Characterization and modeling of a specific transcriptional regulatory network required for multidrug resistance in yeast

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1 Work plan

The objective of this project is to unveil the hierarchy and synergy that lies behind the combined action of the transcription factors (TFs) involved in yeast response to drugs and other chemical aggressions and to develop computational tools for modeling the dynamic of these transcription regulatory networks. The transcription level from a gene results from the balance of the actions of various transcriptional regulators, either promoting or repressing its transcription. However, how transcriptional regulatory networks overlap and cross-talk to influence transcription, under optimal growth conditions or under environmental stress, and how the promoter context affects the binding and action of each transcription factor is scarcely understood. The regulatory network that mediates the activation of the H⁺-antiporter encoding gene FLR1 in the presence of the fungicide mancozeb (widely used in agriculture), was chosen to represent the experimental platform of this project. This option is due to the theoretical and practical importance of this regulatory response and to the previous experience of the BSRG team at Instituto Superior Técnico with this particular system.

Specifically, the main objective of this project is to clarify the hypothesized combinatorial action of the TFs Yap1, Pdr3 and Yrr1, on FLR1 activation and the possible role, in yeast stressed cells, of other selected TFs that have potential binding sites in FLR1 promoter region or that regulate TFs of this network. Gene transcript levels will be compared in wild-type and derived mutant strains, in which the different transcription factors encoding genes were individually deleted, or in double mutants. The time course of the dynamic transcriptional response to mancozeb in the wild-type and in the different mutant strains were analyzed.

In the wet-lab, chromatine immunoprecipitation (IP) and real time-PCR were used to determine the \textit{in vivo} occupancy of gene promoter sequence by the TFs in response to the drug. In the computer lab, we used computational methods and tools for the modeling of the behavior of the FLR1 regulatory network. Based on the models obtained, the regulation of FLR1 in the presence of mancozeb was studied in detail and the simulated behaviors compared with the experimental time course data. The usage of the built model allows to quickly formulate new hypotheses about the regulatory mechanisms controlling the transcriptional activation of FLR1 upon mancozeb induced stress. Additionally, the benefit of having a working model is extended by the ability to simulate \textit{in silico} the system's behaviors with single or double knockouts in a reliable and cost effective manner.
2 Developed work

In order to model the FLR1 regulatory network, we considered previous knowledge on the transcriptional profile of each of the variables of the network registered upon exposure to the fungicide mancozeb, and obtained by quantitative RT-PCR [1]. These variables include, besides FLR1 itself, five transcription factors: Yap1, known to control oxidative stress response, Pdr3 and Yrr1, two regulators of multidrug resistance in yeast, Rpn4, known to control the expression of proteasome genes, required to intensify protein degradation under stress, and a fifth transcription factor, added to the network for the reasons described below. The first four transcription factors were identified as the key players in this network in a previous screening for the participation of all documented, direct or indirect, regulators of FLR1, as indicated by the YEASTRACT database, in its transcriptional activation in yeast cells exposed to mancozeb [1].

2.1 Reconstruction of the FLR1 gene mancozeb response network from experimental data

The transcriptional activation of FLR1 in yeast cells exposed to mancozeb is completely dependent on the Yap1 transcription factor [1]. However, to obtain a full activation of the FLR1 gene Yrr1, Pdr3 and Rpn4 are also required [1]. The effect of Yap1 and Yrr1 in FLR1 transcription is known to be direct, as proven by in vivo ChIP (Chromatine ImmunoPrecipitation) studies [2, 3]. We have made the assumption that it is possible that Pdr3 is also able to bind to the FLR1 promoter region, since a putative binding site (TCCGCGCA) for Pdr3 is found at position -433 of the FLR1 promoter, using the YEASTRACT database [4, 5]. Also, we have made the assumption that Rpn4 should act on FLR1 through the activation of another transcription factor, since there is no existence of potential binding sites for Rpn4 in the FLR1 promoter. This transcription factor appears to be Yap1, whose expression is up-regulated in mancozeb stressed cells in the dependency of Rpn4 [1].

PDR3 up-regulation induced in mancozeb stressed cells is apparently dependent on Yrr1 and Yap1 [1]. There is no evidence of Yrr1 or Yap1 binding to the PDR3 promoter region, and actually, no putative Yap1 binding site is known to occur in the region. However, the effect of Yap1 could take place indirectly through the activation of Yrr1 expression, but this is unlikely since it was observed that in the ∆rpn4, in which there is no mancozeb-induced YRR1 up-regulation, there is still PDR3 transcriptional activation [1]. This has led us to the assumption that PDR3 is regulated simultaneously by Yrr1 and Yap1. Interestingly, in the absence of Yap1 and Yrr1 the up-regulation of PDR3 is completely abrogated.

RPN4 transcriptional activation is also known to depend on both Yrr1 and Yap1 [1]. Also, similarly to what was observed for PDR3 regulatory control, in the absence of Yap1 or Yrr1 the mancozeb-induced up-regulation of RPN4 is completely abrogated [1]. The direct binding of Yap1 to the RPN4 promoter region was previously described by Harbison et al. [6]. Based on the effect of Yrr1 registered upon RPN4 expression and on the occurrence of two Yrr1 binding sites at the RPN4 promoter, a direct effect is assumed herein.
Full mancozeb induced YRR1 transcriptional activation depends on 3 transcription factors: Yap1, Pdr3 and Rpn4 [1]. Rpn4 is assumed to have an indirect effect on Yrr1 expression, since there is no evidence that it binds to the YRR1 promoter nor is there a predicted Rpn4 binding site in its promoter region. YRR1 direct dependency on Yap1 is assumed in this study, given that YRR1 up-regulation is impaired in the absence of Yap1 and the occurrence of a putative Yap1 binding site in the YRR1 promoter. The effect of Pdr3 is also herein assumed to be direct based on expression evidence and on the occurrence of putative Pdr3 binding sites in the YRR1 promoter region.

YAP1 activation is dependent on Yrr1, Rpn4 and an unidentified transcription factor, designated FactorX. Yap1 autoregulation [7] and direct dependency on Rpn4 [6] were previously demonstrated. Although the deletion of Yrr1 was found to have an effect on YAP1 transcriptional activation, this effect was initially assumed to be indirect based on the absence of a putative Yrr1 binding site in the YAP1 promoter region. An additional transcription FactorX had to be added to the final model to account for the discrepancy observed between the simulations obtained from the initial model and the experimental data obtained to test those predictions, as described below. It represents an unknown intermediate between Yrr1 and YAPI transcriptional activation, other than Rpn4.

2.2 Construction of the FLR1 gene mancozeb response model

Due to the lack of biological data, it was not possible to build a quantitative model of this network. However, there is enough data available to build a qualitative model, in order to analyze the dynamical behavior of the regulatory network.

This section describes how the different types of interactions illustrated in Figure 1, that play a role in the FLR1 mancozeb response, can be modeled by means of piecewise-linear differential equations. The resulting model combines six state variables, each corresponding to the total protein concentration and one input variable denoting the concentration of mancozeb (Table 1). Note that the experimental data used in this study [1] concern mRNA and not protein levels. We assume that variations in mRNA and protein levels are the same, since the effect of mancozeb on gene expression occurs mainly at the transcriptional level [1]. A similar approximation is made by Cantone et al. [8], where protein and mRNA levels are assumed to be proportional.

2.2.1 Mancozeb input signal

Mancozeb stress signal has to be sensed by the yeast cell in order to induce the observed transcriptional and physiological response. The exact mechanism(s) by which mancozeb is sensed is still unknown. However, it clearly activates, directly or indirectly, the involved transcription factors. This activation occurs at the transcriptional level [1], but may also occur at the post-translational level. For instance, Yap1 is activated by the formation of intra-molecular disulfide bonds in the presence of oxidants, alkylating or thiol-reactive agents, being preferentially targeted to the nucleus in that activated state [9]. Significantly, it was recently demonstrated that Yap1 is the regulator of over 90% of the proteins up-regulated in response to mancozeb [10] and that mancozeb exerts a strong effect at the level of cystein oxidation [11], a necessary feature for Yap1 activation. Pdr3 is known
to be activated by the direct binding of xenobiotics [12], although this has not been demonstrated for mancozeb. No data has been collected so far on the eventuality of Yrr1 post-translational control, although it seems probable to occur in a similar way to its homologue Pdr3. The presence of the mancozeb signal denotes the mancozeb exposure which stimulates the FLR1 network transcriptional response. The following equation was considered:

$$\dot{u}_{\text{mancozeb}} = 0$$  \hspace{1cm} (1)

A single threshold $\theta_{\text{mancozeb}}$ for the signal was defined considering the inequalities presented in Table 1. We also define a step function $s^+(u_{\text{mancozeb}}, \theta_{\text{mancozeb}})$, to be used by the subsequent state variables, which is evaluated as 1 (0) when the signal is said to be present (absent).

### 2.2.2 FLR1 transcriptional activation

A basal FLR1 mRNA synthesis rate, $\kappa_{flr1}^0$ is considered to account for FLR1 expression in the absence of mancozeb. We denote by $\kappa_{flr1}^1$ the rate of FLR1 mRNA synthesis depending on Yap1 alone, activated in the presence of mancozeb. Also, we denote by $\kappa_{flr1}^2$ the rate of FLR1 mRNA synthesis depending on Yrr1, requiring the presence of Yap1, and by $\kappa_{flr1}^3$ the rate of FLR1 mRNA synthesis depending on Pdr3, activated in the presence of mancozeb, and requiring the presence of Yap1. The rate of degradation of the protein is denoted by $\gamma_{flr1}$. This degradation rate is not known to be regulated by any of the network components, and it is therefore represented as $\gamma_{flr1} x_{flr1}$. The following state equation for FLR1 is obtained:
FLRI transcript levels are defined in the current model with thresholds corresponding to its production rate depending on the influencing step functions. The transcription factors influencing these step functions are Yap1 (θb_yap1, θ1_yap1, θ3_yap1), Yrr1 (θ2_yrr1), and Pdr3 (θpdr3) which need to be present to permit a full FLRI activation upon mancozeb response. Regarding the FLRI threshold inequalities (Table 1), experimental data [1] shows that a ∆yap1 single mutant completely devoid the FLRI transcriptional activation. Also, that a ∆pdr3 (∆yrr1) single mutant provokes a 2-fold (3-fold) reduction of the FLRI transcriptional activation.

### 2.2.3 Pdr3 activation module

The Pdr3 activation module is composed of proteins Pdr3, Yrr1 and Yap1. A basal PDR3 protein synthesis rate, κb_pdr3 is considered to account for PDR3 expression in the absence of mancozeb. Given this interdependency, κ1_pdr3 denotes the rate of Pdr3 protein synthesis depending on Yrr1, activated in the presence of mancozeb, and requiring the presence of Yap1, since in the absence of Yrr1 and Yap1 the up-regulation of PDR3 is completely abrogated. Considering the degradation rate of γpdr3 x_pdr3, the following state equation for PDR3 is obtained:

\[
\dot{x}_{pdr3} = \kappa^b_{pdr3} + \kappa^1_{pdr3} s^+(x_{yrr1}, \theta^1_{yrr1}) s^+(x_{yap1}, \theta^1_{yap1}) s^+(u_{mancozeb}, \theta_{mancozeb}) \\
- \gamma_{pdr3} x_{pdr3}
\]  

Upon mancozeb exposure, PDR3 is transcriptionally activated, reaching the threshold θpdr3. The PDR3 inequalities (Table 1) are justified by the experimental data [1] where a ∆yap1 or a ∆yrr1 mutants prevent the PDR3 transcriptional activation.

### 2.2.4 Yrr1 activation module

The Yrr1 activation module is composed of two transcription factors: Yap1 and Pdr3. κb_yrr1, a basal protein synthesis rate for this transcription factor was considered to account for YRR1 expression in the absence of mancozeb. We further denote by κ1_yrr1 the synthesis rate depending on Yap1 in the presence of mancozeb. κ2_yrr1 the synthesis rate elicited by Pdr3, and by κ3_yrr1 the synthesis rate depending on Yap1. Given the degradation rate γyrr1 x_yrr1, the state equation describing YRR1 expression is as follows:

\[
\dot{x}_{yrr1} = \kappa^b_{yrr1} + \kappa^1_{yrr1} s^+(x_{yap1}, \theta^1_{yap1}) s^+(x_{yap1}, \theta^1_{yap1}) s^+(u_{mancozeb}, \theta_{mancozeb}) \\
- \gamma_{yrr1} x_{yrr1}
\]
\[
\dot{x}_{yrr1} = \kappa_{yrr1}^b + \kappa_{yrr1}^1 s^+(x_{yap1}, \theta_{yap1}^1) s^+(u_{mancozeb}, \theta_{mancozeb}) + \kappa_{yrr1}^2 s^+(x_{pdr3}, \theta_{pdr3}^1) + \kappa_{yrr1}^3 s^+(x_{yap1}, \theta_{yap1}^3) s^+(u_{mancozeb}, \theta_{mancozeb}) - \gamma_{yrr1} x_{yrr1}
\]

Yrr1 protein levels are defined in the current model by three threshold values, \(\theta_{yrr1}^1\), \(\theta_{yrr1}^2\) and \(\theta_{yrr1}^3\). These thresholds are attained by the action of the synthesis rates independently controlled by Yap1 and Pdr3, and the ordering of the inequalities (Table 1) supported by the experimental data (see [1] and Teixeira et al., submitted). The last threshold is reached only when \(\Delta YAP1\) is present at higher concentrations, resulting from a complex feedback loop by which Yrr1 activates \(\Delta YAP1\) and is, then, further up-regulated by Yap1.

### 2.2.5 Rpn4 activation module

The Rpn4 activation module is composed of proteins Rpn4, Yrr1 and Yap1. A basal Rpn4 protein synthesis rate, \(\kappa_{rpn4}^b\) is considered to account for \(RPN4\) expression in the absence of mancozeb. Analogously to \(PDR3\) expression, \(\kappa_{rpn4}^1\) denotes the rate of Rpn4 protein synthesis depending on Yrr1, activated in the presence of mancozeb, and requiring the presence of Yap1. Including the degradation rate \(\gamma_{rpn4} x_{rpn4}\), the following state equation is obtained:

\[
\dot{x}_{rpn4} = \kappa_{rpn4}^b + \kappa_{rpn4}^1 s^+(x_{yrr1}, \theta_{yrr1}^1) s^+(x_{yap1}, \theta_{yap1}^1) s^+(u_{mancozeb}, \theta_{mancozeb}) - \gamma_{rpn4} x_{rpn4}
\]

Similarly to \(PDR3\), upon mancozeb exposure, \(RPN4\) is transcriptionally activated, reaching the threshold \(\theta_{rpn4}^1\). The \(RPN4\) inequalities (Table 1) are justified by the experimental data [1] where a \(\Delta yap1\) or a \(\Delta yrr1\) mutants prevent the \(RPN4\) transcriptional activation.

### 2.2.6 Yap1 activation module

The Yap1 activation module is composed of Rpn4 and an unidentified transcription factor. A basal synthesis rate, \(\kappa_{yap1}^b\) was considered to account for \(YAP1\) expression in the absence of mancozeb stress. \(\kappa_{yap1}^1\) is depending on the presence of mancozeb, and \(\kappa_{yap1}^2\) is depending only on Rpn4 at a high level. An additional synthesis rate, \(\kappa_{yap1}^3\), was added to the current model corresponding to the dependency of an unidentified transcription factor (designated FactorX), which is supported by the fact that the double deletion mutant \(\Delta yrr1\Delta rpn4\) exhibits \(YAP1\) transcript levels qualitatively lower than those observed for the single mutants \(\Delta yrr1\) and \(\Delta rpn4\), suggesting an additive effect of Yrr1
(through FactorX) and Rpn4 in the YAP1 activation. Further details on the inclusion of this new transcription factor will be presented in the following sections. Considering the degradation rate term $\gamma_{yap1} x_{yap1}$, the following state equation is obtained:

$$
\dot{x}_{yap1} = \kappa_{yap1}^b + \kappa_{yap1}^1 s^+(u_{mancozeb}, \theta_{mancozeb}) + \kappa_{yap1}^2 s^+(x_{rpn4}, \theta_{rpn4}^1) + \kappa_{yap1}^3 s^+(x_{factorX}, \theta_{factorX}^1) - \gamma_{yap1} x_{yap1}
$$

The threshold $\theta_{yap1}^1$ is reached by the activation of Yap1 through mancozeb. Upon activation by Rpn4, the threshold $\theta_{yap1}^2$ is attained. Since Yrr1 and Rpn4 exert a cumulative effect on YAP1 up-regulation, as explained later on, an additional threshold, $\theta_{yap1}^3$, is defined as the level reached through the action of Yrr1 on YAP1, mediated by FactorX.

### 2.2.7 FactorX activation module

The FactorX activation module is composed of only Yrr1. FactorX was introduced based on the discrepancy observed between the simulations obtained from the initial model and the experimental data obtained to test those predictions (see [1] and Teixeira et al., submitted). It represents an unknown intermediate between Yrr1 and YAP1 transcriptional activation, other than Rpn4. As such, the synthesis rate, $\kappa_{factorX}^1$, represents the dependency of Yrr1 in the presence of mancozeb. A basal synthesis rate, $\kappa_{factorX}^b$, was considered to account for FactorX expression in the absence of mancozeb stress. Considering $\gamma_{FactorX} x_{FactorX}$ as a degradation rate term, the following state equation is obtained:

$$
\dot{x}_{factorX} = \kappa_{factorX}^b + \kappa_{factorX}^1 s^+(x_{yrr1}, \theta_{yrr1}^1) s^+(u_{mancozeb}, \theta_{mancozeb}) - \gamma_{factorX} x_{factorX}
$$

Threshold $\theta_{factorX}^1$ was defined as the level of FactorX attained upon Yrr1-mediated mancozeb-induced up-regulation. At this level it is proposed to partially control YAP1 up-regulation. FactorX threshold is ordered according to the inequalities presented in Table 1.

### 2.3 Simulation of the FLR1 gene mancozeb response model

The qualitative dynamics of the network was described by six coupled piecewise-linear differential equations and forty eight inequality constraints on the parameter values (Table 1). Using the GNA modeling and simulation tool we performed a qualitative analysis of this model, obtaining a state transition graph of approximately $10^5$ states containing all the simulated behaviors of the system, while the subset of states that are reachable from
\[ u_{\text{mancozeb}} = 0 \]

\[ 0 < \theta_{\text{mancozeb}} < \max_{\text{mancozeb}} \]

\[ x_{\text{flr}1} = \begin{align*}
\kappa_{\text{flr}1} \\
+ \kappa_{\text{flr}1} s^+(x_{\text{yap}1, \theta_{\text{yap}1}}) s^+(u_{\text{mancozeb}}, \theta_{\text{mancozeb}}) \\
+ \kappa_{\text{flr}1} s^+(x_{\text{pdr}3, \theta_{\text{pdr}3}}) s^+(u_{\text{mancozeb}}, \theta_{\text{mancozeb}}) \\
- \gamma_{\text{flr}1} x_{\text{flr}1}
\end{align*} \]

\[ 0 < \kappa_{\text{flr}1} / \gamma_{\text{flr}1} < (k_{\text{flr}1}^b + k_{\text{flr}1}^b) / \gamma_{\text{flr}1} < (k_{\text{flr}1}^b + k_{\text{flr}1}^b) / \gamma_{\text{flr}1} < (k_{\text{flr}1}^b + k_{\text{flr}1}^b) / \gamma_{\text{flr}1} < (k_{\text{flr}1}^b + k_{\text{flr}1}^b) / \gamma_{\text{flr}1} < \max_{\text{flr}1} \]

\[ x_{\text{pdr}3} = \begin{align*}
\kappa_{\text{pdr}3} \\
+ \kappa_{\text{pdr}3} s^+(x_{\text{flr}1, \theta_{\text{flr}1}}) s^+(u_{\text{mancozeb}}, \theta_{\text{mancozeb}}) \\
- \gamma_{\text{pdr}3} x_{\text{pdr}3}
\end{align*} \]

\[ 0 < \kappa_{\text{pdr}3} / \gamma_{\text{pdr}3} < \theta_{\text{pdr}3} < (k_{\text{pdr}3}^b + k_{\text{pdr}3}^b) / \gamma_{\text{pdr}3} < \max_{\text{pdr}3} \]

\[ x_{\text{yrr}1} = \begin{align*}
\kappa_{\text{yrr}1} \\
+ \kappa_{\text{yrr}1} s^+(x_{\text{pdr}3, \theta_{\text{pdr}3}}) s^+(u_{\text{mancozeb}}, \theta_{\text{mancozeb}}) \\
+ \kappa_{\text{yrr}1} s^+(x_{\text{pdr}3, \theta_{\text{pdr}3}}) s^+(u_{\text{mancozeb}}, \theta_{\text{mancozeb}}) \\
- \gamma_{\text{yrr}1} x_{\text{yrr}1}
\end{align*} \]

\[ 0 < \kappa_{\text{yrr}1} / \gamma_{\text{yrr}1} < \theta_{\text{yrr}1} < (k_{\text{yrr}1}^b + k_{\text{yrr}1}^b) / \gamma_{\text{yrr}1} < \theta_{\text{yrr}1} < (k_{\text{yrr}1}^b + k_{\text{yrr}1}^b) / \gamma_{\text{yrr}1} < (k_{\text{yrr}1}^b + k_{\text{yrr}1}^b) / \gamma_{\text{yrr}1} < (k_{\text{yrr}1}^b + k_{\text{yrr}1}^b) / \gamma_{\text{yrr}1} < \max_{\text{yrr}1} \]

\[ x_{\text{rpm}4} = \begin{align*}
\kappa_{\text{rpm}4} \\
+ \kappa_{\text{rpm}4} s^+(x_{\text{yrr}1, \theta_{\text{yrr}1}}) s^+(u_{\text{mancozeb}}, \theta_{\text{mancozeb}}) \\
- \gamma_{\text{rpm}4} x_{\text{rpm}4}
\end{align*} \]

\[ 0 < \kappa_{\text{rpm}4} / \gamma_{\text{rpm}4} < \theta_{\text{rpm}4} < (k_{\text{rpm}4}^b + k_{\text{rpm}4}^b) / \gamma_{\text{rpm}4} < \max_{\text{rpm}4} \]

\[ x_{\text{yap}1} = \begin{align*}
\kappa_{\text{yap}1} \\
+ \kappa_{\text{yap}1} s^+(u_{\text{mancozeb}}, \theta_{\text{mancozeb}}) \\
+ \kappa_{\text{yap}1} s^+(x_{\text{rpm}4, \theta_{\text{rpm}4}}) \\
+ \kappa_{\text{yap}1} s^+(x_{\text{factor}X, \theta_{\text{factor}X}}) \\
- \gamma_{\text{yap}1} x_{\text{yap}1}
\end{align*} \]

\[ 0 < \kappa_{\text{yap}1} / \gamma_{\text{yap}1} < \theta_{\text{yap}1} < (k_{\text{yap}1}^b + k_{\text{yap}1}^b) / \gamma_{\text{yap}1} < (k_{\text{yap}1}^b + k_{\text{yap}1}^b) / \gamma_{\text{yap}1} < (k_{\text{yap}1}^b + k_{\text{yap}1}^b) / \gamma_{\text{yap}1} < (k_{\text{yap}1}^b + k_{\text{yap}1}^b) / \gamma_{\text{yap}1} < \max_{\text{yap}1} \]

\[ x_{\text{factor}X} = \begin{align*}
\kappa_{\text{factor}X} \\
+ \kappa_{\text{factor}X} s^+(x_{\text{yap}1, \theta_{\text{yap}1}}) s^+(u_{\text{mancozeb}}, \theta_{\text{mancozeb}}) \\
- \gamma_{\text{factor}X} x_{\text{factor}X}
\end{align*} \]

\[ 0 < \kappa_{\text{factor}X} / \gamma_{\text{factor}X} < \theta_{\text{factor}X} < (k_{\text{factor}X}^b + k_{\text{factor}X}^b) / \gamma_{\text{factor}X} < \max_{\text{factor}X} \]

Table 1: Piecewise-linear differential equations and parameters inequalities for the FLR1 gene mancozeb response network in *S. cerevisiae*. The model has five state variables corresponding to the concentrations of the transcription factors and the regulated FLR1 gene, as well as one input variable denoting the presence of the fungicide mancozeb in the cell.
an initial state corresponding to the low concentration of the \textit{FLR1} gene, still consists on \(10^2\) states. Even though the qualitative behaviors starting from a particular state are not huge, it is still difficult to analyze them by visual inspection, and formal verification techniques are still required to get a better insight into the transient and asymptotic dynamics of the network.

2.4 Analysis and verification of the \textit{FLR1} gene mancozeb response model

The qualitative behaviors obtained by simulation were exported by the GNA modeling and simulation tool as a state transition graph, to be analyzed and verified for the occurrence of the observed biological behaviors, with the help of the pattern-based property editor \cite{13} implemented in GNA. For the verification stage, we used NuSMV as a model checker plugin.

In this subsection, generic properties concerning the reachability of the attractors of the system are presented, as well as properties concerning the validation of two new biological meaningful interactions identified by the modeling process.

2.4.1 Identification and reachability of attractors

We start by first looking into the existence of attractors in the system, and the conditions for their reachability. Using the attractor search functionality \cite{14} available in the GNA modeling and simulation tool, two attractors were identified. The first attractor identified by means of GNA is a state in the Kripke structure corresponding to an asymptotically stable steady state of the piecewise-linear model. This state, identified as \textit{basal steady state} in Figure 2, is characterized by a low expression of all the transcription factors and the \textit{FLR1} gene. This is due to the fact that the signal corresponding to the entry of mancozeb into the cytosol is absent, corresponding to the following domain in the phase space:

\[
\begin{align*}
    x_{flr1} &= \frac{k_{flr1}}{\gamma_{flr1}}, \\
    x_{factorX} &= \frac{k_{factorX}}{\gamma_{factorX}}, \\
    z_{mancozeb} &= \text{mancozeb} < \theta_{mancozeb}, \\
    x_{pdr3} &= \frac{k_{pdr3}}{\gamma_{pdr3}}, \\
    x_{rpm4} &= \frac{k_{rpm4}}{\gamma_{rpm4}}, \\
    x_{yap1} &= \frac{k_{yap1}}{\gamma_{yap1}}, \\
    x_{yrr1} &= \frac{k_{yrr1}}{\gamma_{yrr1}}
\end{align*}
\]

The second attractor corresponds to another steady state of the piecewise-linear model. This state, identified as \textit{response steady state} in Figure 2, is characterized by a low expression of all the transcription factors and the \textit{FLR1} gene. This is due to the fact that the signal corresponding to the entry of mancozeb into the cytosol is absent, corresponding to the following domain in the phase space:

\[
\begin{align*}
    x_{flr1} &= \frac{k_{flr1} + k_{1flr1} + k_{2flr1} + k_{3flr1}}{\gamma_{flr1}}, \\
    x_{factorX} &= \frac{k_{factorX} + k_{1factorX}}{\gamma_{factorX}}, \\
    \theta_{mancozeb} &= \text{mancozeb} \leq \text{max}_{\text{mancozeb}}, \\
    x_{pdr3} &= \frac{k_{pdr3} + k_{1pdr3}}{\gamma_{pdr3}}, \\
    x_{rpm4} &= \frac{k_{rpm4} + k_{1rpm4}}{\gamma_{rpm4}}, \\
    x_{yap1} &= \frac{k_{yap1} + k_{1yap1} + k_{2yap1} + k_{3yap1}}{\gamma_{yap1}}, \\
    x_{yrr1} &= \frac{k_{yrr1} + k_{1yrr1} + k_{2yrr1} + k_{3yrr1}}{\gamma_{yrr1}}
\end{align*}
\]

This is consistent with the fact that the presence of the mancozeb signal will activate the cascade of regulators that will increase the expression of \textit{FLR1}.

The predicted qualitative evolution of the protein concentrations, correspond to one single path of the simulation graph which represent the molecular events accompanying the transition from the basal steady state to the response steady state upon mancozeb stress (Figure 2).
In order to verify the conditions for the reachability of identified attractors, we start by characterizing them in GNA by means of atomic propositions. The steady state corresponding to a low expression of FLR1 \( (x_{flr} \leq \kappa_{flr}) \) is labeled \( \text{attractor}_{basal} \). The steady state corresponding to a maximal expression of FLR1 \( (x_{flr} \geq (\kappa^b_{flr} + \kappa^1_{flr} + \kappa^2_{flr} + \kappa^3_{flr})/\gamma) \) is labeled \( \text{attractor}_{response} \). Using the pattern-based property editor, we selected the \textit{occurrence} pattern to check for the reachability of both steady states, and we instantiate them with the previously defined atomic propositions, as follows: “It is possible for a state \( \text{attractor}_{basal} \) to occur” and “It is possible for a state \( \text{attractor}_{response} \) to occur”, corresponding to the temporal logic formulas 1 and 2 of Table 2.

The verification was performed choosing the whole phase space for the initial conditions. The model checker returned true for both properties confirming the reachability of these two steady states in the model.

We were also interested in verifying if the occurrence of these steady states was dependent on the presence of the mancozeb signal. We introduced the atomic proposition \( \text{sig}_m \) to denote the presence of mancozeb. Both properties were specified using the \textit{consequence} pattern as follows: “If a state \( \neg \text{sig}_m \) occurs, then it is necessarily followed by a state \( \text{attractor}_{basal} \)” and “If a state \( \text{sig}_m \) occurs, then it is necessarily followed by a state \( \text{attractor}_{response} \)”, corresponding to the temporal logic formulas 3 and 4 of Table 2.

Choosing again all the phase space for the initial conditions, the model checker returned true for both properties, indicating that the attractors are mutually exclusive and dependent on the presence of the mancozeb signal. Which means that the FLR1 response to mancozeb is not bistable, but has a monostable response to each of the possible values for the input variable (mancozeb presence vs. mancozeb absence).

### 2.4.2 Validation of cascade of activation

In order to verify if the model responds to the mancozeb signal following a specific ordering in the cascade of activation, we analyzed the dependency between Yap1, Yrr1 and Flr1 activation. We wanted to ensure that all the behaviors simulated by the model were not reaching a high concentration level of Yrr1 before having reached a high concentration level of Yap1. Additionally, that all the behaviors simulated by the model were not reaching a high concentration level of Flr1 before having reached a high concentration level of Yrr1 first. We started by specifying the atomic propositions \( Y_{ap1}_{high\_level} \) \( (x_{hap1} \geq \theta^3_{ap1}) \), \( Y_{rr1}_{high\_level} \) \( (x_{yrr1} \geq \theta^3_{yrr1}) \) and \( F_{LR1}_{high\_level} \) \( (x_{flr} \geq (\kappa^b_{flr} + \kappa^1_{flr} + \kappa^2_{flr} + \kappa^3_{flr})/\gamma) \), corresponding respectively to the high concentrations levels of Yap1, Yrr1 and Flr1. These two behaviors were then specified using two sequence patterns instantiated with the corresponding atomic propositions, as follows: “A state \( Y_{rr1}_{high\_level} \) is reachable and is necessarily preceded at some time by a state \( Y_{ap1}_{high\_level} \)” and “A state \( F_{LR1}_{high\_level} \) is reachable and is necessarily preceded at some time by a state \( Y_{rr1}_{high\_level} \)”, corresponding to the temporal logic formulas 5 and 6 of Table 2. The model checker returned true for both of these biological properties, validating the generic behavior of the cascade of activation of the FLR1 gene. This is consistent with experimental data which shows the necessity of Yap1...
2.4.3 Pdr3 necessity for FLR1 maximum expression

Like previously mentioned, the full activation of FLR1 is dependent on Yap1, Yrr1, Pdr3 and Rpn4 [1]. The effect of Yap1 and Yrr1 in FLR1 transcription is known to be direct [2, 3]. Rpn4-binding locus cannot be found in the FLR1 promoter region, so Rpn4 is assumed to influence FLR1 through Yap1, whose expression is dependent on Rpn4 in presence of mancozeb [1]. Although the Pdr3-binding locus can be found in the FLR1 promoter region, there is still no experimental evidence to support this hypothesis. We made the assumption that Pdr3 acts as co-transcription factor of Yrr1 when regulating FLR1, and we were interested in verifying if the model accounted for the fact that the full activation of FLR1 is dependent on Pdr3.

In order to answer this question, we first state the biological elements as atomic propo-
sitions in GNA. An atomic proposition $a_{\text{response}}$ was already defined to represent the maximal expression of FLR1. We introduce a new atomic proposition $\text{high}_{\text{Pdr3}}$ to represent a high expression of Pdr3, restricting the concentration values for the variable $pdr_3$ to those above its threshold $x_{pdr_3} \geq \theta_{pdr_3}$.

To account for the temporal ordering of these two events: maximal expression of FLR1 and high expression of Pdr3, a sequence pattern was instantiated with the predefined atomic propositions: “A state | $a_{\text{response}}$ | is reachable and is | necessarily | preceded | at some time | by a state | $\text{high}_{\text{Pdr3}}$”, corresponding to the temporal logic formula 7 of Table 2. The model checker returns true confirming that the maximal expression of FLR1 is dependent on its co-regulation by Pdr3 and Yrr1 transcription factors.

### 2.4.4 Validation of a new Yap1 regulator: FactorX

In order to validate the interactions between the considered transcription factors, two double deletion mutants were performed: $\Delta yrr_1\Delta pdr_3$ and $\Delta yrr_1\Delta rpn_4$ (Teixeira et al. 2010, submitted). Since the single deletion mutant $\Delta yap_1$ provoked an absence of response of all the players in the network, double deletion mutants involving this transcription factor were not performed. The double deletion mutant $\Delta yrr_1\Delta pdr_3$ exhibits YAP1 transcript levels coincident with those found in the $\Delta yrr_1$ single deletion mutant, suggesting that Pdr3 influence on the YAP1 up-regulation is dependent on Yrr1. Regarding the Rpn4 influence on YAP1 up-regulation, the double deletion mutant $\Delta yrr_1\Delta rpn_4$ exhibits YAP1 transcript levels qualitatively lower than those observed for the single mutants $\Delta yrr_1$ and $\Delta rpn_4$, meaning that there is an addictive effect of Yrr1 and Rpn4 in YAP1 up-regulation. However, in the initial considered version of the model we had the assumption that Yrr1 could only influence YAP1 up-regulation through Rpn4. This new information led us to revise a previous version of the network, including a new transcription factor FactorX (bold in Figure 1 and Table 1). Given that there is also no previous evidence for a direct role of Yrr1 in YAPI transcription and that there is no Yrr1 binding site in the YAP1 promoter region, an unknown transcription factor FactorX is proposed to perform this interaction between Yrr1 and YAPI. Other examples can be found in the literature where putative variables are proposed to be included in the model, in order to explain the available biological data [15,16].

In order to verify if the current model accounts for the new biological data, we simulated a $\Delta rpn_4$ knockout in the model by restricting the values to zero of the equation corresponding to Rpn4. We achieved this by adding a step function $s^-$ to each of RPN4 synthesis rates, which were evaluated to zero since the mancozeb signal was present in the simulation. Additionally, we created an atomic proposition $\text{low}_{\text{Yap1}}$ to restrict the concentration values of YAPI to a level where $x_{fis} < (\kappa_{yap_1}^b + \kappa_{yap_1}^3)/\gamma_{yap_1}$. Finally, we instantiated an invariance pattern to verify if YAPI expression is maintained at low levels through the mancozeb response, as follows: “A state | $\text{low}_{\text{Yap1}}$ | must | persist indefinitely”, corresponding to the temporal logic formula 8 in Table 2.

The model checker returned false together with a counterexample showing increase of YRR1 expression, followed by an increase of FactorX expression, with the final increase of expression of YAPI. This confirmed that the extension of the model with a new regulator of YAPI is sufficient to produce the behavior consistent with the biological data. In
particular, the fact that the \textit{RPN4} deletion should not be sufficient to prevent the observation of some \textit{YAP1} expression. The identification of this transcription factor remains to be established.

<table>
<thead>
<tr>
<th>#</th>
<th>Temporal logic formula</th>
<th>Model-checker verdict</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( \text{EF}(\text{attractor}_{\text{basal}}) )</td>
<td>true</td>
</tr>
<tr>
<td>2</td>
<td>( \text{EF}(\text{attractor}_{\text{response}}) )</td>
<td>true</td>
</tr>
<tr>
<td>3</td>
<td>( \text{AG}(\neg \text{sig}<em>m \implies \text{AF}(\text{attractor}</em>{\text{basal}})) )</td>
<td>true</td>
</tr>
<tr>
<td>4</td>
<td>( \text{AG}(\neg \text{sig}<em>m \implies \text{AF}(\text{attractor}</em>{\text{response}})) )</td>
<td>true</td>
</tr>
<tr>
<td>5</td>
<td>( \text{EF} (\text{Yrr1}<em>{\text{mid level}}) \land \neg \text{EF} (\text{Yrr1}</em>{\text{mid level}} \cup \text{Yrr1}<em>{\text{mid level}}) \text{EF} (\text{FLR1}</em>{\text{mid level}}) \land \neg \text{EF} (\text{FLR1}<em>{\text{mid level}} \cup \text{FLR1}</em>{\text{mid level}}) )</td>
<td>true</td>
</tr>
<tr>
<td>6</td>
<td>( \text{EF} (\text{a}<em>{\text{response}}) \land \neg \text{EF} (\text{high}</em>{\text{Pdr3}} \cup \text{a}_{\text{response}}) )</td>
<td>true</td>
</tr>
<tr>
<td>7</td>
<td>( \text{AG}(\text{low}_{\text{gap1}}) )</td>
<td>false</td>
</tr>
</tbody>
</table>

Table 2: Biological properties used to validate the \textit{FLR1} gene mancozeb response model specified in temporal logic formulae. These formulae are an automatic translation from the biological properties specified using the set of biological patterns.
3 Results

Through simulation of the proposed model we have compared the simulated behaviors with the available biological data specified in temporal logic. By performing knockout simulations to the model, we tested the network robustness for perturbations along the cascade of activation. In particular, the comparison between simulated and experimentally obtained data on the expression profiles of FLR1, PDR3, RPN4, and YAP1, in the absence of pairs of the regulators of the network, brought forward the necessity to consider a new transcription factor to mediate the interaction between Yrr1 and the expression levels of Yap1. The inclusion of this new transcription factor in the network reinforces the usefulness of having a model to quickly formulate new hypotheses. Also, the usefulness of the model extends to the easiness of performing new simulations with single or double knockouts in a reliable and cost effective manner.

3.1 Publications resulting from this work

The results from this work were published or are pending publication in a national and international conference and two peer-reviewed international journals, as specified in the following list:

References


