Modeling rapid stomatal closure with Synchronous Boolean Network Approach

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Abstract: The phytohormone Abscisic acid (ABA) is an endogenous messenger in plant abiotic stress responses. Drought stress increases the level of ABA triggering the fastest adaptive physiological response of plants- closure of stomata (guard cells). Understanding gene/protein expression involved in stomatal closure has great importance to genetic modification of plants to survive in severe climatic conditions. However, systems level information that defines the communication pattern of the related network of cellular molecules is not yet known. This study integrates fragmentary information collected from literature to define the dynamics of ABA signaling in rapid closure of stomata through a synchronous Boolean model. Stomatal closure in broad terms is a combined result of organic and inorganic ion regulation to release water from the guard cells through osmosis, and rearrangement of Actin to facilitate resulting stomatal movement.

Our network consists of 57 nodes and their interaction dynamics defined in accordance with past experimental results to regulate stomatal closure. Perturbation analysis was conducted to identify the essential elements crucial for the above mentioned global functions of pumping out water and stomatal movement. It revealed that destruction of ABA receptor complex (PYR/PYL, PP2C and SnRK2 proteins) made stomata insensitive to closure as a result of disruption of signal transmission to downstream regulators.

It was identified that plasma membrane outward ion channels GORK and SLAC1 are crucial for pumping out water by reducing the osmotic load inside the guard cell which facilitates osmosis. Inhibition of MAPK kinases and cytosolic alkalization, as being important regulators of SLAC1, and membrane depolarization, important for GORK, showed drastic effect on the stomatal closure. In contrast, overexpression of plasma membrane H^+-pumping and potassium-in channels inhibit stomatal closure by enhancing the osmotic concentration and there by attracting water inside.

Loss of function of Actin rearrangement resulted in a loss of stomatal closure as structural rearrangement of guard cell are necessary to facilitate the cell shrinkage. Disruption of Reactive Oxygen Species or their regulators (RBOH, PA, PLD or RCN1), SCAB1 protein and overexpression of AtRAC1 showed drastic effects on structural rearrangements.

Perturbation analysis revealed that the number of elements crucial to stomatal closure comprises about 30% of the network; and thus stomatal closure is robust against perturbation in the other 70% of network elements. These results are in agreement with experimental findings and indicate potential redundancy with respect to stomatal closure.

Keywords: *ABA signaling, Stomatal closure, Boolean Network, limit cycles, attractors, oscillations*

1. INTRODUCTION:

Drought tolerance is crucial for plant survival as water is essential for photosynthesis, nutrient uptake, transportation and for cooling. Plants need to have adaptation mechanisms as they cannot move as animals to avoid unfavorable environmental conditions. Further, they need to respond as quickly as possible to sudden onset of droughts to avoid the risk of desiccation by reducing water loss, which increases the survival rate of plants. In achieving this, the most important and the fastest adaptive physiological response is stomatal closure.

Stomatal closure results from the changes in the turgor of guard cells in plant leaves relative to the surrounding epidermal cells with the involvement of metabolic energy and changes in membrane permeability. Dispersion of water decreases the hydrostatic pressure on cell membrane making the cell to shrink. Water moves from a low solute concentration (high osmotic/chemical potential) to an area of high solute concentration until the two areas have an equal ratio of solute: water. This is done by decreasing the solute concentration inside the guard cell through ion discharge to increase the water potential inside the guard to release water through osmosis. Shrinkage of cell is further supported by structural rearrangements of guard cell which facilitates the stomatal movement. When the plants begin to sense a water shortage, mainly in roots, Abscisic acid (ABA) activates several mechanisms and signaling pathways in plant guard cells leading to stomatal closure. When stomata are closed, guard cells are shrunk and have little internal pressure. Guard cells of fully opened and fully closed stomata are both in equilibrium with surrounding cells, even though they all have different internal conditions.

It is important to understand how stress inducible gene/protein signaling networks function to enhance stress tolerance in plants through genetic manipulation. Similarly, the enhancement of plant water use efficiency in drought periods will be a major achievement by engineering guard cell signal transduction network.

This research focuses on the employment of Synchronous Boolean Network modeling of biological networks to better describe the guard cell signaling network, where no up-to-date dynamic model is available. Objectives of this study are to find the most frequently visited states of the network, oscillatory patterns, and important elements and their influence on the global network behaviour.

2. METHODOLOGY:

2.1. Boolean Modeling:

Logic based modeling has been widely used in the literature to map biological networks and to study their functional interactions. They provide a broader qualitative overview of network interactions and are the only tool available in the absence of dynamic data that can help unravel the full complexity of networks. Boolean Network is one of the logic based modeling approaches, which defines the system variables (a gene /protein) in their simplest qualitative form denoted by inactive (0) or active (1) and combine them with a defined Boolean function to represent the influence of a node on another with logical operators "AND", "OR" and "NOT". Operator "AND" represents the association of two or more regulators in a dependent manner where all the regulators are needed for the activation of a node. If the activation of a node can be induced by any of its regulators, Boolean function combine these independent regulators with an "OR" operator. The operator "NOT" represents the negative regulation of an element on a node.

The dynamics of the network are considered as a discrete time series where the values of network nodes at time t is determined by the Boolean rules associated with its regulators and their states at time t-1. If the state of a node i at time t is denoted by x_i^t and the associated Boolean function by B_i with n number of regulators, the dynamics of the Boolean network can be generated as shown in equation 1.

$$x_i^t = B_i(x_1^{t-1}, x_2^{t-1}, \ldots \ldots x_n^{t-1}) \tag{1}$$

In this study, we simulated network dynamics with synchronous approach by simultaneously updating the states of all network nodes after each consecutive time point. The network is simulated for 100 discrete time points with large number of random initial states (10,000) to study the most frequently visited states/attractors of the network. The state of input node ABA was always kept at on (1) state and the target node (stomata) was set at the off (0) state (open) in all initial conditions. Random initiation of initial states of all other nodes was done due to lack of prior information on the initial state of a large number of nodes. The significance of a specific node on global behaviour of the network was studied by setting the state of a particular node at inactive stage (0) over the simulation, which mimics the biological perturbations and/or pharmacological inactivation of a node. The probability of success (stomatal closure) was achieved according to the formula (Eq. 2) specified by Li *et al.* (Li et al., 2006).

$$P(closure)^t = \sum_{j=l}^{N} S_{Stomata(j)}^t / N \tag{2}$$

Where $S^t_{Stomata(j)}$ is the state of the node ''Stomata'' at time t ($S^t_{Stomata(j)} = 1$ if stomata node set at on/active state at time t) in the jth simulation and N is the total number of simulations. Dynamics of the network are produced by the R software package BoolNet (Müssel et al., 2012).

3.   RESULTS:

3.1.   Setup of a comprehensive ABA Network:

We combined fragmentary biological information of stomatal closure as explained below to build up the ABA signalling network, which comprises 57 nodes and 127 interactions (Figure 1). The network was drawn as a sign directed graph where direction of the edges reflects the propagation of signalling flow between signaling molecules such as proteins, small molecules and ion channels represented as nodes and interactions as arrows. In Figure 1, network elements were grouped (and coloured) according to high-level functional aspects described below.

Signal transduction and perception in guard cells occur when ABA activates its receptor (PYR/PYL/RCAR) allowing it to halt the activity of Protein phosphatase C (PP2C) enabling activation of SNF-RELATED KINASE 2(SnRK2) protein (Umezawa et al., 2010) (Green box in Fig.1). Activation of SnRK2 eventually activates plasma membrane slow (SLAC1) and rapid (QUAC) anion channels, and deactivates inward rectifying potassium channel (KAT1) by phosphorylating them (Cyan box). Activation of these ion channels leads to net efflux of anions from the guard cell causing plasma membrane depolarization (Umezawa et al., 2010). Depolarization of the plasma membrane boosts the activity of outward Potassium channel (GORK), together with several other small molecules to facilitate outward movement of water.

SnRK2 physically interacts with Respiratory Burst Oxidase Protein (RBOH) and phosphorylate it to produce Reactive Oxygen Species (ROS) supporting guard cell ionic regulation (Grey box in Fig.1). ROS trigger cytosolic calcium (Ca^{2+}) transients by enhancing the activity of hyperpolarization-activated plasma membrane Ca^{2+} channels (McAinsh & Pittman, 2009). According to a recent review (McAinsh & Pittman, 2009), complex and oscillatory patterns of Ca^{2+} elevations are a result of coordinated action of Ca^{2+} permeable channels, Ca^{2+}ATPases, Ca^{2+}/H^+ antiporters (CAX), Ca^{2+} binding proteins, cyclic ADP-Ribose (cADPR), Inositol-phosphates $InsP_3$, and $InsP_6$. All these elements maintain Ca^{2+} homeostasis by regulating the movement of Ca^{2+} ions between subcellular compartments, between the cell and extracellular environment. Elevated Ca^{2+}, ROS (Merlot et al., 2007), or cytosolic pH (Gonugunta et al., 2009) inhibits the plasma membrane H^+-ATPase (AHA1), which prevents further hyperpolarization of the membrane and shifts the activation potential of KAT1 to large negative potentials preventing opening of stomata. It has been shown that modulation of NO production links with ROS generation and rise in cytosolic pH (Gonugunta et al., 2009).   Additional means of producing NO is directly by Ca^{2+}. In turn, NO modulates Ca^{2+} by regulating cADPR through enhancement of cyclic Guanosine Monophosphate (cGMP).

Further, lipid mediators (Yellow box in Fig.1) generated by phospholipase D (PLD), phospholipase C (PLC) and sphingosine kinase (SphK) have been identified as integral parts of complex ABA signalling cascade (Zhang et al., 2009). ABA increases the activity of SphK enzyme followed by sphingosine-1-phosphate (S1P), Heterotrimeric G-protein α-subunit (GPA1) and PLD leading to the production of Phospatic Acid (PA) from phosphatidyl choline. PA inhibits PP2C, a key negative regulator of ABA signalling at the ABA signal initiation in the cell and binds to AtRBOH to enhance the production of ROS (Yellow box in Fig.1).

Malate contributes to the regulation of stomatal movement as an osmoticum.   However, functional significance or the regulators of Malate control are less studied to date, but reduction in turgor pressure of guard cells requires either conversion of Malate to osmotically inactive starch, or reduction of cytosolic Malate concentration by pumping it out (Penfield et al., 2012).

Actin reorganization (purple box in Fig.1) is one of the principal requirements to facilitate guard cell shrinkage, which is associated with depolymerization, bundling, and nucleation of the cortical Actin filaments of guard cells. Coordination of Actin Binding Proteins (ABPS); especially ARP23 and SCAB1, $Ca2^+$ and ROS facilitate the rearrangements of Actin. Guanosine triphosphatase protein AtRac1 (RAC1) acts as a key negative regulator of the actin depolymerization. ABA prevents the activity of RAC1 through inactivation of PP2C, which positively regulates the activity of RAC1. ABPS negatively act on Actin rearrangement at low $[Ca^{2+}]$, but regulates Actin dynamics through capping and depolymerization at high $[Ca^{2+}]$. However, the functional significance of the Actin rearrangements for stomatal movement has to be further revealed experimentally, but it can be deduced that it provides the required flexibility for the guard cell shrinkage.

3.2. Network Robustness

Robustness is the ability of a system to maintain its original functions despite perturbations. Biologically, it is the tolerance against random attacks. Robustness can be determined from the effect of removal or addition of edges that connect the elements of the network (analogous to genetic mutations), or changes in state of a node (which resembles environmental perturbation) on the behaviour of the system. In this study, we defined robustness as the probability that random permutation of the output values of the chosen transition functions of a node will result in the same network behaviour as the original one. We simulated five batches of 100 perturbed copies of network and compared them with results of the original network. Of them, nearly 60% of the perturbed networks settled in exactly the same attractors as the original network did. With reference to the results of the output node "Stomata", 85% of the perturbed networks functioned in the same way as the original network, having 85% and >75% agreements in length of attractors and length of basin of attractors, respectively.
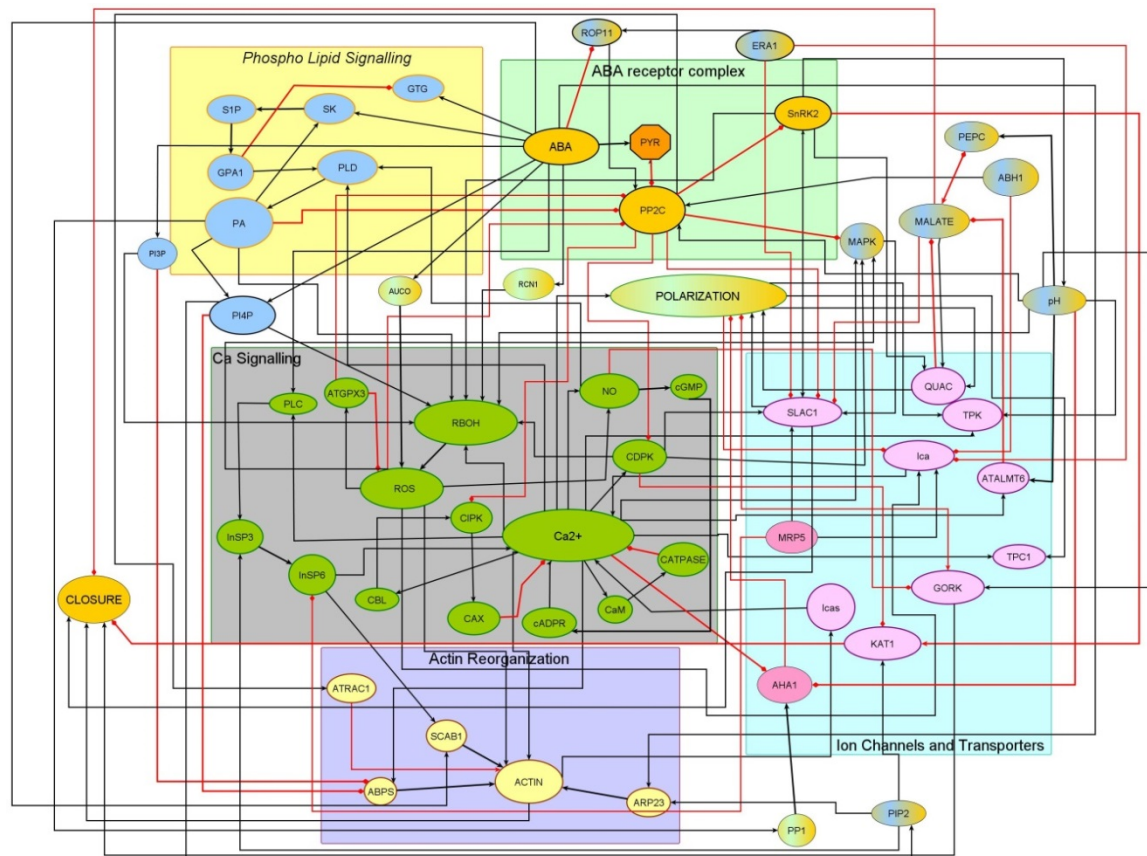

Figure1: ABA signaling network (network created with the yEd graph editor (http://www.yworks.com). Regular arrows (Black) represent positive interactions and diamond-head arrows (Red) represent inhibitory interactions) [segments indicated by colours represent functional categories of ABA signaling network]

3.3. Modelling Dynamics of ABA signalling with Synchronous Boolean Approach

Wild type stomata showed 75% closure as a result of the network simulation with 10,000 random starting conditions. Irrespective of the starting condition, majority of nodes reached a steady state value within two to ten time steps and get frozen either in "on" or "off" state. Temporal dynamics of the network converged to 8 attractor cycles (stable pattern); each consisting of 7 states (Figure 2 shaded part). Attractors are asymptotic behaviour of biological systems where states within an attractor cycle are reached infinitely often. Real biological systems are typically assumed to have short attractor cycles because cyclic attractors can describe oscillatory behavior of a system. As above attractors are not chaotic as they did not depend on the initial conditions, we used them to describe oscillatory behavior of ABA signaling. [These eight attractors vary depending on the combinations of initial states (2^3) of just three network nodes (ERA1, ABH1 and MRP5) where they do not have regulators to change their states throughout the simulations]. Of 57 nodes in the network, 12 nodes showed oscillatory behaviour in all attractors (Figure 2). These nodes are NO, cADPR, cGMP, CDPK, Ca^{2+}, CBL, CaATPase, CaM, CAX, CIPK, ABPS and ATALMT6.

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Figure 2: The most robust limit cycle observed for synchronous Boolean model of ABA signaling (Genes are encoded in the following order: SK, S1P, GPA1, PLD, GTG, PA, RCN1, PI4P, PI3P, PIP2, PLC, InSP3, InSP6, PYR, ROP11, pH, PP2C, SnRK2, RBOH, CuAO, ROS, ATGPX3, NO, cADPR, CGMP, Ica, SLAC1, MAPK, CDPK, Ca^{2+}, CBL, TPC1, GORK, CaATPase, CaM, QUAC, CAX, SCAB1, Icas, CIPK, TPK, PP1, AHA1, ABPS, ACTIN, PEPC, MALATE, AtRAC1, ARP23, Membrane Polarity, ATALMT6, KAT1, Stomata, ABA, MRP5, ERA1 and ABH1)

Oscillations are a result of feedback loops connected with the activities of Ca^{2+} and its regulations, which governs the system behavior through a series of oscillations. Therefore, we evaluated the system to see how significant the effect of Ca^{2+} is on stomatal closure by simply perturbing Ca^{2+} node in the model. Considering the probability of closure, both systems closed stomata with the same probability indicating that ABA system can work independently of calcium. Then, both networks are evaluated for the time taken to achieve the final goal. We found that when the system is under the regulation of calcium, it closes stomata more rapidly than Ca^{2+} knocked out system (Figure 3A). Figure 3A clearly shows that there is very low probability for stomatal closure at 4-8 time steps when no calcium is available in the system. The primary cause of this delay is identified as the time taken to stabilize the activity of SLAC1, the slowly activating anion channel in the plasma membrane (Figure 3B). This finding was supported by the biological fact that ABA induced elevation of Ca^{2+} strongly activates S-Type anion currents by priming its Ca^{2+} sensitivity (Siegel et al., 2009). Modern scientific arguments (Laanemets et al., 2013) about the biological importance of calcium oscillations to guard cell signal transduction also highlights the importance of Ca^{2+} for rapid regulation of SLAC1 and KAT1. However, with this synchronous modeling, we could not find any significant role for Ca^{2+} in regulation of KAT1 either with the state or time taken for stabilization of the node.
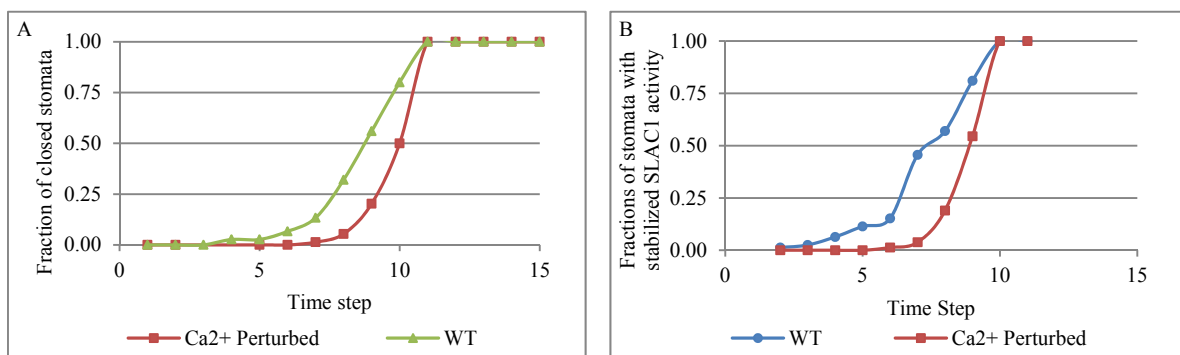

Figure 3: Comparison of Wild Type (WT) and Ca^{2+} Perturbed systems for (A) Time taken to close the stomata and (B) Time taken to activate SLAC1 in ABA induced closure.

3.4. Identification of essential components in the ABA signalling Network

The significance of a specific node on the global behaviour of ABA network was studied by systematically perturbing the system by setting the state of that node from active to inactive stage (or vice versa) throughout the simulation and results of such single node disruptions are discussed in next few paragraphs. As we do not have data to validate the results of the network, significance of biological mutations reported in the literature was used as proof of validity.

Importance of ABA receptor complex (PYR, PP2C and SnRK2) was highlighted in recent literature (Umezawa et al., 2010) as any mutant of them makes the stomata insensitive to ABA induced closure. PP2C, a global negative regulator of stomatal closure, impairs Ca^{2+} signaling, ROS production, SnRK2 and SLAC1 activation. PYR mutants are strong ABA-insensitive phenotypes (Kim et al., 2010) and mutation of SnRk2

blocks all examined ABA responses. Our model produced ABA insensitive mutants, who lack ROS, SLAC1 and Ca^{2+} activities, in response to any of the above three mutations.

Deregulation of ion channel through perturbing either QUAC (Meyer et al., 2010), SLAC1 (Vahisalu et al., 2008) or GORK (Hosy et al., 2003) channel results in severely impaired stomatal closure especially for low ABA concentrations (<10μM). This biological fact is supported by the network results making stomata ABA insensitive with perturbed GORK or SLAC1. This may be because these channels are directly responsible for emission of major osmolytes from the guard cell. Perturbation of QUAC in our model made stomata oscillate in a limit cycle because of deregulation of Malate and thereby SLAC1 but this needs to be validated with biological justification. Involvement of both metabolism and transport of Malate are possible in ABA signalling but a recent study (Penfield et al., 2012) highlighted the importance of Malate export than the metabolism in ABA signalling and the results of our model supports this fact.

In addition to the ion channels, network distinguishes the significance of some of their regulators such as MAPK to the ABA system and this is biologically supported by plants lacking MAPK activity which disrupted SLAC1 activity and sensitivity of stomata to ABA (Jammes et al., 2009). According to the simulation results, over expression of KAT1 made stomata insensitive to ABA, but there are no biological evidence in literature to prove this in reference to sensitivity of closure. However, reduction of inward K^+ currents and stomatal opening (Kwak et al., 2001) are evidenced for *kat1* knocked out mutants. Our results are also consistent with the experimental results that the dominant positive mutants of AHA1 do not close stomata in response to even up to 100 mM ABA (Merlot et al., 2007).

Guard cell cytoskeleton plays a crucial role in regulation of stomata as Actin antagonists interfere with normal stomatal closing responses to ABA. Network results capture the importance of Actin and its regulators ARP23, SCAB1 and AtRAC1 to the regulation of guard cell cytoskeleton.

ROS is another key element identified by network results as a regulator of both ion channel activity and cytoskeleton of the guard cells. Inhibition of ROS production is found to be crucial for rearrangements of microfilaments. Results of the model for perturbing RBOH, the major regulator of ROS production, agreed with the literature as it resulted in 100% ABA insensitive stomata (Suhita et al., 2004) and 50% of them with no calcium induction. According to literature, inhibition of PA, another essential regulator of ROS production partially opposes both ABA-inhibition of stomatal opening and ABA-promotion of stomatal closure (Davies, 2010); our model displays severe reduction of ABA sensitivity (only 24% stomata oscillates without maintaining closure). PLD is involved in the production of PA in response to ABA; biological mutants of PLD lower the production of S1P by 33% (Guo et al., 2012), block the productions of ROS, PA and NO (Zhang et al., 2009), and avoid Cytosolic Alkalization making stomata insensitive to ABA. Mutants of SPHK are 17-30% low in PA and 19% low in S1P production (Guo et al., 2012). In agreement with experimental mutants, our model resulted in severely impaired ABA sensitivity with all the characteristics explained above. With simple synchronous approach used, we could not reproduce the percentage reduction of any molecule but the severity of the effect. Lack of RCN1 in our system resulted in ABA insensitivity to stomatal closure lacking in Ca^{2+} elevation and SLAC1 activity and are supported by the literature (Saito et al., 2008). Further, cytosolic alkalization and depolarization of plasma membrane were two important conditions identified by the model for ABA signaling and supported by the fact that acidification of guard cell cytosol precedes stomatal opening (Gonugunta et al., 2009). Apart from the highlighted nodes, all other nodes do not show significant reduction in stomatal closure when knocked out of the system.

4.   CONCLUSIONS AND RECOMMENDATIONS

Results of our model agree well with the majority of experimental results published in literature suggesting that interpretations and assumptions we made on network topology and Boolean functions are acceptable. Therefore, this model is a good approximation to gain insights into ABA signalling system. It has revealed areas for further experimental exploration; of them, the importance of cytosolic calcium for rapid regulation of SLAC1 channel, in particular, needs further examination. In addition, this study confirms the ability of the simple Boolean approach to reproduce the behaviour of biological networks. However, the network topology discussed in this study cannot be concluded as complete because there may be more unknown intermediate nodes or links which are beyond the current knowledge and understanding of the network. Once new elements or links are found, we need to update the topology and logics of the network accordingly. Similarly, the synchronous approach that we used to update the network is not biologically acceptable to study the real dynamics of systems. Therefore, we need to use an asynchronous update which captures relative time intervals of operation of network nodes.

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