cross activating other events (Schwartz and Madhani 2004; Elion et al. 2005). To understand MAPK specificity, it is therefore essential to understand the interaction between the shared components and the crosstalk amongst other signaling pathways relating to the phenotypic response. Earlier studies have shown that degradation of Tec1p, a transcriptional activator which dimerize with Ste12p to bind to *FLO11* by Fus3p under pheromone inducing conditions and absence of it during invasive growth, determines the specificity (Bao et al. 2004). The Kss1p is also shown to be activated under pheromone induction, however its activation is restricted by mating pathway controlled Fus3p (Bruckner et al. 2004). This raises the question regarding the MAPK cascade differentially activating Fus3p and Kss1p. Studies have demonstrated that magnitude and

duration of MAPK activation encodes specificity. A weak, sustained activation of Kss1 was sufficient to induce invasive growth, whereas a strong transient activation of Fus3p and Kss1p was sufficient for pheromone signaling (Sabbagh et al. 2001; Bruckner et al. 2004; Schwartz and Madhani 2004). Our previous studies demonstrated that MAPK pathway was insensitive and was not limiting in the case of *FLO11* regulation (Neelajan et al. 2007). However, mutation in MAPK pathway affects *FLO11* expression (Mosch et al. 1996; Rupp et al. 1999). Therefore, it is quite possible that signaling through MAP kinase pathway could be weak to maintain the required basal activity under constitutive nutrient limitation during invasive growth.

On other hand, the role and contribution of cAMP pathway towards invasive growth has been ignored in studies relating to specificity of MAPK pathway towards *FLO11* expression. Previous studies mainly pertained to understanding the regulation of transcriptional activator Ste12pTec1p, which binds to the filamentous response elements (FRE) under activating conditions (Madhani and Fink 1997; Sabbagh et al. 2001; Bruckner et al. 2004; Schwartz and Madhani 2004). However, none of these studies correlate the *FLO11* expression to invasive growth by considering the activation status of cAMP pathway. It should also be noted that MAPK pathway is dispensable to *FLO11* expression during amino acid starvation, which activates another protein Gcn4p that does the function of Ste12p (Braus et al. 2003). The cAMP pathway is indispensable to *FLO11* regulation irrespective of activation signals as it is shown to regulate a key transcriptional activator Flo8p (Liu et al. 1996; Braus et al. 2003). Our previous study also demonstrated that cAMP pathway was limiting and was a major controller of *FLO11* expression (Neelajan et al. 2007). The significance of these observation increases with an identification of cross activation of MAPK pathway by cAMP pathway in haploids (Mosch et al. 1999). Hence, the cross activation of MAPK pathway by cAMP pathway is significant to *FLO11* expression.

We observe that cells have programmed to activate MAPK pathway weakly during invasive growth to prevent activation of pheromone signaling. At the same time the crosstalk from cAMP pathway amplifies the weak signal at the downstream of MAPK pathway without actually disturbing the activation status of the MAPK cascade. Such a crosstalk gives the cell an option to keep MAPK pathway activation as minimum as possible without affecting the final phenotypic response, thereby ruling out any possibility to switch on mating specific functions. This suggests that the haploids have found a simple strategy to take advantage of crosstalk from cAMP pathway in overcoming the restriction of MAPK pathway signaling specificity.

On other hand, diploid cells cannot respond to mating pheromone as they do not express components of

pheromone signaling pathway (Liu et al. 1993) and therefore diploids are devoid of the crosstalk from cAMP pathway. This also accounts for the fact that the total concentration of Ste12 in diploids is less compared to haploids. Therefore, the *FLO11* expression is weak in diploids compared to haploids. The minimal activation of MAPK pathway might result in minimal activated Ste12p concentration much below than the threshold concentration due to the limitation in the total Ste12 concentration. This results in a very low *FLO11* expression (about 25%). Whereas, in the presence of crosstalk from cAMP pathway, the expression level increases to 50% which could be sufficient to support less adhesive pseudohyphae growth. It is possible that the effect of crosstalk may not be observed due to a direct consequence of low total Ste12 concentration in diploids. Thus, the difference observed in *FLO11* expression in diploid and haploid may be mainly due to lower total Ste12 concentration.

In addition to cross-activating the MAPK pathway, the activation status of cAMP pathway also controls an inhibitor of *FLO11* expression, the removal of it is primary to *FLO11* expression. Therefore, at gene level, inhibitor can prevent *FLO11* expression even if MAPK pathway gets activated and if cAMP pathway remains to be inactive. Presence of inhibitor in the system helps in deamplifying the signal and therefore requiring higher activated concentration of transcriptional activator. In the absence of inhibitor in the system, the expression takes place at lower concentration of the transcriptional activator. Therefore, presence of inhibitor serves to tighten the regulation of the gene and more importantly it can give resistance to noise or signal spill over from other pathways. Such a regulation will be useful for the genes that needs a tight regulation.

Further, overexpression of Tpk1/3 in haploids and diploids bring about different responses. Overexpression of Tpk1/3 in haploids inhibits *FLO11* expression through a negative feedback on cAMP pathway, however the inhibition is insignificant relative to wild type response (see schematic Fig. 6). Tpk1/3 overexpression also increases the cross-activation of MAPK pathway by an increase in the Ste12pTec1p concentration and will partly activate Flo8p thereby reducing the level of inhibition. The *FLO11* expression would still be inhibited since the Tpk1/3 has lesser effect on inactivating the inhibitor Sfl1 under these conditions. Further, overexpression of Tpk1/3 is essential to inactivate Sfl1 significantly to demonstrate a wild type *FLO11* expression (see schematic Fig. 6). However, in case of diploids, due to limiting Ste12 concentration, the activation of *FLO11* under overexpression condition is lower than that of wild type. Hence, Tpk1/3 functions to inhibit *FLO11* expression under physiological conditions, whereas its positive role on Flo8p and Sfl1 is less significant in the presence of Tpk2.

Fig. 6 Interaction network in haploids. Tpk1/3 exerts negative feedback on cAMP pathway, however their inhibition is not complete. Tpk1/3 functions positively to activate Ste12Tec1 along with Tpk2 thereby helping in MAPK signal amplification. Flo8 phosphorylation by Tpk2 is significant as compared to Tpk1/3 ($Tpk2_{Flo8} \gg Tpk1/3_{Flo8}$). Sfl1 phosphorylation (deactivation) strongly depends on Tpk2. Tpk1/3 has a very less role to play in terms of deactivating Sfl1 as compared to its role in phosphorylating Flo8 ($Tpk1/3_{Flo8} \gg Tpk1/3_{Sfl1}$). Tpk1/3 overexpression or a $\Delta sfl1tpk2$ mutation can demonstrate the positive effect because in the presence of Tpk2 their positive role is not observed. Similarly, $\Delta tpk1/3$ mutation can demonstrate the negative effect of Tpk1/3

In this context, it will be also interesting to study the activation status of cAMP pathway at lower concentration of pheromone which is actually shown to activate invasive growth (Erdman and Snyder 2001). However, higher concentrations of pheromone are shown to inhibit cAMP synthesis and the Ras dependent activity which could eventually help in blocking invasive growth along with pheromone mediated degradation of Tec1 by Fus3p (Arkinstall et al. 1991; Papasavvas et al. 1992). Also, the upstream of pheromone signaling, which is G-protein coupled receptor, and that of invasive growth, which is Ras2p, are programmed to produce strong and weak stimulus of MAPK pathway contributing to its specificity. Finally, we observe that an additional input from cAMP pathway in invasive growth further establishes the specificity of MAPK pathway. The establishment of specificity of a signaling pathway is therefore complex and requires a system level approach including all the components involved to analyze a specific phenotype.

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