Dynamic analysis of the *KlGAL* regulatory system in *Kluyveromyces lactis*: a comparative study with *Saccharomyces cerevisiae*

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Abstract The *GAL* regulatory system is highly conserved in yeast species of *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. While the *GAL* system is a well studied system in *S. cerevisiae*, the dynamic behavior of the *KlGAL* system in *K. lactis* has not been characterized. Here, we have characterized the *GAL* system in yeast *K. lactis* by developing a dynamic model and comparing its performance to its not-so-distant cousin *S. cerevisiae*. The present analysis demonstrates the significance of the autoregulatory feedbacks due to KlGal4p, KlGal80p, KlGal1p and Lac12p on the dynamic performance of the *KlGAL* switch. The model predicts the experimentally observed absence of bistability in the wild type strain of *K. lactis*, unlike the short term memory of preculturing conditions observed in *S. cerevisiae*. The performance of the *GAL* switch is distinct for the two yeast species although they share similarities in the molecular components. The analysis suggests that the whole genome duplication of *S. cerevisiae*, which resulted in a dedicated inducer protein, Gal3p, may be responsible for the high sensitivity of the system to galactose concentrations. On the other hand, *K. lactis* uses a bifunctional protein as an inducer in addition to its galactokinase activity, which restricts its regulatory role and hence higher galactose levels in the medium are needed to trigger the *GAL* system.

Keywords *GAL* system · *Kluyveromyces lactis* · Feedback loops · Galactose · Glucose

Introduction

Organisms live under changing environmental conditions. The successful adaptation to these changes is determined by an appropriate response to external or internal perturbations through the simultaneous expression of a large set of genes. Transcriptional regulation serves as the mechanism to perform these tasks through interplay of various proteins. However, network regulation is hierarchal in nature with controls residing at metabolite, protein, mRNA, and DNA levels (Ruhela et al. 2004). These controls are achieved through complex interactions among the network components. Feedback is the common theme in all these control strategies with complex feedback motifs residing in signaling, metabolic and genetic regulatory networks. These nested feedback loops are key to a robust performance of the regulatory system (Venkatesh et al. 2004). However, other than these feedback loops, there exist other regulatory features such as compartmentalization and stoichiometry, which also play a crucial role in the overall performance of the regulatory system. For example, it has been shown that the nucleocytoplasmic shuttling of repressor protein Gal80p¹ in *GAL* system of *S. cerevisiae* plays a crucial role in the performance of the switch (Verma et al. 2003).

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¹ In this study, all proteins related to *Kluyveromyces lactis* are used with prefix 'Kl' and for *Saccharomyces cerevisiae* they are used as it is. For example, repressor protein in *S. cerevisiae* is denoted as Gal80p and in *K. lactis* it is denoted as KlGal80p.

Although *S. cerevisiae* and *K. lactis* have originated from a common ancestor, they have evolved differently in nature due to their respective environmental niche. The former has evolved in a melibiose environment while the latter in a lactose environment. While the *GAL* regulatory network in these organisms share similarities in the molecular components of the *GAL* system, their compartmentalization and stoichiometry are considerably different from each other (Rubio-Teixeira 2005). For example, the repressor protein Gal80p is nucleocytoplasmic in *S. cerevisiae*, where as in *K. lactis* it is confined to the nucleus. Similarly, the opposite is observed in the case of inducer proteins, Gal3p and KIGal1p, where the former is cytoplasmic and the latter is nucleocytoplasmic protein. Although the regulatory proteins Gal80p and Gal3p/KIGal1p perform similar function in both species, the genes (*GAL3/KIGAL1*, *GAL80*) encoding them have varied binding sites for activator Gal4p in their promoter regions. The activator protein Gal4p is conserved in both species with the exception that its autoregulation (or self-regulation) is observed in the case of *K. lactis* *KIGAL* system, whereas in *S. cerevisiae* the activator is constitutively expressed. Furthermore, the mechanism for activation of inducer protein (Gal3p/KIGal1p) with galactose, and the interaction of inducer with the repressor protein Gal80p is not clearly understood.

In silico modeling studies have been successful in elucidating the network structure of various organisms (Ackers et al. 1982; Chung and Stephanopoulos 1996; Santillan and Mackey 2001). The *GAL* system in *S. cerevisiae* is a well studied regulatory network for which both steady-state and dynamic models have been developed to understand the regulatory structure (Ruhela et al. 2004; Verma et al. 2003). However, such an in-depth dynamic analysis of *K. lactis* has not been reported in literature. Therefore, a detailed dynamic model was developed for the *KIGAL* system of *K. lactis* and further validated using experimental data. The present study provides an opportunity to study the influence of the differences in the regulatory structure in the *GAL* system of *S. cerevisiae* and *K. lactis* on the dynamic performance of the system through a modeling approach. The developed model was used to compare the performance of the *KIGAL* system of *K. lactis* to that of *S. cerevisiae* *GAL* system for which a dynamic model reported in literature (Ruhela et al. 2004). We begin with the description of the key features of the *KIGAL* system in *K. lactis* and followed by its model development.

KIGAL system

The *KIGAL* system in *K. lactis* contains two regulatory genes (*LAC9* or *KIGAL4* and *KIGAL80*), a bifunctional regulatory gene *KIGAL1* and four structural genes (*LAC12*, *LAC4*,

KIGAL7 and *KIGAL10*). The *KIGAL* switch is found in three regulatory states in response to the availability of various carbon sources. In the presence of a non-inducing-non-repressing medium (NINR) such as glycerol or raffinose, the *KIGAL* switch is in an uninduced state. Under such a condition, the KIGal4p activity is inhibited by the binding of KIGal80p protein to the C-terminal activation domain of KIGal4p. In this state, the *KIGAL* genes are poised for induction as they are not subjected to carbon catabolite repression. In the presence of lactose/galactose medium, the *KIGAL* switch is in an induced state. The enzyme permease (Lac12p) transports lactose/galactose into the cytoplasm, which then in combination with ATP activates the protein KIGal1p. The protein KIGal1p, being bifunctional, has both inducer and galactokinase activity. The activated KIGal1p then shuttles into the nucleus and interacts with the repressor protein KIGal80p, thereby relieving the inhibition of KIGal80p on KIGal4p (Anders et al. 2006). In the presence of glucose, the *KIGAL* switch is in a repressed state. In *S. cerevisiae*, glucose represses the *GAL* genes by having a specific repressor protein Mig1p, which binds to the upstream repressor sequences (URS_G) present in the *GAL* genes (Nehlin et al. 1991). In case of *K. lactis*, the repression on *KIGAL4* is Mig1p-independent, since *KIGAL4* has no URS_G in its promoter for Mig1p but glucose indirectly represses the *GAL* system by a Mig1p binding site in *KIGAL1* gene (Dong and Dickson 1997; Lamphier and Ptashne 1992). However, it has been experimentally shown that glucose affects the ability of *KIGAL4* to activate transcription of *KIGAL* genes (Breunig 1989; Flick and Johnston 1990). Thus, while KIGal4p has no Mig1p binding site on its gene promoter, its activity is inhibited in the presence of glucose. The activator Gal4p in yeast contains at least three inhibitory domains in its central region and a glucose response domain between the activator domains, which become active in the presence of glucose, which, however are independent of repressor Mig1p (Stone and Sadowski 1993).

In all of the above three states, the concentration of activator KIGal4p plays a vital role in the induction mechanism of the *KIGAL* system. *KIGAL4* gene contains a UAS_G in its own promoter for binding of KIGal4p resulting in an autoregulatory circuit which causes a two to fivefold increase in KIGal4p concentration in presence of lactose/galactose. This increase is essential for maximal growth rate on lactose and has probably evolved to give the organism a selective advantage in its natural habitat (Czyz et al. 1993). On the other hand, to maintain the repressed state of the *KIGAL4*-controlled genes in a glucose containing medium, the KIGal4p concentration has to be held below a certain threshold concentration (Zachariae et al. 1993). Quantification of the expression of *GAL* genes in *K. lactis* strain using equilibrium considerations for all the molecular interactions in the genetic network has been

Although, the steady-state modeling analysis predicts the system behavior based on molecular mechanisms underlying the network, it fails to capture the temporal dominance of the individual mechanisms in the overall performance of the network. The model also captures cellular growth and the dynamics of substrate consumption. This motivates the development of a dynamic modeling approach. For example, a dynamic model developed for *GAL* system in *S. cerevisiae* illustrates that the autoregulation of regulatory proteins is the key for the dynamic operation of the *GAL* system (Ruhela et al. 2004). Here, the dynamic model was used to identify the relevance of autoregulation of regulatory proteins Gal3p and Gal80p as well as the importance of nucleocytoplasmic shuttling of Gal80p. Further, the dynamic model approach can be used to elucidate the systems-level properties of the network such as bi-stability, sensitive response and robustness. The following section describes the key features of the dynamic model of the *KIGAL* system in *K. lactis*.

Model development

Legend:

- KIGal4p_2 (green circle with 4) $\rightarrow$ KIGal4p (green circle with 4)
- KIGal80p_2 (red circle with 80) $\rightarrow$ KIGal80p (green circle with 80)
- KIGal1p-Gal-int (green circle with 1) $\rightarrow$ KIGal1p (grey circle with 1)
- Lac21p (white circle with 12) $\rightarrow$ Lac21p (white circle with 12)
- KIGal1p-Gal-int (green circle with 1) $\rightarrow$ KIGal1p-Gal-int (green circle with 1)
- Galactose (triangle) $\rightarrow$ Galactose (triangle)

Diagram Description:

The diagram illustrates the GAL4/VP16-based genetic circuit. In the cytoplasm, Galactose (triangles) is converted to Galactose-1-phosphate (12) by the enzyme KIGal80p2 (80). Galactose-1-phosphate (12) is then converted to Galactose-6-phosphate (6) by the enzyme KIGal4p (4). Galactose-6-phosphate (6) is converted to Galactose-1,6-bisphosphate (16) by the enzyme KIGal80p (80). Galactose-1,6-bisphosphate (16) is then converted to Galactose-5-phosphate (5) by the enzyme KIGal1p-Gal-int (1). Galactose-5-phosphate (5) is converted to Galactose (triangles) by the enzyme KIGal1p (1). In the nucleus, Galactose-5-phosphate (5) is converted to Galactose-1-phosphate (12) by the enzyme KIGal4p (4). Galactose-1-phosphate (12) is then converted to Galactose-6-phosphate (6) by the enzyme KIGal80p2 (80). Galactose-6-phosphate (6) is converted to Galactose-1,6-bisphosphate (16) by the enzyme KIGal80p (80). Galactose-1,6-bisphosphate (16) is then converted to Galactose-5-phosphate (5) by the enzyme KIGal1p-Gal-int (1). Galactose-5-phosphate (5) is converted to Galactose (triangles) by the enzyme KIGal1p (1). The diagram also shows the expression of GAL genes D1 and D2 under the control of UAS_G and UAS_GG promoters.

where 0.7 represents the co-response coefficient, which was obtained by fitting the predicted steady state expression level for proteins with two binding sites to

experimental values (Pannala et al. 2010). The above expressions also assume that the dynamics of transcription are fast relative to the time-scales for cell division and growth. The above equations capture the inefficiencies of the translational process, as all the mRNA that is transcribed is not converted to protein. It should be noted that for *S. cerevisiae* a value of 0.5 was set for the exponent in Eq. 3 and 4 to capture the inefficiencies in the translational process (Ruhela et al. 2004).

Since, the gene *KIGAL4* has one binding site for its own protein KIGal4p; the KIGal4p concentration is therefore a function of f_{lp} . Further, the presence of glucose turns off the *GAL* system by repressing the synthesis of KIGal4p, which has been modeled by a Hill equation as follows,

$$R_{KIGal4p} = k_g \times f_{lp} \times D1_t \left(\frac{K_i^{\eta_g}}{K_i^{\eta_g} + Glc^{\eta_g}} \right) \quad (5)$$

k_g , K_i , and η_g represent synthesis rate constant of KIGal4p, inhibitory constant on glucose and Hill coefficient, respectively. The synthesis of the other three regulatory proteins, KIGal1p and KIGal80p and Lac12p depends on the autoregulation of genes with two binding sites (Rubio-Teixeira 2005). Therefore,

$$R_{KIGal1p} = k_{t1} \times f_{2p} \times D2_t \quad (6)$$

$$R_{KIGal80p} = k_{t8} \times f_{2p} \times D2_t \quad (7)$$

$$R_{Lac12p} = k_{t12} \times f_{2p} \times D2_t \quad (8)$$

$R_{KIGal1p}$, $R_{KIGal80p}$, R_{Lac12p} , and k_{t1} , k_{t8} , k_{t12} represent the synthesis rate constants of KIGal1p, KIGal80p and Lac12p and the respective translational kinetic constants.

The model accounts for the transport of extracellular galactose through the cytoplasmic membrane, which is controlled by Lac12p (the first term in Eq. 9 below) as well as Lac12p independent diffusion mechanism (second term) as shown below:

$$\frac{dGal_{int}}{dt} = K_p[Lac12p] \frac{Gal_{ext}}{k_i + Gal_{ext}} + d_c \frac{Gal_{ext}}{k_s + Gal_{ext}} - \frac{\mu_{Gal}}{Y_{Gal}} - \mu_{Gal_{int}} \quad (9)$$

The last two terms represents the consumption and dilution due to growth on galactose. The model is connected to growth on glucose and galactose. The overall growth rate can be written as:

$$\mu = \mu_{Gly} + \mu_{Gal} + \mu_{Glu} \quad (10)$$

Since excess glycerol was used relative to galactose concentration, μ_{Gly} represent growth rate on glycerol and was assumed independent of glycerol concentration, while μ_{Gal} and μ_{Glu} denote growth rates on galactose and glucose,

respectively and are dependent on the concentration of the respective substrates in the medium as follows,

$$\mu_{Gal} = \mu_{Gal,max} \left(\frac{Gal_{int}}{K_0 + Gal_{int}} \right) \quad (11)$$

$$\mu_{Glu} = \mu_{Glu,max} \left(\frac{Glu}{K_{glu} + Glu} \right) \quad (12)$$

The galactose, glucose, and biomass in the medium have been modeled as follows (13)

$$\frac{dGal_{ext}}{dt} = -K_p[Lac12p] \frac{Gal_{ext}}{k_i + Gal_{ext}} X - d_c \frac{Gal_{ext}}{k_s + Gal_{ext}} X \quad (13)$$

$$\frac{dGlu}{dt} = -\frac{1}{Y_{Glu}^X} \mu_{Glu} X \quad (14)$$

$$\frac{dX}{dt} = \mu X. \quad (15)$$

The activation of KIGal1p by intracellular galactose (Gal_{int}) is modeled by assuming a molecular interaction between them with a forward rate constant (k_5) and an equilibrium dissociation constant K_5 to form an activated [KIGal1p- Gal_{int}] complex.

$$[KIGal1p] + [Gal_{int}] \xrightleftharpoons[k_5]{k_5} [KIGal1p - Gal_{int}] \quad (16)$$

The activated [KIGal1p- Gal_{int}] then enters into the nucleus and interacts with monomer and dimer forms of KIGal80p to relieve the inhibition on activator KIGal4p as shown in Fig. 1. The translation of galactose permease (Lac12p) produced by genes with multiple binding sites (D_2) can be described by,

$$\frac{d[Lac12p]}{dt} = k_{t12} f_{2p} D2_t - \mu[Lac12p] \quad (17)$$

where k_{t12} , represents synthesis rate constant.

The complete model consists of 25 differential equations and three algebraic equations, which were solved by MATLAB 7.6 of Math Works Inc. USA. The complete set of model equations are presented in supplementary materials section. The equilibrium dissociation constants were taken from steady-state model (Pannala et al. 2010). The kinetic parameters and distribution coefficient for KIGal1p (K) were fitted to match the steady-state and dynamic experimental data with galactose. The specific growth and half saturation constants were obtained from experimental data. The model has 36 parameters, out of which 27 parameters correspond to reported equilibrium data (such as binding constants between proteins and between proteins and DNA) or experimental data (such as maximum growth rate, yield coefficient) obtained through independent experiments carried out specifically to estimate the parameter. Therefore, only 9 parameters were obtained by fitting parameters to the

dynamic protein expression data. All the parameter values are given in Table S1 in supplementary materials.

Sensitivity of bistable behavior to model parameters

It has been experimentally demonstrated that the GAL system of *S. cerevisiae* shows different steady state expression levels when grown under identical galactose concentrations depending on the preculturing conditions (Acar et al. 2005). In particular, when precultured on galactose, the yeast *S. cerevisiae* shows higher expression levels than when precultured in an NINR medium such as glycerol or raffinose for a given galactose substrate. To quantify the above bistable behavior, we simulated the GAL system of *K. lactis* for the two preculturing conditions namely, on galactose and glycerol. The cells were subsequently grown under various galactose concentrations and the fractional protein expression for genes with two binding sites, f_{2p} (galactose, preculture medium) obtained. The maximum difference between the fractional protein expressions between the two preculturing conditions was used to quantify the degree of bistability,

$$\delta = \max(|f_{2p}(\text{Gal}_{ext}, \text{precultured on galactose}) - f_{2p}(\text{Gal}_{ext}, \text{precultured on NINR})|) \quad (18)$$

Thus, $\delta = 0$ indicates the absence of bistability while $\delta > 0$ indicates a bistable behavior. $\delta = 1$ implies that the cells do not express when precultured on one medium and show complete expression in the other.

It is known that bistable behavior occurs in nonlinear systems which consist of both positive and negative feedback effects. In case of *K. lactis*, the negative regulation is due to *KIGal80p* while *Lac12p*, *KIGal1p* and *KIGal4p* positive regulate the GAL system. Then, we have performed a parametric sensitivity analysis where δ was obtained by perturbing the synthesis rate constants of each of the four proteins described above, individually. Further, a global sensitivity analysis was also performed wherein all the parameters were perturbed simultaneously. A sensitivity coefficient defined as

$$S\delta_i = \frac{\frac{\delta(ki + \Delta ki) - \delta(ki)}{\delta(ki)}}{\Delta ki / ki} \quad (19)$$

was evaluated for the global sensitivity analysis.

Experimental protocol

Strain

K. lactis *GAL80* mutant and wild type strains used in this study were JA6D801 and JA6, respectively (Zenke et al.

1993). The strain was stored in 20% glycerol at -20°C in micro-centrifuge tubes. The cells were precultured in a YPD medium (Yeast extract 10 g/l, peptone 20 g/l, and Dextrose 20 g/l) and streaked out on to the agar plates. A single colony was picked out from agar plate to re-inoculate the YPD broth grown in a shake flask until it reached the exponential phase. Slants were prepared using cultures grown in the shake flask and stored for experimental use. In each experiment, inoculum was prepared by a loopful of culture from the slant.

Medium for preculture

A cotton stoppered 500 ml Borosil® flask containing 100 ml working volume of composition 10 g/l Yeast extract, 20 g/l Peptone and 20 g/l Dextrose/30 g/l Glycerol was used. The pH was adjusted to 5.5 by adding 1 M HCl. The cells were grown in a shake flask at 30°C on a rotary shaker at 240 rpm till optical density (OD_{600}) reached to 1.0–1.5 OD_{600} . Subsequently, the experimental flask was inoculated with 10% (w/v) cell mass of OD_{600} equal to one.

Experimental procedures

Steady state experiments on glucose and galactose were carried out independently in a fed batch mode. Initially *K. lactis* strain was grown in a shake flask of composition similar to the preculture medium until the OD_{600} attained a value between 0.8 and 1.0 in a rotary shaker at 30°C and 240 rpm. After this, experiment was carried out in a fed-batch mode by the addition of glucose/galactose at regular intervals while measuring the concentration of glucose/galactose in the flask. For studies on *K. lactis* *GAL80* mutant strain, different average steady-state glucose concentrations (0–57 mM) were maintained ($\pm 10\%$) in the flask using two standard glucose solutions of concentrations 10 to 50-fold of the required concentration. The protein concentrations of β -galactosidase were measured for different average steady state concentration of glucose. For studies on *K. lactis* wild type strain, different batch experiments were performed using different galactose concentrations (0.002–0.44 M) with glycerol as the background media and the fractional β -galactosidase expression was measured dynamically. The maximum protein expression was noted for cells grown in a 3% glycerol medium without glucose/galactose for a mutant strain lacking *KIGAL80*. The data is provided as the fraction of the maximum value of the β -galactosidase expression. The data obtained from these experiments were tabulated as steady-state fractional protein (β -galactosidase) expressed at different average steady-state glucose/galactose concentrations. All the steady-state and batch experiments were carried out with 3% glycerol as the background medium.

Substrate and enzyme activity measurements

Glucose and galactose were measured using HPLC, using Lachrom L-7490 HPLC system and Biorad Aminex HPX-87H column attached with guard column in series. β -galactosidase activity was measured by taking 2 OD₆₀₀ cells for each measurement and stored in breaking buffer immediately at -20°C for later extraction. The yeast cells were lysed by addition of glass beads (0.5 mm) and activity of β -galactosidase was measured by the method of crude extracts as reported in Adams et al. (Rose and Botstein 1983; Adams et al. 1997). All the experiments were carried out in triplicate and the deviations in the protein expression data are shown by error bars in the results. The fluctuations in the average steady-state concentration of glucose in the fed-batch experiments were within the acceptable limits.

Results

To validate the dynamic model, experiments were performed with the wild-type and the mutant strain lacking *KIGAL80* at different galactose concentrations with glycerol as the background media and the time course of β -galactosidase activity was measured. Note that the β -galactosidase activity represents the protein expression from a *KIGAL* gene with two binding sites for KIGal4p, and its measurement was therefore used to quantify f_{2p} . Firstly, the steady-state data corresponding to fractional protein expression for genes with two binding sites in a mutant strain lacking *KIGAL80* was compared with model predictions at different glucose concentrations. In such a mutant strain lacking *KIGAL80*, the absence of repressor KIGal80p eliminates all interaction with activator KIGal4p and also with KIGal1p, thus making the expression of the *GAL* genes constitutive in absence of glucose. Thus, the *KIGAL* switch responds only to changes in KIGal4p concentration, the synthesis of which is inhibited by glucose as modeled in Eq. 5. The mutant strain was simulated by setting initial condition of Gal80p as well as its synthesis rate constant to zero. Although in literature, equilibrium model was used to obtain steady state profiles for the mutant strain and compared with experimental values, we present here the comparison of the steady state profiles obtained from the dynamic model with the reported experimental values (Pannala et al. 2010).

The squares in Fig. 2a show the experimental data for fractional protein expression for genes with two binding sites in cells containing glycerol (0.32 M) as the background medium and at different glucose concentrations, while the solid line corresponds to model predictions. It is noted from the figure that at low glucose concentrations, the *KIGAL* genes show the maximum expression. All the

subsequent results presented in this study have been normalized with respect to this maximum constitutive expression. It is also observed from Fig. 2a that glucose does not repress the *KIGAL* genes completely, as a 26% expression was observed even at high glucose concentration (solid line). The steady state profiles obtained from the dynamic model compares well with that of the prediction from the model based on equilibrium considerations for the molecular mechanisms as reported by Pannala et al. (2010). Figure 2a also shows the model predictions of a wild-type system at different steady-state glucose concentrations (dashed line) under a high galactose (0.5 M) background. It should be noted that previous studies do not characterize the effect of glucose on the steady state expression of *GAL* genes in a wild-type strain. Here, the presence of the repressor protein KIGal80p makes glucose repress the *KIGAL* genes at high glucose concentrations. The circles show the experimental data. It can be noted that at high galactose and low glucose concentration, the wild-type expression corresponds to 35% of the mutant strain lacking *KIGAL80*. The dynamic model was also able to predict the steady state expression profile for the wild-type strain at various galactose concentrations (Fig. 2b). As indicated before, the maximum expression observed at high galactose concentration was only 35% of the maximum. The observation suggests that either the repressor protein KIGal80p concentration is very high in the nucleus or the inducer protein KIGal1p is limiting in its function to sequester the repressor protein.

Having validated the steady-state predictions of the dynamic model, we now validate the dynamic model predictions. Figure 2c shows the comparison between the dynamic predictions of fractional protein expression of genes with two binding sites with experimentally obtained dynamic protein expression levels for *K. lactis* wild-type precultured on glycerol for three different galactose concentrations namely, 0.44 M (model: solid line; experiment: circles), 0.16 M (model: dashed line; experiment: squares) and 0.02 M (model: dotted line; experiment: diamond). At $t = 0$, the expression level is about 2.5%, which is observed in a glycerol medium grown until 10 h. Upon exposing the cells to a galactose concentration of 0.44 M (solid line), the genes fully express themselves in about 20 h. When exposed to 0.16 M galactose concentration the switch responds similar to that observed for previous case with a small decrease in the final steady-state expression values. Further, for a low galactose concentration of 0.02 M the system reaches the steady-state in about 10 h. Figure 2d shows the comparison of model predictions and experimental protein expressions for a mutant strain of *K. lactis* lacking *KIGAL80* in a glycerol medium. Here, the cells were precultured in a glycerol medium till exponential phase (optical density, OD₆₀₀, between 0.8 and 1.0). At

Fig. 2 **a** steady-state protein expression levels for genes with two binding sites (f_{2p}) with varying steady-state glucose concentrations (Pannala et al. 2010). Solid line and squares represents model predictions and experimental validation, respectively for a *K. lactis* strain lacking gene *KIGAL80*. Dashed line and circles represent model predictions and experimental validation, respectively for *K. lactis* wild-type strain. **b** steady-state protein expression levels for genes with two binding sites (f_{2p}) with varying galactose concentrations. Solid line represents dynamic model predictions, while circles represent corresponding experimental validation. Model predictions for dynamic performance of the *GAL* system. **c** Fractional protein expressions of genes with two binding sites (f_{2p}) in a wild-type strain for three galactose concentrations, namely 0.44 M (solid line), 0.16 M (dashed line), and 0.02 M (dotted line) and symbols show the

corresponding experimental validation. **d** Fractional protein expressions of genes with two binding sites (f_{2p}) (solid line) in a mutant strain lacking *KIGAL80* in a glycerol media (30 g/l). Circles show the corresponding experimental data on glycerol for genes with two binding sites. The dashed line shows the variation of total KIGal4p concentration. **e** Fractional protein expressions of genes with two binding sites (f_{2p}) in a mutant strain lacking *KIGAL80* in a mixed medium of glucose and glycerol. The dashed line shows the model predictions with experimental validation (circles) for glucose and glycerol concentrations of 0.027 and 0.32 M, respectively. The solid line shows the model predictions with experimental validation (squares) for glucose and glycerol concentrations of 0.1 and 0.32 M, respectively

$t = 0$, the cells were transferred to a fresh medium containing glycerol, where the initial fractional protein expression was 20%. Since, repressor KIGal80p was absent, the switch expresses to its maximum expression levels (solid line) as the total KIGal4p concentration increases due to autoregulation (see Fig. 2d, dashed line). It can be seen from the Fig. 2d that the model predictions for a *KIGAL80* mutant strain matches well with the experimental data. Further, the dynamic model was simulated for a *KIGAL80* mutant strain to find the effect of glucose on the response of the *GAL* system. Here, the model was simulated for a medium containing both glucose and glycerol. Figure 2e shows experimental and model predictions for a

glucose concentration of 0.027 M, with 0.32 M glycerol in the background. As seen from the figure, the protein expression remains at a basal level for 6 h due to repression by glucose. Once glucose is completely consumed, the switch expresses to its maximum levels (dashed line). A similar behavior was observed for a glucose concentration of 0.1 M (solid line). Here, the protein expression remains at its basal level for 10 h, until the presence of glucose in the media. Thus the model was not only able to predict the wild-type behavior for growth on galactose and glucose individually but also the mutant behavior for growth on glucose and galactose as well as mixed substrates, thereby capturing the entire range of system behaviors.

The validated dynamic model of the *KIGAL* system was further used to obtain insights into the regulatory design of the system. The *KIGAL* system in *K. lactis* contains four feedback loops that regulate the genes *KIGAL4*, *KIGAL1*, *KIGAL80*, and *LAC12*. Of these, regulation of gene *KIGAL80* forms a negative feedback on the *KIGAL* system, while the regulation of the remaining genes act as positive feedback loops. We used the dynamic model to study the effect of these feedback loops on the steady-state performance of the *KIGAL* system. We begin the analysis by eliminating the autoregulation of the transcriptional activator KIGal4p in the model thereby making the expression of its gene constitutive by choosing the value for the synthesis rate constant k_g in Eq. 5 appropriately to reflect the constitutive synthesis rate. The maximum synthesis rate of KIGal4p is achieved when f_{lp} is set equal to unity in Eq. 5. The dashed line in Fig. 3a shows the response of *KIGAL* genes to the constitutive expression of KIGal4p and compared with the response of the wild-type (solid line). In this case, the observed maximum expression levels were higher than the wild-type due to the higher synthesis rate of KIGal4p. Similarly constitutive basal expression levels of *K. lactis* can be simulated by setting f_{lp} equal to unity and choosing a small value for rate constant k_g . On reducing the value of k_g by 16-fold, the corresponding response of the system is shown by the dotted line in Fig. 3a. It is seen that the *KIGAL* gene response shows a twofold reduction relative to the wild-type at high galactose concentrations. Thus, the results indicate that the autoregulation of *KIGAL4* plays a major role in the operation of the *KIGAL* switch to produce the maximum induction levels.

In order to study the effect of KIGal4p autoregulation on the dynamic expression of the *KIGAL* system, the model was simulated assuming that the cells were precultured on glycerol and grown on a 0.4 M galactose medium. The solid line in Fig. 3b shows the fractional protein expression for genes with two binding sites for a wild-type. The cell reaches an expression level of 35% in 20 h and sustains the level until about 60 h, at which time the galactose in the medium is completely utilized. Thus the expression levels drop to a new steady-state corresponding to the preculturing in the glycerol medium (identical to initial condition at $t = 0$). The dashed line indicates the expression levels for a cell when KIGal4p is constitutively expressed using a synthesis rate constant identical to that in the wild-type. Here, the expression levels rise to about 40% and are maintained until about 60 h after which it drops to expression levels observed in NINR medium. The higher expression levels relative to wild-type are consistent with steady-state results discussed above. When the constitutive expression of KIGal4p is reduced by 16-fold using $k_g = 7.5 \text{ h}^{-1}$, the expression levels are not maintained at steady-state. The continued reduction in the expression

levels after reaching a maximum is due to inadequate intracellular galactose needed for KIGal1p activation.

The dashed line in Fig. 3c shows the fractional protein expression of genes with two binding sites (f_{2p}) when autoregulation of *KIGAL1* is removed and expressed constitutively with a rate constant same as in the wild-type. Here, since the concentration of the inducer KIGal1p is very high, it sequesters the repressor KIGal80p from the activator-DNA complex thereby relieving repression at a lower galactose concentrations compared to wild-type (solid line). In the case when autoregulation of *KIGAL80* alone is removed and expressed constitutively at its maximum expression levels using the rate constant identical to that in wild-type, the *KIGAL* switch completely shut off (dash dotted line in Fig. 3c). This is due to the limited availability of KIGal1p whose synthesis is autoregulated, which cannot overcome the repression by the high constitutive levels of KIGal80p. However, the switch can be turned on by reducing the constitutive KIGal80p concentration levels. For the case when both KIGal1p and KIGal80p are constitutively expressed to their maximum levels, the model predicts that the switch is induced to its maximum protein expression levels at higher galactose concentration relative to the wild-type (dotted line in Fig. 3c). The ultrasensitive behavior is evidenced by the fact that a Hill equation representation of the stimulus–response relationship yields a Hill coefficient value of 7. Here, the unregulated KIGal1p requires the high galactose concentrations to sequester the abundant repressor protein. In such a mutant strain although the response is ultrasensitive, the organism does not show growth up to 0.4 M of galactose concentration. Thus, it is noted that for the induction of the switch on galactose either both, KIGal1p and KIGal80p, have to be autoregulated or both should be expressed constitutively.

The dynamic performance for the various mutants discussed above is shown in Fig. 3d. The dashed line shows the performance of the *KIGAL* system when KIGal1p alone is not autoregulated and expressed constitutively at maximum induced wild-type levels. Here, the cells show a rapid response due to the large amounts of KIGal1p available for induction and reaches a new steady-state which is maintained until galactose is depleted completely at about 60 h. The dash-dotted line in Fig. 3d shows the response when *KIGAL80* alone was not autoregulated. Here, the regulated amount of KIGal1p is insufficient to sequester the high concentration of KIGal80p leading to the failure of the system in response to galactose. The dotted line in the Fig. 3d shows the performance of the system when both KIGal1p and KIGal80p are not autoregulated and expressed constitutively at maximum induced wild-type levels. Here, the system shows a delay due to the time required for the KIGal1p to sequester the excessive KIGal80p. The thin

Fig. 3 **a** Effect of autoregulation of gene *KIGAL4* on the steady-state performance of the *GAL* genes with two binding sites (f_{2p}) with varying galactose concentrations. **b** Effect of autoregulation of gene *KIGAL4* on the dynamic performance of the *GAL* genes with two binding sites (f_{2p}) for galactose concentration of 0.4 M. In both figures **a** & **b**, dashed line shows the response of a *KIGal4p* constitutively expressed strain with the value of synthesis rate constant equal to wild-type. The dotted line shows the response of a constitutively expressed *KIGal4p* strain with synthesis rate reduced by 16-fold. Solid line represents the wild-type behavior. **c** Effect of autoregulation of *KIGAL1* and *KIGAL80* on the steady-state performance of the *GAL* switch with varying galactose concentrations. **d** Effect of autoregulation of *KIGAL1*, *KIGAL80*, and *LAC12* on the dynamic performance of the *GAL* system for galactose concentration of 0.4 M. Here in both figures **c** & **d**, the dashed line shows the response of a *KIGal1p* constitutively expressed strain with synthesis rate constant made equal to the wild-type level. The dotted line shows

the response when both *KIGal1p* and *KIGal80p* were constitutively expressed to their wild-type induced levels. The dash-dotted line shows the response when *KIGal80p* alone constitutively expressed at wild-type levels. The thin and thick solid line figure **d** shows the dynamic response when *Lac12p* expressed constitutively at wild-type levels and wild-type response, respectively. **e** Predictions of the dynamic model for steady-state protein expressions for genes with two binding sites (f_{2p}) with varying steady-state galactose concentrations for a *LAC12* not autoregulated strain. The solid lines shows the response of the switch when *Lac12p* constitutively expressed and the synthesis rate constant equal to wild-type (the first solid line from top) and when synthesis rate decreased fourfold (second solid line). Dotted line and dash-dotted line represent when *Lac12p* synthesis rate constant value decreased by tenfold and 100-fold, respectively of the wild-type. The dashed line shows the response of a *LAC12* deletion strain

solid line in Fig. 3d shows the response of the system when permease *Lac12p* was expressed constitutively at wild-type levels. Here, the system responds at a faster rate compared to that of wild-type case (thick solid line) due to the large amount of permease available even at $t = 0$ h.

Since galactose is transported into the cytoplasm both by a *Lac12p* facilitated transport mechanism (*Lac12p*) and by a diffusion mechanism as shown in Eq. 9; it is of interest to ascertain the role of the feedback loop implemented through

regulation of *Lac12p*. The dashed line in Fig. 3e shows the fractional protein expression of genes with two binding sites for a mutant strain lacking *LAC12*. In this case, galactose is transported only through diffusion mechanism; the system shows a graded response with varying galactose concentrations giving subsensitive response with a Hill coefficient value of 0.8. Further, at the other extreme, when permease *Lac12p* is expressed constitutively in silico to its maximum expression levels using a synthesis rate constant as in the

wild-type case, the system shows the wild-type expression levels as shown by top most solid curve with a Hill coefficient value of 1.25 in Fig. 3e. At intermediate constitutive synthesis rates of Lac12p, the GAL system shows a graded response for the decreased synthesis rate constant values (lower solid line, $k_{i12} = 125 \text{ g}^{-1} \text{ dm}^3 \text{ h}^{-1}$; dotted line, $k_{i12} = 50 \text{ g}^{-1} \text{ dm}^3 \text{ h}^{-1}$ and dash-dotted line, $k_{i12} = 5 \text{ g}^{-1} \text{ dm}^3 \text{ h}^{-1}$). Thus, the presence of the positive feedback brought about by Lac12p is critical for the observed wild-type response at various galactose concentrations.

It has been experimentally observed that the GAL system of *S. cerevisiae* shows a bistable behavior when exposed to different preculturing conditions (Acar et al. 2005). To verify if such a phenotype exists for the *K. lactis* KIGAL system, we simulated the dynamic model for two different preculturing conditions, that is, glycerol and galactose and subsequently grown on galactose. In the case of preculturing on galactose, the wild type model was simulated for a high galactose concentration for 28 h and the end points were taken as the initial condition for the subsequent growth on galactose. Our results indicate that, unlike as in *S. cerevisiae*, here the wild-type strain does not exhibit bi-stable behavior as shown in Fig. 4a. Experiments were performed with cells precultured on glycerol and galactose and subsequently grown on galactose. The experimental results confirmed that the steady state expression was the same at various galactose concentrations in both the cases indicating absence of bi-stable response (results not shown). On constitutive expression of KIGal80p using a synthesis rate constant lowered by 14-fold relative to the wild-type, the model predicts a bi-stable response (see Fig. 4b) indicating that a weak negative feedback through the constitutive expression of KIGal80p and a strong positive feedback by KIGal1p is essential for a bi-stable response. This fact is corroborated by observing that upon elimination of any of the positive feedback loops (through KIGal4p, KIGal1p and Lac12p) the bi-stable behavior vanishes as was the case with the wild-type (see Fig. 4a) (Vinod et al. 2008).

The above analysis indicates that while the wild-type strain does not show bistable behavior, a strain lacking KIGal80p feedback with a low synthesis rate for KIGal80p does show bistability. In order to quantify the role of parameters in the manifestation of bistable behavior, a parametric sensitivity study was performed by evaluating the metric ' δ ' (see Eq. 18) for parameters perturbed from their nominal values one at a time. Figure 5a shows the variation of δ at different values of the KIGal4p synthesis rate constant (k_g) for the wild-type strain (solid line) and for the strain with KIGal80p expressed constitutively (dashed line). It can be seen that the wild-type strain does not show a significant bistable behavior for all values of k_g considered whereas the mutant strain shows bistable behavior at higher values of k_g including at the nominal value of $k_g = 126 \text{ g}^{-1} \text{ dm}^3 \text{ h}^{-1}$.

Figure 5b, c and d show the variation of δ for different values of the synthesis rate constant of Lac12p, KIGal1p and KIGal80p respectively. It is clear that the synthesis rate constant for Lac12p does not influence the extent of bi-stability while the rate constants of KIGal1p and KIGal80p significantly affect the bistable behavior. Enhancing the KIGal1p (positive regulator) synthesis rate constant while decreasing the KIGal80p synthesis rate constant (negative regulator) induces bistable behavior. Bistable behavior is also observed in the mutant if the half saturation constant K_0 in the growth expression is enhanced (see supplementary material). A random perturbation in all other model parameters except for k_g , k_{i12} , k_{i8} , k_{i1} and K_0 did not alter the bistable behavior in the wild-type or the mutant (see supplementary material). Thus, only synthesis rate constants of KIGal1p and KIGal80p influence the bistable behavior for the wild-type. In case of the mutant, the bistable behavior is altered by k_g and K_0 in addition to synthesis rate constants of KIGal1p and KIGal80p. To rank the relative importance of the parameters rate constants for the synthesis of KIGal4p, KIGal80p and KIGal1p, a global sensitivity analysis was performed. Figure 5e and f shows the sensitivity coefficient with standard deviation for the

Fig. 4 Effect of preculturing on the steady-state performance of the GAL system for genes with two binding sites (f_{2p}) when precultured on glycerol (dashed line) and galactose (solid line) and subsequently transferred to a galactose medium. **a** Wild-type response. **b** KIGAL80 not autoregulated strain

Fig. 5 Parametric sensitivity of the bistable behavior for the wild-type strain (solid line) and a mutant strain wherein KIGal80p is constitutively expressed (dashed line). The ordinates correspond to the metric δ , which represents the maximum separation between the fractional protein expressions (f_{2p}) of *K. lactis* when precultured on galactose and when precultured on glycerol. Effect of **a** rate constant for the synthesis of KIGal4p. **b** Rate constant for the synthesis of Lac12p. **c** Rate constant for the synthesis of KIGal1p. **d** Rate constant for the synthesis of KIGal80p. Global analysis to study the sensitivity to parameters k_3 , k_4 , k_g , k_{18} and k_{11} for **e** wild-type and **f** the mutant. Parameter 1— k_3 ; 2— k_4 ; 3— k_g ; 4— k_{18} ; 5— k_{11}

five parameters obtained by simulating the model with 500 random realizations of the 5 parameters while maintaining all other parameters at their nominal values. The results indicate that in both the wild-type as well as the mutant strain, bistable behavior is most sensitive to the synthesis rate constant of KIGal4p when the mean of sensitivity is used for ranking the parameter importance.

Comparative study with *GAL* system of *S. cerevisiae*

A similar model development for the *GAL* system of *S. cerevisiae* has been reported in the literature (Ruhela et al. 2004). Since the two species of yeast are related evolutionarily with similar regulatory components, it is of

interest to compare the steady-state and dynamic performance of the *GAL* system of the two organisms. The dynamic modeling strategy discussed above was used to compare the performance of *GAL* systems in both, mutant (lacking gene *GAL80*) and wild type strains of *S. cerevisiae* and *K. lactis*. Experimental studies have reported that the wild-type *GAL* system in *S. cerevisiae* shows bistable behavior at intermediate galactose concentrations (Acar et al. 2005). Further, it is known that such short term memory of *GAL* system in *S. cerevisiae* was due to the presence of positive feedback loop of Gal3p. Here, the *K. lactis* dynamic model used to simulate such response by preculturing on galactose and glycerol. The system shows no bistability for any galactose concentration (Fig. 4a). A similar study performed on *GAL* system of *S. cerevisiae* shows a bi-stable behavior for intermediate galactose concentrations (Acar et al. 2005). It has been shown in this case that the positive feedback loop provided by Gal3p is responsible for the observed bistability. In *K. lactis*, since Gal3p is absent and KIGal1p functions as the inducer, the system seems to have lost the short term memory.

The *GAL* system in both species functions in a coordinated way with inherent feedback loops. Both species have identical repressor proteins (Gal80p and KIGal80p) which share 70% identical residues. However, the gene producing KIGal80p in *K. lactis* contains two binding sites in its promoter for activator KIGal4p and is confined to the nucleus. Whereas, the *S. cerevisiae* repressor gene contains only one binding site in its promoter for Gal4p and is a nucleocytoplasmic protein. A similar analogy was observed for inducer proteins KIGal1p and Gal3p with *KIGAL1* gene contains two binding sites and KIGal1p is nucleocytoplasmic protein, whereas, Gal3p is cytoplasmic and *GAL3* contains one binding site for activator Gal4p. It is therefore of interest to compare the performance of the *GAL* systems of both species to analyze how these changes in the structure affect the performance. In the case when both regulatory proteins KIGal1p and KIGal80p were not autoregulated, the *KIGAL* gene response in *K. lactis* required higher galactose concentrations to express compared to wild-type case due to the large amount of unregulated Gal80p concentration present in the nucleus (dotted line in Fig. 3c). A similar response was observed in *S. cerevisiae* (result not shown). When *GAL80* alone was not autoregulated the *GAL* system switches off in both species indicating the importance of *GAL80* autoregulation (dash-dotted line in Fig. 3c) (Ruhela et al. 2004).

Further, the dynamic model can be simulated to determine temporal relevance of each protein for a given galactose concentration. Figure 6a and b shows the comparison of fractional protein expression of genes with two binding sites (f_{2p}) and total protein concentrations with time for *KIGAL* system of *K. lactis* (solid lines) and *S. cerevisiae* (dashed lines). Figure 6a shows the dynamic

Fig. 6 Dynamic performance of *GAL* system in *S. cerevisiae* and *K. lactis* for a high galactose concentration of 0.4 M. **a** Fractional protein expression of genes with two binding sites (f_{2p}) with galactose provided in batch mode. **b** Total nuclear concentration of Gal80p in *K. lactis* and in *S. cerevisiae*. Here, the dashed line for *S. cerevisiae* is scaled by factor 10 for clarity. **c** Steady-state total nuclear concentrations of KIGal1p and Gal80p in *K. lactis* and *S. cerevisiae*, respectively with varying galactose concentrations. In all of the cases above dashed line represents *S. cerevisiae* and solid line represents *K. lactis*

comparison of fractional protein expression of genes with two binding sites (f_{2p}). Here, the cells were precultured in a glycerol medium and exposed to a high galactose concentration (0.44 M for *K. lactis* and 0.44 M for *S. cerevisiae*) at $t = 0$. As seen from the figure the expression levels reached maximum after about 20 h in both species but the maximum level of expression is different. *K. lactis* requires high galactose concentration (0.44 M) to express at maximum level of 35%, while *S. cerevisiae* needs only 0.1 M to reach its maximum expression levels of 82% (Verma et al. 2003). The expression levels drop once galactose is consumed completely after about 60 h in *K. lactis*, whereas, in *S. cerevisiae* the expression levels drop after about 35 h. Since the *GAL* gene expression is due to the interplay of the regulatory proteins, it is of interest to see how concentration of these protein varies with time for a given steady-state galactose concentration. Figure 5b shows the total nuclear concentration of the KIGal80p (solid line) and *S. cerevisiae* (dashed line) for a steady-state galactose concentration of 0.4 M. The high increase in nuclear concentrations of KIGal80p from 37 nM at $t = 0$ to 420 nM after complete induction in *K. lactis* makes the system express moderately compared to that in *S. cerevisiae*, where the nuclear Gal80p concentrations vary between 0 and 2.2 nM (the dashed line in Fig. 6b is scaled by a factor 10 for clarity of representation).

Figure 6c shows the steady-state total nuclear concentrations for KIGal1p and Gal80p with varying galactose concentrations in *K. lactis* and *S. cerevisiae*, respectively.

As seen from the figure, the steady-state total nuclear Gal80p concentration is very high at low galactose concentrations and drop relatively to a small value as the concentration of galactose increased (dashed line) resulting in shuttling of Gal80p to the cytoplasm. On the other hand, the nuclear concentration of KIGal1p is zero at low galactose concentrations and increases to a high value for increased galactose concentrations (solid line).

In silico re-engineering of *GAL* system

The developed dynamic models for both yeast species have been re-engineered in silico to interchange the nucleocytoplasmic shuttling of regulatory proteins. In the dynamic model of the re-engineered *K. lactis* KIGAL system, the inducer protein (KIGal1p) is made to reside in the cytoplasm while the repressor protein (KIGal80p) shuttles between the nucleus and the cytoplasm, thus mimicking the design prevailing in *S. cerevisiae*. Here, the distribution coefficient (K) is defined as the ratio of KIGal80p in the cytoplasm to the nucleus. Figure 7 shows the response of fractional protein expression for two binding site genes for these different scenarios. The modification in shuttling of regulatory proteins in KIGAL system of *K. lactis* makes the fractional protein expression of genes with two binding sites express to about 20% for a distribution coefficient value of 0.4, which is also used in *S. cerevisiae* model (solid line, Fig. 7a). However, an increase in the shuttling coefficient to $K = 2.4$ makes the re-engineered *GAL* switch

Fig. 7 In silico reengineering of *GAL* system in *S. cerevisiae* and *K. lactis* by interchanging the compartmentalization of regulatory proteins. **K. lactis GAL system:** repressor KIGal80p made to translocate to the nucleus while KIGal1p confines to the cytoplasm. **a** Dynamic performance of altered *K. lactis* *GAL* system response for genes with two binding sites for distribution coefficient values of $K = 0.4$ (solid line) and $K = 2.4$ (dashed line) **b** Steady-state performance of *K. lactis* *GAL* system response for genes with two binding sites for distribution coefficient values of $K = 0.4$ (solid line) and $K = 2.4$ with varying galactose concentrations. *S. cerevisiae* *GAL*

system: Repressor Gal80p made confined to the nucleus while Gal3p translocates to the nucleus in *GAL* system of *S. cerevisiae*. **c** Dynamic performance of altered *S. cerevisiae* *GAL* system response for genes with two binding sites for a unchanged parameter system (solid line) and for a change in dissociation constant, $K_3 = 1 \times 10^{-10}$ (dashed line). **d** Steady-state performance of altered *S. cerevisiae* *GAL* system response for genes with two binding sites for a unchanged parameter system (solid line) and for a change in dissociation constant, $K_3 = 1 \times 10^{-10}$ with varying galactose concentrations

Fig. 8 In silico re-engineering of *GAL* system in *S. cerevisiae* and *K. lactis* for both regulatory proteins Gal3p/Gal1p and Gal80p shuttle across nuclear membrane. **a** Re-engineered *GAL* system for *S. cerevisiae* to include shuttling of Gal3p to nucleus. The solid line shows the experimental validation for Gal3p distribution coefficient of 3.2 and dashed line shows the response of the system for a

decreased Gal3p distribution coefficient of 0.12. **b** Re-engineered *GAL* system for *K. lactis* to include shuttling of KIGal80p to cytoplasm. The solid line shows the experimental validation for KIGal80p distribution coefficient of 0.012 and dashed line shows the response of the system for an increased KIGal80p distribution coefficient of 1.4

behave like *S. cerevisiae* GAL system with expression levels reaching about 78% (dashed line). In this case, it was observed that the re-engineered system requires larger time (60 h) to reach the maximum expression levels than in the wild-type *S. cerevisiae* GAL system. The corresponding steady-state performance the GAL system for varying steady-state galactose concentration was shown in Fig. 7b for genes with two binding site (f_{2p}). Here, for increased distribution coefficient ($K = 2.4$) (dashed line, Fig. 7b), the expression levels were increased even at low galactose concentrations due to the higher quantity of KIGal80p that shuttles into the cytoplasm.

A similar study on GAL system of *S. cerevisiae* is performed by making the inducer Gal3p shuttle between the nucleus and the cytoplasm and Gal80p confined to the nucleus. In such a re-engineered system, the response was switched off for a K value of 8.0, which is also used in *K. lactis* model (solid line, Fig. 7c) and the system response was unchanged by changing distribution coefficient value. However, the system was observed to be sensitive to the reduced affinity for [Gal80p–Gal4p] complex. The nominal *S. cerevisiae* system has a dissociation constant value for Gal80p–Gal4p interaction of $K_3 = 1 \times 10^{-13}$, by increasing K_3 value to $K_3 = 1 \times 10^{-10}$, the GAL system performance was observed similar to that of *K. lactis* KIGAL system (dashed line, Fig. 7c). Further, the corresponding model simulations for steady-state response of genes with two binding sites are shown in Fig. 7d. As seen from the figure, the system shows no response when parameters were not changed from the original system for any galactose concentrations (solid line). However, by increasing the dissociation constant for [Gal80p–Gal4p] complex, the GAL gene response was observed similar to *K. lactis* (dashed line, Fig. 7d).

Recent experimental studies on *S. cerevisiae* GAL system have indicated that the inducer protein Gal3p can also shuttle into the nucleus and interact with the repressor protein Gal80p to relieve inhibition on activator protein Gal4p (Wightman et al. 2008). To mimic the experimental findings, we re-engineered the GAL system in *S. cerevisiae* to include the nucleocytoplasmic shuttling of both repressor (Gal80p) and inducer (Gal3p) proteins. Figure 8a shows the model predictions for the re-engineered system. The distribution coefficient of Gal80p was similar to the original model ($K = 0.4$), while the distribution coefficient for Gal3p was regressed to match the experimental data (Verma et al. 2003). Solid line in Fig. 8a shows that the model predictions were able to predict the experimental steady-state data on varying galactose concentrations for a Gal3p distribution coefficient value of 3.2 (here, the distribution coefficient for Gal3p is defined as the ratio of activated Gal3p in the cytoplasm to the nucleus), while the distribution coefficient for Gal80p was kept unchanged as

in the original model. Here, the observed total nuclear Gal3p concentration of 0.05% at high galactose concentration indicates only a small portion of the total Gal3p shuttles into the nucleus. The system out-performs the nominal system when distribution coefficient for Gal3p was decreased to 0.12 (10% of total Gal3p shuttles into the nucleus) as shown by dashed line in Fig. 7a. Subsequently a similar study was performed on KIGAL system of *K. lactis* to include the nucleocytoplasmic shuttling of both repressor and bifunctional protein. The model predictions show that the *K. lactis* KIGAL system also shows fractional protein expressions under conditions of both KIGal1p and KIGal80p shuttling for a KIGal80p distribution coefficient value of 0.012 (solid line in Fig. 8b) and the KIGal1p distribution coefficient was kept similar to the original unchanged model. Here, the distribution coefficient for KIGal80p is defined as the ratio of KIGal80p in the cytoplasm to the nucleus. The KIGAL system shows the observed experimental behavior with 10% of the total KIGal80p shuttling into the cytoplasm. The fractional protein expressions can be increased by increasing the distribution coefficient value to 1.4 as shown by dashed line in Fig. 8b. The analysis shows that the GAL system in both organisms can perform under conditions of shuttling of both proteins as reported experimentally for *S. cerevisiae* case but with an appropriate value of distribution coefficient. However, a similar situation of KIGal80p shuttling has not been reported experimentally for the *K. lactis* KIGAL system (Anders et al. 2006).

Discussion

A dynamic modeling strategy is used here to understand the complex interactions in the KIGAL system of *K. lactis* and is subsequently compared its performance to that of *S. cerevisiae*. The performance of the KIGAL system in *K. lactis* is dependent on the interplay of its regulatory proteins and their relative concentrations. The maximum expression levels observed in wild type strain after induction with galactose (see Fig. 2b) are very low (only 35%) compared to that of a mutant strain lacking KIGAL80 expressed under no glucose or in a NINR medium (see Fig. 2a). The lowered expression levels in wild-type strain can be explained in two ways. Since, in *K. lactis* the repressor protein KIGal80p is nuclear, the proximity for interaction with activator KIGal4p is greater and it forms a competition between KIGal1p and KIGal4p to interact with repressor. Further, the protein KIGal1p is a bifunctional protein which has both galactokinase and inducer activity. This leads to a decisive conflict in the organism: what fraction of the KIGal1p must be refrained for inducer function. A steady-state model analysis revealed that, only

10% of the total KIGal1p is used for inducer function leading to a lowered expression levels observed in *K. lactis*. In case of *S. cerevisiae*, the species resolved this issue by undergoing a whole genome duplication to create a new regulatory protein Gal3p which has only inducer activity. In addition, the inducer protein in *S. cerevisiae* sequesters Gal80p into the cytoplasm leading to a small concentration of repressor in the vicinity of the activator. Further, in the presence of glucose, the *KIGAL* system of *K. lactis* is not completely repressed (see Fig. 2a) due to the autoregulation of gene *KIGAL4*, whereas in *S. cerevisiae* which is not autoregulated shows complete repression of *KIGAL* genes (Ruhela et al. 2004).

The regulatory proteins Gal80p and Gal3p/Gal1p in the *GAL* system are autoregulated by their respective genes. However, the number of binding sites in their promoter regions varies from *S. cerevisiae* to *K. lactis*. Both genes *GAL80* and *GAL3* in *S. cerevisiae* are controlled by a single binding site, whereas in *K. lactis*, both *KIGAL80* and *KIGAL1* are controlled by genes with two binding sites. It is interesting to see how these changes in the structure of the *GAL* system reflect in the steady-state response. The dynamic model predictions for steady-state protein expression levels shows that when autoregulation of both genes is removed, the response of the *GAL* system in both the species are repressed completely at low galactose concentrations due to higher repressor concentrations. However, when repressor gene alone is not autoregulated, both species respond similarly. Here, the unregulated repressor is in a large enough concentrations in both the species to keep the system under repressed conditions. The result indicates that, in both species the repressor gene must be autoregulated along with the inducer gene with the same number of binding sites in their promoter regions for activator to make the system function normally. Further, in both species, the synthesis of transporter proteins (Gal2p/Lac12p) are autoregulated by their respective genes with two binding sites for activator in their promoter regions. Elimination of regulation of the transporter gene suggests that the *GAL* system response can be tuned in both organisms. The system response shows a graded response when Lac12p is expressed constitutively to a low value (see Fig. 3e), indicating the importance of the positive feedback responsible for the switch like response observed in both organisms in addition to other mechanisms. However, the graded response observed for *K. lactis* was not as steep as it was observed for *S. cerevisiae* case (Hawkins and Smolke 2006).

The dynamic model can be used to analyze the effect of preculturing on steady-state response of the *GAL* gene expression. The *K. lactis* *KIGAL* system does not show bistable behavior for different preculturing conditions. However, the system shows bistability for a strain where *KIGAL80* is not autoregulated (Fig. 4b). In the case of wild

type *K. lactis* system, the negative feedback by KIGal80p is much stronger both due to its nuclear localization and stronger control by multiple binding sites for KIGal4p leading to a loss of bistability. In case of *S. cerevisiae*, the *GAL* system shows bistability, which is due to the presence of positive feedback by inducer Gal3p (see Fig. 4a). This is consistent with the experimental observation that the *GAL3* is responsible for the short term memory observed in *S. cerevisiae* (Kundu and Peterson 2010). Parametric sensitivity analysis indicated that the synthesis rate constants for KIGal1p and KIGal80p, imparting positive and negative feedbacks, respectively, are the dominant parameters influencing bistability (Vinod et al. 2008).

Further, the dynamic model provides the opportunity to compare how autoregulation of all these regulatory proteins affects the dynamic performance of the *GAL* system in both organisms. Since, synthesis of activator protein KIGal4p is autoregulated in *K. lactis*, the removal of autoregulation and a constitutive expression with a reduced synthesis rate here makes the response drop at a faster rate than observed for wild-type system (see dotted line in Fig. 3b) due to insufficient amount of activator concentration. When both regulatory proteins KIGal80p and KIGal1p are independently expressed, the system responds with an initial lag both in *K. lactis* (see Fig. 3d), and in case of *S. cerevisiae* (Ruhela et al. 2004). The lag observed here is due to the time required for the Gal3p to sequester the excessive Gal80p from the nucleus in case of *S. cerevisiae* and due to the high concentrations of KIGal80p in the nucleus in case of *K. lactis*. In this case where both regulatory proteins are not autoregulated, the system shows ultrasensitive behavior, either fully expressed or fully repressed in both of the organisms. In the case when synthesis of inducer alone is not autoregulated, both systems show rapid response due to high constitutive concentrations of inducer. However, the maximum expressions observed in both species were similar to their wild-type response. The analysis shows that the autoregulation of regulatory proteins is crucial for the nominal response for both the systems. The temporal comparison of total concentrations of individual regulatory proteins in *K. lactis* and *S. cerevisiae* suggests that even though *K. lactis* has higher basal protein concentrations (Fig. 6b), the time required to reach the maximum is longer (Fig. 6a) due to a large increase in the nuclear concentration of KIGal80p with time (see Fig. 6b). In case of *S. cerevisiae* the opposite is observed leading to higher expression levels due to moderate increase in the total concentration of Gal80p (Fig. 6b). However, the relative total concentrations are very low in case of *S. cerevisiae* compared to that of *K. lactis*. Further, in silico re-engineering study shows that, interchange of nucleocytoplasmic shuttling of regulatory proteins in *K. lactis* was able to show response with an increased distribution coefficient, where as a similar effect was not

observed for *S. cerevisiae* system due to high affinity between the Gal80p and Gal4p proteins, indicating the differences in affinities for regulatory proteins in two systems.

Recent studies indicate that in addition to the nucleocytoplasmic shuttling of Gal80p, the inducer Gal3p also shuttles to the nucleus (Wightman et al. 2008). To study the effect of nucleocytoplasmic transport of Gal3p on the response of GAL switch, the *S. cerevisiae* model was re-engineered to include the transport of Gal3p. The analysis estimated the distribution coefficient for the nucleocytoplasmic transport of Gal3p to be 3.2, which indicates that only 0.05% of the total Gal3p will reside in the nucleus. Thus, although Gal3p shuttles to the nucleus, the shuttling of Gal80p is the key mechanism for the operation of the switch. A similar analysis with *K. lactis* indicated that the shuttling of KIGal80p into the cytoplasm, although feasible, is not a key mechanism that influences the performances of the KIGAL switch.

In summary, the developed dynamic model predicts the steady-state behavior of the KIGAL system for various scenarios establishing the importance of a particular mechanism involved in the operation of the switch. The analysis predicts the importance of various inherent systems-level properties such as autoregulation of regulatory proteins, bistability and sensitivity arising out of the molecular interactions. The dynamic model can be used to compare the performance of two homologous yeast species to evaluate the role of regulation and compartmentalization of proteins on the phenotypic response. Further, the comparative studies revealed that the *K. lactis* KIGAL system operates with higher basal level concentrations of regulatory proteins compared to *S. cerevisiae*. The in silico re-engineering study shows that the differences in affinities for regulatory protein interactions also responsible for the observed phenotypic responses. Thus, though the GAL system in yeast species *K. lactis* and *S. cerevisiae* share similarities in some of the molecular components, the interactions among them varied considerably due to evolutionary pressures, giving a different system response. The presence of a dedicated inducer due to genome duplication in *S. cerevisiae* resulted in a highly sensitive to galactose. Whereas, in *K. lactis* the bifunctional inducer, KIGal1p and the autoregulation of KIGal4p yielded higher basal levels of regulatory proteins in response to galactose. However, it would be interesting to study the response of the KIGAL system to lactose since the lactose medium constitutes a niche environment for *K. lactis*.

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