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A modelling approach to quantify dynamic crosstalk between the pheromone and the starvation pathway in baker's yeast

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Note
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Cells must be able to process multiple information in parallel and, moreover, they must also be able to combine this information in order to trigger the appropriate response. This is achieved by wiring signalling pathways such that they can interact with each other, a phenomenon often called crosstalk. In this study, we employ mathematical modelling techniques to analyse dynamic mechanisms and measures of crosstalk. We present a dynamic mathematical model that compiles current knowledge about the wiring of the pheromone pathway and the filamentous growth pathway in yeast. We consider the main dynamic features and the interconnections between the two pathways in order to study dynamic crosstalk between these two pathways in haploid cells. We introduce two new measures of dynamic crosstalk, the intrinsic specificity and the extrinsic specificity. These two measures incorporate the combined signal of several stimuli being present simultaneously and seem to be more stable than previous measures. When both pathways are responsive and stimulated, the model predicts that (a) the filamentous growth pathway amplifies the response of the pheromone pathway, and (b) the pheromone pathway inhibits the response of filamentous growth pathway in terms of mitogen activated protein kinase activity and transcriptional activity, respectively. Among several mechanisms we identified leakage of activated Ste11 as the most influential source of crosstalk. Moreover, we propose new experiments and predict their outcomes in order to test hypotheses about the mechanisms of crosstalk between the two pathways. Studying signals that are transmitted in parallel gives us new insights about how pathways and signals interact in a dynamical way, e.g., whether they amplify, inhibit, delay or accelerate each other.

Cells respond to their environment based on external cues. A great variety of receptors exist that are able to sense all kinds of stimuli and trigger corresponding responses in the cell through signalling pathways. However, life is complex and in order to make the right decisions concerning growth, proliferation, stress response, etc., cells must not only be able to process multiple information in parallel but also to combine and integrate this information. It can be expected that a cell's response to multiple stimuli is not just the sum of the individual responses but that signals suppress or amplify each other according to their respective importance. This is achieved by wiring signalling pathways in such a way that they can interact with each

Abbreviations
PP, double phosphorylated; FREP, filamentation response element product; K, kinase; MAP, mitogen activated protein; PREP, pheromone response element product.

other, a phenomenon often called crosstalk. Many different ways of pathway interactions have been described in the literature [1–3]. An important question in cell biology is how these systems transduce different extracellular stimuli to produce appropriate responses despite or in exploitation of pathway interactions.

There have been attempts to quantify crosstalk in signalling networks. In one study crosstalk was categorized by a classification of the input-output relations of signalling networks [4]. Quantification consisted of counting the occurrence of each category in a pairwise comparison of pathways. Another study quantified the degree of crosstalk between two pathways by relating the number of realized interactions between two pathways to the number of hypothetically possible interactions [5]. This definition was restricted to pathways that do not share components. Both studies considered topological and structural properties of signalling networks and did not account for temporal and dynamic aspects. Another study analysed the steady state properties of two simple dynamic three-step kinase cascades with a shared component and concluded that with the proposed wiring scheme selective activation was possible without physical separation of the two cascades [6]. However, an analysis of the temporal behaviour of the two cascades shows that both pathways will always be activated even though not at the same time but subsequently. Thus, in order to understand crosstalk mechanisms, the dynamic behaviour of interacting pathways is important, even more because it is the transient dynamic behaviour that is important in signalling rather than the static or steady state features.

A recent study addressed this problem proposing measures of dynamic crosstalk [7]. By analysing the activation of pathways by the intrinsic and an extrinsic stimulus, respectively, they defined measures for pathway specificity and fidelity. These measures give useful insights into how pathways interact with each other. However, it is important to note that these measures refer to responses to one stimulus at a time. These measures give no clue of how signals interact while being transmitted concomitantly. It can be expected that signals amplify or inhibit each other, when transmitted at the same time. Thus, to understand how signals interact dynamically it does not suffice to study each signal in isolation but also to study the cell's response to multiple stimuli at the same time.

The aim of this study was twofold. First, we wanted to map existing literature to a mathematical model to study the dynamic behaviour of two experimentally well characterized pathways and their interactions, i.e., the pheromone and filamentous growth pathway in bakers yeast. Second, we wanted to analyse and compare measures of dynamic crosstalk.

The mathematical model described here has been submitted to the Online Cellular Systems Modelling Database and can be accessed free of charge at http://jjj.biochem.sun.ac.za/database/schaber/index.html.

Discussion

We developed a dynamic mathematical model that represents current knowledge about the wiring of the pheromone pathway and the filamentous growth pathway in yeast. We concentrated on the main dynamic features and the interconnections between the two pathways and on a limited temporal scope. Moreover, we defined new measures of dynamic crosstalk, analysed their relations and conducted simulation studies to explore the contributions of several pathway interactions to crosstalk. As the kinetics of the considered reactions are largely unknown, our results must be viewed with respect to the chosen set of parameters. However, the important dynamic features of the model resembled what is known from experiments and were robust to single parameter perturbation (Fig. 3).

We defined new measures of crosstalk, i.e., intrinsic specificity S_i and extrinsic specificity S_e that yield a better understanding of how the two pathways dynamically interact because they consider the combined response of several signals. Crosstalk, in our view, is not something that cells must avoid but rather it is indispensable in order to trigger the appropriate response to multiple simultaneous stimuli. Thus, it is instructive to analyse signal transduction of several pathways in parallel, because this is what the cell has to face.

The new crosstalk measures characterize how the cells integrate different signals when being transmitted concomitantly. Concerning the pheromone response, they indicate that both signals amplify each other. This result could already be anticipated from the wiring scheme of the pathways, because it contains no direct inhibition of the pheromone pathway by the filamentous growth pathway. In the case of the filamentous growth pathway, however, we saw a crossinhibition by the pheromone pathway. This result was not clear just by studying the wiring scheme, because we considered several promoting and inhibiting influences of the pheromone pathway on the filamentous growth pathway, whose overall effect is not obvious. Our new crosstalk measures complement already existing crosstalk measures and give additional information by a single number that integrates complex time courses in a conceivable and interpretable way. However, it must be stressed that our proposed interpretations of the new crosstalk measures only mirror a phenomenological description of the considered outputs. If the wiring

scheme is not known, these measures do not allow deriving conclusions about actual molecular interactions. Sensitivity analysis indicated that the new crosstalk measures are more stable than the other crosstalk measures, probably because by integrating both inputs they mutually buffer sensitivities of the other pathway.

For the pheromone pathway the Komarova-specificity S_K is less than one, meaning that the pheromone stimulus activates its extrinsic response stronger than its intrinsic response. This result is not intuitive. It exemplifies that activation profiles of different components can hardly be compared because in the model these depend strongly on the parameters, and biologically an access of component A over component B does not necessarily mean that component A has a stronger impact than component B.

In experimental and theoretical studies, the crosstalk measures C (or F), S_i and S_e (Table 1) can relate the activation profile of one specific component to different stimuli and allow drawing a conclusion about how pathways interact in a dynamical way and how signals are thereby modulated.

The newly proposed crosstalk measures S_i and S_e can be generalized to more than two interacting pathways. Suppose we have n stimuli f_1, …, f_n corresponding to n intrinsic responses X_1, …, X_n. The intrinsic specificity of pathway k, $S_i(k)$, i.e., a measure of how extrinsic signals influences the intrinsic signals when acting in parallel, can be defined as

$$S_i(k) = \frac{X(f_k)}{X(f_1, \ldots, f_n)}$$

and the extrinsic specificity of pathway k, $S_e(k)$, i.e., a measure of how the intrinsic signal influences the extrinsic signals when transmitted in parallel, can be defined as

$$S_e(k) = \frac{X(f_1, \ldots, f_{k-1}, f_{k+1}, \ldots, f_n)}{X(f_1, \ldots, f_n)}$$

From the Monte Carlo analysis we conclude that it is most instructive to use the time integral I as a measure for activation. First, the integral is biologically meaningful, because it represents the total amount of activated species, which were produced during the presence of a stimulus. It virtually combines both amplitude and time of a response. Second, it was correlated to the maximal concentration, thus the maximal concentration did not give much additional information in our model. Moreover, the integral is also more easily computed than the maximum as there are not pitfalls like local maxima, and it was in our cases more intuitive. In terms of signal timing we found the time of reaching the first maximum more useful than the signalling time τ as it gave a good measure of how fast a first significant response was, rather than the time of an average response.

In the literature we could not find experiments where a pheromone stimulus and a starvation stimulus were applied in parallel, although from our viewpoint this would be an interesting experiment concerning crosstalk. A prediction of our model for the phenotype that would result from such an experiment is not possible, because the model was not built for such a purpose. Specifically, we disregarded the Ras-dependent activation of the filamentous growth pathway, and additionally, most described effects depend on unknown parameters. Moreover, in our model the pheromone response will always be transient, irrespective of the length of the pheromone stimulus, because activated Ste11 is degraded without being newly synthesized (Fig. 4). Nevertheless, it would be informative to test experimentally several features that are predicted by the model. On the one hand, the model predicts that a pheromone stimulus inhibits at least transiently the starvation-induced activation of Kss1 and FREP. On the other hand, a starvation stimulus is anticipated to amplify Fus3 activation by a pheromone stimulus. Moreover, we identified leakage of activated Ste11 as the most influential source of crosstalk. Crosstalk of activated Ste11 was stronger than crossinhibition by degradation of Ste12/Tec1 induced by activated Fus3. The model also predicts that activating both pathways at the same time results in amplification of the pheromone response and inhibition of the filamentous growth response compared to a single stimulus, indicating that the pheromone response is in this case the dominant factor. In an experiment where cells are first starved until a certain level of activated Kss1 is reached and then a pheromone stimulus is applied, the model predicts a lower pheromone response and a weakened inhibitory effect of the pheromone pathway on the filamentous growth response compared to the effects caused by application of both stimuli at the same time. This result depends of course on the chosen set of parameters, but exemplifies how such a study can lead to new hypotheses about the relative contribution of distinct mechanisms to overall crosstalk. In the model no cell cycle-dependent processes are considered and to test the model predictions by experiments we recommend using synchronized cells, e.g., by counter-flow centrifugal elutriation [39].

We strongly believe that if we want to understand how pathways interact and crosstalk dynamically, measurements of pathway activation with both pathways being active are indispensable.

Model development and simulations

The pheromone and the filamentous growth pathway

In this study we employ mathematical modelling techniques to analyse dynamic mechanisms and measures of crosstalk. We illustrate our approach by giving an example of two signalling pathways in the budding yeast *Saccharomyces cerevisiae*, i.e., the mating response, initiated by pheromone, and the filamentous growth response, triggered by glucose starvation or nitrogen depletion [8–10].

Budding yeast may be present in one of two haploid cell types that are able to mate. Pheromones released by one type bind to a receptor of the respective other type. The receptor activates a heterotrimeric G protein that transmits the signal from the cell surface to intracellular effectors with the help of the membrane-associated protein Ste20 [11,12]. Elements of the signal transduction are the activation of a scaffold protein-bound mitogen activated protein (MAP) kinase (K) cascade consisting of the scaffold protein Ste5, the MAPKKK Ste11, the MAPKK Ste7 and the MAPK Fus3, and the phosphorylation and activation of nuclear proteins controlling cell polarity, transcription and progression through the cell cycle [2,13,14]. The signal transduction prepares the cell for fusion with the mating partner. Gene transcription is necessary to produce proteins involved in processes like cell fusion and in the signalling cascade. In the following, these proteins are called pheromone response element products (PREPs). Their transcription is regulated by the transcriptional activator Ste12 and its repressors Dig1/Rst1 and Dig2/Rst2 [15–19] (Fig. 1).

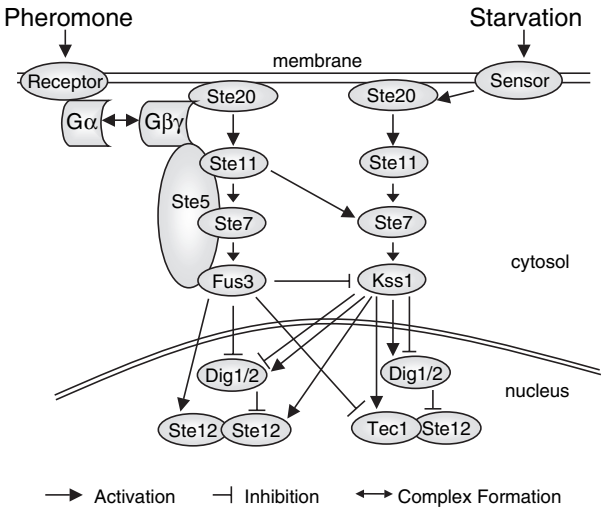


Fig. 1. Schematic overview of the pheromone (left) and the filamentous growth pathway (right) depicting pathway interactions. Components may have a promoting or inhibiting influence, depending on their activation state.

Bakers yeast is a fungus that occurs in distinct morphologies in response to different stimuli. In haploid cells, the switch from normal growth to so-called invasive or filamentous growth leads to enhanced cell–cell adhesion and agar penetration. The stimuli causing this change in cell shape are, for example, glucose depletion, alcohols or low levels of pheromone [20]. The signalling pathway of filamentous growth consists of two branches, the cAMP branch and a MAPK branch. Here, only the latter is regarded. Like in the pheromone pathway, a receptor activates a G protein, which is competent to initiate a MAP kinase cascade via Ste20. That cascade consists of the MAPKKK Ste11, the MAPKK Ste7 and the MAPK Kss1. Double phosphorylated Kss1 (Kss1PP) is able to shuttle into the nucleus and influence filamentous growth-intrinsic genes regulated by the transcription factors Ste12 and Tec1 and the repressors Dig1/Rst1 and Dig2/Rst2. The produced proteins are called filamentation response element products (FREPs) in the following (Fig. 1).

There are several ways in which the two roughly presented pathways can crosstalk or communicate with each other that can both complement and counteract each other. We will consider those for which there is strong evidence and we find most important:

- It has been shown that pheromone activated Ste11 can leak out from the scaffold complex and can activate the filamentous growth cascade [21]. This can result in a crossactivation. In the same paper it is demonstrated that the invasive growth pathway can also leak into the mating pathway. However, activation of Fus3 by the filamentous growth pathway is weak and therefore neglected in the following.
- The scaffold complex of the pheromone pathway can activate both Fus3 and Kss1, potentially activating both the mating and the filamentation response [22–27]. However, the amount of phosphorylated Kss1 is attenuated by double phosphorylated Fus3 (Fus3PP) [25]. This way, an activation of the filamentous growth response by a pheromone stimulus is reduced. The mechanism causing this process is still unknown, but it seems to be necessary that Fus3PP exceeds a certain threshold concentration to regulate the level of Kss1PP [25].
- In the pheromone pathway, Ste11 that is activated and released from the scaffold is unstable and rapidly degraded by an ubiquitin-dependent mechanism. Activated Fus3 may promote this through feedback phosphorylations. Thus, the possibility of an activation of other pathways by activated Ste11 is decreased [28,29], but still detectable [21].
- Phosphorylated Kss1 is able to phosphorylate Ste12, but to a lower extent than Fus3PP [30] resulting in the potential crossactivation of PREPs by the filamentous growth pathway [26,27].
- Fus3PP induces Tec1 ubiquitination and degradation [25,30–32] and thereby reduces crossactivation of filamentous growth response by pheromone activated Kss1.

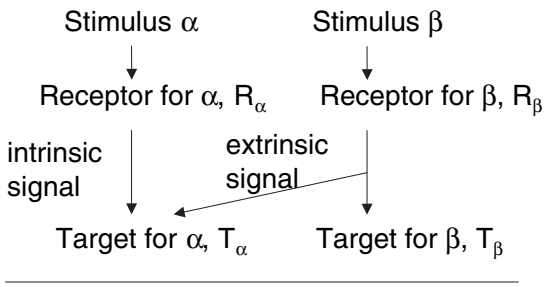
Definition of crosstalk measures

We assume that a signalling pathway has certain targets it activates and that each target can be assigned a specific or intrinsic stimulus and signal, whose major target it is, and nonspecific or extrinsic stimuli and signals, whose minor target it is (Fig. 2). This leads to an intuitive first description of the term crosstalk, i.e., the activation of a certain pathway component by an extrinsic stimulus. We define crosstalk C of the considered pathway with another pathway as the activation of a pathway component by the extrinsic stimulus e relative to the activation by the intrinsic stimulus i, i.e.,

$$C = \frac{X(e)}{X(i)}$$

where $X(e)$ and $X(i)$ denote some activation measures of the considered pathway by stimulus e and i, respectively (Fig. 2, for definition of activation measures see below). This definition is the reciprocal of the pathway fidelity introduced by Komarova *et al.* [7]. Given the intuitive understanding that the activation by the extrinsic signal $X(e)$ is smaller than the activation by the intrinsic signal $X(i)$, this results in a measure between zero and one for no and strong crosstalk, respectively. Of course, we can also get $C > 1$, meaning that the activation by the extrinsic signal is stronger than the activation by the intrinsic signal.

As stated above, cells may be subjected to multiple stimuli at a time that can call for conflicting responses. In this case, the cell has to combine signals to trigger the appropriate response. Therefore, we introduce the two new measures, i.e., the intrinsic specificity S_i and the extrinsic specificity S_e.



$$X(\alpha) = f(T_\alpha(t) \mid R_\alpha = \text{on}, R_\beta = \text{off})$$

$$X(\beta) = f(T_\alpha(t) \mid R_\alpha = \text{off}, R_\beta = \text{on})$$

$$X(\alpha, \beta) = f(T_\alpha(t) \mid R_\alpha = \text{on}, R_\beta = \text{on})$$

Fig. 2. (Upper) Illustration of the definition of intrinsic and extrinsic signal. The stimulus α is recognized by a specific receptor R_α, which transduces a signal to a specific (intrinsic) target T_α. The stimulus β is recognized by a specific receptor R_β, which transduces a signal to a specific (intrinsic) target T_β but can also transduce a signal to T_α, to which it is defined as an extrinsic signal. (Lower) Activation X of T_α is a function f of the time course of T_α, given a certain combination of present stimuli. The function f can be the integral or the maximal concentration, for instance.

We define intrinsic specificity S_i as the activation of the target of the considered pathway by the intrinsic stimulus i relative to the activation by both stimuli i and e, i.e.,

$$S_i = \frac{X(i)}{X(i,e)}$$

where $X(i,e)$ is the pathway activation when both stimuli are present (Fig. 2). The intrinsic specificity is a measure of how the intrinsic signal is influenced by the extrinsic signal when both are transmitted concomitantly. $S_i < 1$ means that the combined signal of i and e yields a stronger response than the intrinsic signal alone, and indicates that the extrinsic signal amplifies the intrinsic signal when both are transduced, i.e., it points to crossactivation. The smaller S_i, the stronger is the amplification by extrinsic signals and, thus, the less is the specificity of activation concerning the intrinsic signal. In cases where $S_i > 1$, the activation by the intrinsic signal is stronger than the integrated response and indicates that when both signals are transmitted the extrinsic signal inhibits the intrinsic signal, which can be called a crossinhibition. The greater S_i, the stronger is the inhibition by the extrinsic signal and, thus, the pathway is activated more specifically by the intrinsic signal alone.

We can also define a measure of how the extrinsic signal is affected by the intrinsic signal, when both are transmitted, i.e., the extrinsic specificity S_e:

$$S_e = \frac{X(e)}{X(i,e).}$$

If $S_e > 1$, we encounter a situation where both signals together produce a smaller activation than the extrinsic signal alone. This indicates that the intrinsic signal inhibits the extrinsic signal, i.e., there is a crossinhibition. The larger the value of S_e the stronger the inhibition by the intrinsic signal and, thus, the more specific the pathway is activated by an extrinsic signal alone. A value of $S_e < 1$ hints to a situation where the intrinsic signal amplifies the extrinsic signal. The lower S_e the less specific is the pathway activation in relation to an extrinsic signal. A number close to zero shows a dominance of the intrinsic signal over the extrinsic signal or a weak crossactivation, and a number close to one shows a dominance of the extrinsic signal over the intrinsic signal, i.e., a strong crossactivation.

Table 1 gives an overview of these measures and proposed interpretations of their respective values. Both measures of crosstalk should always be considered in parallel. Table 2 lists how the combinations of both crosstalk measures can be interpreted.

The definitions above only consider activation measures explicitly and not the input stimuli. These activation measures relate to time series of protein activation profiles

Table 1. Crosstalk measures and their interpretations. $X(i)$, $X(e)$ and $X(i,e)$ are measures for the activation of pathway X by the intrinsic, the extrinsic and both stimuli, respectively. C pathway crosstalk, S_i intrinsic specificity, S_e extrinsic specificity.

Crosstalk measure	Values	Interpretation
$C = \frac{X(e)}{X(i)}$	0	No crosstalk
	< 1	Crosstalk, extrinsic activation weaker than intrinsic activation
	> 1	Crosstalk, extrinsic activation stronger than intrinsic activation
$S_e = \frac{X(e)}{X(i,e)}$	0	No crosstalk
	< 1	Crossactivation, intrinsic signal amplifies extrinsic signal, low specificity to extrinsic signal
	> 1	Crossinhibition, intrinsic signal inhibits extrinsic signal, high specificity to extrinsic signal
$S_i = \frac{X(i)}{X(i,e)}$	< 1	Crossactivcation, extrinsic signal amplifies intrinsic signal, low specificity to intrinsic signal
	> 1	Crossinhibition, extrinsic signal inhibits intrinsic signal, high specificity to intrinsic signal

Table 2. Combinations of crosstalk measures and their interpretations. $X(e)$, $X(i)$ and $X(i,e)$ are measures for the activation of pathway X by the extrinsic, the intrinsic and both input signals, respectively. S_i, intrinsic specificity, S_e extrinsic specificity.

	$X(e) > X(i,e)$ $S_e > 1$	$X(e) < X(i,e)$ $S_e < 1$
$X(i) > X(i,e)$ $S_i > 1$	Mutual signal inhibition	Intrinsic signal dominance
$X(i) < X(i,e)$ $S_i < 1$	Extrinsic signal dominance	Mutual signal amplification

obtained by western blot analysis or time series of mRNA expression profiles obtained by microarrays, for example. These profiles are much easier to compare between pathways than input stimuli, like, for instance, a pheromone and a starvation stimulus, simply because they have the same units. It is not clear what would be the strength of a pheromone stimulus compared to a starvation stimulus, whereas the activation of a kinase or gene expression under two different conditions can be much better compared. Obviously, the measure of activation of a pathway by a single stimulus, like $X(i)$, and to several stimuli, like $X(i,e)$, can only be obtained by distinct time series experiments. In order to calculate the crosstalk measures the readouts from both experiments must be comparable, not only by using, in this case, the same input stimulus i in both experiments, but also by relating the readout in a quantitative way. In the case of western blots this can be achieved by blotting the protein activation time series of both experiments on the same gel. In the case of microarrays the signal values must be comparable not only between time points for one experimental condition, but also between experimental conditions by appropriate normalization techniques.

The mathematical model

The balance between two opposing goals guided the mathematical model development, i.e., to be as comprehensive and yet as parsimonious as possible. Including as many components as possible makes the model more realistic but at the same time more difficult to analyse and comprehend. Moreover, almost all parameters and kinetic constants are unknown and thus, augmenting the model also increases its arbitrariness. Therefore, we included only those components that are involved in crosstalk and the most important dynamic processes, so that the typically observed dynamic behaviour could be captured (Figs 3 and 4). We omitted, e.g., the MAPKK Ste7 because it is not yet clear whether it is involved in crosstalk, and for the dynamics we consider here it is negligible. We also omitted the G protein cycle for the sake of simplicity, and we consider phosphorylation reactions to be irreversible. Moreover, we only consider the cell response up to a time point where the first proteins are being synthesized, and neglect all processes that are important for morphological changes. We also assume that within this time frame degraded Ste11 is lost from the system and is not resynthesized. We therefore run the simulations only until a time point of six hours. For a more detailed model of the pheromone pathway see Kofahl and Klipp [33] and a diagram of such a comprehensive combined model is depicted in the supplementary material. In the following, the concentration of compounds and reactions will be numbered with a preceding 'c' or 'v', respectively (Fig. 3).

The scaffold protein Ste5 (c_1) and the MAPKKK Ste11 (c_2) reversibly form a complex (c_3, reactions v_1 and v_{27}) that is able to bind to Gβγ (c_4) after a pheromone stimulus α (reaction v_2). The complex Gβγ–Ste5–Ste11 (c_5) binds the MAPK Fus3 (c_6) or Kss1 (c_{12}) (reactions v_3 and v_9, respectively). The phosphorylation events of the MAPK cascade are lumped into one step (reactions v_4 and v_{10}, respectively) resulting in the activated complexes c_8 and c_{14}. The phosphorylated MAPKs Fus3PP (c_9) and Kss1PP (c_{15}) are able to dissociate from the scaffold protein (reactions v_5 and v_{11}), which still forms a complex

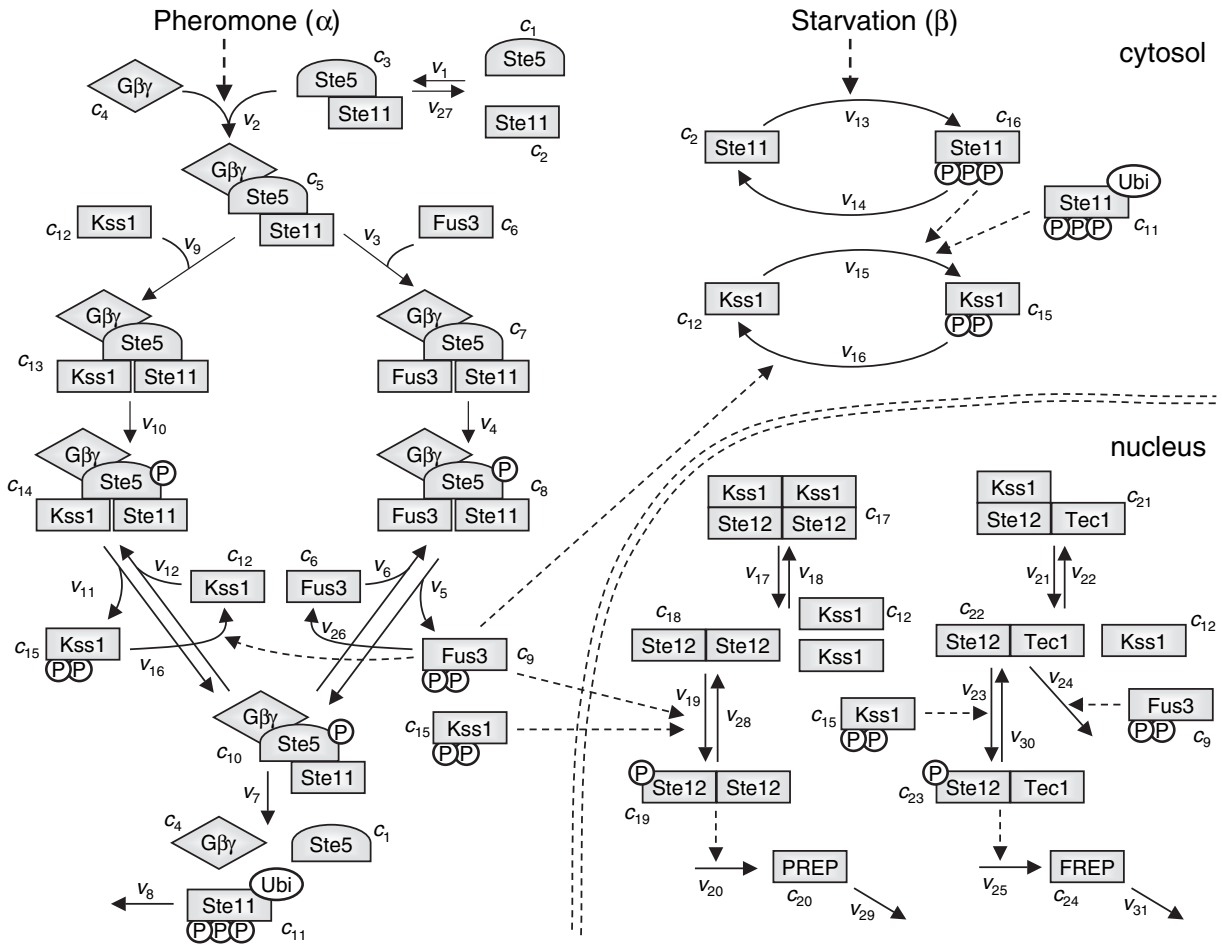


Fig. 3. Graphical representation of the mathematical model including all components and reactions considered (for a mathematical representation as a set of ordinary differential equations refer to the supplementary material). Proteins and reactions are annotated by their model name. The distinction between cytosol and nucleus is only depicted for illustrative reason and is not reflected in the model. Solid arrows indicate conversions whereas dotted arrows indicate promoting influences on the respective reaction.

with the other components (c_{10}), allowing further binding of unphosphorylated MAPKs and release of phosphorylated MAPK molecules (reactions v_6 and v_{12}). The complex c_{10} can decompose into Gβγ (c_4), Ste5 (c_1) and ubiquitinated activated Ste11 ($Ste11PPP_{ubi}$, c_{11}) (reaction v_7). $Ste11PPP_{ubi}$ in conjunction with activated Ste11 (Ste11PPP) can phosphorylate Kss1, resembling leakage of activated Ste11 into the filamentous growth pathway. The phosphorylated MAPKs become dephosphorylated (reactions v_{16} and v_{26}). Fus3PP enhances the dephosphorylation of Kss1PP (v_{16}).

Even though the processes involving the transcription factors take place in the nucleus we do not explicitly model different reaction compartments or transport processes. The transcriptional activator Ste12 is able to form homodimers (c_{18}) or heterodimers with Tec1 (c_{22}). Both dimers can reversibly bind to Kss1 (reactions v_{17} and v_{18}; v_{21} and v_{22}, respectively). Kss1PP can activate c_{18} and c_{22} (reactions v_{19} and v_{23}). The active form of Fus3 exerts different influences on the transcription factors. While Fus3PP activates c_{18} (reaction v_{19}), it induces degradation of Tec1 (reaction v_{24}). The active forms of Ste12/Ste12 and Ste12/Tec1 (c_{19} and c_{23}) activate gene expression of target genes (reactions v_{20} and v_{25}).

In response to a stimulus that activates the filamentous growth pathway by a hitherto not completely identified molecular mechanism, here named β, the MAPKKK Ste11 (c_2) is activated (reaction v_{13}) and Ste11PPP (c_{16}) is produced. Ste11PPP can be deactivated (reaction v_{14}) and/or activates Kss1 (c_{12}) (reaction v_{15}). Kss1PP generated by this signalling pathway acts like Kss1PP produced by the pheromone response pathway.

There are some processes enabling crosstalk corresponding to the processes described above:

- Ste11PPP phosphorylated in the pheromone pathway ($Ste11PPP_{ubi}$) can also phosphorylate Kss1 unbound to

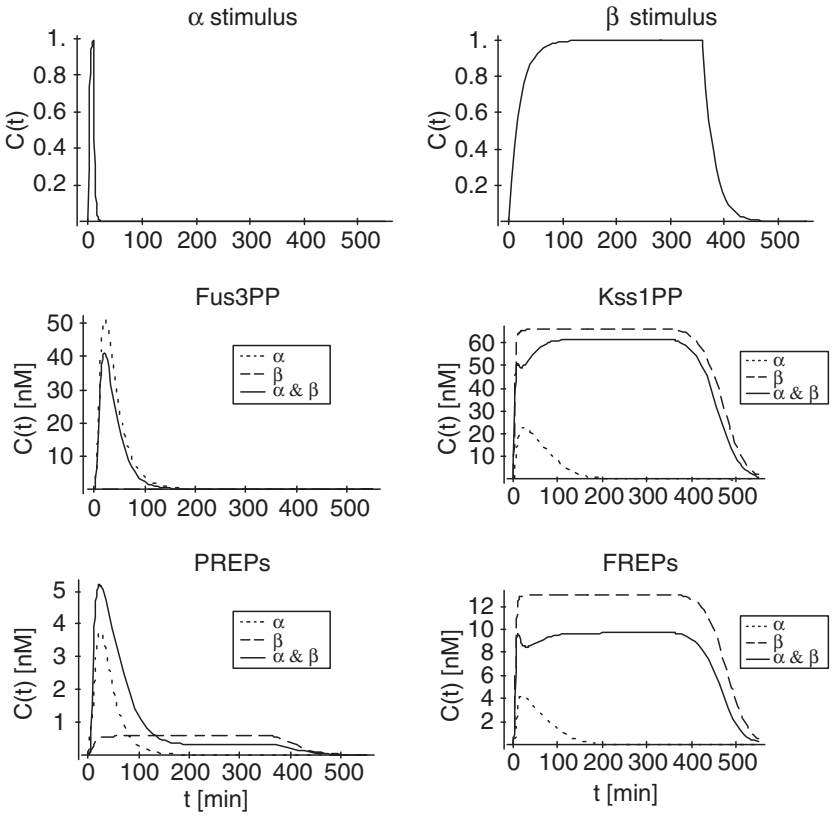


Fig. 4. Concentration profiles of pathway output components. Fus3PP and PREPs are the main targets of the pheromone pathways whereas Kss1PP and FREPs are the main targets of the filamentous growth pathway. For each component, the time curves are displayed for the case that only pheromone is present (α), that only a starvation signal is present (β) or that both are active (α & β).

Ste5 and, thus, leaks from the pheromone pathway and enters the filamentous growth pathway (reaction v_{15}).

- Both pathways activate Kss1. However, Fus3PP promotes Kss1PP dephosphorylation and thereby reduces crossactivation (reaction v_{16}).
- Ste11PPP is degraded as $Ste11PPP_{ubi}$ (reaction v_8).
- On the one hand, Kss1PP activates both Ste12/Ste12 and Ste12/Tec1 (reactions v_{19} ad v_{23}), however, activation of the former is not as potent as activation of the latter. On the other hand, Kss1 binds to both Ste12/Ste12 and Ste12/Tec1 and thereby inhibits their activation.
- Fus3PP induces degradation of Tec1 (reaction v_{24}) inhibiting crossactivation.

For a listing of the model equations and parameters refer to the Supplementary material.

As little was known about the kinetic parameters they were all set to unity in a first step. Systematic parameter fitting like in other models of yeast signalling [34] was not feasible because of lack of data. In order to map the dynamic model behaviour to what is known from the few available experiments (see below), some parameter adjustments were made. Qualitative information that was available about the relation of certain reaction velocities was incorporated into the model by increasing or decreasing kinetic parameters by a factor of 10 (see remarks to the model parameters in the Supplementary material). Due to the lack of knowledge about the kinetics all reactions were modelled as either first or second order mass action kinetics. Initial values for the concentrations were derived from Yeast GFP Fusion Localization Database (http://yeastgfp.ucsf.edu [35], Table S1). The model was implemented in MATHEMATICA 5.1® (www.wolfram.com), and can be downloaded as an SBML file from the journal website.

It must be noted that diploid cells lack a receptor for pheromone and, thus, the pheromone pathway is not responsive in diploid cells. The filamentous growth pathway, however, is responsive in diploid cells, even though the phenotype upon starvation is different. Therefore, there is no crosstalk between the two pathways in diploid cells and the model works only for haploid cells. Nevertheless, the model for the filamentous growth pathway can also be used for diploid cells.

Dynamic model behaviour

The dynamic behaviour of the model was tested by a qualitative comparison of the model results to available data. Three so-called standard runs were employed: (a) only application of α factor, (b) only application of β stimulus, and (c) application of both stimuli. The application of α factor was modelled by a smoothened step function of 10 min duration resembling receptor activation and subsequent deactivation by receptor internalization and other

negative feedbacks. The β stimulation was modelled as a smoothened step function of 6 h duration because starvation was supposed to act on a larger time scale than α factor treatment. The simulation time was 12 h (Fig. 4).

Figure 4 displays the simulated temporal concentration profiles of α and β stimulus, Fus3PP, Kss1PP, PREPs and FREPs for the three standard runs. As can already be deduced from the model structure, activated Fus3 can only be produced by a pheromone stimulus and not by a starvation signal. When both pathways are activated less Ste11 is available for the pheromone pathway, therefore the concentration of Fus3PP decreases. Nevertheless, PREP production is slightly stronger and lasts longer when both signals are active. This is due to the combined activation of Fus3PP and Kss1PP on the PREPs and less Ste12 inhibition by nonactivated Kss1 (complex c_{17}). The temporal profile of Fus3PP follows well the experimental evidence where a peak of activated Fus3 was observed after 20 min and a decay to half of the maximal concentrations was seen after 90 min [25,28]. Fus3PP and Kss1PP show similar dynamics upon a pheromone stimulus as has also been shown in experiments [25]. Kss1 becomes rapidly activated by all stimuli but to a different extent. While the response to starvation is strongest and follows the time course of the stimulus, the response to pheromone is weaker and more transient, which is in accordance with experimental data [25]. The response to both stimuli is of intermediate strength and duration. The PREPs time course upon α stimulus has the same shape as the Fus3PP time course. In experiments, a longer activation of mating response reporter genes and mRNA was observed [25,36,37]. The PREPs also become weakly activated upon a starvation stimulus without pheromone signal. This was also observed in experiments [25]. The activation profile of FREPs has the same shape as the Kss1PP profiles.

Performance of crosstalk measures

In our example, activation of a pathway by an extrinsic stimulus is defined as either the activation of the pheromone response by a starvation stimulus or, vice versa, the activation of the filamentous growth response by a pheromone stimulus. Activation is quantified by four different measures derived from the time curves of PREPs and FREPs, respectively, i.e., the time integral I, the first local maximum M, the time of the first local maximum t_M and signalling time τ [38]. For reasons of comparison we also calculated the recently proposed measures of pathway specificity (called Komarova-specificity S_K in the following) and fidelity F [7]. The calculated measures depicted in Tables 3 and 4 refer to the standard simulations described above (Fig. 4).

In Table 3 the crosstalk measures from the pheromone pathway perspective are listed, i.e., the intrinsic stimulus is α and the extrinsic stimulus is β. The time integral for the intrinsic signal is smaller than for the extrinsic signal, which is reflected by a crosstalk $C > 1$, indicating a stronger activation by the extrinsic signal than by the intrinsic signal. This is counterintuitive. However, the integral has its largest value when both signals are transmitted at the same time. The crosstalk measure extrinsic specificity S_e tells us that the combined signal is stronger than the extrinsic signal alone ($S_e < 1$), indicating that the intrinsic signal amplifies the extrinsic signal. This can also be seen in the PREPs time curves of Fig. 4. The intrinsic specificity $S_i < 1$ also indicates a crossactivation, where this time the extrinsic signal amplifies the intrinsic signal. Thus, we can hypothesize a mutual crossactivation of both signals (Table 2). Pathway fidelity $F < 1$ again shows that the pathway is activated more strongly by its extrinsic stimulus than by the intrinsic stimulus. The Komarova-specificity for the integral is smaller than one. Following the interpretation of Komarova *et al.* [7], this means that in our model the pheromone stimulus promotes the FREP activation more than its own output.

The crosstalk measures for the maximal concentration of a component give a different picture. Here, the crosstalk C is lower than one and accordingly the pathway fidelity F is

Table 3. Crosstalk measures for the pheromone pathway (PREPs). Here α is the intrinsic signal whereas β is the extrinsic signal. $X(\alpha)$, $X(\beta)$ and $X(\alpha,\beta)$ are the respective activation measures by the pheromone (intrinsic) signal, the filamentation (extrinsic) signal and both. C, S_i, S_e are the crosstalk measures for crosstalk, intrinsic and extrinsic specificity, respectively, as described in the text and in Table 1. F is the pathway fidelity, the reciprocal of C, and $S_K = X(\alpha)/Y(\alpha)$ is the pathway specificity, where $Y(\alpha)$ is the activation of the target of filamentous growth pathway by the pheromone signal. The latter two quantities were defined in Komarova *et al.* [7].

X	$X(\alpha)$	$X(\beta)$	$X(\alpha,\beta)$	C	S_e	S_i	F	S_K
Integral	174.9	231.6	423.9	1.32	0.5	0.4	0.7	0.5
Maximum	3.8	0.6	5.2	0.1	0.1	0.7	6.6	0.9
t_M	23.6	359.2	22.8	15.2	15.7	1.0	0.1	1.2
τ	42.7	217.5	98.0	5.1	2.2	0.4	0.2	0.7

Table 4. Crosstalk measures for the filamentous growth pathway (FREPs). Here β is the intrinsic signal whereas α is the extrinsic signal. $X(\alpha)$, $X(\beta)$ and $X(\alpha,\beta)$ are the respective activation measures by the pheromone (extrinsic) signal, the filamentation (intrinsic) signal and both. C, S_i, S_e are the crosstalk measures for crosstalk, intrinsic and extrinsic specificity, respectively, as described in the text and in Table 1. F is the pathway fidelity, the reciprocal of C, and $S_K = X(\beta)/Y(\beta)$ is the pathway specificity, where $Y(\beta)$ is the activation of the pheromone pathway by a starvation signal. The latter two quantities were defined in Komarova *et al.* [7].

X	$X(\alpha)$	$X(\beta)$	$X(\alpha,\beta)$	C	S_e	S_i	F	S_K
Integral	324.3	6141.2	4393.4	0.1	0.1	1.4	18.9	26.5
Maximum	4.3	13.0	9.6	0.3	0.4	1.4	3.0	22.7
t_M	20.2	352.8	11.7	0.1	1.7	30.2	17.5	1.0
τ	60.4	244.7	240.4	0.2	0.3	1.0	4.1	1.1

high. The extrinsic specificity S_e and intrinsic specificity S_i are both below one, indicating a situation of mutual cross activation. The Komarova-specificity S_K is also low. Note that when considering maximum and integral the crosstalk measures of Komarova *et al.* [7] come to opposing conclusions, whereas our new crosstalk measures result in a consistent interpretation.

Interpretation of the crosstalk measures concerning the temporal measure t_M again yields different conclusions. In this case, $C > 1$ and $F < 1$ denote a delay of reaching the maximal PREPs concentration when activated by its extrinsic signal. $S_i = 1$ shows that the extrinsic signal does not influence the timing of the response to the intrinsic signal, but $S_e > 1$ can be interpreted as an acceleration of the combined signal compared to the extrinsic signal alone. $S_K > 1$ indicates that the pathway activates its extrinsic output faster than its intrinsic output. This is also seen in Fig. 4 where the maximal concentration of the FREPs is reached faster than the maximal concentration of the PREPs after a pheromone stimulus.

The signalling time τ that can be interpreted as the time of the mean activation [38], depicts larger values as t_M. As for t_M, the intrinsic signal is faster than the extrinsic signal, however, the timing of the combined signal is between the intrinsic and the extrinsic signal, which results in $S_i < 1$. $S_K < 1$ means that the intrinsic output is activated faster than its extrinsic output.

In Table 4 the crosstalk measures from the filamentous growth pathway perspective are listed. All considered activation measures (I, M, t_M, and τ) are smaller for the extrinsic stimulus (α) than for the intrinsic stimulus (β). Contrary to Table 3, the response to the combined signal is between the intrinsic and the extrinsic response, except for t_M. It can be hypothesized that there is a weak crosstalk ($C < 1$). From $S_i > 1$ follows that the extrinsic signal inhibits the intrinsic signal. This can also be seen in the FREPs time curves in Fig. 4. However, the intrinsic signal dominates the extrinsic signal when both are transmitted ($S_i > 1$ and $S_e < 0.5$). Concerning t_M, again the combined signal results in an acceleration of both individual signals ($S_i > 1$, $S_e > 1$). Contrary to the effect observed for the pheromone pathway the intrinsic signal exhibits slower dynamics than the extrinsic signal ($C < 1$). The filamentous growth stimulus exhibits in both pathways similar dynamics ($S_K = 1.1$).

Sensitivity analysis

A sensitivity analysis gives an impression about how certain properties of the model depend on the choice of parameter values. A sensitive parameter, i.e., whose change has great impact on a property of interest, indicates where measurements should be made with care or where the model should be refined. Especially, when parameters are unknown and set arbitrarily, as in our case, a sensitivity analysis is indispensable.

The model response was robust with respect to perturbation of most parameters (for details see Supplementary material). The sensitive parameters upon a pheromone stimulus, i.e., those affecting Fus3PP and PREPs, were those affecting the dephosphorylation and breakdown rates of Fus3PP, PREPs and the scaffold complex c_{10} (v_{26}, v_{29}, v_7), respectively, as well as the synthesis rates of the inactive transcription complexes c_{17} and c_{18} (v_{18}, v_{28}). Regarding the filamentous growth pathway, only the FREPs breakdown rate was sensitive (v_{31}) (Table S3). The fact that parameters affecting dephosphorylation rates were sensitive indicates an important role of phosphatases in pathway activation and regulation.

Concerning the crosstalk measures, many more parameters were sensitive, especially for C, F, and S_K. The reactions involved in Kss1 activation (v_{15}, v_{16}) and transcription factor activation (v_{19}, v_{23}) were sensitive with respect to many crosstalk measures. Notably, the crosstalk measures involving only single stimulus activation measures (C, F, S_K) proved to be much more sensitive than our new activation measures (S_i, S_e). Only S_e was sensitive in three instances (Table S4).

Monte Carlo simulation

In addition to the parameter sensitivity of the model behaviour, we were interested in correlations between different crosstalk and activation measures for varying parameters. In the Monte Carlo study, we picked the values of 34 kinetic parameters randomly from an interval between a minimal (0.01) and a maximal value (10). For each random parameter set we calculated the corresponding crosstalk measures according to the employed activation measures as in Tables 3 and 4. This was done 500 times. As a measure of correlation we used the Spearman's rank correlation coefficient r_S, because it is robust against outliers and can also measure nonlinear correlations as long as they are monotonous. While the normal correlation coefficient uses the actual data values, the Spearman's rank correlation is based on the rank of the sorted data.

First, we calculated correlations between the different activation measures for each crosstalk measure, respectively. For all crosstalk measures there was a strong correlation between the integral and the maximum (mean $r_S = 0.9 \pm 0.1$) and a medium correlation between t_M and τ ($r_S = 0.3 \pm 0.2$ and $r_S = 0.7 \pm 0.0$ for PREPs and FREPs, respectively). The other activation measures were only weakly correlated and the results were similar for PREPs and FREPs (Table S2).

Then, we calculated correlations between the different crosstalk measures for each activation measure, respectively. Apart from the obvious nonlinear correlation between C and F (the one is the reciprocal of the other), the correlations differed considerably between activation measures, PREPs and FREPs, and crosstalk measures (Table 5).

Table 5. Mean Spearman's rank correlation coefficients r_S and their respective standard deviations between crosstalk measures for Monte Carlo simulations of 500 runs. The mean was taken over the activation measures integral *I*, maximal concentration *M*, and the time measures t_M and τ, separately for PREPs and FREPs, which are depicted in the upper triangle and the lower triangle of the table, respectively.

		PREPs *C*	S_e	S_i	*F*	S_K
FREPs	*C*		0.9 ± 0.1	−0.4 ± 0.3	−1 ± 0.0	−0.2 ± 0.2
	S_e	0.5 ± 0.4		−0.4 ± 0.3	−1.0 ± 0.0	−0.1 ± 0.1
	S_i	−1.0 ± 0.0	−0.4 ± 0.4		0.5 ± 0.2	0.1 ± 0.1
	F	−1.0 ± 0	−0.5 ± 0.4	1.0 ± 0.0		0.1 ± 0.1
	S_K	0.0 ± 0.0	−0.1 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	

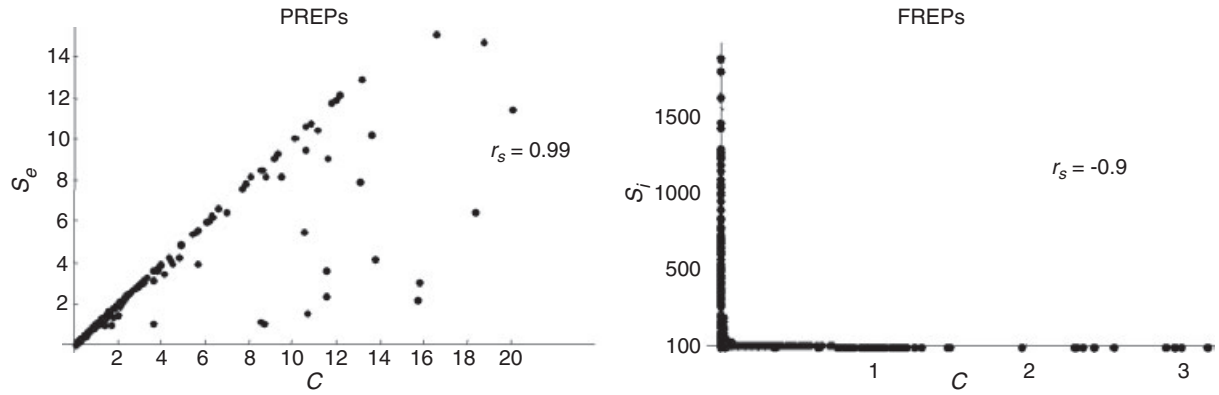


Fig. 5. Correlation between crosstalk measures for the integral as activation measure. Each dot represents one Monte Carlo simulation (see text). r_S denotes the Spearman's rank correlation coefficient.

However, there were two strong correlations between crosstalk measures irrespective of the employed activation measure. This was the correlation between *C* and S_e for the PREPs and between *C* and S_i for the FREPs (Fig. 5). Of course, all measures that are correlated with *C* are also correlated with *F*.

Considering the definitions of the crosstalk measures, the strong correlation between *C* and S_e for the PREPs indicates that $X(\alpha) \approx X(\alpha,\beta)$, meaning that the pheromone response is almost equal to the combined response. This is indeed the case as can be seen in Fig. 4. The strong reciprocal correlation between *C* and S_i for the FREPs indicates the same. Thus, in both pathways the pheromone response seems to dominate the combined response independently of the chosen parameter set. In the pheromone pathway, the pheromone signal dominates because of the small influence of the extrinsic (filamentous growth) signal and in the filamentous growth pathway it dominates the combined signal, because of its strong inhibitory role (reaction v_{16} and v_{24}). Thus, our conclusion from the standard run, that the filamentous growth response dominates the combined response regardless of inhibition (above), depends on the particular choice of parameters, and in general the pheromone activation is similar to the combined activation, i.e., $X(\alpha) \approx X(\alpha,\beta)$ in the filamentous growth pathway. However, in the filamentous growth pathway it is mostly $X(\alpha) < X(\alpha,\beta)$, i.e., $S_e < 1$ (results not shown).

Model simulations and predictions

One advantage of a mathematical model is its ability to easily conduct virtual experiments and generate predictions addressing biological questions. This way it is, for instance, possible to explore the contributions of different crosstalk mechanisms to the overall response. From the perspective of the filamentous growth pathway, we analyse the relative contributions to crosstalk by (a) activation of Kss1 through pheromone activated Ste11 (c_{11}, reaction v_{15}), by (b) enhanced deactivation of Kss1PP through Fus3PP (reaction v_{16}), and by (c) degradation of Ste12/Tec1 induced by Fus3PP (reaction v_{24}) by setting a single or several of the corresponding parameters to zero (Table 6). The first process can be regarded as a crossactivation whereas the latter two are crossinhibitions instead. The results are displayed in Table 6.

Not surprisingly, shutting off the leaking of activated Ste11 from the pheromone pathway to the filamentous growth pathway (simulation experiment 1) substantially lowers extrinsic specificity (S_e) compared to the standard run. This is a sign of decreased crossactivation leading to

Table 6. Crosstalk specificities S_i and S_e for the filamentous growth pathway (FREPs, using the integral as activation measure) corresponding to different simulation experiments. 1, no activation of Kss1 by activated Ste11 leaking from the pheromone pathway ($k_{30} = 0$); 2, no dephosphorylation of Kss1PP triggered by Fus3PP ($k_{28} = 0$); 3, no degradation of Ste12/Tec1 induced by Fus3PP ($k_{24} = 0$). Std, standard run as displayed in Table 4.

	Std.	1	2	3	1 + 2	1 + 3	2 + 3	1 + 2 + 3
S_e	0.07	0.01	0.13	0.07	0.03	0.01	1.3	0.03
S_i	1.4	1.4	1.3	1.1	1.3	1.1	1.1	1.1

stronger crossinhibition. Shutting off dephosphorylation of Kss1PP induced by Fus3PP (simulation experiment 2) enhances crossactivation and lowers crossinhibition (lower S_i and higher S_e). Inhibiting degradation of Ste12/Tec1 triggered by Fus3PP (simulation experiment 3) only had a notable effect by decreasing crossinhibition (lower S_i). Notably, neither the second nor the third process nor both together could compensate for the effect of the first. This identifies leakage of activated Ste11 from the pheromone pathway as the most prominent of the three considered crosstalk processes. However, it has to be emphasized that even in the case of shutting off both inhibitory processes (Column '2 + 3', Table 6) the overall response is still a crossinhibition ($S_i > 1$) even though not as strong as in the standard run. This is because both pathways sequester Ste11 when both stimuli are present, and therefore the filamentous growth pathway cannot be fully activated in this situation.

The availability of Ste11 also plays a role when we study the effect of a delayed pheromone stimulus ($\alpha_t = 30$ min, $\alpha_e = 40$ min). When the filamentous growth pathway is active, a fraction of Ste11 is already phosphorylated and is no longer available for a subsequent pheromone response. In this case the pheromone response is smaller and consequently also its inhibitory effect on the filamentous growth pathway (results not shown).

A hypothetical experiment with a proteasome inhibitor ($v_8 = 0$) results in a prolonged activation of Kss1, PREPs and FREPs by a pheromone stimulus and consequently a higher mutual crossactivation.

The sensitivity analysis showed that an altered sensitivity of PREPs and FREPs to Ste12 promotor activity (v_{20} and v_{25}) has a linear effect on their activation, i.e., the parameter sensitivity is equal to one concerning the integral and maximum due to the linear kinetics involved. However, calculating the quotient of the two integrals as in the case of S_K leads to reciprocal effects and thus to a much higher sensitivity concerning this crosstalk measure (Table S4).

Different signal intensities (α, β) had only marginal effects in our implementation because of rapid saturation of the activation reactions. In experiments different signal intensities did have an effect, of course. It must be stressed, however, that quantitative predictions cannot be achieved with this model given the qualitative nature of the parameters.

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Supplementary material

The following supplementary material is available online:

Doc. S1. (A) Detailed description of the mathematical model. (B) Sensitivity analysis.

Fig. S1. Extended model scheme of the pheromone and the filamentous growth pathway.

Table S1. Nonzero steady state concentrations used as initial concentrations for the simulations.

Table S2. Mean Spearman's rank correlation coefficients r_S and their respective standard deviations between activation measures for Monte Carlo simulation.

Table S3. Sensitivity analysis of activation measures (AM), time integral I, first local maximum M and the time of the first local maximum t_M of simulated time courses of Fus3PP, Kss1PP, PREPs and FREPs with respect to all parameters.

Table S4. Sensitivity analysis of crosstalk measures (CM) C,F,S_K, S_i and S_e with respect to the activation measures (AM), time integral I, first local maximum M and the time of the first local maximum t_M of simulated time courses of PREPs (PRs) and FREPs (FRs) with respect to all parameters.

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