

Boolink: a graphical interface for open access Boolean network simulations and use in guard cell CO₂ signaling

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Abstract

Signaling networks are at the heart of almost all biological processes. Most of these networks contain large number of components, and often either the connections between these components are not known or the rate equations that govern the dynamics of soluble signaling components are not quantified. This uncertainty in network topology and parameters can make it challenging to formulate detailed mathematical models. Boolean networks, in which all components are either on or off, have emerged as viable alternatives to detailed mathematical models that contain rate constants and other parameters. Therefore, open-source platforms of Boolean models for community use are desirable. Here, we present Boolink, a freely available graphical user interface that allows users to easily construct and analyze existing Boolean networks. Boolink can be applied to any Boolean network. We demonstrate its application using a previously published network for abscisic acid (ABA)-driven stomatal closure in *Arabidopsis* spp. (*Arabidopsis thaliana*). We also show how Boolink can be used to generate testable predictions by extending the network to include CO₂ regulation of stomatal movements. Predictions of the model were experimentally tested, and the model was iteratively modified based on experiments showing that ABA effectively closes *Arabidopsis* stomata at near-zero CO₂ concentrations (1.5-ppm CO₂). Thus, Boolink enables public generation and the use of existing Boolean models, including the prior developed ABA signaling model with added CO₂ signaling components.

Introduction

Intracellular signaling networks are essential in almost all biological processes. These networks are often complex, involving a large number of many components (or nodes)

that are interconnected. To gain insights into these networks, it is possible to construct mathematical models. One of the strengths of these mathematical models is the ability to develop predictive outcomes of experimental

perturbations (Phillips, 2015; Shou et al., 2015). These perturbations can be much more easily implemented in simulations than in experiments. Removing or changing a component or connection between components is a trivial task in simulations, but it is usually a task that requires lengthy wet lab experimental procedures. Predictions developed through models can enable narrowing the parameters for subsequent wet lab examinations. Wet lab examinations, in turn, can be used to iteratively update and correct mathematical models. Furthermore, mathematical models can be used to test potential biological mechanisms or to pinpoint the most important components of a signaling network (Brodland, 2015).

One way of constructing mathematical models for signaling networks is to create a rate–equation model. In such a model, the concentrations for network components can take on zero and positive rational values, and their change is governed by differential equations involving rate constants and the concentration of diverse components (Melke et al., 2006; Muraro et al., 2011; Wang et al., 2017). For example, the computational platform OnGuard can incorporate known ion transporters and channels of the guard cell network and can provide simulations of stomatal responses (Hills et al., 2012). The concentrations of soluble signaling components, however, are often not well-defined, and the strength and kinetics of many of the connections between different nodes of the network are either poorly understood or not known at all. This results in considerable uncertainty in parameter values, especially rate constants. This is particularly the case for signal transduction networks of soluble components for which rate constants are more difficult to define in a cellular context than, for example, metabolic flux networks (Feist et al., 2007).

An alternative to these “analog” networks is to formulate the problem in terms of Boolean networks (Kauffman, 1969). In these binary networks, each node can be either “ON” (1, or high) or “OFF” (0, or low) (Bornholdt, 2008; Wang et al., 2012; Schwab et al., 2020). The state of the nodes is then determined by an update rule, which involves information from the upstream nodes, and a single-time step corresponds to updating all nodes. The most commonly applied update rules are synchronous and asynchronous. For synchronous updates, the future state of each node depends on the state of the network at the current time step. In the case of asynchronous updates, the future state of a node depends on the states of nodes already updated in the current time step and on the previous states of those nodes that are not yet updated. In our application, the order of asynchronous updates is chosen in a random fashion. In Boolean networks, the regulation is no longer encoded in terms of rate constants, which may or may not be quantified by experiments, but in terms of NOT, AND, and OR logic gates. For example, in the case of an AND gate, a downstream node will be turned on (i.e. 0 transitions to 1) if and only if the upstream node is on.

Despite the significant simplification associated with the binarization, Boolean networks have been shown to be able to predict behavior in a wide variety of networks, including genetic networks (Herrmann et al., 2012; Kauffman, 1969), protein networks (Dahlhaus et al., 2016), synthetic gene networks (Zhang et al., 2014), and cellular regulatory networks (Albert et al., 2017; Lau et al., 2007; Li et al., 2004). However, the price one has to pay for the simplification is the loss of dynamic information. Moreover, the order in which the update rules are applied can critically affect the outcome of the network. Therefore, the networks are mainly used to probe the attractors of the network, defined as the states representing the long-term behavior of the system (Schwab et al., 2020).

In plants, Boolean networks have been applied to genetic networks to investigate, for example, possible crosstalk and microbe response (Genoud and Métraux, 1999; Timmermann et al., 2020). Boolean logic has also been extensively used to study ABA-induced stomatal closure in Arabidopsis (*A. thaliana*; Li et al., 2006; Albert et al., 2017; Waidyarathne and Samarasinghe, 2018; Maheshwari et al., 2019; Maheshwari et al., 2020). This ABA signal transduction network contains a large number of components (>80), with many unknown rate constants, and is thus challenging to encode using an analog model. In a series of studies, it was shown that the formulation of a Boolean network for ABA-induced stomatal closure was able to confirm interesting experimental data (Albert et al., 2017; Maheshwari et al., 2019, 2020). Furthermore, it was shown that the Boolean network could function as a vehicle to generate predictions. Specifically, predictions were generated through perturbations that either removed nodes or set nodes permanently to the “ON” state. Some of these predictions were subsequently tested and validated in quantitative experiments (Albert et al., 2017; Maheshwari et al., 2019).

The aforementioned studies have clearly demonstrated the potential value of casting signaling pathways into Boolean networks. However, encoding these networks, especially the ones with a large number of components, might present a significant impediment to widespread use of Boolean networks to probe, analyze, and understand signaling networks. Motivated by the challenge of creating Boolean networks that can be used by the community for independent simulations, we present in this article an open-source, user-friendly algorithm that can simulate Boolean networks, which can be easily formulated by the users. This algorithm, which we term Boolink, uses a graphical user interface (GUI) and allows the users to define nodes and their internode connections, add nodes, subtract nodes, introduce mutations, and analyze the results. Unlike other algorithms that also use GUIs, the equations are implemented in the programming language C++, which results in computationally efficient simulations. A further comparison between Boolink and existing algorithms is provided in the “Discussion”. Boolink can be freely downloaded from the GitHub repository (<https://github.com/dyhe-2000/Boolink>).

GUI or <https://github.com/Rappel-lab/Boolink-GUI>). User-friendly instructions for downloading and using Boolink are provided in the [Supplemental data](#). Although Boolink can be applied to any Boolean network, we verify its use using the ABA signaling network described by [Albert et al. \(2017\)](#). We then describe an extension of this network that incorporates input from the stimulus CO₂ and examine the ABA-induced stomatal closing, while clamping CO₂ concentration in the leaf to very low levels ([Raschke, 1975](#); [Zhang et al., 2018](#)). Finally, we test predictions from this extended network using quantitative experiments, and results from these experiments were used to update possible testable CO₂ input mechanisms into the network.

Results

Algorithm

Our algorithm, Boolink, simulates a Boolean network with user-defined variables and interactions. Each node of the network corresponds to a Boolean variable that can be in either of the two states: 0 (OFF) or 1 (ON). In biophysical terms, an ON state of a node corresponds to a concentration of its active form that is high enough to effect change through its interactions in the system. Conversely, an OFF state corresponds to a low concentration, which is not able to effect change. Nodes can flip their states because of their interactions with other nodes, and these interactions are encoded using an update equation. Some nodes denoting external conditions or inputs remain fixed, but affect the states of other nodes.

An update equation for a given node relates its future state to the current states of all upstream nodes. While there are several ways of formulating an update equation, our algorithm uses the so-called Sum of Products form that is intuitive and easy to formulate ([Kime and Mano, 2003](#)). This form consists of a series of terms linked by the OR logic operator. Each of these terms is a product, which contains Boolean variables connected by the AND operator. Note that these variables may be preceded by the operator NOT. A typical example used here to exemplify Boolean logic and extracted from the model of [Albert et al. \(2017\)](#), reads:

$$\text{AnionEM} = \text{SLAC1} | \text{SLAH3} \& \text{QUAC1}$$

where the symbols “|” and “&” stand for OR and AND, respectively. In this example, the future state of node AnionEM, which represents the anion efflux through the plasma membrane, is determined by the current states of three other nodes that encode two major classes of anion channels in guard cells: SLAC1, SLAH3, and QUAC1. Specifically, AnionEM is set to 1 or remains 1 if either (1) SLAC1 is 1 or (2) both SLAH3 and QUAC1 are 1 ([Albert et al., 2017](#)). In this simplification, if neither condition is met, AnionEM remains 0 or is set to 0. Using Boolink, this Boolean logic step, and any other step in the network, can be easily modified by users, and outcomes can be simulated. The [Supplemental data](#) has a brief primer on Boolean

algebra and several illustrations for formulating Boolean equations using the Sum of Products form.

In a typical simulation in Boolink, there are a few nodes that affect the nodes downstream of them but do not have any nodes upstream. As such, their states remain unchanged during the simulation. These nodes are called “input” nodes. There is typically one “output” node denoting the product or end state of the pathway, which can be monitored to determine the function of the network. For example, in the case of the ABA signaling network, ABA, Nitrite, GTP, etc., are inputs, and the output node, termed “Closure”, represents stomatal closure ([Albert et al., 2017](#)). The initial states of other network nodes can be specified, based on prior knowledge, or can be assigned a random (0 or 1) value.

Once the initial states of all the nodes are specified, the program evaluates the update equations in a random order and exactly once for each node, with the output node evaluated last. The update for a single node is based on the current state of the network so that some of its upstream nodes may already have been updated in the same time step. This so-called asynchronous updating is motivated by the fact that many of the reaction rates are unknown, resulting in non-deterministic outcomes. A single-time step in the simulation corresponds to each node being updated once. The number of time steps, corresponding to one iteration over all the nodes, can be chosen by the user and should be large enough for the system to reach a steady state. Furthermore, the user also specifies the number of simulations, each with randomly chosen initial conditions. All parameters, equations, and initial conditions can be easily entered into Boolink using an intuitive graphical interface. Finally, Boolink is able to display graphs of the dynamics of one or more nodes, and all variables are stored for later analysis.

Simulations

Boolean network introductory and training example

As a first example, we investigate a very simple Boolean network, shown in [Figure 1A](#). This network contains an input node (IN), an output node (OUT), and three intermediate nodes X, Y, and Z. The input node does not depend on any other nodes, which is simply written as $\text{IN} = \text{IN}$. Furthermore, X and Y reinforce each other, and X inhibits Z through a NOT link (denoted by the symbol $\sim$ in the equations and flat arrowheads in [Figure 1A](#)), while IN inhibits X; Y inhibits OUT and Z activates OUT. For both X and OUT, the two inputs combine according to the AND logic as denoted by a “&”. The Boolean update equations can thus be simply written as follows:

$$\begin{aligned}\text{IN} &= \text{IN} \\ \text{X} &= \text{Y} \& \sim \text{IN} \\ \text{Y} &= \text{X} \\ \text{Z} &= \sim \text{X} \\ \text{OUT} &= \sim \text{Y} \& \text{Z}\end{aligned}$$

The dynamics of this network can be worked out by hand or, since it only contains three nodes, can be analyzed

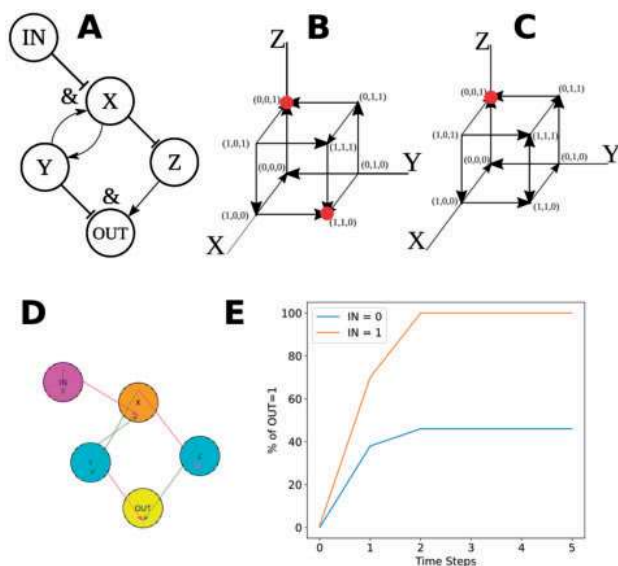


Figure 1 Simple Boolean network and its implementation into Boolink. A, Schematic of the network. Pointed arrowheads indicate positive regulation and flat arrowheads indicate negative regulation. For nodes X and OUT, the two inputs combine using AND logic, indicated by the symbol '&'. B, State space and dynamics, represented by arrows, in the absence of input ($IN = 0$). The two possible attractors, (0,0,1) and (1,1,0), are indicated by red dots. C, State space and dynamics in the presence of input ($IN = 1$). The only attractor, (0,0,1), is indicated by a red dot and leads to $OUT = 1$. D, Representation of the Boolean model in the Boolink GUI. Green and magenta arrows indicate positive and negative regulation, respectively. An arrow within the node indicates self-regulation. In this representation, one can “search” for upstream and downstream nodes of a given node. Here, X (colored in orange) has two downstream nodes, colored in cyan, and one upstream node, colored in magenta. Other node(s) are colored in yellow. E, Percentage of $OUT = 1$ versus time step in an asynchronous update scheme starting with randomly assigned initial states for the intermediate nodes when the input is present ($IN = 1$, orange line) and absent ($IN = 0$, blue line).

graphically. One feature of an asynchronous update scheme is that the updated state is always the “nearest neighbor” of the previous state. Therefore, the evolution of the states can be represented as a continuous trajectory through the state space, with dimensions equal to the number of nodes. This is shown in Figure 1, B and C, for our simple model in the absence ($IN = 0$; B) and in the presence of input ($IN = 1$; C). The 3D state space is spanned by X, Y, and Z, and all possible states of the intermediate nodes are points in this 3D space that are connected by arrows according to the rules of the Boolean network. By following these arrows, the attractors for the model can be determined. For example, in the absence of input (Figure 1B), the state $X = 1$, $Y = 0$, and $Z = 0$, compactly written as (1,0,0), can transition to either (0,0,0) or (1,1,0). Since no arrows originate from (1,1,0), this is an attractor of the system: this state will remain unchanged indefinitely. Furthermore, the only permissible transition from (0,0,0) is to (0,0,1), which can easily be seen as an attractor as well. Since only (0,0,1) leads to the ON

state of the OUT node, we find that $OUT = 1$ in 50% of the possible initial conditions. However, in the presence of input ($IN = 1$), it is easy to verify that the only possible attractor is (0,0,1), and thus, $OUT = 1$ for all initial conditions. Calculations and further analysis on this simple network are provided in the Supplemental data.

The implementation in Boolink is shown in Figure 1D, obtained after specifying the input files in the subfolder “sample_data_files/simple_network_data_files” of the repository (<https://github.com/dyhe-2000/Boolink-GUI> or <https://github.com/Rappel-lab/Boolink-GUI>). Here, the green/magenta arrows indicate positive/negative regulation and an arrow within the IN node indicates self-regulation. In Boolink, all nodes are visualized in yellow by default. However, connections between a particular node and other network nodes can be easily visualized by double-clicking on the specific node, after which it changes its color to orange. Upstream nodes will then change their color to magenta, while downstream nodes will turn cyan. In the example of Figure 1D, this procedure has been carried out by clicking on node X. Note that the color scheme for the nodes can be changed by the user. (See Supplemental data section 2, “Downloading and Running Boolink”).

This network was simulated using Boolink, choosing five time steps and 50 different sets of initial conditions. The results of these simulations are shown in Figure 1E, where we plot the state of the output node, expressed as the percentage of runs in which $OUT = 1$, in the absence (blue line, $IN = 0$) and presence (orange line, $IN = 1$) of input. Note that time in these plots indicates the iteration number. The state of the simulation that is of physiological relevance is the steady state; here it is reached after two steps. Consistent with the arguments presented above, the simulations show that this percentage is 50% when $IN = 0$ and 100% when $IN = 1$.

ABA network

Next, we applied Boolink to the ABA-induced stomatal closure network formulated by Albert et al. (2017). The input file for this network, containing all components by name and their interactions, can be found in the subfolder “sample_data_files/ABA_data_files/” of the repository for the Boolean equations and for the names of the nodes (<https://github.com/dyhe-2000/Boolink-GUI> or <https://github.com/Rappel-lab/Boolink-GUI>). The reconstruction of the published ABA signaling network (Albert et al., 2017), within the Boolink interface here, will enable any user to use and manipulate components of this network and develop experimental predictions and to modify the Boolean network depending on experimental outcomes or to predict outcomes for modified network models. A screenshot of our implemented network encoded within the Boolink GUI is presented in Figure 2, with the input ABA node shown in red and the “Closure” output node shown in green. This network contains 81 nodes, including input and output nodes, and was constructed by and adapted from Albert et al.

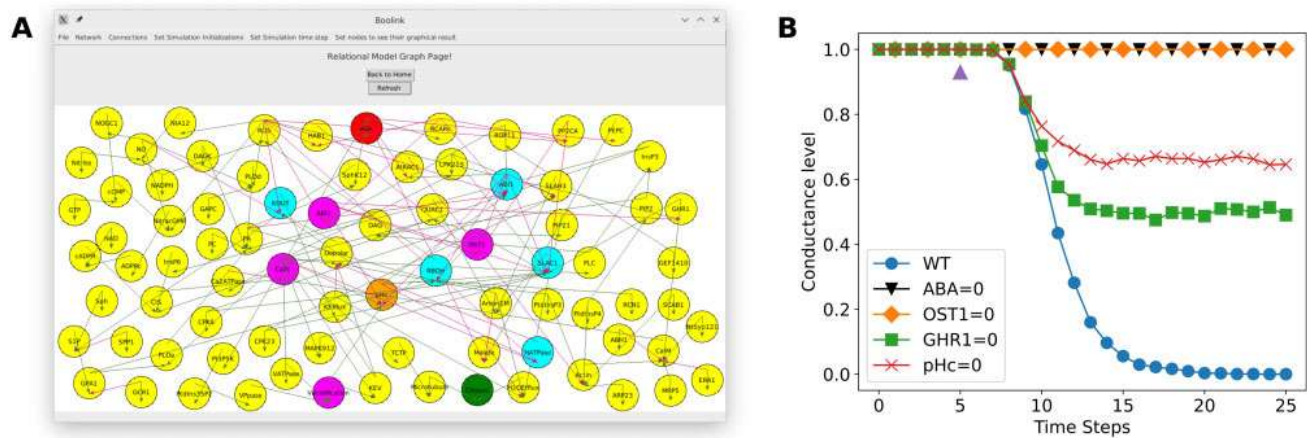


Figure 2 Implementation of ABA signaling into Boolink A: Visualization of the Boolean network for ABA-induced stomatal closure (Albert et al., 2017), rendered here by the Boolink GUI. The input ABA and output Stomatal Closure are colored in red and green, respectively. The node denoting cytoplasmic pH (pHc), colored in orange, has its upstream nodes colored in magenta and downstream nodes in cyan. Connections for any node can be viewed by double-clicking on the node of interest (see Results). B: Simulated stomatal conductance as a function of time steps in the simulation for wild type (with and without ABA) and the mutants *ost1* and *ghr1*, and alteration of cytosolic pH (pHc) based on the model of Albert et al. (2017) (see Results). A conductance level of 1 corresponds to maximal relative stomatal conductance while 0 represents complete stomatal closure. The violet triangle shows the point in the simulation where ABA is introduced (except for the case ABA=0).

(2017) following an extensive survey of more than 100 peer-reviewed articles. As in the simple example, the interactions between nodes in the GUI are color-coded, with green arrows representing positive interactions and magenta arrows representing negative interactions. Using the Boolink GUI, the user can move nodes around by simply dragging them to a new location. Furthermore, to facilitate examining inter-node connections, double-clicking on a node reveals all downstream and upstream interactions of that node (Figure 2A). (See <https://github.com/dyhe-2000/Boolink-GUI> or <https://github.com/Rappel-lab/Boolink-GUI>; detailed instructions can be found in the Supplemental data).

Results of the Boolink simulations for 25 time steps and averaged over 2,500 initial conditions are presented in Figure 2B. In this and subsequent curves, following Albert et al. (2017), we chose to illustrate the predicted stomatal conductance level as a function of simulation (time) step. However, and as discussed above, this time step does not equate to “real time”, and only the steady state following a change in network architecture or input can be compared to experimental results. This conductance level, computed as 1-Closure, varies between 0 (corresponding to closed stomata and Closure = 1) and 1 (corresponding to open stomata and Closure = 0) and facilitates comparison with experiments in which the stomatal conductance is presented (Figures 3 and 4). In the absence of ABA, simulated by setting the input node ABA to 0 throughout the simulation, the output node Closure is 0 for all time steps, corresponding to no stomatal closure and a conductance level of 1 (black triangle curve). In the presence of ABA, modeled by changing ABA from 0 to 1 at time step five, the network reaches a conductance level of 0 (stomatal closure) after approximately 15 time steps (blue curve, labeled as wild-type [WT]). By implementing the model of Albert et al. (2017),

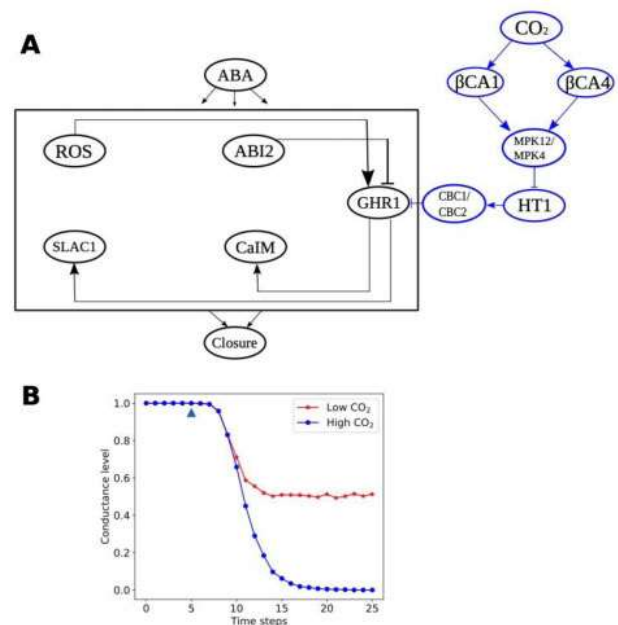


Figure 3 Stomata 2.0 and Boolink. A, ABA-driven stomatal closure model extended with a CO₂ branch, indicated in blue, which positively regulates GHR1. The box denotes all the intermediate nodes of the original ABA network shown in Figure 2, with only GHR1 and its immediate upstream and downstream nodes shown. B, Predicted relative stomatal conductance levels obtained by implementing Stomata 2.0 into Boolink for two concentration levels of CO₂: low (CO₂ = 0; red line and symbols) and high (CO₂ = 1; blue line and symbols). The triangle shows the point in the simulation where ABA is introduced (ABA = 1).

we have also computed the relative stomatal conductance for several mutants, labeled in Figure 2B, following the introduction of ABA after five time steps. Knocking out the

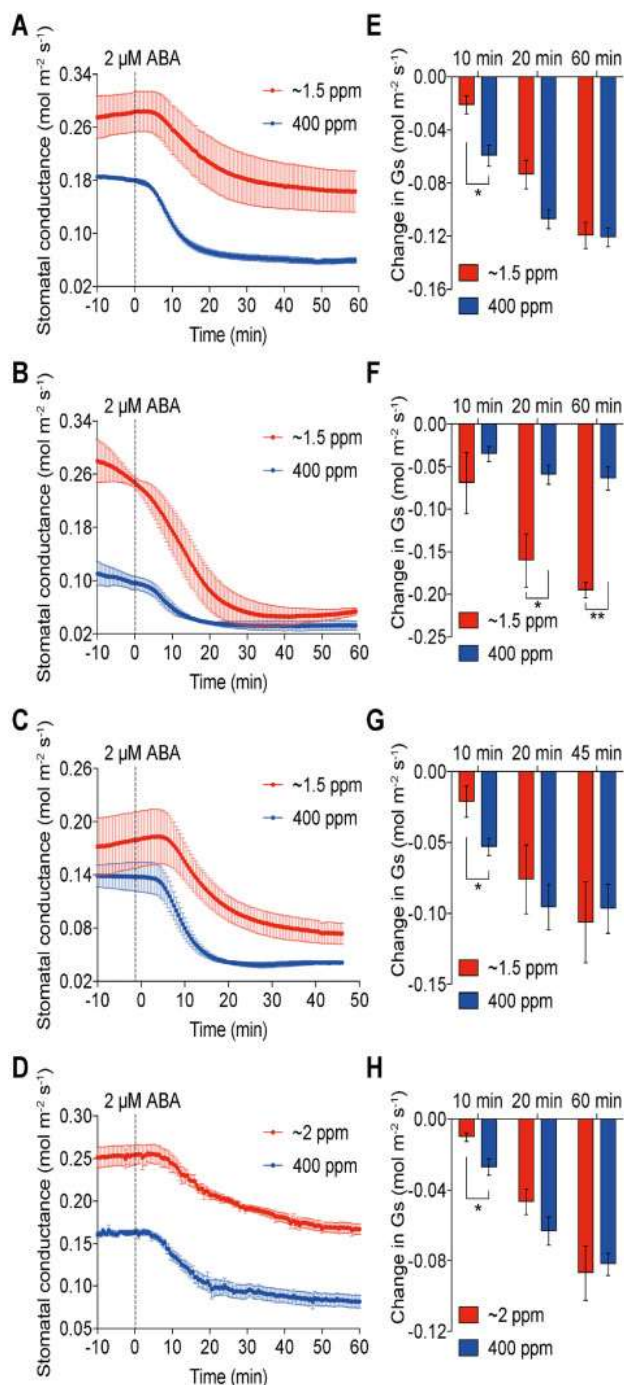


Figure 4. ABA-mediated stomatal closing responses of WT leaves during CO₂ “starvation”. A–C, Intact excised leaves of WT plants ($n = 3$ to 4 independent leaves per treatment and experimental set) were equilibrated at 400 ppm CO₂ or ~1.5 ppm CO₂ for 60 min prior to stomatal conductance measurements. Stomatal conductances were measured with a LI-6400XT Portable Photosynthesis System. D, Time-resolved stomatal conductance responses to ABA in intact excised leaves ($n = 5$ independent leaves per treatment) equilibrated at ~2 ppm CO₂ or 400 ppm CO₂ for 2 h prior to ABA application. Experiments were carried out using a LI-6800 Portable Photosynthesis System. In each experimental set, 2- μ M ABA was applied through the transpiration stream via the petiole at time = 0 min. CO₂ concentrations in the intercellular spaces of leaves (C_i) equilibrated under CO₂ starvation were computed using the gas exchange analyzer (see

Open Stomata 1 (OST1) protein kinase, which corresponds to forcing the node OST1 to 0 at all times, results in a conductance level that remains 1 (no stomatal closure) after setting ABA = 1 (orange diamond curve). Furthermore, knocking out guard cell hydrogen peroxide resistant 1 (GHR1) results in a conductance level of 0.5 (50% stomatal closure) following the introduction of ABA (green square curve) and manipulating the cytosolic pH through the node pHc leads to a conductance level of approximately 0.65 (red X curve). These control predictions are identical to the ones obtained in the original publication (Albert et al., 2017), further validating reconstruction of this network in our interactive graphical user interface.

CO₂ network

We next applied our GUI to examine and explore the guard cell signaling network. We used the original ABA network of Albert et al. and first extended it with a putative branch that models the input of CO₂. Elevated CO₂ triggers stomatal closure and some elements of CO₂ signaling overlap with those of ABA signaling, whereas others affect stomatal closure through separate pathways (Hsu et al., 2018; Merilo et al., 2015; Zhang et al., 2020). Based on previous experimental data, we modeled the CO₂ branch to be upstream of GHR1 in the ABA network (Hörak et al., 2016; Jakobson et al., 2016). The added branch contains CO₂ as input, which is then catalyzed by the beta carbonic anhydrases β CA4 and β CA1 in parallel (Hu et al., 2010, 2015). These then activate the node Mitogen-Activated Protein Kinase 4 and 12 (MPK4 and MPK12) via mechanisms that are yet unknown. This node inhibits the negative-regulator of CO₂-induced stomatal closing, the high temperature 1 (HT1) protein kinase, which, in turn, regulates the convergence of blue light and CO₂ 1 and 2 (CBC1 and CBC2) protein kinases either directly or indirectly (Hashimoto et al., 2006; Hörak et al., 2016; Hiyama et al., 2017). Finally, CBC1/CBC2 enters the ABA network through an assumed inhibitory link to GHR1 (Figure 3A).

The above summarized CO₂ branch can be translated into the following Boolean equations:

$$\begin{aligned} \text{CO}_2 &= \text{CO}_2 \\ \beta\text{CA1} &= \text{CO}_2 \\ \beta\text{CA4} &= \text{CO}_2 \\ \text{MPK12/MPK14} &= \beta\text{CA4}|\beta\text{CA1} \\ \text{HT1} &= \sim \text{MPK12/MPK14} \\ \text{CBC1/CBC2} &= \text{HT1} \\ \text{GHR1} &= \sim \text{ABI2\&ROS\&} \sim \text{CBC1/CBC2} \end{aligned}$$

Note that the equation for GHR1 is adapted from Albert et al. and takes into account the existing connections from

“Materials and methods”), with C_i values <20 ppm (A–C) and <3 ppm (D) before application of ABA. E–H, Changes (differences) in stomatal conductance at the indicated time points after ABA application compared to 0 min. Data present mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$ Student’s t test in E–H.

the ABA network (from ABI2 and ROS) and the new input from the CO₂ branch. This simplified CO₂ signaling model, which can be accessed in the online Boolink repository, termed Stomata 2.0, includes presently identified and confirmed early CO₂ signaling mechanisms that have been found to function in the CO₂ signaling pathway upstream of the merging with the ABA-induced stomatal closing pathway (Hsu et al., 2018; Zhang et al., 2018, 2020).

Based on this model, we then determined how the extended network responds in simulations to ABA under high and low CO₂ conditions (Figure 3B), simulated by setting the input node for CO₂ to either 0 (very low concentration) or 1 (high concentration) and again averaging over 2,500 initial conditions. For CO₂ = 1, the introduction of ABA at time step five results in a decrease of conductance level from 1 to 0 (Figure 3B, blue curve), identical to the WT response shown in Figure 2 (blue curve). This can be understood as the added CO₂ branch having little or no effect on the ABA network since CBC1/CBC2 is 0 when CO₂ = 1. Note that in this model, the starting steady-state stomatal conductance of 1 is similar at each background CO₂ concentration, which will be addressed in updated simulations further below. When simulating very low CO₂ conditions, our simulations predicted that introduction of ABA also induces stomatal closure and, thus, a decrease in the conductance level. However, the ABA-induced conductance level in the presence of low (nominally 0) CO₂, was found to be reduced from 1 to 0.5 (Figure 3B, red curve).

To test our predictions experimentally, we analyzed ABA-mediated stomatal closure under either 400 ppm or near-zero ppm (~1.5 ppm) CO₂ by conducting gas-exchange experiments with ABA application to the transpiration stream of excised intact leaves (Ceciliato et al., 2019). Our results showed that application of 2-μM ABA induced robust stomatal closure in leaves exposed to 400-ppm CO₂ as expected (Figure 4, A–D, blue curves). As stomatal responses are known to show biological noise, and as ABA-induced stomatal closing in *Arabidopsis* spp. has not been previously analyzed at near-zero CO₂, we conducted four independent sets of experiments (Figure 4). In all four experiments, leaves exposed to 1.5-ppm CO₂ showed robust stomatal closing in response to 2-μM ABA, with a degree of expected biological variation (Figure 4, A–D, red curves). By analyzing the steady-state stomatal conductance in leaves, it appears that the response to ABA at low CO₂ was reduced in three of these experiments (Figure 4, A, C, and D). We also compared the difference (change) in steady-state stomatal conductance before and after applying ABA (Figure 4, E–H). This analysis shows that independent of whether leaves are exposed to 400-ppm CO₂ or 1.5–2-ppm CO₂, the ABA responses had a similar magnitude in three of the experiments, and there was a stronger ABA response in one of the experiments (Figure 4F). Furthermore, our data show that in the absence of ABA, leaves exposed to low CO₂ show a higher stomatal conductance than leaves exposed to 400-ppm CO₂. This is consistent with a reduction in

stomatal conductance upon CO₂ elevation. In addition, analyses of an early time point of the ABA response, 10 min after ABA addition, show a slightly, but significantly, slowed initial ABA response in three of four experiments when compared with controls exposed to 400-ppm ambient CO₂ (Figure 4, E, G, and H). Taken together, our experimental data show that the steady-state stomatal conductance responses to ABA in *Arabidopsis* remain, to a large degree, intact even at very low, near-zero CO₂ concentration and as an approximation indicate a steady-state additive effect of low CO₂ on the stomatal conductance prior to ABA exposure.

A comparison of our experimental results and model predictions in the absence of ABA reveals that Stomata 2.0 (Figure 3B) is not able to fully capture the dependence of the steady-state conductance levels on CO₂. This suggests that additional modifications of the ABA network are required to reproduce the observed reduction of stomatal conductance in the presence of CO₂. To explore possible modifications that result in model predictions that are more consistent with our experimental steady-state response results, we utilized the ability of Boolink to easily modify, simulate, and visualize modified Boolean network outcomes. We found that we were able to better reproduce experimental data if we modified the Boolean equations for four network components (Figure 5A). Specifically, we modified the nodes cytosolic calcium (Ca2c), which is linked in the ABA model (Albert et al., 2017) to Ca²⁺ influx across the plasma membrane (CaIM), microtubule depolymerization (microtubule), and water efflux through the plasma membrane (H2Oefflux) to:

$$\begin{aligned} \text{Ca2c} &= \sim \text{Ca2ATPase} \ \& \ (\text{CIS} \mid \text{CaIM}) \mid \underline{(\text{ABA} \& \text{CO}_2)} \\ \text{CaIM} &= \sim \text{ABH1} \ \& \ (\text{NtSyp121} \mid \text{MRP5} \mid \text{GHR1}) \mid \sim \text{ERA1} \mid \text{Actin} \mid \underline{\text{CO}_2} \\ \text{Microtubule} &= \text{TCTP} \mid \text{Microtubule} \ \& \ \underline{\text{ABA}} \\ \text{H2Oefflux} &= \text{AnionEM} \ \& \ \text{PIP21} \ \& \ \text{KEfflux} \ \& \ \sim \text{Malate} \mid \underline{\text{CO}_2} \end{aligned}$$

where the modifications are underlined. Introducing a CO₂ dependence on calcium signaling was motivated by experimental evidence that Ca2c was involved in CO₂-induced stomatal closure (Webb et al., 1996; Schwartz et al., 1988; Schulze et al., 2021). Furthermore, findings that anion efflux and water efflux functions in the CO₂ response and that GHR1 functions in CO₂-induced stomatal closure were included (Hörak et al., 2016; Jakobson et al., 2016). Addition of an ABA component to microtubule function was motivated based on recent findings (Rui and Anderson, 2016; Qu et al., 2018). These modifications, their associated Boolean logic operations, and their effect on stomatal closure are further detailed in the Supplementary data. When simulating this modified and updated network, termed Stomata 2.1, we found that the steady-state conductance level now depends on CO₂ and is reduced for high CO₂ conditions (Figure 5B). Furthermore, and also approximately consistent with the experiments, the introduction of ABA reduces stomatal conductance by similar absolute conductance changes for both low and high CO₂ conditions (Figure 5B). The Stomata 2.1 network is able to reproduce experimental results better.

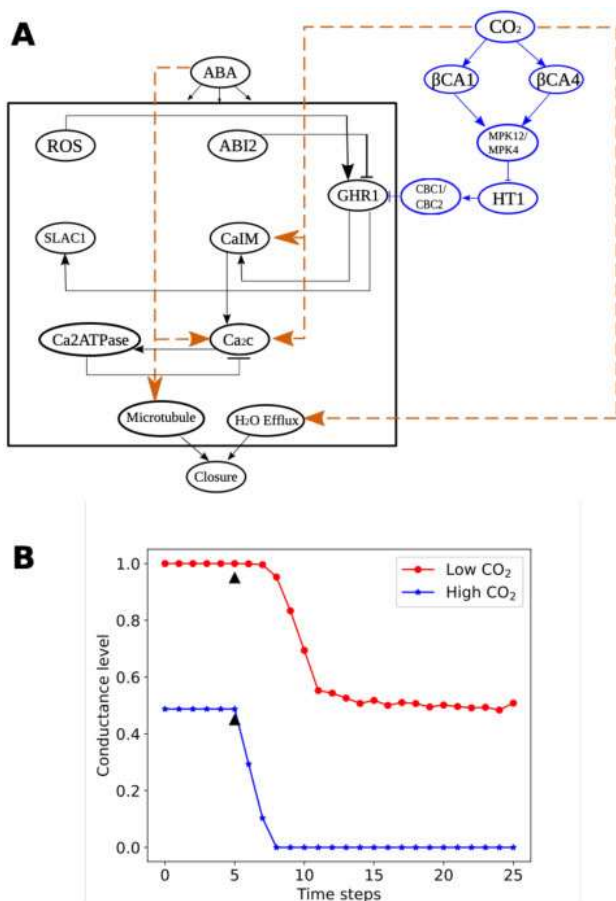


Figure 5. Stomata 2.1 and Boolink. A, ABA-driven stomatal closure model extended with a CO_2 branch, indicated in blue, which positively regulates GHR1, and additional modifications represented by the orange links (read the study for details). B, Predicted relative stomatal conductance levels obtained by implementing Stomata 2.1 into Boolink for two concentration levels of CO_2 : low ($\text{CO}_2 = 0$; red line and symbols) and high ($\text{CO}_2 = 1$; blue line and symbols). The triangle shows the point in the simulation where ABA is introduced.

We have included the network files for both Stomata 2.0 and 2.1 in the folder “sample_data_files/in the Boolink repositories”. We anticipate that community members will be able to use Boolink as a starting point to easily introduce modifications and iteratively test predictions.

Discussion

Large signaling networks are common in biology, in general, and plant physiology. The published ABA signaling network we implemented into Boolink, for example, contains more than 80 components (Albert et al., 2017). The vast majority of the interaction strengths and kinetic parameters between these components are not known, making it difficult to formulate mathematical models of these networks. Motivated by the simplicity and utility of Boolean networks and the challenges associated with formulating detailed rate equation-based models for these large networks, we have presented here a software package with a GUI that can simulate, visualize, and plot the results of a user-defined

Boolean network. Our package, named Boolink, is free to use and distribute and is built from free and open-source software. The interface is intuitive, and users do not require extensive coding knowledge to use it. The [Supplementary data](#) contains detailed instructions on downloading, installing, and running Boolink on Windows, Mac, and Linux-based machines. In addition to this open-source version, we also packaged the software in a “Docker container”, which allows execution of Boolink in an even more facile and direct computer operating system-independent fashion. Instructions on how to obtain the Docker container is also provided in the [Supplementary data](#).

Boolink platform and advantages

As reviewed by Schwab et al. (Schwab et al., 2020), a number of computational platforms exist that are able to simulate Boolean networks and can be useful in certain applications. Boolink, however, is distinct from these platforms for several reasons, making it uniquely capable of analyzing the large-scale Boolean networks addressed in this study. First of all, many of the existing packages, including BoolSim (Garg et al., 2008), ChemChains (Helikar and Rogers, 2009), MaBoSS (Stoll et al., 2012), Pint (Paulevé, 2017), BooleanNet (Albert et al., 2008), BoolNet (Müssel et al., 2010), and PyBoolNet (Klarner et al., 2017), do not use GUIs and cannot visualize the network. Obtaining an intuitive understanding of networks without a graphical interface becomes increasingly difficult as the number of nodes increases. Furthermore, having the Boolink GUI allows users to determine connections between nodes with a simple click on the graphical representation. Furthermore, the packages SQUAD (Di Cara et al., 2007), SimBoolNet (Zheng et al., 2010), and Polynome (Dimitrova et al., 2011) either are no longer available or use Java versions that are no longer supported. In addition, CellNetAnalyzer (Klamt et al., 2007), is implemented in MATLAB and is, thus, not open source. Also, BooleSim (Bock et al., 2014) and CellNOptR (Terfve et al., 2012) only use synchronous updating and cannot implement the evaluation of nodes in an asynchronous order. Finally, ViSiBool (Schwab and Kestler, 2018), GINsim (Gonzalez et al., 2006), and The Cell Collective (Helikar et al., 2012) can only run a single initialization at a time. This means that performing the computational studies reported in Figures 3 and 4, which used 2,500 randomly chosen initial conditions, would require manually starting 2,500 separate simulations.

To further distinguish Boolink from existing packages, we have performed a benchmarking study to compare the computational efficiency of our platform with that of two existing packages, GINsim and The Cell Collective, that are GUI-based, are open source, can perform asynchronous updating, and can plot results. Both of these packages are implemented using Java, which can be expected to be less efficient than our C++-based platform (Hundt, 2011; Prechelt, 2000). To benchmark GINsim, we used a published Boolean network comprising 18 nodes that models the cellular cycle in budding yeast (Irons, 2009). Computing 2^{18}

asynchronous updates for all possible states of this network took 35.83 s in GINsim. Simulating the same network in Boolink and for 50 time steps and 2,500 initial conditions, corresponding to $2500 \times 50 \times 18 = 2.25 \times 10^6$ asynchronous updates, took 8.2 s. Comparing the Central Processing Unit (CPU) time per update reveals that Boolink is faster than GINsim by a factor of 38: 3.64×10^{-6} s per update versus 1.37×10^{-4} s per update. To benchmark The Cell Collective, we simulated our simple five-node network (Figure 1) for one initialization and 10,000 time steps. This simulation required approximately 1 min and 45 s or around 0.01 s per time step using The Cell Collective. In contrast, Boolink was able to complete this simulation in 0.338 s, with a difference in speed of approximately 340. This considerable advantage in computational efficiency can become particularly attractive for large-scale networks that need to be run multiple times, as is the case for our CO₂ signaling network. Coupled to a user-friendly graphics interface, our C++ implementation should thus provide a fast and user-friendly platform for the exploration of Boolean networks. Furthermore, our platform is independent of operating system and can be installed on Linux, Windows, and Mac operating systems. The explicit instructions and the Docker container we provide should facilitate this installation.

Boolink uses asynchronous and random order of updates, which is best suited to simulate a network of chemical reactions in which the outcome of one reaction then affects the outcome of another in the near future (hence asynchronous), and when the relative rates of different reactions in the network are unknown (hence random order of update). Besides nodes and connections, users may also specify the number of time steps or iterations to run the simulations and the number of initial conditions to get a statistically robust sample output. Once a system is defined, it may be visualized as a network in Boolink. Visualization includes identifying the upstream and downstream nodes of a given node and the type of connections (activating or inhibiting) between them, by simply double-clicking on the node of interest. The steady states of nodes of the system after simulation can be quickly plotted within Boolink. The trajectory of the entire simulation is stored in a NumPy array; a Jupyter notebook is provided with the package, which can be used to further analyze the system starting from the NumPy array, including producing publication-ready plots of the simulation.

Guard cell CO₂ signaling iterative modeling

We first tested and verified Boolink using a published advanced model for stomatal closure in guard cells as mediated by ABA and verified that our simulations were consistent with those of Albert et al. (2017). We then extended the network to include the effects of CO₂ on stomatal movements. Previous research indicated that CO₂ might mediate signal transduction via the OST1 protein kinase, as *ost1* mutant leaves were impaired in their stomatal response to CO₂ elevation (Merilo et al., 2013; Xue et al., 2011). However, more recent studies unexpectedly showed that, in

contrast to ABA, CO₂ elevation does not activate the OST1 protein kinase (Hsu et al., 2018; Zhang et al., 2020). This research further provided experimental evidence that basal OST1 protein kinase activity and basal ABA signaling are required for WT-like CO₂-induced stomatal closure (Hsu et al., 2018; Zhang et al., 2020; Figures 3 and 4). Thus, merging CO₂ signaling into the extant ABA signaling model is reasonable. The biochemical link by which CO₂ signaling merges with ABA signaling is proposed to lie downstream of the OST1 protein kinase but remains unknown. In the present study, to test a simplified model merging the ABA signaling and CO₂ signaling networks, we modeled this link to occur at the level of the transmembrane receptor-like (pseudo)kinase GHR1 (Hua et al., 2012; Sierla et al., 2018).

The simulations of this simplified network predicted that the response to ABA should depend on the CO₂ concentration (Figure 3). This prediction was then subsequently analyzed, and ABA-mediated stomatal closure of intact *Arabidopsis* spp. leaves was measured while leaves were either exposed to ambient 400-ppm CO₂ or near-zero (1.5 ppm) CO₂ (Figure 4). Interestingly, our data show that leaves exposed to 1.5-ppm CO₂ exhibited a robust response to ABA. Under low CO₂ conditions, the stomatal conductance remained higher prior to ABA application at a steady state than at 400-ppm CO₂ (Figure 4A). When comparing true steady-state stomatal conductance responses, it appears that CO₂ and ABA may in part have additive responses in *Arabidopsis* as a first-order approximation. (Note that basal ABA signaling amplifies or accelerates the CO₂ response [Hsu et al., 2018], such that the starting stomatal conductance was much higher at low CO₂ due to the lack of CO₂-induced stomatal conductance reduction [Figure 4A]).

In contrast to our experimental data (Figure 4), Stomata 2.0 predicted identical conductance levels for low and high CO₂ concentration in the absence of ABA (Figure 3B). In an illustration of the use of Boolink, we modified the ABA network further, with as goal to incorporate CO₂ dependence on steady-state conductance levels when ABA is absent. Creating this updated network, Stomata 2.1, was greatly facilitated by the ability of Boolink to easily implement changes and generate predictions. We introduced CO₂ dependence on calcium signaling based on experimental evidence that Ca²⁺ is involved in CO₂-induced stomatal closure (Schwartz et al., 1988; Schulze et al., 2021; Webb et al., 1996). Furthermore, it is well-established that anion efflux and water efflux from guard cells are essential for the CO₂-induced reduction in stomatal conductance. Furthermore, findings that GHR1 functions in CO₂-induced stomatal closure (Hörak et al., 2016; Jakobson et al., 2016) were expanded to include GHR1 predictions of the original ABA signaling model (Albert et al., 2017). Addition of an ABA component to microtubule function was motivated based on recent findings on roles of guard cell microtubules (Qu et al., 2018; Rui and Anderson, 2016). The output of Stomata 2.1 was able to better incorporate effects of CO₂ and the present experimental data (Figure 5B). As described

earlier, important gaps exist in the understanding of the CO₂ signaling pathway, including that the primary CO₂ or bicarbonate sensors remain unknown in guard cells and the mechanisms by which HT1, CBC1, and CBC2 link to one another and to stomatal closing mechanisms are unknown. Expansion of the present model will be required.

In the present study, ABA responses were analyzed at near-zero CO₂ in the C3 model plant *Arabidopsis*. The robust decrease of steady-state stomatal conductance at near-zero CO₂ by ABA addition was also found in the C3 species such as Oats (*Avena sativa*), Cotton (*Gossypium hirsutum*), and Cocklebur (*Xanthium strumarium*) but was not found in the C4 species such as Amaranth (*Amaranthus powellii*) and Maize (*Zea mays*; Dubbe et al., 1978), suggesting a species variability in the response. The converse response was also analyzed in which CO₂ responses were observed in the absence of exogenously added ABA in these C3 and C4 species (Dubbe et al., 1978). Interestingly, a variation among species was detected, in which either CO₂ responses proceeded in the C4 species or were impaired in the C3 species (Dubbe et al., 1978). These findings are consistent with recent findings that CO₂ signaling requires basal ABA signaling and would be predicted to depend on variation in basal levels of ABA in guard cells depending on plant species and growth conditions (Hsu et al., 2018; Zhang et al., 2020). These classical findings (Dubbe et al., 1978; Raschke, 1975) corresponds to a model in which ABA and basal ABA signaling plays an important role for other stomatal closing stimuli.

Our proposed additions to the existing ABA network, which illustrate the potential use of Boolink, are meant as a starting point for further explorations, and further research is needed to determine the precise mechanism by which CO₂ signaling merges with ABA signal transduction. Nevertheless, several improvements can be suggested. First, it is conceivable that CO₂ affects yet unknown mechanisms. Second, the CO₂ pathway may contain feedback loops, which can be easily implemented within Boolink (Figure 1; Dubbe et al., 1978). Finally, we should point out that Boolean networks do not incorporate explicit rate constants and contain nodes that can only take one of two values (0 or 1). Therefore, these networks are not able to address the time dependence of responses nor how they respond to graded inputs.

Importantly, the GUI platform and stomatal signaling model we developed can be used and altered by the public community of users to generate diverse testable predictions to add expanded components, or to modify the ABA and CO₂ signaling models. Furthermore, the methods and open-access software tools we have presented can be of interest to the wider life sciences and plant biology community interested in physiological pathways.

Materials and methods

Software

Boolink is implemented using Python and C++ and can be freely downloaded from the GitHub repository ([https://](https://github.com/dyhe-2000/Boolink-GUI)

github.com/dyhe-2000/Boolink-GUI and <https://github.com/Rappel-lab/Boolink-GUI>). It requires a current version of Python and C++ and a detailed manual, including installation instructions, is provided in the GitHub repository. These instructions are provided for Windows, Mac, or Linux-based computers. Boolink can also be run as a Docker container, a self-contained environment that includes all the required packages and utilities, on MacOS and Linux-based systems. The advantage of this method is that users only need to install the desktop client for Docker and not the dependencies like C++ and Python. A detailed explanation of instructions to install the Docker client and the required script to run the container are given in the [Supplementary data](#) and in the GitHub repository.

Experiments

Plants of the *Arabidopsis* (*A. thaliana*) accession Columbia (Col-0) were grown as described by Hsu et al. (2018). Stomatal conductance measurements in response to ABA were performed in detached intact leaves of 5.5- to 7-week-old plants in *Arabidopsis* leaves following the procedure described previously (Ceciliato et al., 2019) using a LI-6800 Portable Photosynthesis System with an integrated Multiphase Flash Fluorometer (6800-01A; LI-COR Biosciences, Lincoln, Nebraska, USA). Detached leaves were clamped in the leaf chamber and kept at ~1.5 ppm or 400 ppm CO₂, 135 $\mu\text{mol m}^{-2} \text{s}^{-1}$ red light combined with 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ blue light, $70 \pm 0.5\%$ or $65 \pm 0.5\%$ relative air humidity, 21°C heat exchanger temperature, and 500 $\mu\text{mol s}^{-1}$ incoming air flow rate for at least 2 h until stomatal conductance equilibrated and stabilized. Stomatal conductances were recorded every 30 s under ~1.5-ppm or 400-ppm CO₂ for 10 min. ABA (2 μM) was then added to the transpiration stream via the petiole, and stomatal conductances were recorded as shown in the figures. In each independent set of experiments, intact leaves from independent plants were analyzed per experimental condition.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Text. Contains a primer on Boolean logic, details of Stomata 2.1, and installation and operating instructions of Boolink.

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