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Network Structure and Robustness of Intracellular Oscillators

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Abstract: Sustained oscillations play a key role in many intracellular functions, such as circadian time keeping, cell cycle control and calcium signalling. The oscillations are in all cases driven by feedback interactions taking place in biochemical reaction networks. While a single feedback loop in principle is sufficient to generate such oscillations, experimental evidence reveal that more complex network structures, involving multiple feedback loops, underly intracellular oscillations. One hypothesis frequently set forth is that a multi-loop structure is motivated by the need for robustness to internal and external perturbations. We here consider robustness analysis of several recently published models of circadian clocks to determine the role of the underlying network structure in providing robust stability of the oscillators. The robustness analysis is based on adding dynamic perturbations to the network interactions, similar to that used in robust control theory. To elucidate the role of various interactions in providing robust oscillations, we consider blocking specific interactions. Biologically, this contrasts the often considered gene knockouts and implies that genes are persistently expressed. We find that different models have highly different active structures and also differ significantly in their robustness. While some models essentially rely on a single loop in generating robust oscillations, other models have more intricate structures in which some loops provide oscillations and other serve to increase the robustness. Other models again have redundant loops that provide failure tolerance in the face of large perturbations, such as gene knockouts.

1. INTRODUCTION

Most intracellular oscillators, such as cell cycle controls, circadian clocks and calcium signalling pathways, have an underlying network structure consisting of multiple intertwined feedback loops. It is not clear why these oscillators have evolved to such complex network structures, but a hypothesis frequently set forth is that multiple feedback loops enhances the robustness of the network, e.g., in the presence of gene mutations (Cheng et al. (2001); Lee et al. (2000); Preitner et al. (2002); Ueda et al. (2001); Wagner (2005)). In this paper we investigate this hypothesis closer by studying the robustness of several recently published models of circadian clocks in mouse (Leloup and Goldbeter (2004)) and *Arabodopsis thaliana* (Locke et al. (2006, 2005); Zeilinger et al. (2006)).

Robustness, the ability to maintain function in the face of external and internal environmental changes, is an important property of biological systems (Kitano (2004)). In general, robustness can here either mean insensitivity of function characteristics (robust performance) or the persistence of a qualitative behavior (robust stability), in the presence of perturbations. In either case, proper assessment of robustness requires that both the characteristic behavior of interest as well as the allowable set of perturbations are well defined (Stelling et al. (2004b)). For the case of oscillators, robust performance concerns the sensitivity of characteristics such as oscillation period and amplitude (Stelling et al. (2004a)), while robust stability concerns the persistence of oscillations (Wang et al. (2007)). In this paper we focus on robust stability.

The perturbations typically considered when analyzing the robustness of biochemical models are almost exclusively parametric. That is, the set of allowable perturbations consists of perturbations of the model parameters, such as reaction rate constants (Hong et al. (2007); Stelling et al. (2004a); Kim et al. (2006)). Usually, only single parameter perturbations are considered. In this paper, we consider perturbations applied directly to the network interactions. For instance, the considered perturbations can include perturbing the strength in the regulation of a gene by a transcription factor, or perturbing the transport rate of a protein between cytoplasm and nucleus. We allow the perturbations to be dynamic, and compute the destabilizing perturbations using results from robust control theory.

The main aim when applying the proposed robustness analysis, apart from quantifying the robustness of the models, is to elucidate the mechanisms providing for the oscillations and their robustness. In particular, we want to determine if the complete networks are important for robust oscillations, or if specific substructures can be identified. Furthermore, it is of interest to determine if similar structures are employed in different models. For this purpose, we employ blocking of specific interactions. Blocking of interactions between two components imply that changes in the activity of one the components does not lead to changes in the activity of the other component,

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and is in contrast to the commonly considered gene knockouts in which a component is removed from the network altogether.

We start the paper by presenting the method we employ for robustness analysis and interaction blocking. The circadian clock models presented in (Leloup and Goldbeter (2004); Locke et al. (2006, 2005); Zeilinger et al. (2006)) are then analyzed using the proposed method, and their overall network structures as well as the substructures active in providing robust oscillations are compared and discussed.

2. ROBUSTNESS ANALYSIS USING DYNAMIC NETWORK PERTURBATIONS

We employ concepts from robust control theory to assess robustness in terms of persistence of a regular periodic solution. Dynamic perturbations are added directly to the interactions between the network components, and we determine the minimal dynamic perturbation that changes the stability, and hence the qualitative behavior, of the network. Rather than considering destabilization of the limit cycle itself, we focus on stability of the underlying steady-state. In particular, if a perturbation translates the underlying steady-state into a structurally unstable equilibrium, corresponding to a Hopf bifurcation point, then this corresponds to a limit cycle collapsing into the steady-state. For cases in which the Hopf point is supercritical, the collapse implies that the limit cycle behavior disappears. If the Hopf is subcritical, an unstable limit cycle collapses with the steady-state and the stable limit cycle will persist, but now coexisting with a stable steady-state. To eliminate the limit cycle, a nonlinear dynamic perturbation converting the Hopf into a supercritical one can be determined. This is not considered here. Most models of circadian clocks have supercritical Hopf points, and we will therefore focus on this case here.

Consider a nonlinear ODE model of a biochemical network

$$\dot{\mathbf{x}} = \mathbf{f}(\mathbf{x}(t), \mathbf{p}) \tag{1}$$

where $\mathbf{x}$ denotes the state variables (activities or concentrations of components) and $\mathbf{p}$ denotes the model parameters. For parameters $\mathbf{p}^*$, steady-states $\mathbf{x}^*$ are obtained from $\mathbf{f}(\mathbf{x}^*, \mathbf{p}^*) = 0$. We consider the unstable steady-state, $\mathbf{x}^*_{\mathbf{uss}}$, underlying the stable oscillations[3]. A linear model is obtained from linearization of (1) at $\mathbf{x}^*_{\mathbf{uss}}$

$$\Delta\dot{\mathbf{x}} = A\Delta\mathbf{x}(t) \tag{2}$$

where $\Delta\mathbf{x}(t) = \mathbf{x}(t) - \mathbf{x}^*_{\mathbf{uss}}$ denotes deviations from steady-state and $A = \frac{\partial \mathbf{f}}{\partial \mathbf{x}}|_{\mathbf{x}^*_{\mathbf{uss}}, \mathbf{p}^*}$ is the Jacobian matrix. The off-diagonal elements of A represent interactions between the state variables and the diagonal elements are related to internal dynamics, such as self-degradation.

To analyze the impact of the interactions on the stability properties, the interactions between the components are separated from the internal dynamics according to

$$\boldsymbol{\Delta}\dot{\mathbf{x}} = \tilde{A}\boldsymbol{\Delta}\mathbf{x}(t) + (A - \tilde{A})\boldsymbol{\Delta}\mathbf{u}(t) \tag{3}$$

[3] We here assume that such a state exist, which always holds for systems with supercritical Hopf bifurcations.

where $\tilde{A}$ is a diagonal matrix containing the diagonal of A, and the effect of other components $\boldsymbol{\Delta}\mathbf{u}(t)$ is independent of $\boldsymbol{\Delta}\mathbf{x}(t)$. Eq. (3) will be referred to as the open-loop system.

The open-loop system (3) can be transformed into a frequency response $L(j\omega)$ from $\boldsymbol{\Delta}\mathbf{u}$ to $\boldsymbol{\Delta}\mathbf{x}$

$$L(j\omega) = (j\omega I - \tilde{A})^{-1}(A - \tilde{A}) \tag{4}$$

where I is the identity matrix and $L(j\omega)$ is the response to a sinusoidal stimuli with frequency ω. Particulary, if $\Delta u(t) = \sin\omega t$, then $\Delta x(t) = |L(jw)|\sin(\omega t + \arg L(j\omega))$ in stationarity.

The dynamic interactions between components are recovered by introducing the unity feedback

$$\boldsymbol{\Delta}\mathbf{u}(t) = \boldsymbol{\Delta}\mathbf{x}(t) \tag{5}$$

recovering the original system (2), the closed-loop system.

If there are no autocatalytic effects in the model, the internal dynamics of the components are stable. This implies that the diagonal elements of A are strictly negative and that the open-loop system (3) hence is stable. The instability of $\mathbf{x}^*_{\mathbf{uss}}$ is then a result of the feedback interactions between the components.

According to the Nyquist stability criteria, the stability of the linear closed-loop system (2) can be inferred from the open-loop systems (3). The restriction that the open-loop system is stable simplifies the generalized Nyquist stability criteria so that we can conclude that the closed-loop system is unstable when at least one of the eigenvalue loci of the frequency response (4) encircles the point +1 in the complex plane (+1 as (5) denote positive feedback). A more intuitive way of viewing this is that the amplification of the open-loop system should be greater than one ($|L(j\omega)| > 1$), when the phase is zero ($\arg L(j\omega) = 0$), for the feedback to introduce instability.

To determine the smallest perturbation that will change the qualitative network behavior, i.e., the smallest perturbation required to stabilize $\mathbf{x}^*_{\mathbf{uss}}$, we add dynamic perturbations to the model strucure. In the frequency domain, a dynamic perturbation is represented by a complex number at each frequency. The complex perturbations can be translated into differential equations in the time domain, and these can then be added as perturbations in the nonlinear model (1). A dynamic perturbation can represent e.g., a neglected intermediate reaction step, a time delay or diffusion. Addition of perturbations to the network structure can be made in numerous ways. In each case, the robustness analysis aims at determining the smallest size, in a norm sense, of the applied dynamic perturbation that will change the stability of the network. Below we discuss how different structural perturbations can be used to quantify the overall robustness, determine specific network fragilities and elucidate the impact of substructures within the network.

2.1 Overall robustness

The overall robustness of the network is quantified by perturbing the activities, or concentrations, of all components simultaneously. With relative perturbations, this corresponds to perturbing the feedback in (5) according to

$$\boldsymbol{\Delta}\mathbf{u_p} = (I + \Delta_I)\boldsymbol{\Delta}\mathbf{u} \tag{6}$$

where Δ_I is an $n \times n$ diagonal complex valued perturbation matrix with diagonal elements Δ_i $(i = 1, ..., n)$ and n is the number of state variables. The smallest size relative perturbation Δ_I that will move one eigenlocus of (4) to $+1$ at a given frequency will have magnitude

$$\frac{1}{\mu} = \bar{\sigma}(\Delta_I(j\omega)) \tag{7}$$

where μ is the structured singular value and $\bar{\sigma}(\Delta_I(j\omega))$ denotes the maximum singular value of the perturbation matrix Δ_I, i.e., the magnitude of the largest element of Δ_I [4]. The perturbation $\Delta_I(j\omega)$ can be interpreted as a global effect on the dynamics of the system, e.g., a temperature change.

2.2 Robustness to changes in single components

Addition of a relative perturbation to the direct effect of a single component on all other network components provides information on how the network handles changes in the properties of individual components. The perturbation to the feedback (5) is in this case added according to

$$\Delta u_{p,i} = (1 + \Delta_i)\Delta u_i;\ \Delta u_{p,k} = \Delta u_k;\ k \neq i \tag{8}$$

The minimal relative perturbation changing the qualitative behavior is given by $\frac{1}{\mu_i} = |\Delta_i|$, where Δ_i can be interpreted as a global change in the dynamics of the i:th component, e.g., as a result of a gene mutation.

2.3 Robustness to specific pairwise interactions

Addition of a perturbation to the direct interaction between two components provides more detailed insight into how different parts of the network handle perturbations, and can be used to determine specific network fragilities. This corresponds to adding a relative dynamic perturbation Δ_{ik} to a single element of L_{ik}

$$L_{p,ik}(j\omega) = L_{ik}(j\omega)(1 + \Delta_{ik}(j\omega));\ k \neq i \tag{9}$$

The minimal relative perturbation of the pairwise direct interaction between component k and component i changing the qualitative behavior is given by $\frac{1}{\mu_{ik}} = |\Delta_{ik}|$, where Δ_{ik} is a perturbation of the effect of component k on i.

Apart from quantifying robustness, the structured perturbations above can also be used to determine components and interactions important for the function in question. For instance, if a small relative perturbation of the dynamics of a single component changes the qualitative behavior of the network, this component can be assumed to be an important part of the mechanism underlying the oscillations. Also, individual and pairwise interactions with a μ-value less than one can be removed without affecting the stability of the network, and can hence be assumed to be of little importance for the existence of network function (but may be important for robustness or for other specific properties of the function). Note that we by "removing" do not imply complete removal of the biochemical component, but that the interactions are blocked.

[4] It is necessary to verify that the perturbation moves the encircling locus, and not some other locus, to $+1$. Otherwise, the computed perturbation size is only a lower bound.

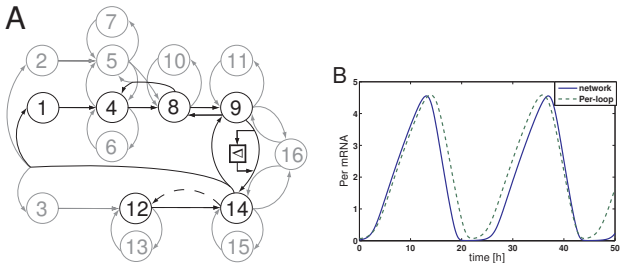

Fig. 1. A. The network underlying circadian oscillations in mice (Leloup et al., 2004). States and interactions determined to be important for the oscillations are highlighted (parameter set 1). Dashed lines shows interactions important for the robustness. B. Simulations of circadian oscillations in *Per* mRNA concentration with full model (solid) and *Per*-loop only (dashed).

All computations of the structured singular value in general only provide lower and upper bounds. However, we here consider scalar or diagonal complex perturbations and then these bounds are in general relatively tight. For all examples below, the lower and upper bound overlap so that we obtain an exact value for μ, and hence the smallest destabilizing perturbation, in all cases.

2.4 Blocking of interactions

Blocking of interactions are correspond to applying $\Delta_{ik} = -1$ to the network structure. This corresponds to removal of the direct effect of component k on i. For a gene i regulated by a transcription factor k, the perturbation $\Delta_{ik} = -1$ would thus correspond to constitutive (constant) expression of the gene and contrast complete removal of the component, i.e., a knockout mutation. Knockout mutations have been used extensively and successfully to gain insight into biomolecular systems. Complete removal of a component, however, disrupts the network and the dynamics of the original system can not be investigated. Removal of gene regulation, together with knockdown approaches using RNAi and molecular genetic screens for mutations affecting the dynamics (Pomerening et al. (2005); Sato et al. (2006)), all leave the underlying network intact and are examples of dynamic perturbations to the structure of the system.

3. CIRCADIAN OSCILLATIONS IN MAMMALS AND PLANTS

We here apply the robustness analysis outlined above to four recently proposed models of circadian clocks in mammals and plants. The aim is to quantify the robustness and determine the subnetwork structures underlying the circadian oscillations and their robustness.

3.1 Circadian Oscillations in Mice

A model for the gene regulatory network responsible for circadian oscillations in mammals is proposed in (Leloup and Goldbeter (2003, 2004)). The model includes a total of five genes and their products. However, only three of

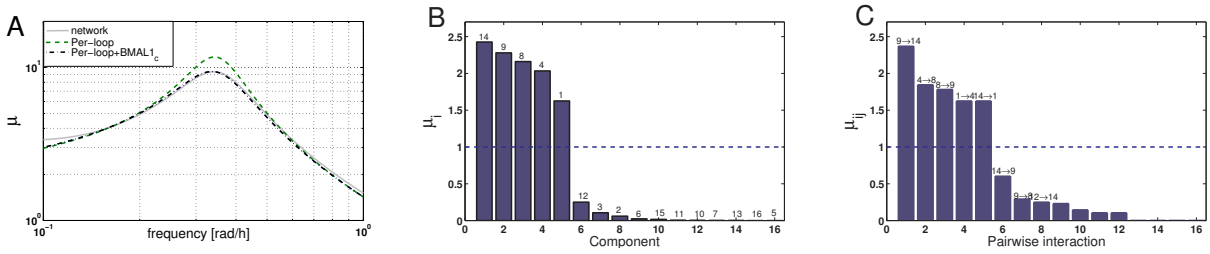

Fig. 2. Mammalian oscillations, parameter set 1: A. The structured singular value μ for the overall network (solid), for the loop involving only the *Per*-gene (dashed), and for the *Per*-loop interacting with Bmal1 protein in cytoplasm (dash-dot). B. Structured singular value μ_i for perturbation of individual components. C. Structured singular value μ_{ik} for perturbation of specific pairwise direct interactions.

the genes are considered to be directly involved in generating the oscillations, while two genes are assumed to be constantly expressed. The network corresponding to the three active genes, *Cry, Per* and *Bmal1*, involves 16 states and is illustrated in Fig. 1A. The states correspond to the concentrations of the gene mRNAs and the various proteins and protein complexes. Leloup et al. (2004) consider 4 different parameter sets. We here analyze the model with parameter sets 1 and 2 in Leloup and Goldbeter (2004).

Parameter set 1: The overall robustness measure μ (7) of the network is shown in Fig. 2A. As can be seen, the robustness measure μ peaks at about 9 around the frequency $\omega = 0.33$, corresponding to the circadian frequency. This implies that the network can tolerate up to 11% simultaneous variations in all concentrations without loosing the oscillations. Thus, the network can be considered reasonably robust.

Fig. 2B shows the results of the robustness analysis for perturbations of individual components. The results show that the interactions involving all components except $1, 4, 8, 9, 14$, who all have $\mu_i > 1$, can be removed without loosing the circadian oscillations. This suggests that the oscillations are generated by interactions between 5 components, out of a total of 16. This is also supported by the robustness analysis for specific pairwise interactions in Fig. 2C, showing that the loop $1-4-8-9-14-1$, corresponding to the *Per*-gene loop, is critical for the oscillations. Figure 1B shows simulations of the oscillations in *Per* mRNA concentration for the full network and the network with only the 5-component *Per*-loop active, i.e., with all other interactions blocked. The simulation supports the result from the robustness analysis. The generation of the oscillations appears to be confined to a small subnetwork of the overall network.

To examine if the remaining network plays a role in providing robustness of the oscillations, we recomputed the overall robustness measure (7) with only the 5 component *Per*-loop active. As can be seen from Figure 2A, the μ-value increases to 11, and hence the remainder of the network contributes somewhat to the robustness. From Fig. 2B we see that the one component apart from the *Per*-loop components with the largest impact on the oscillations is component 12 (BMAL1-protein in cytoplasm). As seen from Fig. 2A, including this component in addition to the *Per*-loop improves the robustness to the degree that the resulting 6-component subnetwork has robustness essentially equal to the overall 16-component network. The structure corresponding to the 6-state network is highlighted in Fig. 1A.

Parameter set 2: For parameter set 2, the overall robustness in terms of the μ-value is shown in Fig. 3B. The μ-value for the complete network now peaks at a value about 22, and hence the network with parameter set 2 is significantly less robust than with parameter set 1. Robustness analysis for perturbations in single components reveals that components 12 and 3 now are important in addition to the *Per*-loop components $1, 4, 8, 9, 14$. This corresponds to the substructure highlighted in Figure 3A. In this case, the *Per*-loop in addition to component 12 are required to generate the oscillations, while the *Bmal1*-loop involving components $14 - 3 - 12$ improves robustness. As can be seen from Fig. 3B, the *Bmal1*-loop increases the robustness by a factor close to 2 and represent the only network interactions apart from the *Per*-loop that provide for the robustness.

3.2 Circadian Oscillations in Arabidopsis

Significant efforts have been put into identify the genes that underly circadian oscillations in the plant *Arabidopsis thaliana*. Locke et al. (2005) proposed a model involving 4 genes, two of which are still unidentified. Recently, Locke et al. (2006) and Zeilinger et al. (2006) refined this model by adding one and two genes, respectively. It is important to stress that the refinement and tuning of these models have mainly been directed by experimental observations concerning various properties, such as phase differences, in the circadian oscillator. We here focus on the robustness of these models, and unravel the subnetwork structures that underly the oscillations and their robustness.

Locke model 1 (2005): Originally this model was proposed as a two-loop model with 4 genes, and a total of 13 components (Fig. 4A). The overall robustness in terms of μ is shown in Fig. 4B. The μ-value peaks at around 46, close to the circadian frequency. Thus, the network can only tolerate about 2% simultaneous (worst-case) variation in the component activities before loosing the circadian oscillations, and is hence significantly less robust than the mammalian model above. From the robustness analysis with perturbations of single components we find that all components, except component 13, are critical for the oscillations. As can be seen from Fig. 4A, the component

interactions form a single negative feedback loop with a parallell path between component 3, a transcription factor, and component 4, a gene. In terms of specific interactions, we find that all interactions are required for oscillations, with the exception of the effect of component 3 on component 2 and the effect of component 6 on component 10. The latter interaction does however have a significant impact on the robustness, but in a negative way. As seen from Fig. 4B, removing this interaction improves the robustness almost by a factor 2.

The analysis of the robustness with respect to perturbations of specific interactions also reveals that the most fragile connection is the effect of component 8 on component 9, corresponding to the transport of the unknown gene X protein product from cytoplasm to nucleus. A relative perturbation of 13% in this interaction at the circadian frequency is sufficient to remove the oscillations.

Locke model 2 (2006): This model is essentially a direct extension of the previous model, in which one more gene has been added to provide for another feedback loop. The main purpose of this extra loop is, according to Locke et al. (2006), to predict the oscillations that has been observed experimentally when the main gene *LHY* (component 1 in both models) has been knocked out. Thus, the extra gene loop is supposed to provide a redundant mechanism for generating circadian oscillations. The modified network structure with 16 states is shown in Fig. 5A. Figure 5B shows the overall robustness in terms of μ. Note that the computed μ in this case only provides an upper bound, or lower bound on the size of the required perturbation, since in this case there are two eigenvalue loci of the open-loop system (4) that encircles the $+1$ point in the complex plane. A perturbation that stabilizes the steady-state must exceed the perturbation computed using μ, and hence the μ-value is an upper bound in this case. Since the maximum μ-value in Fig. 5B is 35 we can thus conclude that this network is more robust than the one in Locke et al. (2005).

A robustness analysis with perturbations of single components and interactions confirms that there are two distinct subnetworks in the model that each provide circadian oscillations. See also Fig. 5A. When both subnetworks are in function, the oscillations will entrain due to the weak connections that exist between the two subsystems. If one of the subnetworks is perturbed so that the corresponding oscillations disappear, then the circadian oscillations will persist. Figure 5B show that we need to perturb interactions in both subnetworks to remove the circadian oscillations. Thus, the network contains redundancy which provide for failure tolerance.

Zeilinger model (2006): Similar to Locke et al. (2006), Zeilinger et al. (2006) extend the model proposed in Locke et al. (2005) by adding extra genes. In this case, two genes are added to yield a network with a total of 19 states. We here analyze the final tuning of the model presented in Zeilinger et al. (2006), called model C. The corresponding network structure is shown in Fig. 6A. Note that although the basis for this model is similar to the previous model, the structure appears to be quite different. The overall robustness in terms of μ is shown in Fig. 6B. The peak-value of μ is 910 around the circadian frequency, indicating that the network is highly fragile to some perturbation.

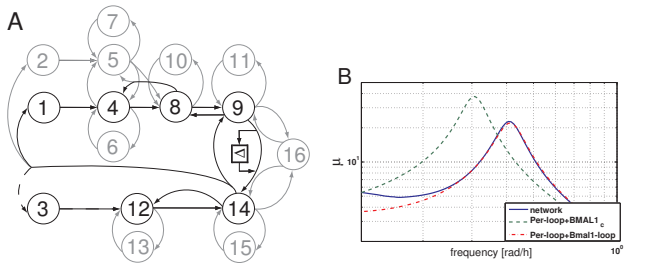

Fig. 3. Mammalian oscillations, parameter set 2: A. Overall network with interactions providing oscillations highlighted. Dashed lines show interactions affecting robustness. B. Structured singular value for overall network and subnetworks.

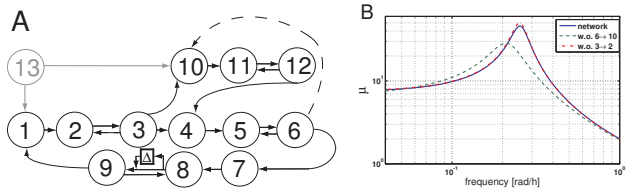

Fig. 4. *Arabodopsis* circadian clock (Locke et al. 2005). A. Network structure. B. Structure singular value for overall robustness. The impact of blocking specific interactions on the overall robustness is also shown.

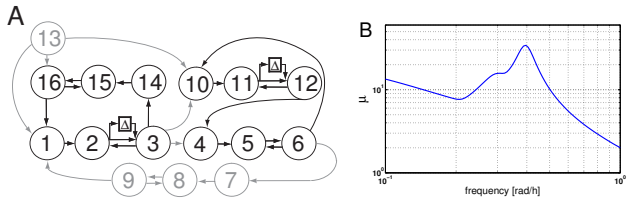

Fig. 5. Modified *Arabodopsis* clock (Locke et al. 2006). A. Network structure. B. Overall robustness measure μ (upper bound only).

Robustness analysis for perturbations in individual components and interactions reveal that the only components that are active in providing the oscillations, as well as their robustness, are the 6 components related to the two genes *TOC1* and Y (Fig. 6A). The identified subnetwork corresponds to a single feedback loop with local feedback between the proteins in the cytoplasm and nucleus. Robustness analysis for specific interactions reveals that the network is highly fragile with respect to changes in these latter local feedback loops. For instance, a relative change of less than 0.4% in the effect of protein in nucleus on protein in cytoplasm will completely remove all oscillations.

4. CONCLUSIONS

Sustained oscillations play a key role in many intracellular functions. Such oscillations can in principle be generated by a single feedback loop. However, the biochemical net-

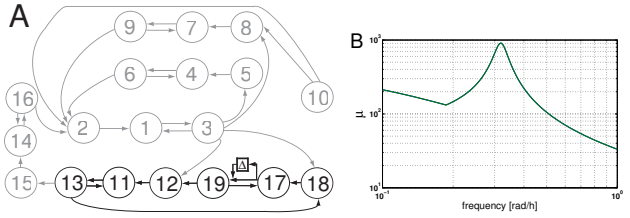

Fig. 6. Modified *Arabodopsis* clock (Zeilinger et al. 2006). A. Network structure. B. Overall robustness measure μ.

works underlying oscillatory functions in the cell typically involve a number of intertwined feedback loops. A common hypothesis is that the complex structure is motivated by the need for robustness to external and internal perturbations. In this paper we propose a method for robust stability analysis of networks providing sustained oscillations. The method is based on adding relative dynamic perturbations to the interactions between the network components, and then computing the smallest size perturbation that changes the behavior in a qualitative sense. By adding perturbations in a strategic fashion to the various components and interactions of the network, the role of specific interactions and subnetworks in generating the oscillations can be determined. Furthermore, by employing a method termed interaction blocking, we can determine the interactions and substructures that provide robustness of the oscillations.

The proposed analysis method is applied to four recently proposed models of circadian oscillations in mammals and plants. The results reveal that for most models, only a relatively small active substructure of the overall biochemical network accounts for the oscillations and their robustness. Furthermore, the active substructures differ significantly between different models. In some models, a single negative feedback loop provides for the oscillations, while other have several intertwined loops in which some loops generate oscillations while other serve to improve robustness. Other models again have redundant mechanisms that provide for circadian oscillations and hence have a built-in failure tolerance, e.g., to gene knockouts.

An important purpose of the paper is to demonstrate the usefulness of the proposed analysis method to determine the overall robustness, and detect important substructures, of models for sustained intracellular oscillations. A potential use of the proposed method is in tuning models for robustness, so that they reflect the robustness observed in biological systems.

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