

1 **Title**

2 **A dynamic model of the ABA Signaling pathway with its core components: translation rate**
3 **of PP2C determines the kinetics of ABA-induced gene expression**

4 **Authors: Ruth Ndathe¹, Renee Dale², and Naohiro Kato^{1*}**

5 1. Department of Biological Sciences, Louisiana State University, Baton Rouge, United
6 States

7 2. Donald Danforth Plant Science Center, St. Louis, MO, United States

8 *Corresponding Author

9 TEL: +1(225) 578-2004 EMAIL: KATO@LSU.EDU

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27 **Summary**

28 The abscisic acid (ABA) signaling pathway is the key defense mechanism against drought stress
29 in plants, yet the connectivity of cellular molecules related to gene expression in response to ABA
30 is little understood. A dynamic model of the core components of the ABA signaling pathway was
31 built using ordinary differential equations to understand the connectivity. Parameter values of
32 protein-protein interactions and enzymatic reactions in the model were implemented from the data
33 obtained by previously conducted experiments. On the other hand, parameter values of gene
34 expression and translation were determined by comparing the kinetics of gene expression in the
35 model to those of ABA-induced *RD29A* (response to desiccation 29A) in actual plants. Based on
36 the analyses of the optimized model, we hypothesized that the translation rate of PP2C (protein
37 phosphatase type 2C) is downregulated by ABA to increase the ABRE (ABA-responsive element)
38 promoter activity. The hypotheses were preliminarily supported by newly conducted
39 experiments using transgenic *Arabidopsis* plants that carry a luciferase expression cassette driven
40 by the *RD29A* promoter (*RD29A::LUC*). The model suggests that identifying a mechanism that
41 alters PP2C translation rate would be one of the next research frontiers in the ABA signaling
42 pathway.

43

44 **Introduction**

45 Plants possess defense mechanisms against drought (Basu *et al.*, 2016; Kumar *et al.*, 2018;
46 Takahashi *et al.*, 2020a). One of the major mechanisms is the abscisic acid (ABA) signaling
47 pathway. ABA is a phytohormone that is produced under the drought stress conditions (Zeevaart
48 & Creelman, 1988; Sauter *et al.*, 2001; Ikegami *et al.*, 2008). The ABA signaling pathway has
49 been well-characterized, leading to downstream ABA responses such as stomatal closure and gene
50 expression that help the plant to acquire drought stress resistance (Steuer *et al.*, 1988; Fujii *et al.*,
51 2009; Umezawa *et al.*, 2009). The most upstream of the core components in the ABA signaling
52 pathway is ABA-receptors named pyrabactin resistance/pyrl-like/ regulatory components of ABA
53 receptors (PYR/PYL/RCAR) that bind ABA and in turn interact with different protein phosphatase
54 2Cs (PP2Cs), namely aba insensitive1/2 (ABI1/ABI2), hypersensitive to aba1/2 (HAB1/HAB2),
55 aba-hypersensitive germination 3 (AHG3/PP2CA), and highly aba induced 1/2/3 (HA1/2/3).
56 Without the PYR interaction, these PP2Cs inhibit SNF1-related protein kinase 2s (SnRK2s) that
57 include SnRK2.2, SnRK2.3 and SnRK2.6. (Rodriguez *et al.*, 1998; Gosti *et al.*, 1999; Merlot *et*
58 *al.*, 2001; Saez *et al.*, 2004; Ma *et al.*, 2009; Melcher *et al.*, 2009; Nishimura *et al.*, 2009; Park *et*
59 *al.*, 2009; Santiago *et al.*, 2009; Yin *et al.*, 2009; Soon *et al.*, 2012). Activated SnRK2s
60 phosphorylate ABA-responsive elements (ABRE) binding factors 1/2/3/4 (ABF1/2/3/4). These
61 phosphorylated transcription factors bind ABREs on a regulatory region of ABA-induced genes
62 (Choi *et al.*, 2000; Uno *et al.*, 2000; Yoshida *et al.*, 2015). Alternatively, the activated SnRK2,
63 namely SnRK2.6 kinase, phosphorylate the slow-anion channels (SLAC1) leading to their
64 activation and subsequently lead to stomatal closure due to anion and K⁺ efflux and eventual solute
65 loss from the guard cells (Schroeder *et al.*, 1984; Geiger *et al.*, 2009; Lee *et al.*, 2009; Albert *et*
66 *al.*, 2017).

67 The ABA signaling pathway has been mathematically modeled to help understand the
68 ABA signaling pathway in guard cells leading to stomatal closure (Li *et al.*, 2006; Albert *et al.*,
69 2017; Maheshwari *et al.*, 2019; Maheshwari *et al.*, 2020). These works have led to the
70 determination of new predictions and hypotheses in the ABA signaling pathway, for example, the
71 role of feedback regulation, ROS, Ca²⁺, pH, and heterotrimeric G-protein signaling in ABA-
72 induced stomatal closure (Li *et al.*, 2006; Albert *et al.*, 2017; Maheshwari *et al.*, 2019). In addition,

73 the additive effect of ABA and salt stress on ABA and drought-responsive expression of genes
74 was also explained using mathematical modeling (Lee *et al.*, 2016).

75 The ABA signaling pathway has additional regulatory mechanisms, which are feedback
76 and post-translational regulations. The feedback regulation involves upregulation of PP2C genes,
77 which eventually results in enhanced deactivation of SnRK2s (Rodriguez *et al.*, 1998; Saez *et al.*,
78 2004; Fujita *et al.*, 2009; Wang *et al.*, 2019). It also includes the upregulation of ABF genes, which
79 increases ABF expression (Wang *et al.*, 2019). These regulatory elements are thought to affect
80 gene expression kinetics. The post-translation regulation involves phosphorylation of PYL by the
81 target of rapamycin (TOR) protein kinase (Wang *et al.*, 2018). On the other hand, Raptor, the TOR
82 associated protein, is phosphorylated by SnRK2s, leading to TOR kinase inhibition (Wang *et al.*,
83 2018). In another study, TOR was found to suppress ABA-responses by phosphorylating
84 *Arabidopsis thaliana* yet another kinase (AtYAK1) (Forzani *et al.*, 2019) that is a positive
85 regulator of ABA-mediated signal responses (Kim *et al.*, 2016). Therefore, TOR was proposed to
86 be a post-translation regulator in the ABA signaling pathway. E3-ligases are another post-
87 translational regulator which promotes the degradation of ABA signaling components, including
88 PP2CA (Wu *et al.*, 2016), SnRK2.6 (Ali *et al.*, 2019), and PYL5/7/8/9 (Zhao *et al.*, 2017).

89 Network connectivity of these additional regulatory mechanisms to the core components
90 is little understood. Dynamic modelling can allow us to better understand their role in the ABA
91 signaling pathway. Dynamic modelling is a powerful tool that integrates extensive experimental
92 data of pathway components, improving our understanding of the signaling pathway dynamics and
93 making novel hypotheses and predictions (Poolman *et al.*, 2004; Aldridge *et al.*, 2006; Janes &
94 Yaffe, 2006; Thakar *et al.*, 2007). *In vitro* parameters for many of the interactions of the core
95 components in the ABA signaling pathway have been experimentally determined, allowing us to
96 create a dynamic model.

97 The purpose of this study is to build a dynamic model consisting of the core components
98 with fixed parameter values that were previously obtained by experiments. Approximate curve
99 fitting of the model output to actual plant data was conducted by optimizing parameter values of
100 transcription and translation, which were not determined previously. In this report, we describe
101 how we built, optimized, and validated the model. The resulting model suggested two novel

102 hypotheses, which were supported by preliminary experiments. This model can be expanded to
103 investigate the roles of additional regulatory mechanisms in future studies.

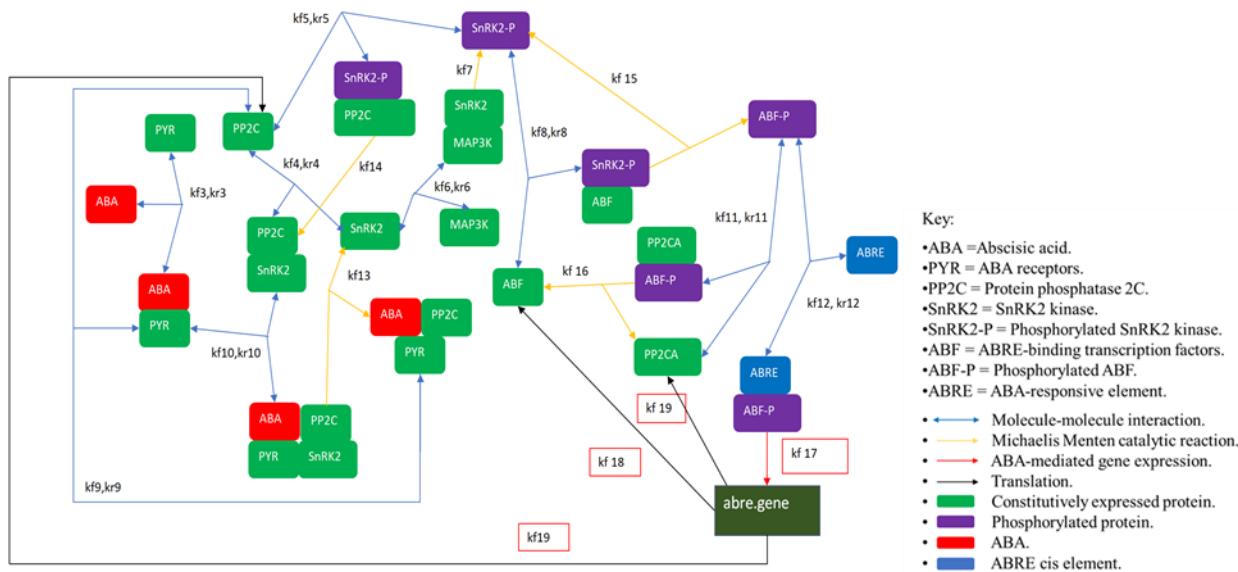
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105 **Description**

106 **Construction of the dynamic model**

107 A previous study defined a minimal set of core components that activate the ABFs, leading
108 to ABA-induced gene expression in the ABA signaling pathway (Fujii *et al.*, 2009). The
109 components are ABA, PYR/PYL/RCAR, PP2Cs (ABI1/2 and HAB1/2), SnRK2s (SnRK2.2/3/6),
110 ABFs (ABF2/3/4), and ABRE. Other studies have determined that the PP2CA phosphatases
111 dephosphorylate phosphorylated ABFs (Antoni *et al.*, 2012; Lynch *et al.*, 2012). In addition,
112 another study identified MAP3K phosphorylates SnRK2s (Takahashi *et al.*, 2020b). These two
113 reactions were included in the model. We also included the feedback regulation in which the
114 expression of PP2C, PP2CA, and ABF genes are upregulated by the ABRE promoter activity
115 (Wang *et al.*, 2019). A set of 21 ordinary differential equations representing biochemical reactions
116 of each component were constructed based on the law of mass action (Fig. 1). Homologous
117 proteins with redundant function are modeled as a single protein. Initial values of variables and
118 values of parameters in the equations were obtained from the literature (Table 1). The equations,
119 initial conditions (concentrations), and parameter values were then compiled and numerically
120 analyzed with MATLAB R2020b SimBiology (MathWorks) with default settings.

121



122

123 **Figure 1. A schematic mass-action model of the ABA-signaling pathway with its core components.** Rectangles
 124 and arrows represent variables and reactions, respectively. Identifiers of parameters in each reaction are shown as kf
 125 or kr with unique number. Parameters optimized in this study are indicated with a red frame. The values of each
 126 parameter are shown in Table 1.

127 In the model, we assumed:

- ABA signal transduction occurs through molecule-molecular interactions; where the molecule could be a protein, a hormone, or DNA.
- Enzymatic reactions follow Michaelis-Menten kinetics.
- All molecules freely diffuse in the cell.
- The cell volume is $50 \mu\text{m}^3$.
- The Michaelis constant is $K_M = \frac{k_{off} + k_{cat}}{k_{on}}$, where k_{off} is the dissociation rate constant, k_{cat} is the catalytic rate constant, and k_{on} is the association rate constant.
- A molecule associates with another molecule at a rate constant of, $k_{on} = 1000 \mu\text{M}^{-1}\text{s}^{-1}$ (Milo & Phillips, 2015).
- Proteins are generated by reactions of gene expression and protein translation, then subject to degradation.
- The concentration of a protein in a cell remains at $0.1 \mu\text{M}$ at a steady state without ABA activation and feedback regulation.

141 • A gene (mRNA) is expressed from a pair of gene loci that have a constitutively active
142 promoter, then subjected to degradation.
143 • A gene (mRNA) that is expressed by a feedback regulation has an additional regulatory
144 element (ABRE) in the same gene loci that have a constitutively active promoter.

145 In numerical analysis, the model was first run for 300 equivalent hours with the variable ABA
146 (representing intracellular ABA) set at 0 μ M. This allows the system to reach a quasi-steady state.
147 After the 300 equivalent hours, the variable ABA was set to 100 μ M. Changes of all variables in
148 the model from the quasi-steady state was then monitored for another 300 equivalent hours. In this
149 report, the time point when the variable ABA is changed is presented as time zero.

150 **Optimization of parameters, validation of the model, and analyzing identifiability of model
151 parameters**

152 To optimize selected model parameters, we approximately curve fit model output to
153 experimental data. We focused on changes in the variable abre.gene, representing accumulated
154 mRNA expressed from the ABRE promoter. Three parameters, 1. transcription of ABA-induced
155 genes, 2. translation of feed-backed ABF, 3. translation of feedbacked PP2C and PP2CA, were
156 manually changed to obtain qualitatively good fits to experimental data. The remaining model
157 parameters were unchanged (fixed). To validate the model, we quantitatively evaluated changes
158 of the variable abre.gene. Fold changes calculated by the model were compared to data previously
159 published or data newly obtained in this study. To analyze identifiability on the dynamics of the
160 variable abre.gene, we conducted sensitivity analysis using Calculate Sensitivity in Model
161 Analyzer in SimBiology with default settings.

162

163 **Results**

164 **Parameter values were obtained by literature curation**

165 We curated previously published data to define parameters in the model of the ABA
166 signaling pathway that activates the ABF, resulting in the activation of the gene promoter
167 containing ABRE cis element. The summary of our curation is shown below (Table 1).

168 **Table 1. Curated values from literature and the values chosen as parameters for the model.** Each reaction in the
 169 model was shown with the respective parameter and the source from which the value was obtained.

Description.	Reference.	Value found in the literature.	Parameter name in the model.	Value used in the model.	Fixed in the model*.
Transcription of constitutively expressed genes	(Hausser <i>et al.</i> , 2019)	< translation rate	kf1	1 hr ⁻¹	✓
Translation of constitutively expressed genes	(Hausser <i>et al.</i> , 2019)	< 10,000 hr ⁻¹	kf2	4.5 hr ⁻¹	✓
ABA and PYR binding	(Dupeux <i>et al.</i> , 2011)	$K_D = 65 \mu\text{M}$	kf3 kr3	1000 $\mu\text{M}^{-1} \text{s}^{-1}$ 65000 s^{-1}	✓
PP2C and SnRK2 binding	(Soon <i>et al.</i> , 2012)	IC_{50} 2 μM – 8 μM	kf4 kr4	1000 $\mu\text{M}^{-1} \text{s}^{-1}$ 0.1 s^{-1}	✓
PP2C and SnRK2-P binding	(Xie <i>et al.</i> , 2012)	$K_M = 0.097 \mu\text{M}$	kf5 kr5	1000 $\mu\text{M}^{-1} \text{s}^{-1}$ 97 s^{-1}	✓
SnRK2 and MAP3K binding	(Ghose, 2019)	$K_M = 23 \mu\text{M}$	kf6 kr6	1000 $\mu\text{M}^{-1} \text{s}^{-1}$ 23000 s^{-1}	✓
Phosphorylation of SnRK2 by MAP3K	(Ghose, 2019)	$k_{cat} = 14 \text{ s}^{-1}$	kf7	14 s^{-1}	✓
SnRk2-P and ABF binding	(Xie <i>et al.</i> , 2012)	$K_M = 19.3 \mu\text{M}$	kf8 kr8	1000 $\mu\text{M}^{-1} \text{s}^{-1}$ 19300 s^{-1}	✓
PYR.ABA and PP2C binding	(Dupeux <i>et al.</i> , 2011)	$K_D = 30 \text{ nM}$	kf9 kr9	1000 $\mu\text{M}^{-1} \text{s}^{-1}$ 30 s^{-1}	✓
PYR.ABA and PP2C.SnRK2 binding	(Dupeux <i>et al.</i> , 2011)	$K_D = 30 \text{ nM}$	kf10 kr10	1000 $\mu\text{M}^{-1} \text{s}^{-1}$ 30 s^{-1}	✓
ABF-P and PP2CA binding	(Pan <i>et al.</i> , 2015)	$K_M = 11.15 \mu\text{M}$	kf11 kr11	1000 $\mu\text{M}^{-1} \text{s}^{-1}$ 11150 s^{-1}	✓
ABF-P and ABRE binding	(Geertz <i>et al.</i> , 2012)	K_D of DNA-protein binding 2 nM - 2 μM	kf12 kr12	1000 $\mu\text{M}^{-1} \text{s}^{-1}$ 2 s^{-1}	✓
Release of SnRK2 from ABA.PYR.PP2C.SnRK2 complex.	(Bar-Even <i>et al.</i> , 2011)	Average k_{cat} of enzyme reaction 10 s^{-1}	kf13	10 s^{-1}	✓
Dephosphorylation of SnRK2-P	(Xie <i>et al.</i> , 2012)	$k_{cat} = 0.924 \text{ s}^{-1}$	kf14	0.924 s^{-1}	✓
Phosphorylation of ABF by SnRK2-P	(Xie <i>et al.</i> , 2012)	$k_{cat} = 0.04 \text{ s}^{-1}$	kf15	0.04 s^{-1}	✓
Dephosphorylation of ABF-P by PP2CA	(Pan <i>et al.</i> , 2015)	$k_{cat} = 1.04 \text{ s}^{-1}$	kf16	1.04 s^{-1}	✓
Transcription of ABA induced genes	(Hausser <i>et al.</i> , 2019)	< translation rate	kf17	10 hr ⁻¹	
Translation of feed-backed ABF	(Hausser <i>et al.</i> , 2019)	< 10,000 hr ⁻¹	kf18	200 hr ⁻¹	
Translation of feedbacked PP2C and PP2CA	(Hausser <i>et al.</i> , 2019)	< 10,000 hr ⁻¹	Kf19	200 hr ⁻¹	
Degradation of mRNA	(Hausser <i>et al.</i> , 2019)	mRNA degradation in HEK293 cells 0.06 hr ⁻¹	kf20, kf21	0.06 hr ⁻¹	✓

Degradation of protein	(Hausser <i>et al.</i> , 2019)	Protein decay rate in Hela cells 0.05 hr ⁻¹	kf22 to kf38	0.05 hr ⁻¹	✓
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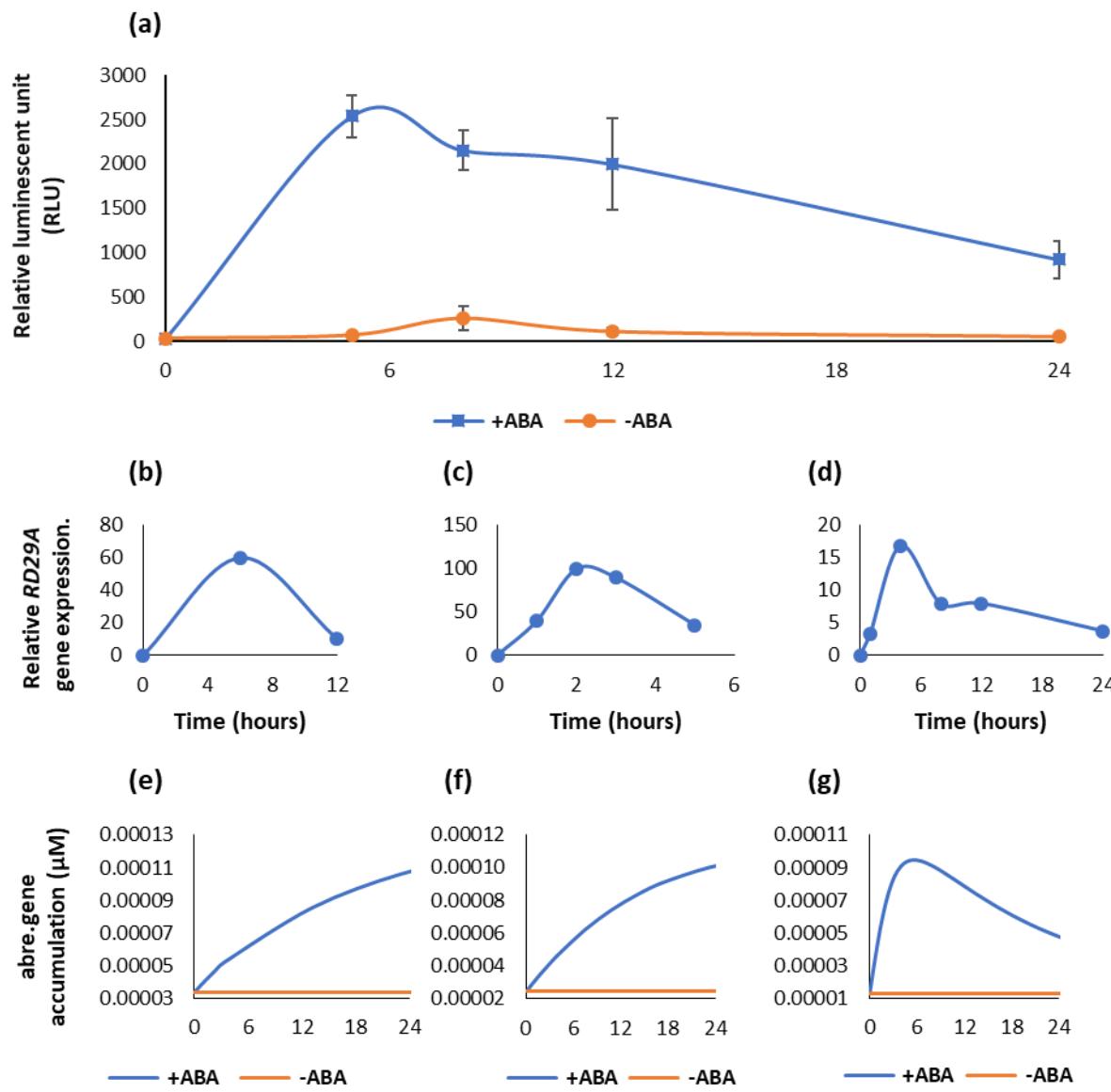
170 ***Fixed in the model:** ✓ indicates the value used in the model was not altered during model optimization

171 While parameter values for protein-protein interactions and enzymatic reactions were
172 characterized in *in vitro* studies using recombinant proteins, no studies related to parameter
173 values of DNA-protein binding, gene expression, protein translation and degradation were found
174 for the ABA signaling pathway. To this end, we implemented parameter values from studies
175 using non-plant eukaryotic organisms. These parameters have a wide range to select from: 1.
176 equilibrium dissociation constant between ABF-P (phosphorylated ABF) and the ABRE
177 promoter (from 2 nM to 2 μ M) (Geertz *et al.*, 2012), 2. translation rate of protein from mRNA
178 expressed by the ABRE promoter (less than 10,000 hr⁻¹) (Hausser *et al.*, 2019), 3. transcription
179 rate of the ABRE promoter (slower than the translation rate) (Hausser *et al.*, 2019). We selected
180 the values of translation and transcription rates for genes at 4.5 hr⁻¹ and 1 hr⁻¹, respectively, and
181 2nM for (ABF-P)-(ABRE) binding. This is because an average rate of gene transcription in
182 multicellular eukaryotes is 1 hr⁻¹ (Hausser *et al.*, 2019) while an average concentration of
183 proteins involved in a signal transduction is 0.1 μ M (Milo & Phillips, 2015). Setting translation
184 rate at 4.5 hr⁻¹ and transcription rate at 1 hr⁻¹ makes the concentration of a protein at quasi-steady
185 state to 0.1 μ M without ABA and feedback regulation in our model. The affinity of (ABF-P)-
186 (ABRE) binding was set at 2 nM to curve-fit kinetics of the variable abre.gene with actual gene
187 expression (Fig. 2). Protein degradation was set at 0.05 hr⁻¹ (Hausser *et al.*, 2019). Equilibrium
188 dissociation constant between SnRK2 (non-phosphorylated SnRK2) and PP2C was set at 100
189 pM, representing complete inhibition of SnRK2 kinase activity by PP2C at an equal molar
190 concentration (Soon *et al.*, 2012).

191 **The transcription rate of genes expressed by the ABRE promoter and the translation rate of
192 feedback loop components ABF, PP2C, and PP2CA were optimized in the model to capture
193 observed dynamics in experimental data**

194 To understand the connectivity of the components, we compared the kinetics of gene
195 expression in the model and experimental data in actual plants. Namely, we compared the
196 simulation data of the variable abre.gene, which represents the accumulation of genes expressed
197 by the ABRE promoter, to four independent data sets that were experimentally obtained using
198 actual plants. One set of data was obtained by our new experiments using transgenic *Arabidopsis*

199 *thaliana*. The transgenic plants carry the *RD29A::LUC* gene expression cassette that has been used
200 to study the activity of the ABRE promoter (Zhan *et al.*, 2012). The activity of ABRE promoter
201 can be monitored by luminescence in near real-time in plants. The other three sets were obtained
202 from previously published data that show a change in *RD29A* gene expressed from the native
203 ABRE promoter in the genome of either *Arabidopsis thaliana* (Lee *et al.*, 2016; Song *et al.*, 2016)
204 or *Oryza sativa* (rice) (Singh *et al.*, 2015). Kinetics of the gene expression in the plants and the
205 variable abre.gene were compared within the first 24 hours (Fig. 2).



206

207 **Figure 2. Dynamic model agrees with ABA-induced gene expression in real plants after optimization.** (a)
208 Kinetics of luciferase activity in the *RD29A::LUC* plant after exposing to 200 μM ABA (+ABA) or DMSO for control

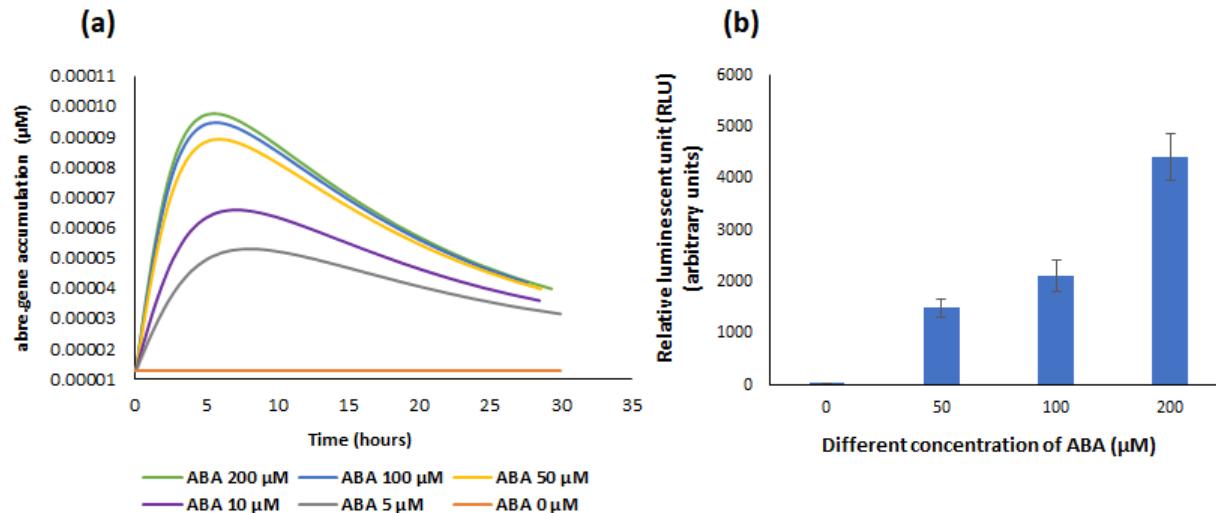
209 (-ABA). The graph shows a mean of three independent experiments. Error bars represent standard error from the
210 mean. (b) Kinetics of *RD29A* gene accumulation in the previously published data with 50 μ M ABA in rice (Singh *et*
211 *al.*, 2015). (c) Kinetics of *RD29A* gene accumulation in the previously published data with 100 μ M ABA in
212 Arabidopsis (Lee *et al.*, 2016). (d) Kinetics of *RD29A* gene accumulation in the previously published data with 10 μ M
213 ABA in Arabidopsis (Song *et al.*, 2016). (e) Model output without feedback regulation ($k_{f17} = 1 \text{ hr}^{-1}$). (f) Model
214 output with feedback regulation (adding reactions $k_{f18} = 4.5 \text{ hr}^{-1}$ and $k_{f19} = 4.5 \text{ hr}^{-1}$). (g) Model output with feedback
215 regulation and optimized parameters ($k_{f17}=10 \text{ hr}^{-1}$, $k_{f18} = 200 \text{ hr}^{-1}$, $k_{f19} = 200 \text{ hr}^{-1}$).

216 Experimental data from the transgenic *RD29A::LUC* plants showed transient activation of
217 the ABRE promoter with an initial increase and then a decrease after 5 hours (Fig. 2a). Similar
218 transient expression of the *RD29A* gene were observed in non-transgenic plants, Arabidopsis and
219 rice (Fig. 2b, c, d) (Singh *et al.*, 2015; Lee *et al.*, 2016; Song *et al.*, 2016). When we simulated
220 kinetics of the variable abre.gene in the model without the feedback regulation on ABF, PP2C,
221 and PP2CA (parameters k_{f18} and k_{f19}), the kinetics were logarithmic upon adding ABA (Fig.
222 2e). Addition of the feedback regulation had minor impact on the kinetics (Fig. 2f). We then
223 optimized the parameters so that kinetics of the gene expression in the model qualitatively agree
224 with that in actual plants (Fig. 2g). We namely altered the three parameters, the transcription rate
225 constant of the ABRE promoter (parameter k_{f17}) and the translation rate constants of ABF and
226 PP2Cs (parameter k_{f18} and k_{f19} , respectively) (Fig. 1 & Table 1). These three parameters had not
227 been determined previously, and studies in other eukaryotic cells indicate wide ranges of
228 reasonable values (Table 1). Hence, we selected the values within the ranges that made the kinetics
229 of the variable abre.gene best fit to the actual plant data. The values $k_{f17}=10 \text{ hr}^{-1}$, $k_{f18}=200 \text{ hr}^{-1}$,
230 and $k_{f19}=200 \text{ hr}^{-1}$ fitted the kinetic curve with the actual plant reasonably (Fig. 2a, g).

231 **Approximation of the model was validated by determining model responses to different doses**
232 **of ABA or a set of gene null-mutations**

233 To validate the model, we first compared the ABA-dose-dependent response in actual
234 plants to the dynamics of the variable abre.gene (Fig. 3). In the model, changes of the variable
235 abre.gene increased in an ABA-dose dependent manner in the range from 0 to 200 μ M (Fig. 3a).
236 With the *RD29A::LUC* transgenic plants, changes of luminescence increased in an ABA-dose
237 dependent manner in the range from 0 to 200 μ M (Fig. 3b). This suggested that the model is
238 approximated to actual plants with respect to ABA sensitivity although the response in the model
239 seems to have narrower sensitivity against the ABA concentration (i.e., from 0 to 50 μ M)

240 compared to that in the actual plants (i.e., from 0 to 200 μ M) (Fig. 3b) (Gampala *et al.*, 2001; Lee
241 *et al.*, 2016).



242 **Figure 3. ABRE-promoter activity increases with a function of ABA concentration in the model as it is observed
243 in actual plants. (a)** Model output of the variable abre.gene with different values of the variable ABA. **(b)** Relative
244 luminescence unit in 25-day-old *RD29A::LUC* plants was determined at 5 hours after spraying different concentrations
245 of ABA. The bars represent the mean relative luminescence of three replicates with error bars representing standard
246 error from the mean (15 seedlings).

247 We also validated changes of the variable abre.gene in gene-knockout simulations.
248 Namely, we simulated expression of a gene from the ABRE promoter in gene null-mutations of
249 *pyr*, *pp2c*, *snrk2*, and *abf*, which were previously studied (Fujita *et al.*, 2009; Rubio *et al.*, 2009;
250 Nishimura *et al.*, 2010; Yoshida *et al.*, 2015). We simulated knockout mutations by setting the
251 translation rate constant (kf2) to zero for the variable PYR, PP2C, SnRK2, and ABF. In addition,
252 we also set the translation rates of the feedback regulations kf18 and kf19 to zero for ABF and
253 PP2Cs, respectively. The mimicked null-mutant in *pyr*, *snrk2*, and *abf*, all showed reduced levels
254 of the variable abre.gene while the mimicked null-mutant in *pp2c* showed elevated levels (Table
255 2).

256 **Table 2. Mutant simulations show similar output to actual mutated plants with respect to the ABRE promoter
257 activity.** Mutant simulations were made on the model with the variable ABA set at 100 μ M. Highest concentration of
258 the variable abre.gene at each of the simulation was recorded. Relative expression of the *RD29A* gene in actual plants
259 was curated from previously published literatures.

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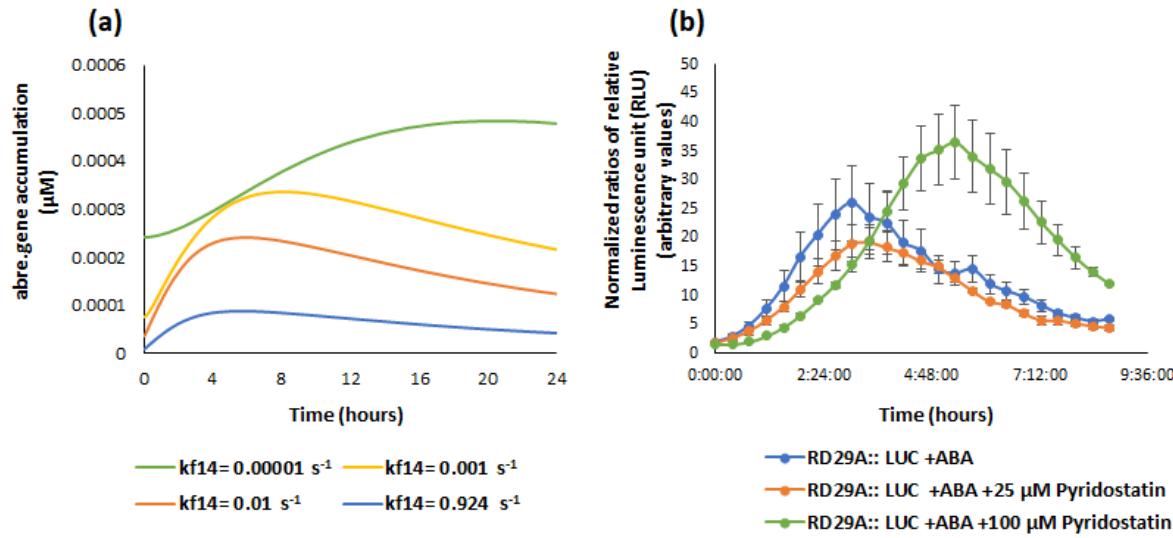
Variable set to 0 in the model	Highest abre.gene concentration in the model (μM)	Knockout genes in actual plants	RD29A gene expression in the knockout plants exposed to ABA	Reference
None	0.000089	None (wild type)	transient	(Song <i>et al.</i> , 2016)
PPC2	0.011166	<i>pp2ca/hai1</i>	constitutive and high	(Antoni <i>et al.</i> , 2012)
PYR	0.000008	<i>pyr1/pyl1/pyl2/pyl4</i>	impaired	(Park <i>et al.</i> , 2009)
SnRK2	0.000000	<i>snrk2.2/ snrk2.3 snrk2.6</i>	impaired	(Thalmann <i>et al.</i> , 2016)
ABF	0.000000	<i>areb1/areb2/abf3</i>	impaired	(Thalmann <i>et al.</i> , 2016)

262

263 Experimental data in actual plants shows that *pyr* null-mutants are impaired in ABA-
264 induced gene expression (Park *et al.*, 2009; Nishimura *et al.*, 2010; Gonzalez-Guzman *et al.*, 2012).
265 Similarly, experimental data on *snrk2.2/ snrk2.3/ snrk2.6* triple knockout mutants showed that the
266 expression of ABA-induced genes was impaired (Fujii & Zhu, 2009; Fujita *et al.*, 2009; Thalmann
267 *et al.*, 2016). Triple *areb/abf* mutants were found to have reduced ABA-induced gene expression
268 (Yoshida *et al.*, 2015; Thalmann *et al.*, 2016). On the other hand, null-mutants of *pp2cs* in actual
269 plants show a higher and constitutive ABA response (Rubio *et al.*, 2009; Antoni *et al.*, 2012).
270 Based on the two validations described above, we concluded that the model constructed, and
271 parameters implemented in the model are approximated to actual plants.

272 **Model simulation and actual plants agree with respect to the activity of ABRE promoter in
273 a condition where PP2C phosphatase activity is inhibited**

274 With the validated model, we examined a relationship between the phosphatase activity of
275 PP2C and the activity of the ABRE promoter, which was not examined before. First, we simulated
276 expression kinetics of the ABA induced gene in which the phosphatase activity of PP2C was
277 decreased. Namely, we decreased the catalytic rate constant of PP2C (kf14). We changed the value
278 from the original 0.924 s^{-1} (Xie *et al.*, 2012) to 10^{-5} s^{-1} , progressively, and tracked changes of the
279 variable abre.gene for the first 24 hours after changing the variable ABA from 0 to 100 μM (Fig.
280 **4a**).



281 kf14: catalytic rate constant of PP2C.

282 **Figure 4. Model simulation and actual plants agree with respect to the activity of ABRE promoter in a condition**
 283 **where PP2C phosphatase activity is inhibited.** (a) Model simulation for changes in the variable *abre.gene*. The
 284 parameter in catalytic rate constant of PP2C (kf14) is progressively reduced from 0.924 s^{-1} to 10^{-5} s^{-1} . Notice the levels
 285 of the variable *abre.gene* increased as the parameter value was reduced. At the same time, the time when the variable
 286 *abre.gene* reached the maximum, was delayed. (b) Changes of luminescence in the *RD29A::LUC* transgenic plants.
 287 The plants were exposed to pyridostatin, an inhibitor of PP2C phosphatase. The *RD29A::LUC* plants were treated
 288 with 100 μM ABA, 100 μM ABA + 25 μM pyridostatin, or 100 μM ABA + 100 μM pyridostatin. Luminescence
 289 values were normalized against control (DMSO + 25 μM or 100 μM pyridostatin). Data shown is means of three
 290 independent replicates with error bars derived from standard error from the mean. Notice the levels of normalized
 291 luminescence intensity was increased and the peak time point was delayed on addition of 100 μM pyridostatin.

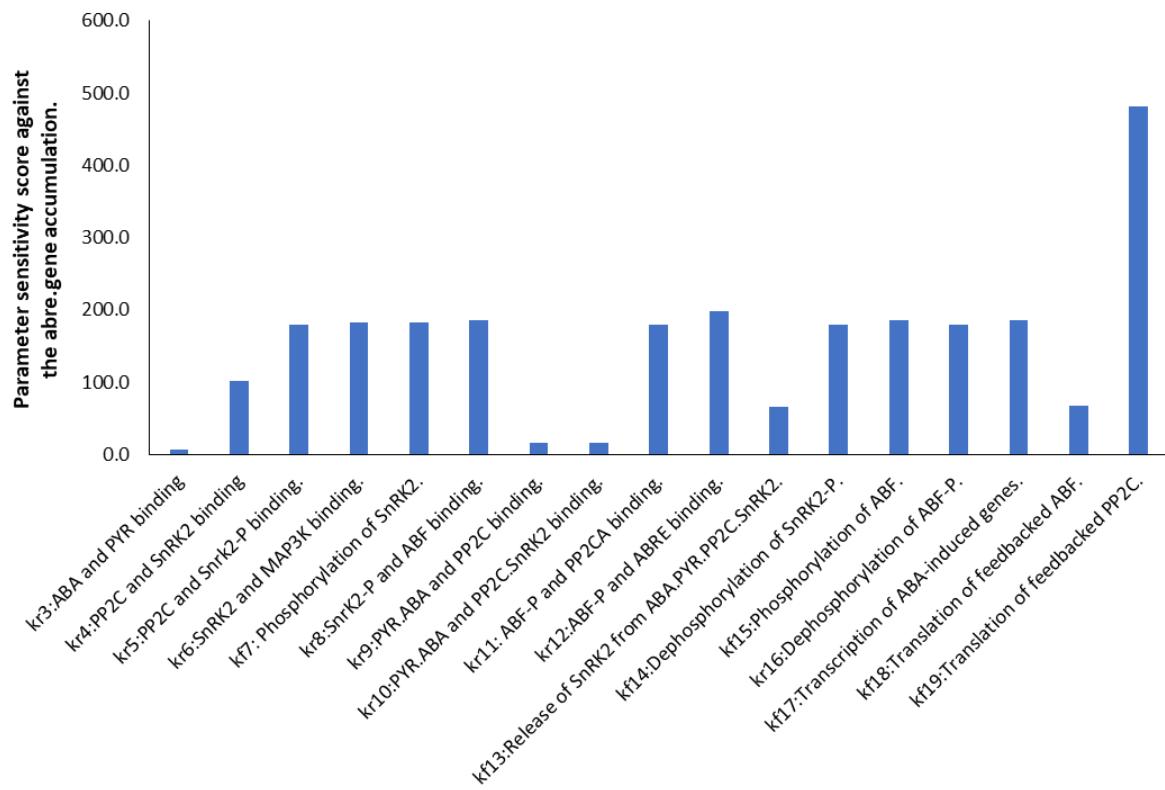
292 On reduction of catalytic rate constant, the variable *abre.gene* increases, and the peak time
 293 point is delayed (Fig. 4a). Based on the prediction, we hypothesized that inhibition of the PP2C
 294 phosphatase activity would increase expression of the ABA induced gene and delay its peak time.
 295 To examine the hypothesis, we conducted an experiment with the *RD29A::LUC* transgenic plants
 296 and pyridostatin hydrochloride, a recently identified chemical inhibitor that is specific for the
 297 PP2C phosphatase activity against SnRK2 (Janicki *et al.*, 2020). On addition of 100 μM but not
 298 25 μM pyridostatin hydrochloride, an increase in luminescence as well as a delay of the peak
 299 time was observed, indicating inhibitor-concentration dependent changes (Fig. 4b). We also
 300 examined the *CAMV35S::LUC* transgenic plants in which a constitutive promoter from a
 301 Cauliflower Mosaic Virus drives the expression luciferase (Rosin *et al.*, 2008). We observed no
 302 significant difference between the plants, in which pyridostatin hydrochloride was added or not

303 added, in peak time and luminescence (Fig. S1). This confirmed that the change in luminescence
304 kinetics was not due to the alteration of luciferase enzymatic activity, but due to the differential
305 activity of the ABRE promoter. Based on these model predictions and biological experiments, we
306 concluded that inhibition of the PP2C phosphatase activity would increase the ABRE promoter
307 activity and delay its peak time.

308

309 **A new hypothesis: ABA downregulates a translation rate of PP2C to increase the ABRE
310 promoter activity**

311 To understand important parameters in the ABA signaling pathway with respect to the
312 ABRE promoter activity, we conducted a sensitive analysis of key parameters against the
313 variable abre.gene in the model.



314

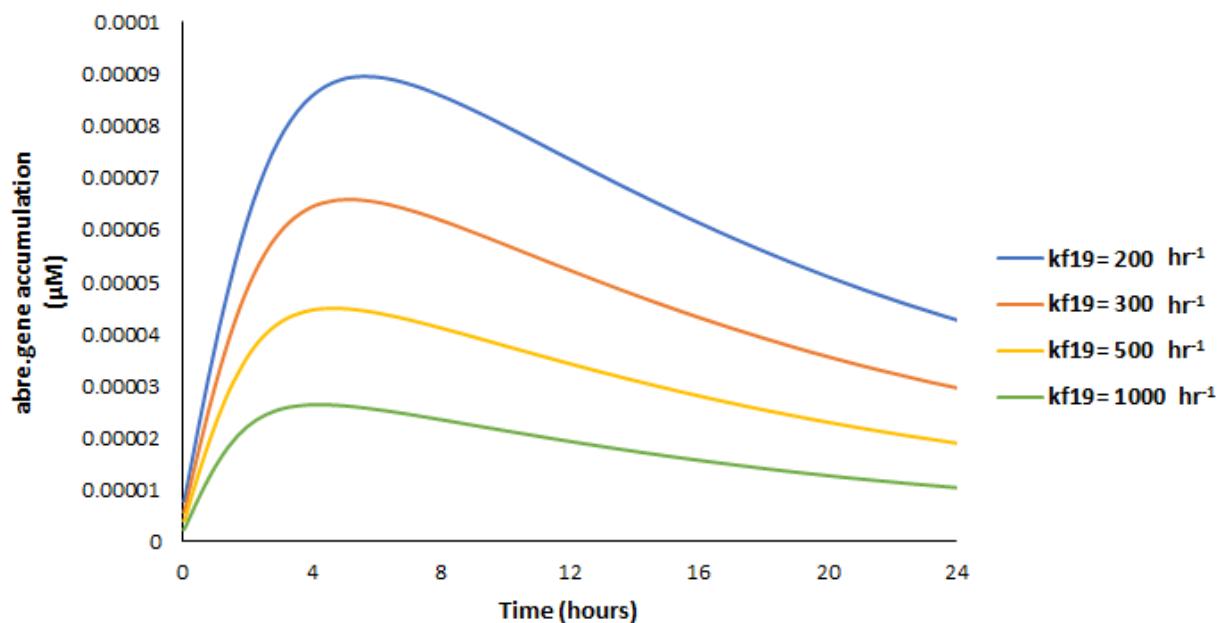
315 **Figure 5. Sensitivity analysis identified the parameter of translation rate constant in feed backed PP2Cs is the
316 most sensitive to the kinetics of the variable abre.gene.** A sensitivity analysis was conducted against the variable
317 abre.gene using the calculate sensitivity function in the model analyzer in SimBiology.

318 The analysis found that while most of the selected parameters are equally sensitive,
319 parameters related to ABA and PYR binding were least sensitive. The parameter related to

320 translation of feedbacked PP2Cs, which was optimized in this study to curve-fit the kinetics of the
321 variable abre.gene, had the highest sensitivity (Fig. 5).

322 To determine how the translation rate constant of PP2Cs affects the ABRE promoter
323 activity, we changed the PP2C translation rate (kf19) and tracked the resulting kinetics of the
324 variable abre.gene. We found that the PP2C translation rate (kf19) affects not only the maximum
325 of variable abre.gene but also the peak time when the highest value of the variable abre.gene is
326 achieved (Fig. 6). These dynamics are similar to the changes of the parameter in the PP2C
327 enzymatic activity (kf14; Fig. 4a).

328



kf19= translation rate constant of feed backed PP2C.

329

330 **Figure 6. Increase of the translation rate constant of PP2C reduces the variable abre.gene but expedites the**
331 **peak time.** The parameter kf19 (translation rate constant of feed backed PP2C) was changed from the original 200 hr⁻¹
332 to 300, 500, and 1000 hr⁻¹. Notice the level and the peak time point of the variable abre.genes changed with a function
333 of translation rate constant.

334 Learning that the kinetics of the variable abre.gene is largely affected by the translation
335 rate of the feedbacked PP2Cs in the model, we wondered whether the translation rate is affected
336 by ABA in actual plants. To this end, we searched literature that studied changes of the translation
337 rate. We found that while direct measurement of the translation rate in eukaryotic cells has been

338 conducted only in yeast and animal cells (Schwanhäusser *et al.*, 2011; Weinberg *et al.*, 2016),
339 indirect measurement has been conducted in plants as well (Fujita *et al.*, 2019).

340 In the indirect measurement, using ribosomal profiling, a ratio of ribosome-protected
341 mRNA fragments over total mRNA extracted from cells are measured at a given time point. In
342 theory, a higher ratio of ribosome-protected mRNA over total mRNA indicates higher translation
343 rate at a given time point. We found in a previously conducted study with a DNA microarray that
344 translation rates in all PP2Cs involved in the ABA signaling pathway (namely ABI1, ABI2, HAB1,
345 PP2CA) are downregulated due to dehydration (Table 3) (Kawaguchi *et al.*, 2004). This suggests
346 that the translation rate in PP2Cs may indeed be downregulated by ABA. Because a microarray
347 used in the study does not contain a completed set of gene probes, change in translation rate of
348 ABFs involved in the ABA signaling pathway (namely ABF2, ABF3, and ABF4) is not conclusive.
349 On the other hand, a study with a deep RNA-sequencing technology, in which all extracted
350 mRNAs are measured by sequenced frequency, showed that the translation rates of ABFs involved
351 in the ABA signaling pathway (ABF2, ABF3, and ABF4) are all up-regulated while that of the
352 PP2Cs (data for ABI2 is not available) are little changed upon exposure of exogenously added
353 TOR inhibitor (Scarpin *et al.*, 2020) (Table 3). The study concluded that the plant TOR specifically
354 controls the translation of a set of mRNAs that possesses 5' oligopyrimidine tract motifs (5'TOPs),
355 which results in alteration of translation in other genes as well.

356 **Table 3. Changes of translation rate in PP2Cs and ABFs identified in the previously published data.**

mRNA species.	Relative changes in relative translation rate with dehydration, compared to a control condition (Kawaguchi <i>et al.</i>, 2004).	Relative changes in relative translation rate with TOR inhibition, compared to a control condition (Scarpin <i>et al.</i>, 2020).
ABI1	0.92	1
ABI2	0.95	Data not available
HAB1	0.80	0.92
PP2CA	0.98	1.13
ABF2	Data not available	1.39
ABF3	0.97	1.32

ABF4	Data not available	1.15
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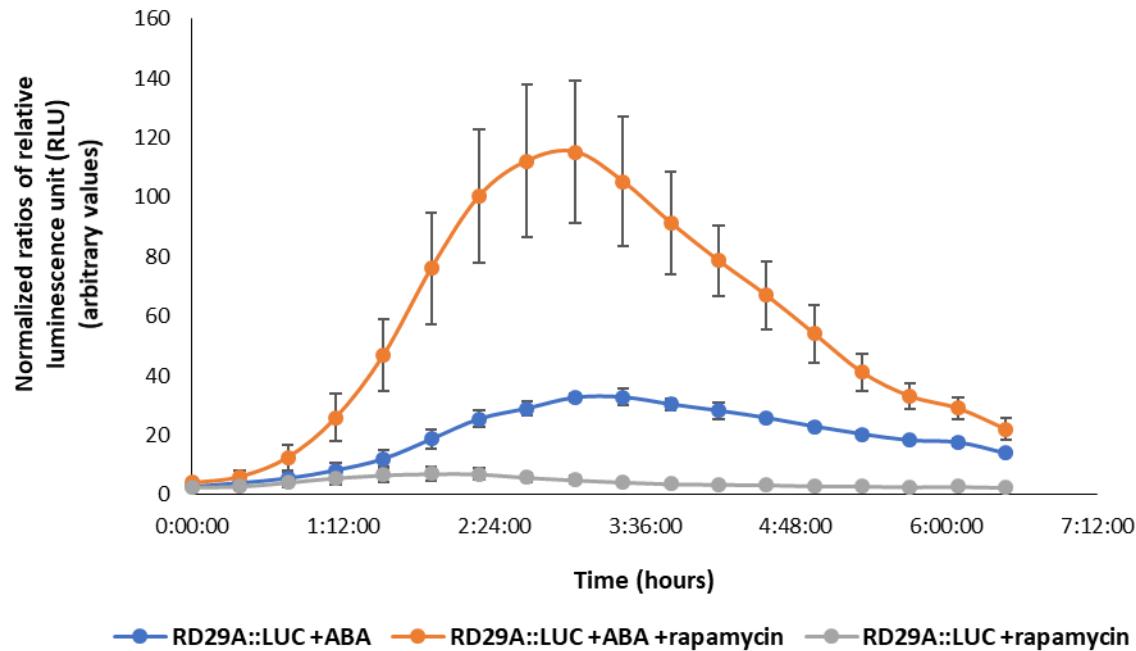
357

358 Based on the sensitive analysis on our model and the two previous studies described above,
359 we hypothesized that ABA downregulates a translation rate of PP2C to increase the ABRE
360 promoter activity.

361 **Combinational exposure of ABA and TOR inhibitor upregulates activity of the ABRE
362 promoter**

363 We further hypothesized that the combinational exposure of ABA and TOR inhibitor up-
364 regulates activity of the ABRE promoter. The rationale is as follows. First, upon ABA exposure,
365 transcription of PP2Cs and ABFs are both upregulated due to the feedback regulation (Wang *et*
366 *al.*, 2019). Secondly, the translation rate of PP2Cs is down regulated by a yet unknown mechanism
367 (Kawaguchi *et al.*, 2004), resulting in diminishing the effect of up-regulation of the transcription
368 of PP2Cs. Thirdly, by exposing a TOR inhibitor, translation rate of ABFs is increased while that
369 of PP2Cs is not changed (Scarpin *et al.*, 2020). We assumed the increase of the ABF translation
370 occurs independent from the role of TOR in suppression of PYR-ABA binding activity (Wang *et*
371 *al.*, 2018). As a result, by exposing ABA and a TOR inhibitor, the activity of the ABRE promoter
372 increases, compared to when only ABA is exposed to plants.

373 To examine the hypothesis, we analyzed the ABRE promoter activity in the *RD29A::LUC*
374 transgenic plants. As a control, we analyzed the *CAMV35S::LUC* transgenic plants. We exposed
375 the plants to ABA only and ABA and rapamycin, the TOR inhibitor (Xiong & Sheen, 2012). When
376 the plants were exposed to ABA alone, luciferase intensity was increased as expected (Fig. 7).



377

378 **Figure 7. Combinational exposure of ABA and rapamycin increases the ABRE promoter activity.** Normalized
379 luminescence in the *RD29A::LUC* transgenic plants are shown. The plants were exposed to 200 μ M ABA alone or
380 200 μ M ABA + 10 μ M rapamycin or 10 μ M rapamycin only. Luminescence values were normalized against control
381 (DMSO only). Data shown is means of three independent replicates with error bars derived from standard error from
382 the mean.

383 When the plants were exposed to both rapamycin and ABA, the luciferase intensity was
384 about 4-fold higher than that when plants were exposed to ABA alone at the maximum. When the
385 *RD29A::LUC* transgenic plants were exposed to rapamycin alone, luciferase activity was little
386 altered (Fig. 7). When the *CAMV35S::LUC* transgenic plants were examined with the identical
387 conditions, no significant difference was observed among the different exposures (Fig. S2). This
388 result supported our hypothesis that combinational exposure of TOR inhibitor and ABA up-
389 regulates activity of the ABRE promoter.

390

391 Discussion

392 Here we presented a model of the ABA signaling pathway describing the activation of
393 ABF and resulting activation of the ABRE promoter (Fig. 1). The model was built with fixed
394 parameter values of protein-protein interactions and enzymatic kinetics that were obtained by *in*

395 *vitro* experiments from the literature. The model suggests that the feedback regulation of PP2C
396 and ABF allows the transient upregulation of the ABRE promoter. Without the feedback, the
397 model predicts that ABRE expression activity would be logarithmic and not show the transient
398 increase (Fig. 2e). Based on the model prediction, we hypothesized that inhibition of the PP2C
399 phosphatase activity on SnRK2 would increase expression of the ABA induced gene and delay its
400 peak time. The hypothesis was supported by biological experimentation using transgenic
401 Arabidopsis plants (Fig. 4b). The model also predicted that the translation rate for PP2C in the
402 feedback regulation is the most sensitive parameter for activation of the ABRE promoter while
403 parameters related to ABA and PYR binding were least sensitive (Fig. 5). The reason parameters
404 related to ABA and PYR binding were least sensitive is evident because we assume extremely
405 high concentration of ABA (100 μ M) is exposed to plants, while a production of endogenous ABA
406 during abiotic stress would be in a nM range (Dubas *et al.*, 2013). We found out that a high value
407 of the translation rate not only reduces the ABRE promoter activity but also expedites the time
408 point when the promoter activity reaches the maximum (Fig. 6). This suggested that the translation
409 rate of PP2C would be one of the most important factors that determine the kinetics of the ABRE
410 promoter activity. In the past, accumulation of mRNA and post-translational modification of
411 proteins are thought to define activity of the ABRE promoter (Nordin *et al.*, 1993; Joo *et al.*, 2021).
412 However, our model and biological experimental data suggest that changes in translation rates
413 would also largely determine the activity of the ABRE promoter (Fig. 7). Our literature search
414 found out that the translation rate of PP2Cs is downregulated during dehydration (Table 3). This
415 suggests that activity of the ABRE promoter would be regulated by not only upregulation of the
416 gene expression but also downregulation of the protein translation on PP2Cs.

417 We are aware that not only translation rate but also degradation rate of proteins, which are
418 not investigated in this study, are important in the ABA signaling pathway (Wu *et al.*, 2016; Ali *et*
419 *al.*, 2019). Hence, changes of protein degradation rate by ABA must be quantitatively analyzed to
420 conclude the role of translation rate in the ABA signaling pathway. We are also aware that ABFs
421 are not the only transcription factors that bind to the ABRE promoter (Song *et al.*, 2016). Hence,
422 the activity of the ABRE promoter does not depend only on ABF activation in actual plants,
423 whereas in the model we consider the activity of ABF only. To fully understand kinetics of the
424 ABRE promoter activity in actual plants, further expansion of the model to include other
425 transcription factors is required. Furthermore, quantitative predictions in the current model

426 somewhat disagrees with real plant data. For instance, when an ABA-concentration dependent
427 response of the ABRE promoter was determined, the response range was narrower in the model
428 than in actual plants (Fig. 3). Optimization of parameter values fixed in this study or the expansion
429 to include other factors driving the ABRE promoter may be required to improve model
430 performance.

431 Nevertheless, our model successfully builds off existing work to represent the relationship
432 between the ABA signaling pathway and ABRE gene expression. As demonstrated here, the
433 model is useful to generate novel hypotheses. The model suggests new avenues of experimental
434 inquiry. In particular, our analysis proposes that investigating alteration of translation rates in
435 proteins, such as PP2Cs, is the next frontier in the research field of ABA signaling pathway and
436 downstream promoter activity.

437

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440 **Author Contribution**

441 Conceptualization and methodology, N.K. Validation, R.N. and R.D. Experiments, R.N. Formal
442 analysis, R.N. and N.K. Writing—original draft preparation, R.N. and N.K. Writing—review and
443 editing, R.N. R.D. and N.K. Funding acquisition, N.K. All authors have read and agreed to the
444 published version of the manuscript.

445 **Data Availability**

446 .sbproj file (MATLAB SimBiology Project File) that includes a model diagram, ODE equations,
447 initial values, parameters, simulations for Figures 2, 3, 4, 5, 6, and Table 2 are available as
448 supplement files.

449

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