

## Problem: Network properties underlying seed germination control

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### SUMMARY

We sought to gain a mechanistic understanding of the control of seed dormancy and germination by hormone balance. The field has matured to a stage where most of the key genes are known, and competing hypotheses have been proposed to explain how hormone balance works in seeds. During the meeting we simplified a more complex model of seed germination (Figure 1), reducing it to a tractable network. We then showed that if considered as a set of competing protein complexes the network took on the properties of a switch. Results from two models of the reduced network, which incorporated the biological switching phenomena, were found to be in good agreement with both wild and mutant phenotypic data. Our models made the novel prediction that one complex in particular was key to promoting germination, and this prediction can now be tested in the laboratory.

### INTRODUCTION

Seed germination control requires the assimilation of signalling from multiple input pathways. Environmental signals such as light, control the level of the hormones gibberellin (GA) and abscisic acid (ABA) in seeds. For a long time it has been hypothesised that the ‘balance’ of the germination-promoting hormone GA and the germination inhibiting hormone ABA controls the decision to germinate. Importantly, recent work shows that the pathways regulating germination are also important for plant growth control: therefore the function of the germination- controlling network has wide-ranging implications for plant development.

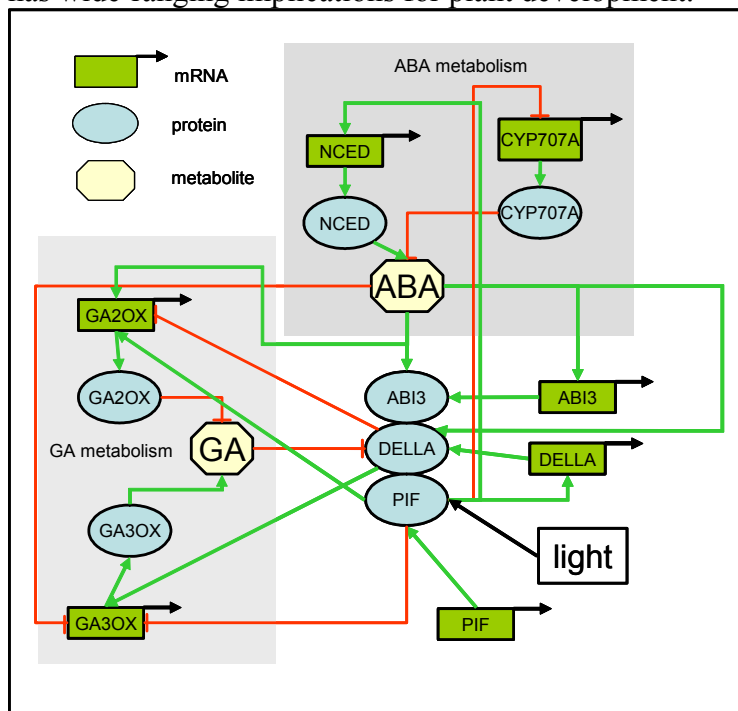


Figure 1. The germination-controlling network proposed by Penfield. A putative nuclear complex of transcriptional regulators controls GA and ABA metabolism in seeds

At the centre of the germination control process is a nuclear complex that acts largely by chromatin remodelling and subsequent transcriptional regulation. In *Arabidopsis* germination is dependent on light. Light causes shifts in GA and ABA levels that lead to the degradation of the DELLA and ABSCISIC ACID INSENSITIVE 3 (ABI3) transcription factors, both of which are central repressors of germination with mutants displaying identical phenotypes (Penfield et

al., 2006). However, recent work shows that the PHYTOCHROME INTERACTING FACTOR (PIF), DELLA and ABI3 proteins may all exist in a single nuclear complex. So we have a situation where a complex regulates plant hormone levels that in turn regulate the stability and composition of the same complex (see Figure 1). This may be a mechanism to ensure coordinated action at the tissue level, or it might be crucial to generating the polarised binary germinating/non-germinating output states of the network. We have previously hypothesised that the balance of GA and ABA is regulated through opposite effects on DELLA protein stability (Penfield et al., 2006). Other groups have shown that ABA represses GA biosynthesis by transcriptional repression of GA-synthesising enzymes, and suggested that this is how hormone balance works (Seo et al., 2006).

In addition, these proteins are linked by the ability of PIF and ABI3 to bind TIMING OF CAB EXPRESSION 1 (TOC), a component of the plant circadian clock (Millar et al., 1995; Kurup et al., 2000; Yamashino et al., 2003). Recent unpublished work (from the Penfield lab) suggests that the circadian clock is of central importance in germination control, and that the regulation of GA synthesis is rhythmic in seeds, peaking at dawn (Figure 2).

Key to the success of any model is that it should reproduce the following characteristics:

1. Loss of DELLA or ABI3 should always lead to germination, regardless of GA or ABA levels.
2. Increasing ABA should increase DELLA protein levels independently of the regulation of GA biosynthesis, as in Penfield et al. (2006).
3. Eliminating ABA should increase GA-levels (Seo et al., 2006).
4. The model should be able to produce rhythmic increases in GA levels. These should be sensitive to ABA levels and to PIF concentration.

## RESULTS

During the course of the study group we made good progress on the problem. Advancement was step-wise and the approach to answering the questions posed became divided into two principle approaches. The path we followed is best explained by outlining a series of key advances as described below.

**Key Advance 1: reduction of the network to its core components.** After an initial discussion we considered that the central proteins of the signalling network, ABI3, PIF, DELLA and TOC1, could form heterodimers as predicted by previous work. These heterodimers would essentially compete with other for free proteins for their production (Figure 3). It also became increasingly clear through discussions that the network should be simplified so that all synthesis and degradation of GA and ABA could be ignored, at least to begin with. Therefore we reduced the regulation of GA and ABA to single variables, which input into the system by modifying the abundance of free DELLA and free ABI3 proteins respectively as shown in Figure 3. Transcription and translation were ignored, introducing single terms for the synthesis and degradation of each protein.

**Key advance 2: the proposition that the PIF-TOC complex was key to germination control and GA regulation.** The variables represented by PIF in the scheme are very complex. There are up to 12 PIF-encoding genes in Arabidopsis, and

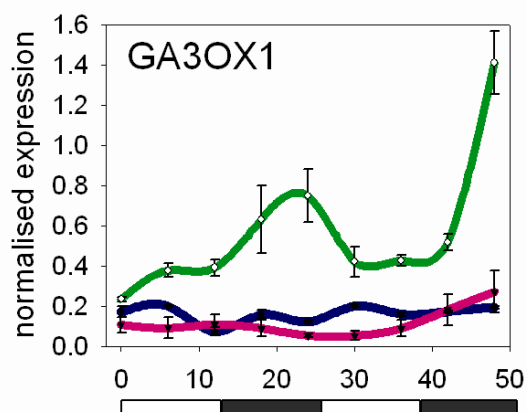


Figure 2. The expression of the GA-biosynthetic gene *GA3OX1* follows a diurnal rhythm in seeds.

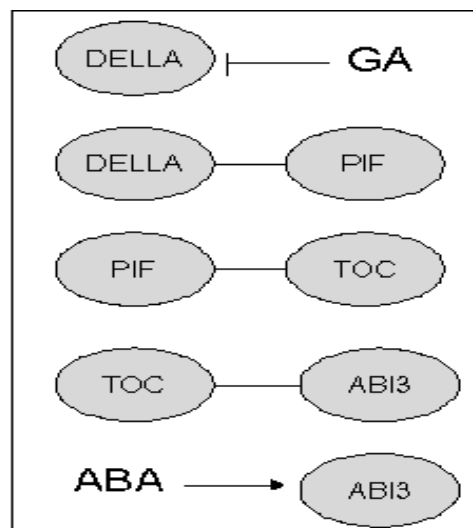


Figure 3. Three possible nuclear heterodimeric complexes were identified. GA and ABA inputs were reduced to the regulation of free DELLA or ABI3.

the resulting PIF proteins do not all share the same properties. Several are expressed in seeds. One unifying concept in all work pertaining to PIF in seeds is that PIF controls GA biosynthesis, either positively or negatively (e.g. Penfield et al., 2005). The one PIF we have shown to be important in germination control is a positive regulator of GA-biosynthesis, and we therefore decided to include this one in the model. The next key advance was proposed by Savage: using a simple logical analysis of the network, it was hypothesised that the PIF-TOC complex was key to promoting GA biosynthesis in seeds (Figure 4). This produced a network that satisfied many criteria for a model of hormone balance. Firstly we assumed (and this assumption is supported by microarray data) that DELLA and ABI3 were first present in excess over PIF and TOC. Loss of either DELLA or ABI3 now leads to a radical increase in the concentration of the PIF-TOC complex [PIFTOC], GA and germination. This satisfies key criterion 1. Adding exogenous ABA reduces [PIFTOC] by the sequestering of TOC by ABI3. This releases free PIF which increases [PIF-DELLA]. The PIF-DELLA complex is assumed to be GA-stable (in contrast to free DELLA which is degraded by GA), so ABA will in effect, stabilise DELLA. This satisfies key criterion 2, because ABA can now affect DELLA stability without directly regulating GA. If we now consider a situation in which ABA is eliminated, this should release TOC from ABI3-TOC. Thus [PIFTOC] increases, with the effect of promoting GA synthesis. Total DELLA then drops rapidly because of increasing GA, and because the increased competition for PIF with TOC releases more free DELLA for destruction by GA. We also assumed that PIFTOC directly or indirectly inhibits ABA levels, as suggested by published and unpublished data (Seo et al., 2006; Penfield unpublished). This has the satisfying effect of amplifying any perturbation of ABA levels (Figure 4). Savage finally showed that the model could be logically reduced to a feedback loop involving only [PIFTOC], GA and ABA.

### Key Advance 3: dynamic simulation of network function

Our group took two complementary approaches to dynamic modelling. Savage advocated the use of difference equations. These seem suited to modelling germination because the network controlling germination always has a predictable set

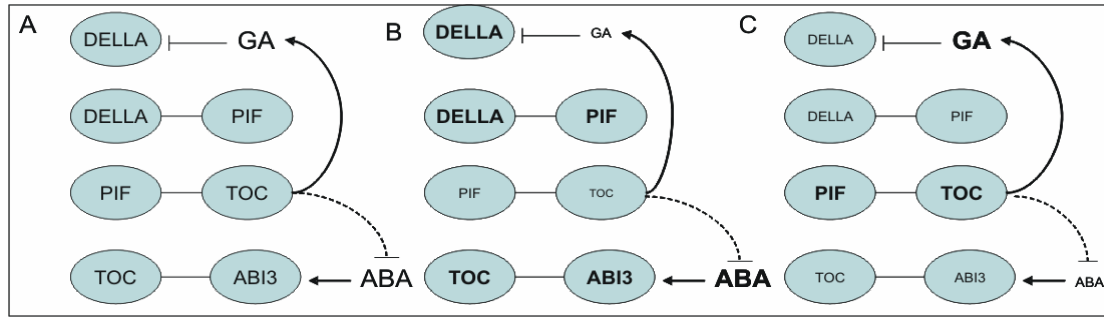


Figure 4. The effect of manipulating GA and ABA on our reduced network mimics the behaviour in seeds. A. Unperturbed network, all components roughly equally abundant. B. ABA added. Components in bold are up-regulated, those in small type are down-regulated. Note that ABA acts to increase DELLA as has been shown experimentally (Penfield et al., 2006). C. GA added. Adding GA depletes DELLA releasing free PIF to make more PIF-TOC complex.

of initial conditions. TOC is a central component of the circadian oscillator and expressed in its rhythmically fashion. This pattern of expression was simulated as a daily pulse of TOC at dusk as described in the literature. PIF is sensitive to complex environmental regulation, so for simplicity we considered a situation where PIF levels increased over-time, to cover all possible levels of PIF activity. These simulations (Figure 5) showed that the system could produce GA rhythmically, as suggested by experiment (Figure 2), dependent on increases in [PIF]. They also predicted a rhythmic decline in [ABA], also dependent on [PIF]. This is not seen experimentally. This suggests that unlike GA, ABA levels are not directly inhibited by PIF-TOC. A hypothesis that is supported by unpublished data from Penfield, which indicates that further clock genes are important in regulating ABA levels.

Further simulations included adding a rhythmic synthesis of ABA in the morning, as suggested by experiment. Observation suggests that rhythmic ABA production should eliminate GA. This could not be simulated by the difference equation model in the time frame available, although this could just be a parameter issue.

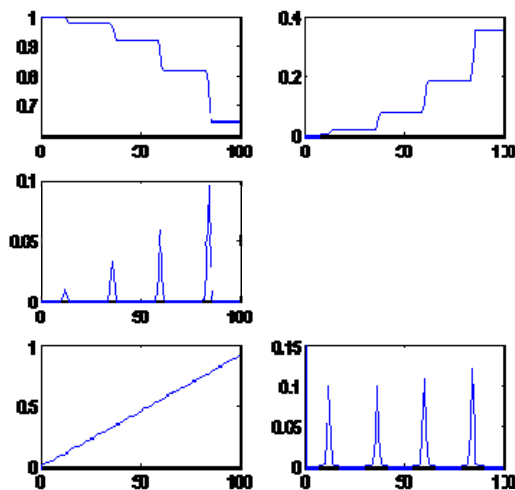


Figure 5. The simulation of network over time (hours) from imbibition of the seed. GA levels increase rhythmically, proportional to [PIF] and [TOC]. ABA levels decline rhythmically.

King and Fozard developed a deterministic ordinary differential equation (ODE) model of the network, as shown in Figure 6. In the long term this approach is attractive because it can easily be integrated with the plant circadian clock models developed by Andrew Millar and David Rand (Locke et al., 2006). The system was simplified through the assumption that the half life of the mRNA is shorter than the timescale of the various protein half-lives. If this assumption holds, the mRNA transcription rate is

$$\begin{aligned}
\dot{P} &= \mu_P - \lambda_P P - \nu_{PT} TP - \nu_{PD} PD + \gamma_{PT}[PT] + \gamma_{PD}[PD] \\
\dot{A} &= \frac{\mu_A \Phi_A(t)}{1 + k_A [PT]} - \lambda_A A \\
\dot{G} &= \frac{\mu_G ([PT] + \theta D)}{1 + k_G A} - \lambda_G G \\
\dot{D} &= \frac{\mu_D}{1 + k_D G} - \lambda_D D - \nu_{PD} PD + \gamma_{PD}[PD] \\
\dot{a} &= \mu_a A - \lambda_a a - \nu_{Ta} Ta + \gamma_{Ta}[Ta] \\
\dot{T} &= \mu_T \Phi_T(t) - \lambda_T T - \nu_{PT} TP - \nu_{Ta} Ta + \gamma_{Ta}[Ta] + \gamma_{PT}[PT] \\
[\dot{Ta}] &= \nu_{Ta} Ta - \gamma_{Ta}[Ta] \\
[\dot{PD}] &= \nu_{PD} PD - \gamma_{PD}[PD] \\
[\dot{PT}] &= \nu_{PT} PT - \gamma_{PT}[PT]
\end{aligned}$$

Figure 6. Differential equations describing our network behaviour. Variables are:  $P$  (PIF),  $A$  (ABA),  $G$  (GA),  $D$  (DELLA),  $a$  (ABI3),  $T$  (TOC),  $[Ta]$  TOC-ABI3 complex,  $[PD]$  PIF-DELLA complex,  $[PT]$  PIF-TOC complex.

DELLA is inhibited by GA, and so occurs at a rate  $\mu_D/(1+k_D G)$ ; the production of GA requires either the PIF-TOC complex of DELLA, and is inhibited by ABA, so proceeds at a rate  $\mu_G([PT]+\theta D)/(1+k_G A)$ . The protein TOC is associated with the circadian clock of the seed, here treated as an external system with synthesis of TOC at a rate  $\mu_T \Phi_T(t)$ , where  $(\Phi_T(t))$  is a function of time alone. The synthesis of ABA is thought to be possibly coupled to the clock, but is inhibited by the PIF-TOC complex, and so occurs at a rate  $\mu_A \Phi_A(t)/(1+k_A [PT])$ . The TOC production function is taken to be given by:

$$\Phi_T(t) = K_T (e^{-(t-36)^2} + e^{-(t-60)^2} + e^{-(t-84)^2} + \dots),$$

Corresponding to an initial pulse centred on  $t=36$ , repeating every 24 hours thereafter. The ABA production function is either taken to be constant ( $\Phi_A(t)=1$ ) or

$$\Phi_A(t) = K_A (e^{-(t-24)^2} + e^{-(t-36)^2} + e^{-(t-96)^2} + \dots), \quad (1)$$

$P$	PIF	0.2
$A$	ABI	5.0
$G$	GA	0.2 (5.0)
$D$	DELLA	5.0
$a$	ABI3	5.0
$T$	TOC	0.1
$[Ta]$	TOC-ABI3	0.1
$[PD]$	PIF-DELLA	0.1
$[PT]$	PIF-TOC	0.1

Table 1. The initial conditions. Table shows initial values (3<sup>rd</sup> column) for each variable specified in columns 1 and 2.

approximately equal to the degradation rate; the behaviour of the system can be described in terms of protein levels, with transcription/translation dynamics being accounted for by the expressions used for synthesis rates. The hormones GA and ABA are not produced directly from translation of mRNA, but through the action of enzymes. By assuming that the half life of the enzymes are so short they may be ignored, the dynamics can be approximated through the synthesis (and degradation) rates for the levels of GA and ABA. The synthesis of PIF is modelled as occurring at a constant rate  $\mu_P$ ; the synthesis of

Which is similar to  $\Phi_T(t)$  but with the first pulse at  $t=24$ . Complex assembly rates are given by the law of mass action (proportional to the product of the concentrations of the components). Protein degradation and complex dissociation occur at rates proportional to the substrate concentration. The resulting system of equations is shown in Figure 6. The system of equations is dimensional with time being measured in hours. The period of interest is from  $t=0$  to  $t=100$ . The parameter values used are shown in Table one and the initial conditions in Table 2. These were thought to correspond to

Synthesis rate constants	
$\mu_G, \mu_D, \mu_a$	1
$\mu_A$	20 (4, 2)
$\mu_P$	1 (0.1, 0.2)
$\theta$	0.01
Constants in production rates $\Phi_T(t), \Phi_A(t)$	
$K_A, K_T$	1
Degradation rate constants	
$\lambda_P, \lambda_A, \lambda_a, \lambda_G$	0.1
$\lambda_T$	1
Inhibition constants	
$k_D$	1
$k_A$	5
$k_G$	0
Complex assembly rate constants	
$\nu_{[PD]}, \nu_{[PT]}, \nu_{[Ta]}$	1
Complex degradation rate constants	
$\gamma_{[PD]}, \gamma_{[PT]}, \gamma_{[Ta]}$	1

Table 2. The values for the parameters used in the equations in Figure 6.

the state of the seed shortly after imbibition. The initial levels of GA and PIF were thought to be low, while those of ABA, DELLA and ABI3 were thought to be high. Simulations were run using the MATLAB stiff solver ode15.

Initial simulations showed that large rhythmic increases in GA could be produced (Figure 7), as seen in the experimental situation shown in Figure 2. In common with the real-life situation, these increases in GA were out of phase with TOC expression. Rhythmic increases in GA were observed as soon as ABA levels dipped below a critical threshold, also in agreement with observations. Increasing PIF alone was enough to tip the network into a germinating state, as long as there is some free TOC (Figure 7). This agrees with observations that overexpressing

several PIFs in seeds results in loss of dormancy.

Other simulations were less successful. Adding an oscillatory ABA synthesis (as observed) could not absolutely repress the rhythmic increases in GA levels. This is partly because the rhythm of ABA synthesis is out of phase with TOC rhythms (as suggested by experiment). It is clear that these simulations underline the fact that

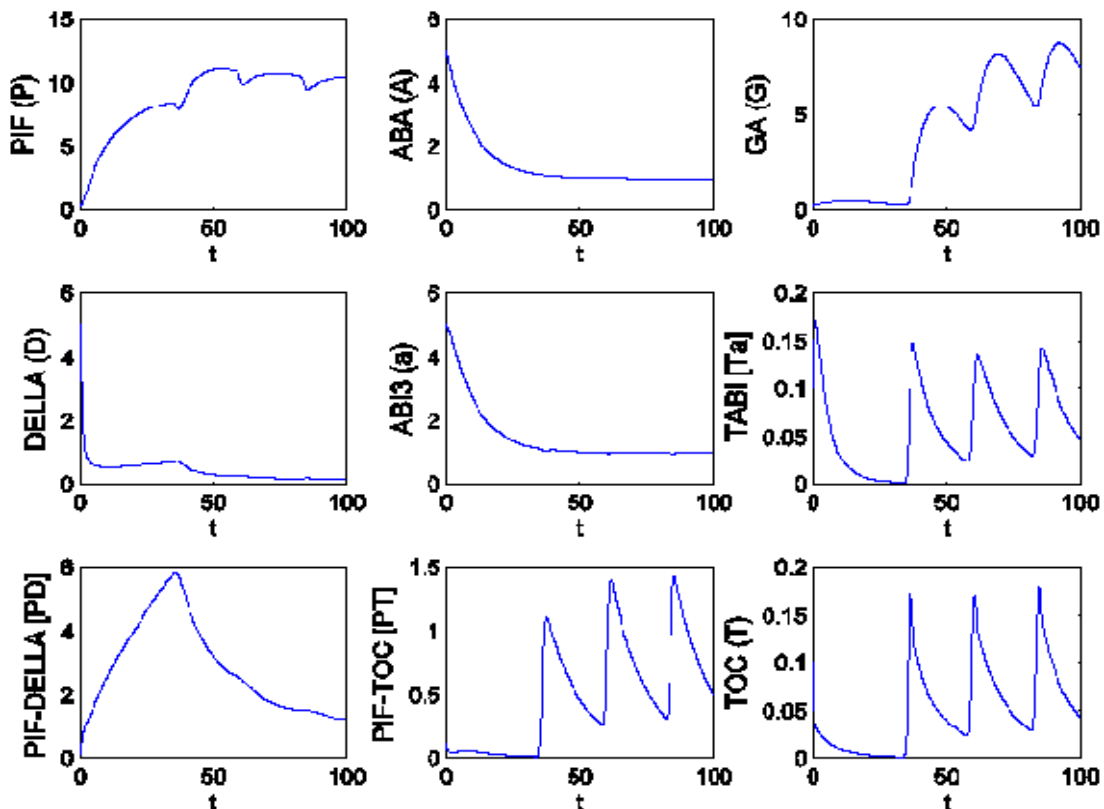


Figure 7. ODE simulation of the germination-controlling network over time (Hours). Rhythmic increased in GA are observed, dependent on the amplitude of TOC and PIF abundance. GA production is delayed until ABA levels have fallen because ABI3 is binding TOC and preventing TOC from binding PIF.

coincidence between ABI3 and TOC expression is crucial to dormancy in our model.

### **Conclusions and further perspectives**

We have proposed a novel scheme for the control of seed dormancy and germination through the regulation of hormone balance. Using two approaches we have shown that the network we propose has many of the desired characteristics that have previously been shown to be salient features of the germination control process. Our novel hypothesis also predicts several features of the behaviour of the network, and importantly predicts the results of several perturbations that have not yet been examined. One of these is that TOC1 overexpression should result in low dormancy, especially if combined with PIF overexpression. A second is that if ABI3 expression is not coincident with TOC1, this should be sufficient to trigger germination. Since the MPSSG meeting the Penfield lab has discovered that, at least in the case of ABI3 transcription, a shift in the peak of ABI3 occurs in germinating seeds compared to dormant seeds. Without the work from the study group, the significance of this result would not be at all obvious. Clearly it has now become important to gather data on GA and ABA levels themselves, and on protein abundance and stability during seed germination. This, along with further model development and experimental verification could easily form the backbone of a competitive grant application to the BBSRC. Because our network also regulates plant growth rate, biomass production and vigour, this work could be of wide general importance in plant biology.

### **Thoughts on further CPIB/Garnet MPSSG meetings**

My horizons have been considerably broadened by the MPSSG experience and I will thoroughly recommend future participation to anyone. The success of future meetings in generating profitable collaborations for CPIB clearly depends on the quality of the problems considered, both in terms of tractability and biological or functional significance. My strong view is that these considerations should be given extra weight by the organising committee over diversity of problem type. Further consideration should also be given to the availability of MATLAB in the discussion rooms.

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