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Dynamics of the Genetic Regulatory Network for *Arabidopsis thaliana* Flower Morphogenesis

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We present a network model and its dynamic analysis for the regulatory relationships among 11 genes that participate in *Arabidopsis thaliana* flower morphogenesis. The topology of the network and the relative strengths of interactions among these genes were based from published genetic and molecular data, mainly relying on mRNA expression patterns under wild type and mutant backgrounds. The network model is made of binary elements and we used a particular dynamic implementation for the network that we call semi-synchronic. Using this method the network reaches six attractors; four of them correspond to observed patterns of gene expression found in the floral organs of *Arabidopsis* (sepals, petals, stamens and carpels) as predicted by the ABC model of flower morphogenesis. The fifth state corresponds to cells that are not competent to flowering, and the sixth attractor predicted by the model is never found in wild-type plants, but it could be induced experimentally. We discuss the biological implications and the potential use of this network modeling approach to integrate functional data of regulatory genes of plant development.

1. Introduction

As experimental data on gene function and regulatory interactions accumulate in biological model systems, the need of formal and modeling paradigms for functional inference and integration of large data sets is becoming imminent. In this paper we put forward a first trial to apply dynamic analyses of a genetic regulatory network model to integrate molecular and genetic data of *Arabidopsis thaliana* genes involved in flowering morphogenesis. In contrast to cascade or hierarchical models of regulatory genes, that are widely used in molecular biology (see for example Kornfeld, 1997), models of genetic regulatory networks consider direct and indirect feedback regulatory relationships (Garzon, 1990). Such feedback regulatory interactions are analogous to those present in metabolic routes, in which the product of a reaction regulates its own synthesis rate. The product of a certain gene might also regulate its own transcription rate directly or indirectly. Feedback loops make genetic regulatory network models dynamic systems, which may have fixed or periodic activity patterns. Moreover, feedback loops, characteristic of genetic regulatory networks, constitute the necessary mechanism to explain multiple equilibria and homeostasis of a given network (Thieffry *et al.*, 1995; Thomas, 1991; Thomas & D'Ari, 1990; Thomas *et al.*, 1995).

Transcriptional and post-transcriptional regulation are central issues for understanding the origin of cellular differentiation. Each cell type can be identified by its molecular profile (i.e. by the pattern of all molecular markers present in the cell), and in theory the cellular identity might be determined by describing all the active genes in the cell (Kauffman, 1969, 1991, 1993). Gene activity, in turn, depends on

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the presence of one or several transcription factors, which are themselves gene products, creating in this way a functional interdependence among a large group of genes. These groups of genes regulating the activity of each other are known as genetic regulatory networks. Formal dynamic analyses of these networks can be useful to explore the possible long-term genetic activation patterns (called attractors) of a given genotype. Such attractors have been identified with the different cell types of an organism (Kauffman, 1993; Zuckerkandl, 1994), thus in order to achieve cellular differentiation it would be sufficient to give a perturbation to the genetic regulatory network to change from one attractor to another. The perturbation might be in the form of a morphogen or an environmental factor, and the identification of such signaling factors or molecules is a matter of intensive experimental research (see Wolpert, 1996).

Many studies have explored the behavior of partial hypothetical regulatory networks (Kauffman, 1993; Clarket *et al*., 1993), but there are few published applications of this dynamic approach to specific biological systems. Most of the published applications are for *Drosophila melanogaster* (Burstein, 1995; Reinitz & Sharp, 1995; Spirov, 1996) for which abundant genetic and molecular data has accumulated over the past few years. From this kind of model it has been possible to acquire insights that could have not been reached otherwise. For example, these models have suggested that a small group of homeotic proteins are sufficient to coordinate morphogenesis (Burstein, 1995), that the connectivity of the network determines a metabolic pathway (McAdams & Shapiro, 1995), or that it is possible to find a correlation among biochemical regulators and morphogenesis even when the mechanism is not completely known (Mjolsness *et al*., 1991). Network models have been useful to make inferences on the evolutionary pathway of a group of regulatory genes (Spirov, 1996), or even to suggest missing components of a regulatory system (Loomis & Sternberg, 1995).

During recent years much has been learned about the genetic and molecular basis of flower morphogenesis in *Arabidopsis thaliana* (Coen, 1991; Weigel, 1995; Yanofsky, 1995). We used published genetic and molecular data for 11 genes to construct a genetic regulatory network for *Arabidopsis thaliana* flower morphogenesis and we provide analyses of the dynamic behavior of this network. To our knowledge, this is the first genetic regulatory network model for a plant, or part of a plant. We are particularly interested in the dynamics of the genetic regulatory network and we explore the hypothesis that the attractors of a network correspond to the activation states of specific cell groups. In this paper, we specifically addressed if the four gene activation states predicted by the ABC model of *Arabidopsis* flower morphogenesis (Meyerowitz, 1994a; see later) can be recovered as stable activation states of the regulatory network model that we put forward. We found that the dynamics of the network predicts six stable states: the four gene activation states of the ABC model, an activation state of cells in vegetative tissue, and anactivation state not found in wild-type plants.

1.1. *ARABIDOPSIS THALIANA* FLOWERS AND THE ABC MODEL

Flowers of *Arabidopsis thaliana* (hereafter *Arabidopsis*) are formed by four concentric whorls of flower organs made of, from outside to inside: four sepals (whorl 1), four petals (whorl 2), six stamens (whorl 3) and two fused carpels (whorl 4). This particular organization can be disrupted by mutations in different genes, and the analysis of such mutations have led to the proposition of a combinatorial model that has been used extensively to describe the morphology of *Arabidopsis* flowers in wild-type and mutant plants (Fig. 1). The so-called ABC model (Coen & Meyerowitz, 1991; Meyerowitz 1994a) postulates the existence of three different activities (A, B and C) which are each active in two adjacent whorls, and their combination determines the identity of the organs that develop in the flowers. According to the model, the presence of activity A will determine the differentiation of sepals, a combination of activities A and B will result in petals, while the presence of both B and C will give rise to stamens, and finally activity C alone results in the formation of carpels. Additionally, the ABC model postulates a mutual inhibition between activities A and C, such that when function A is absent function C substitutes it and vice versa.

There are several specific genes related to the three above mentioned activities. *APETALA*1 (*AP*1) and *APETALA*2 (*AP*2) have been considered A function genes (Bowman *et al*., 1991, 1993. In the *Arabidopsis* literature, proteins are abbreviated using uppercase letters, wild-type genes with uppercase italics, and mutated genes with lowercase italics), because plants mutated in either gene yield flowers lacking sepals and petals. However, while molecular data confirmed the expected spatial distribution of *AP*1 mRNA according to the ABC model (Mandel *et al*., 1992), *AP*2 mRNA is present throughout the flower and is also present in non-floral organs (Jofuku *et al*., 1994). Even though *AP*2 might be regulated at a

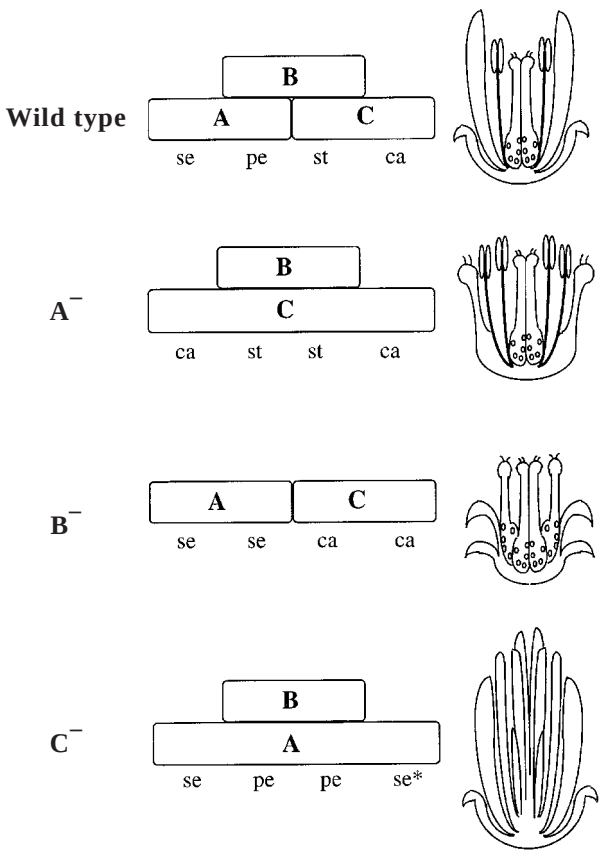

FIG. 1. The ABC combinatorial model proposes that the four different floral organs are determined by the specific combination of three different functions or activities. Activity A specifies sepals, activities A and B specify petals, activities B and C specify stamens, and activity C alone specifies carpels. Additionally, the ABC model postulates a mutual inhibition between activities A and C, such that when the function A is absent function C takes its place and vice versa. The rightmost figures are schematic representations of *Arabidopsis thaliana* flowers, which in wild-type plants (top) are composed from the outside to the inside of four sepals, four petals, six stamens and two carpels. Mutants in genes that confer each of the three floral activities and their effect on floral morphology are shown. se = sepals, pe = petals, st = stamens, ca = carpels, se* = an iteration of sepals, petals and petals.

post-transcriptional level, for simplicity we will consider *AP*1 as the only A function gene. On the other hand, it is known that *APETALA*3 (*AP*3) and *PISTILLATA* (*PI*) constitute the B activity (Krizek & Meyerowitz, 1996). Finally, *AGAMOUS* (*AG*) is the only reported C function gene (Bowman *et al*., 1991; Sieburth *et al*., 1995; Yanofsky, 1995; Yanofsky *et al*., 1990). Even though some authors have suggested minor modifications to the ABC model (Ma, 1994; Ray *et al*., 1994) based on new molecular data, the model has been very robust for describing overall flower morphology of mutant (Coen & Meyerowitz, 1991; Ma, 1994; Meyerowitz, 1994a) and transgenic plants (Krizek & Meyerowitz, 1996; Mizukami & Ma, 1992). Moreover, the molecular and genetic mechanisms implied in this model seem to be conserved in virtually all angiosperms (Bowman, 1997; but see Vergara & Alvarez-Buylla, 1997).

2. The Network Model

We reviewed the literature looking for molecular and/or morphological data that could reveal the regulatory interactions among 11 genes involved in flowering morphogenesis of *Arabidopsis*. Four of these genes are the ABC genes described above. Based on this information we constructed the genetic regulatory network that we present as the NET model in Fig. 2. Most of the postulated gene interactions represent regulatory interactions at the transcriptional level. Each element in the network represents one gene with one exception: the element referred to as *BFU* in the network implementation denotes a protein heterodimer formed by AP3 and PI, this complex forms an active transcription factor (see later), therefore this interaction is represented by merging arrows acting over *AP*3 and *PI*. Further

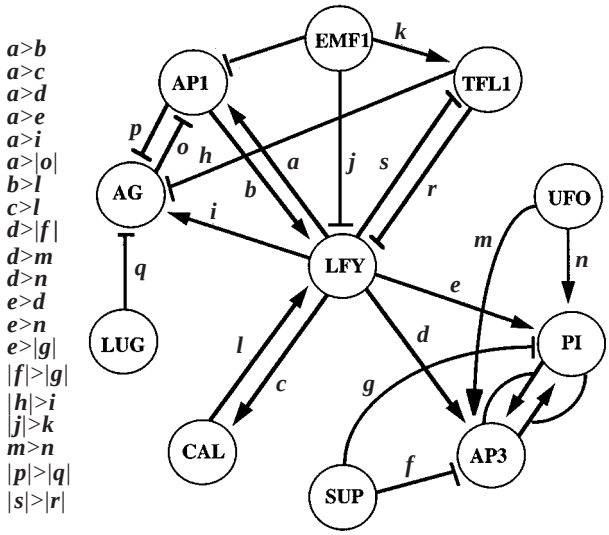

FIG. 2. The NET model. Arrows represent activations and flat-end lines represent inhibitions. Genes included are *EMBRYONIC FLOWER* 1 (*EMF*1), *TERMINAL FLOWER* 1 (*TFL*1), *LEAFY* (*LFY*), *APETALA*1 (*AP*1), *CAULIFLOWER* (*CAL*), *LEUNIG* (*LUG*), *UNUSUAL FLORAL ORGANS* (*UFO*), *APETALA*3 (*AP*3), *PISTILLATA* (*PI*), and *SUPERMAN* (*SUP*). Lowercase letters indicate the weight of interactions while inequalities at the left are the relative values inferred from experimental data. Absolute values represent the effect of de-repression, for example $|h| > i$ means that the absence of the repression of *TFL*1 has a stronger impact over *AG* expression than its activation by *LFY*.

more, molecular data enabled us to obtain the sign of each interaction (activation or inhibition, arrow-head or dash-head, respectively), and the *relative strength* of many interactions. We explain case by case the logic that led us to each of these relative values.

2.1. IMPLEMENTATION OF THE NET MODEL

We wanted to know if the dynamics of the genetic regulatory network leads to attractors that correspond to the genetic activities proposed in the ABC model for each of the four floral organs. Despite the great advances in the studies of gene expression of *Arabidopsis*, the published molecular data is still qualitative. Nevertheless, we have used this data to put forward the first network architecture and we explore the final stable patterns of gene activity (attractors of the network).

We do not have data regarding rates of transcription but rather know only if a gene is either active or inactive at certain time intervals. Furthermore, the spatial resolution of such data does not provide information on the expression dynamics of genes inside individual cells. Therefore, we cannot construct a state transition diagram to infer the network connectivity as has been achieved in some studies of neural networks (Glass & Young, 1979), or biochemical control networks (Glass & Kauffman, 1973). However, the molecular data at hand provide enough information to establish gene connectivity and a first proposition of the relative effect of some interactions (see later). Consequently, we decided to use a network realization that takes into account both gene activation states and relative interaction weights, the implementation takes the form of a difference equation:

$$x_i(t+1) = \mathbf{H}\left(\sum_{j=1}^{N} w_{ij}x_j(t) - \theta_i\right) \qquad (1)$$

where $\mathbf{H}$ is the Heaviside step function:

$$\mathbf{H}(x) = \begin{cases} 1 & \text{if} \quad x > 0 \\ 0 & \text{if} \quad x \leqslant 0 \end{cases}$$

The vector state indicating the activity of the n elements (genes) of the network is represented by $\mathbf{X} = (x_1, x_2, \ldots x_n)$ where $x_i \in \{0, 1\}$; meaning that a gene can only be in either of two states, active (1, maximum transcription rate) or inactive (0). In this network implementation a gene x_i becomes active if the weighted input of all the genes regulating it exceeds a certain activation threshold θ_i. Biologically, thresholds represent the amount of activators/inhibitors needed for turning on/off the transcription of that gene. Since we do not have any experimental evidence regarding the magnitude of such thresholds, we used integer values to keep computation simple, i.e. $\theta_i \in \mathbf{Z}$. A similar situation applies for the weights (w_{ij}s) of every interaction, thus $w_{ij} \in \mathbf{Z}$. Regarding the weights or relative strengths of the interactions, however, we based their sign (positive for activations and negative for inhibitions) and *relative* magnitudes on experimental data unless otherwise indicated (see next section and inequalities in Fig. 2). The relative values that we propose here imply differences in the transcription factors' efficiency, concentration, or both. Although we propose here a simple network model with on–off elements, previous theoretical work has yielded maps to compare some shared dynamic features between continuous and discrete networks (Glass, 1975; Glass & Kauffman, 1973). Therefore, the model we present here could be used, in principle, to construct more realistic continuous models, that should still keep the same long-term dynamic behaviors.

For our purposes, the thresholding behavior implied in eqn (1) was adequate because we were able to obtain the gene expression patterns observed in the flower, namely, four stationary states corresponding to the four regions of the ABC model that give rise to the floral organs (see ahead). Nevertheless, such implementation does not allow for interactions like those shown in Fig. 2 between the products of *AP*3 and *PI*. To circumvent this problem we introduced a network element (*BFU*, for B function) receiving inputs only from *AP*3 and *PI*, in such a way that it becomes active if and only if *AP*3 and *PI* are both active (this is a logical AND function, see experimental data later). Finally, we assigned numeric values to all thresholds and interactions between elements of the network. In networks, however, different values might result in different dynamics, therefore we tried to find the lowest integer values that result in the long-term activation patterns observed in the *Arabidopsis* flower. Remember that our objective is to evaluate if the *architecture* of the network, rather than the specific values, is compatible with the observed experimental data. For that reason, we used the lowest numerical values in our model to avoid as much as possible hypotheses that cannot be supported by experimental data at the moment.

All interactions among network elements depicted in Fig. 2 are presented in matrix $\mathbf{W}$, where each element w_{ij} represents the weight of the interaction from gene x_j to gene x_i. Likewise, in vector $\boldsymbol{\theta}$ the θ_is represent the thresholds of activation for each network element x_i. The order of the elements are

*EMF*1, *TFL*1, *LFY*, *AP*1, *CAL*, *LUG*, *UFO*, *BFU*,*AG*, *AP*3, *PI* and *SUP*.

$$\mathbf{W} = \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & -2 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ -2 & -1 & 0 & 2 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ -1 & 0 & 5 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 \\ 0 & 0 & 2 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & 0 \\ 0 & -2 & 1 & -2 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 3 & 0 & 0 & 0 & 2 & 1 & 0 & 0 & 0 & -2 \\ 0 & 0 & 4 & 0 & 0 & 0 & 1 & 1 & 0 & 0 & 0 & -1 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \end{bmatrix} \quad \boldsymbol{\theta} = \begin{bmatrix} 0 \\ 0 \\ 3 \\ -1 \\ 1 \\ 0 \\ 0 \\ 1 \\ -1 \\ 0 \\ 0 \\ 0 \end{bmatrix}.$$

The numerical values of matrix $\mathbf{W}$ and vector $\boldsymbol{\theta}$ were obtained using a genetic algorithm. We implemented a program that used only mutations, where a particular value of w_{ij} or θ_i chosen at random was changed for another one that fulfilled the following restrictions: (a) inequalities in Fig. 2; (b) network topology (i.e. no interactions were created or disappeared); and (c) the sign of interactions in Fig. 2. Such procedure was done with a population of 20 networks. After each mutation, every network was studied to examine if it had as stable states the four gene activation patterns corresponding to the ABC model and to non-flowering. After this step, each network was assigned with a fitness value. The fitness value was a linear function of the number of desired fixed points. Finally, to avoid local maxima during the selection step, besides the network with the highest fitness value, a randomly chosen network was kept. These two networks were used to replenish the network population, and the cycle of mutation, assignment of fitness values and selection were repeated until the population fitness reached a maximum.

The final issue regarding the implementation of the NET model concerns how to solve the transition from the vector state $\mathbf{X}(t)$ to $\mathbf{X}(t+1)$. The easiest way is the synchronous approach (as in Kauffman, 1969, 1991) in which eqn (1) is applied to all network elements at the same time. This dynamic description is problematic from the biological point of view, because it implies the unlikely situation in which all the genes respond exactly at the same time. Conversely, the asynchronous approach (as in Thomas, 1991) consists in solving eqn (1) for one network element at each time step, once the order in which the elements to be solved has been specified. This asynchronous approach is also problematic for our purpose, because the order for solving eqn (1) might change the gene activation pattern and the long term stable states, and there is still no experimental data on the precise order of activation of individual genes. We therefore decided to introduce a combined, biologically inspired approach, henceforth named semi-synchronic. In this method, the elements of the network are divided into groups. Hence, eqn (1) is solved synchronically for elements within the same group, and asynchronically for elements in different groups (see Fig. 3). We used experimental data to decide the order of activation of the different groups. Therefore, we think that this method is more appealing from the biological point of view, than previous ones.

Experimental biologists have grouped the genes related to flowering into a hierarchy of four sets of genes depending on their time of activation as the transition to flowering and flower morphogenesis proceeds. For the genes included in our network, *EMF*1 and *TFL*1 belong to the first group of genes to become active, namely the group of early and late floral genes (Coupland, 1995). The next set of genes to become active is the group of meristem identity genes (Weigel *et al*., 1992), in which *LFY*, *AP*1 and *CAL* are included. Then, the so-called cadastral genes become active (Weigel & Meyerowitz, 1993a), from

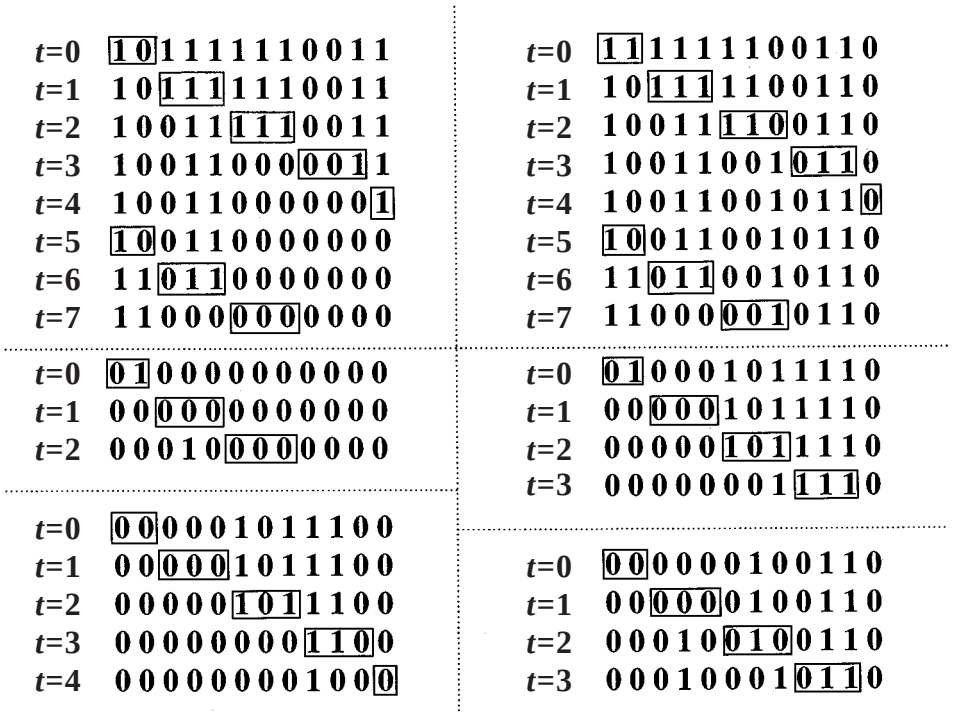

FIG. 3. A sample of the network activation patterns. These are some of the initial states (marked by $t = 0$) that eventually lead to one of the six fixed points. Rectangles indicate the group of element to which eqn (1) is applied to obtain the following network activation state. The order of the network elements here and throughout the text is from left to right *EMF*1, *TFL*1, *LFY*, *AP*1, *CAL*, *LUG*, *UFO*, *BFU* (B function, see text for details), *AG*, *AP*3, *PI* and *SUP*.

which *LUG* and *UFO* are representatives. It is important to mention that *LUG* and *UFO* are included in the same category due to their effect on flower morphology rather than to their spatial and temporal expression patterns, since *LUG* is not yet cloned and *UFO* is active at all developmental stages (Lee *et al*., 1997). Finally, the ensemble of organ identity genes (Ma, 1994), represented in our model by *AG*, *AP*3 and *PI*, become active. It is important to say that *SUP* was first considered as a cadastral gene, but it becomes active after the organ identity genes (Sakai *et al*., 1995). Based on these considerations, we have divided the 12 network elements into five groups: the first containing *EMF*1 and *TFL*1; the second *LFY*, *AP*1 and *CAL*; the third *LUG*, *UFO*, and *BFU*; the fourth *AG*, *AP*3 and *PI*; and the fifth with only *SUP*.

Another important biological consideration while performing the dynamic analysis, concerns the activation state of *EMF*1. This gene is part of the proposed floral repressor (Haughn *et al*., 1995; Weigel, 1995), which supposedly controls the transition from vegetative to reproductive growth, and it is proposed to be under the influence of many upstream genes (Coupland, 1995). To incorporate such an external influence, we decided to fix the activation state of *EMF*1 (either 0 or 1) throughout each dynamic cycle. In this way, we simulated the effect of an external factor not incorporated in the network. We achieved this by changing $w_{1,1}$ from 0 to1.

2.2. MOLECULAR BASIS OF THE NET MODEL

LEAFY (*LFY*) is known to be positively regulated by *AP*1 and *CAULIFLOWER* (*CAL*), because its mRNA is reduced in *ap*1 *cal* plants (Bowman *et al*., 1993; Kempin *et al*., 1995; Weigel & Nilsson, 1995). However, *AP*1 and *CAL* products are not needed simultaneously to activate *LFY* because single and double mutants for these two genes have different floral morphologies and *LFY* mRNA levels (Bowman *et al*., 1993; Gustafson-Brown *et al*., 1994). Therefore, *AP*1 and *CAL* regulations over *LFY* are part of two independent pathways. Conversely, *AP*1 mRNA (Weigel & Nilsson, 1995) onset is delayed in *lfy* mutants. The relative reduction of *AP*1 mRNA in *lfy* plants is more pronounced than the reduction of *LFY* mRNA in *ap*1 plants. Therefore, we propose that the up-regulation of *AP*1 by *LFY* is stronger than the up-regulation of *LFY* by *AP*1 ($a > b$; see Fig. 2). Furthermore, the phenotype of 35*S*::*LFY ap*1 plants

(Weigel & Nilsson, 1995; 35*S*::*LFY* is a molecular construction that over-expresses *LFY*) is between that of wild-type and 35*S*::*LFY* plants, suggesting that a large function of *LFY* is to activate *AP*1. We incorporated this in the NET model by making the relative value of such activation greater than any other activation from *LFY* to any other gene (i.e. $a > c, a > d, a > e$ and $a > i$). Unpublished data suggest that a similar regulatory interaction to that documented between *LFY* and *AP*1 exists between *LFY* and *CAL* (Savidge & Yanofsky, unpublished data; see also Fig. 6 in Weigel & Nilsson, 1995). In order to maintain the symmetry of the relationship between *LFY* and *AP*1/*CAL*, we made the activation of *LFY* over *CAL* stronger than that of *CAL* over *LFY* ($c > 1$ in the NET model). Finally, because *ap*1 plants have a more pronounced mutant phenotype than *cal* plants we made $b > 1$.

TERMINAL FLOWER 1 (*TFL*1) is a repressor of *LFY* (Okamuro *et al*., 1993; Weigel *et al*., 1992), because in *tfl*1 mutants, both *AP*1 and *LFY* mRNAs are ectopically expressed (an expression found outside the wild-type pattern, Bowman *et al*., 1993; Gustafson-Brown *et al*., 1994). But the effect of *TFL*1 over *AP*1 seems to be mediated by *LFY*, because *tfl*1 *ap*1 mutants have an additive phenotype (Bowman *et al*., 1993; Shannon & Meeks-Wagner, 1993). If the morphology of double mutants is the summation of the phenotypes of the individual single mutants, we consider that one of the two genes is not directly downstream of the other. Besides, *tfl*1 *lfy* double mutants (*tfl*1-10 *lfy*-16) have determinate growth and a flowering time similar to that of the *tfl*1 single mutant (Shannon & Meeks-Wagner, 1993), suggesting that *LFY* and *TFL*1 are in the same pathway, one downstream of the other. Conversely, *LFY* seems to inhibit *TFL*1 transcription, because 35*S*::*LFY* plants are very similar to *tfl*1 mutants (Shannon & Meeks-Wagner, 1991; Weigel & Nilsson, 1995). The *ap*1-1 mutation largely attenuates the 35*S*::*LFY* phenotype, nevertheless those plants still form terminal flowers (Weigel & Nilsson, 1995) as in *tfl*1 mutants, indicating that *LFY* inhibits *TFL*1 using an *AP*1-independent pathway. However, 35*S*::*LFY* plants produce a terminal flower before and at a shorter inflorescence stem size than *tfl*1 mutants, therefore, we postulate that the inhibition of *TFL*1 over *LFY* is weaker than the inverse inhibition ($|s| > |r|$). In other words, the effect caused by the disinhibition of *LFY* in the *tfl*1 mutants is not sufficient to cause the dramatic effects seen with the constitutive expression of *LFY* in 35*S*::*LFY* plants. Such contrast might be due to a difference in the levels of the LFY protein, to the activation or inhibition of other downstream genes, or a combination of both. This mutual inhibition between *LFY* and *TFL*1 is supported by the fact that their spatial domains of mRNA expression are contiguous but they do not overlap (Bradley *et al*., 1997).

There is much evidence indicating that *LFY* activates both B activity genes, *AP*3 and *PI* (Goto & Meyerowitz, 1994; Jack *et al*., 1994; Weigel *et al*., 1992; Weigel & Meyerowitz, 1993a,b). However, in *lfy* mutants *PI* mRNA reduction is more pronounced than *AP*3 mRNA reduction, therefore suggesting that the activation of *PI* by *LFY* is stronger than that of *AP*3, ($e > d$). Likewise, the gene *UNUSUAL FLORAL ORGANS* (*UFO*) activates *AP*3 and *PI* (Lee *et al*., 1997; Levin & Meyerowitz, 1995; Wilkinson & Haughn, 1995), but in this case *AP*3 mRNA levels are more dramatically reduced in *ufo* mutants than levels of *PI* mRNA ($m > n$). Finally, morphological analyses of mutant flowers suggest that *LFY* is a stronger activator of B function genes than *UFO* ($e > d, e > m, e > n, d > m, d > n$, and $m > n$). It is necessary to mention that *UFO* has a role in cell proliferation or organ initiation (Lee *et al*., 1997), therefore it is quite probable that the effect of this gene over *AP*3 and *PI* is not a direct regulation of expression. Nevertheless, the dynamic study presented here might be valid even when some of the proposed regulatory interactions turn out to be indirect mediated by other products or processes (see discussion). It has been proposed that *UFO* and *LFY* might act as coregulators (Lee *et al*., 1997). However, since the single and double mutants have different phenotypes, the putative interaction between LFY and UFO are not yet clear and therefore we decided to wait for more experimental results before including such interaction in our model.

In Fig. 1, merging arrows between AP3 and PI indicate that a dimer of the proteins encoded by these two genes is formed and that it maintains the activities of both *AP*3 and *PI* (Goto & Meyerowitz, 1994; Jack *et al*., 1994). Finally, *SUP* inhibits both *AP*3 and *PI* (Bowman *et al*., 1992; Goto & Meyerowitz, 1994; Shultz *et al*., 1991); and once more, *AP*3 mRNA expression is the most affected one in *sup* mutants (Sakai *et al*., 1995) suggesting that $|f| > |g|$. In *sup* mutants, in contrast to wild-type, *AP*3 mRNA is partially expressed in the fourth whorl during late stages of flower development. *PI* mRNA, on the other hand, is present in the fourth whorl from the onset of its expression, as normally does, but is maintained at detectable levels throughout development in contrast to the pattern of mRNA expression in wild-type (Sakai *et al*., 1995). These changes in mRNA expression of *AP*3 and *PI* are less drastic than

those observed in *lfy* mutants, where *AP*3 and *PI* mRNAs are abolished completely, therefore suggesting that $e > |g|$ and $d > |f|$ in Fig. 2. Here again, *SUP* seems to have a role in cell division (Sakai *et al*., 1995) rather than a direct regulation of *AP*3 and *PI* gene expression. Its inclusion in the NET model, however, reveals the possible existence of another, yet undiscovered, inhibitor of the B function genes (see Discussion).

The mechanism by which *AP*1 and *AP*2 (activity A) inhibit *AG* (activity C) is still uncertain, with the extra problem that most of the genetic analyses are reported for weak *ap*1 and *ap*2 alleles. It is known that *AP*2 is needed for the inhibition of *AG* in the whorls that will give rise to sepals and petals, but not in those where stamens and carpels will arise even though *AP*2 messenger is expressed throughout the flower (Jofuku *et al*., 1994). However, since *AP*1 mRNA is present in the two outer whorls (Mandel *et al*., 1992) many authors have suggested that a combination of both AP1 and AP2 are needed for the inhibition of *AG* (Bowman *et al*., 1993; Liu & Meyerowitz, 1995; Ma, 1994). Nevertheless, these regulatory interactions are still controversial and the mechanism is far from clear. However, our purpose is to investigate the dynamic properties of the genetic regulatory network, and *AP*2 seems to be a constitutively expressed gene in the *Arabidopsis* flower. Therefore, for simplicity we will assume that *AP*1 inhibits *AG*, keeping in mind that *AP*2 and perhaps other factors are also needed.

AG is activated by *LFY* (Weigel & Meyerowitz, 1993c), and inhibited by *LEUNIG* (*LUG*; Liu & Meyerowitz, 1995; Meyerowitz, 1994b). However, single *ap*1 mutants have more severe floral transformations than single *lug* mutants ($|p| > |q|$). *AG* itself provides an important feedback regulation by means of its inhibitory effect over *AP*1 (Gustafson-Brown *et al*., 1994). But the strongest regulation over *AP*1 seems to come from LFY (rather than from *AG*) because flowers of 35*S*::*AG* plants (Mizukami & Ma, 1992) start diverging morphologically from wild-type flowers when *LFY* expression diminishes in the center of the flower primordium (Weigel *et al*., 1992). Hence the up-regulation of *AP*1 by *LFY* seems to be stronger than the down-regulation of *AP*1 by *AG* ($a > |o|$). An experimental way to test this hypothesis is to observe flower development in 35*S*::*AG lfy* plants, our model predicts that such flowers would diverge morphologically from wild-type earlier than their 35*S*::*AG* counterparts, since the former plants would not have the activation of *LFY* over *AP*1, while the inhibition of *AG* over *AP*1 would always be present. There are other possible experiments of this kind that might help to unravel genetic interactions, for example if 35*S*::*AG ap*1 plants start diverging from wild-type while *LFY* diminishes its expression in the center of the flower, that would indicate that there is another gene acting downstream of both *AG* and *LFY* but mainly regulated by *LFY*.

Finally, concerning *AG*, it seems that *TFL*1 inhibits *AG*. Flowers of *ap*1 *ap*2 double mutants have an inflorescence-like morphology and also lack a proper central pistil. For example, the fourth whorl of *ap*1-1 *ap*2-2 (strong) mutants is formed by carpels that often fail to fuse and have sectors of stamen tissue [Bowman *et al*., 1993, see Fig. 8(c)], suggesting an alteration of the C activity. However, *tfl*1 *ap*1 *ap*2 mutant flowers do have pistil (Shannon & Meeks-Wagner, 1993), suggesting that the *tfl*1 mutation re-establishes the C activity. Given that *AG* is the only reported C function gene, we infer that the *tfl*1 mutation causes an increase in *AG* mRNA, and hence in wild-type plants *TFL*1 would be normally inhibiting *AG*. This inhibitory relationship is not mediated by *LFY*, because *tfl*1 *lfy* plants flower at the same time as *tfl*1 plants do (Shannon & Meeks-Wagner, 1993), indicating that the *LFY* product is not needed. Furthermore, *lfy* plants flower later and *tfl*1 earlier than wild-type plants; then if *tfl*1 *lfy* plants flower at the same time as *tfl*1 mutants, we can say that *AG* expression is more severely affected by the lack of *TFL*1 product than by the lack of *LFY* product, thus establishing that $|h| > i$.

EMBRYONIC FLOWER 1 (*EMF*1) is the most upstream gene we have considered. Actually, *EMF*1 has not been cloned and we rely only on morphological data of the mutant plants to make inferences about possible regulatory interactions involving this gene. *EMF*1 is considered as part of the so-called floral repressor (Haughn *et al*., 1995; Weigel, 1995), and it may fulfil such a function by two, non-excluding possibilities: either *EMF*1 inhibits the floral promoting genes (*LFY* and *AP*1) or it activates the other floral repressing gene (*TFL*1). The morphology of mutants suggests that both pathways may be operating. First, *lfy* mutants grown under short photoperiod have a stronger mutant phenotype than the same plants grown under continuous light (Schultz & Haughn, 1993). Actually, *lfy* plants under short photoperiods have hardly any flowers, just like *lfy ap*1 plants under long photoperiods (Haughn *et al*., 1995; Weigel *et al*., 1992). This suggests that under short photoperiods *AP*1 expression is absent or very reduced in *lfy* plants, thus acquiring a *lfy ap*1-like phenotype. This putative inhibition might be carried out by either *EMF*1 or *TFL*1, the late-flowering genes. However, *TFL*1 mRNA is found just

below the inflorescence dome and absent in the regions that will give rise to flowers (Bradley *et al*., 1997), thus *TFL*1 mRNA is never present in the regions where *AP*1 mRNA eventually appears, making very unlikely that *TFL*1 would repress *AP*1. Such spatial considerations leaves only *EMF*1 the possible repressor of *AP*1.

Indirect evidence suggests that *EMF*1 activates *TFL*1. Long day grown *emf*1 *tfl*1 plants flower just like *tfl*1 single mutants (Yang *et al*., 1995), suggesting that *TFL*1 is directly downstream of *EMF*1. Additionally, pistils are the most prominent organs in *emf*1 flowers (Yang *et al*., 1995), suggesting that those mutants over-express *AG*, presumably due to the absence of the inhibitory effect of *TFL*1. Finally, *EMF*1 could also be directly inhibiting *LFY*, because *emf*1 *lfy* plants develop *lfy*-like flowers with leaf-like organs, but do not develop *emf*1-like flowers (Yang *et al*., 1995), again suggesting that *LFY* is directly downstream of *EMF*1. Furthermore, it has been shown that the gene *CONSTANS* (*CO*) activates *LFY* (Simon *et al*., 1996) mediated by the inhibition of *EMF*1 (Coupland, 1995). This is, *CO* inhibits *EMF*1 which in turn inhibits *LFY*. Finally, the *emf*1 mutation causes an early flowering which is more similar to that presented by 35*S*::*LFY* than *tfl*1 plants. This morphological evidence suggests that the morphology of *emf*1 plants is due to an over-activation of *LFY* rather than to a lack of *TFL*1 expression ($|j| > k$).

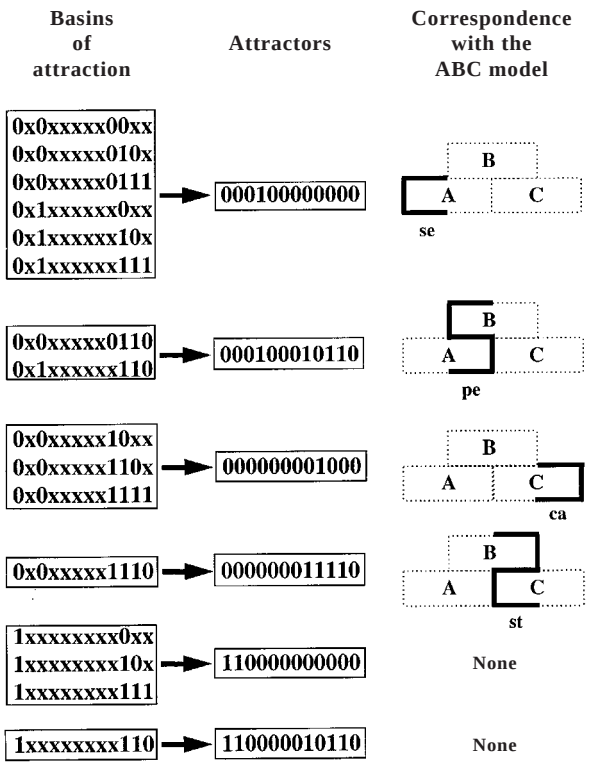

FIG. 4. Attractors, basins of attraction of the NET model and their correspondence with the ABC model. A basin (left column) is composed of all those initial states that eventually lead to a particular attractor (central column). **0** and **1** represent inactive and active genes respectively, while **x** means that an element might be either active or inactive. Four attractors have a direct equivalence with the four regions of the ABC model (right column), therefore it is possible to infer the floral organs that will develop from those fixed-point of the NET model.

2.3. DYNAMICS OF THE NET MODEL

We made an exhaustive examination of the dynamics of the model. We tested all possible initial states ($2^{12} = 4096$ states) and we used the semi-synchronic method for solving eqn (1) for a sufficiently long time period ($t = 200$) to find the final activation states of the network. In such a way we obtained the attractors and basins of attraction of the NET model (Fig. 4). Six stable fixed points were obtained, five of them with a clear biological meaning. Instead of writing the network activity state as a vector, like $\mathbf{X}(t) = (0, 0, 0, 1, 0, 0, 0, 1, 0, 1, 1, 0)$, we will simply write it down as a 12-digit binary number, i.e. 000100010110.

The first attractor (000100000000) corresponds to the exclusive activation of *AP*1, the A function gene. As discussed above, this pattern of activation is found in the first whorl of flowers, where sepals develop. The second fixed point (000100010110) is a stable activation of genes *AP*1, *AP*3 and *PI*, and the presence of the AP3/PI protein complex. This attractor corresponds to the activation of A and B functions that determine petal formation in the second whorl. We also obtained a fixed point (000000011110) representing the stable activation of *AG*, *AP*3, *PI* and the presence of the AP3/PI complex, (i.e. B and C functions active). This activation state corresponds to that proposed by the ABC model for the third whorl cells that differentiate into stamens. The fourth attractor (000000001000) corresponds to an exclusive activation of *AG*, the only C function gene. This activation pattern is found in cells of the fourth whorl that give rise to carpels. The last biologically meaningful attractor (110000000000) is a stable activation of the floral inhibitor genes *EMF*1 and *TFL*1, characteristic of cells that will not become part of flowers. Heretofore, the dynamics of the NET model agrees with the well supported ABC model (Meyerowitz, 1994a).

The NET model has a sixth attractor (110000010110) with stable expression of *EMF*1, *TFL*1, *AP*3, *PI* and the complex of AP3/PI. In this

case we obtained the stable expression of the flowering inhibition genes at the same time as the presence of floral B function genes and products. This attractor does not agree with any observed gene expression pattern in wild-type plants, but we think that it can be experimentally induced. *AP*3 and *PI* can be turned on despite the activation of the *EMF*1 and *TFL*1, because of the mutual positive feedback present between *AP*3 and *PI* (see Fig. 2). If for any reason the AP3/PI dimer is present at any stage of development, and in the absence of an inhibitory signal (notice that *SUP* is inactive in this basin of attraction), the B function genes will be expressed. The model predicts, therefore, that the presence of the AP3/PI dimer will be enough to originate and sustain their own expression as long as the proteins do not diffuse or degrade. Nevertheless, flowers would not appear in this putative region of induced *AP*3 and *PI* expression because meristem identity genes (mainly *LFY* and *AP*1) will remain inactive. This type of experiment has not been performed but plants over-expressing both B function genes (Krizek & Meyerowitz, 1996) showed AP3 protein expression in some vegetative tissues.

3. Discussion

Many genes involved in *Arabidopsis thaliana* flowering and flower morphogenesis have been cloned in recent years (Weigel, 1995; Yanofsky, 1995). Messenger RNA expression patterns, altogether with their related phenotypes are accumulating fast and providing valuable functional information regarding such genes. Some interesting pictures of the complex regulatory interactions established by these genes are slowly arising (see for example Theißen & Saedler, 1995), but these schemes are mostly hierarchical and static. In this paper we provide a first example of how nonlinear dynamic models can help us understand the molecular mechanisms underlying flowering and flower morphogenesis.

The network presented here provides a first provisional architecture that yields as stable states the A, B and C activities and their combinations as proposed in the widely used ABC model (Fig. 1). This network model is, therefore, a first proposition of at least some of the molecular mechanisms underlying the ABC model. We do not claim that the NET model incorporates all the important genes involved in determining the cellular fate in flower morphogenesis. The inclusion of further genes would alter the network topology but it could be possible to maintain the four attractors that represent the activation states characterizing the regions where sepals, petals, stamens or carpels differentiate.

In this work, our main goal was to know if the molecular data available for genes related to *Arabidopsis* flower morphogenesis could be synthesized in a dynamic model compatible with the ABC model. Our goal in the long run is, however, to obtain a more general dynamic model that includes the ABC model as a particular case. In the meantime, the NET model provides with both a tool for further theoretical analysis, and a guide for future experimental work. For example, we are currently looking for the minimal network representation that still has the four activation regions represented by the ABC model. This exercise might prove useful for the experimental biologist working with *Arabidopsis* in order to distinguish direct from indirect regulatory relationships among these genes. Further simulations on this NET model may be used also to identify interactions for which the quantitative value is critical to recover the expected stable states. Special effort might be put to obtain quantitative data for such interactions.

The attractors of the NET model (Fig. 4), show that two types of elements can be distinguished: those which initial states affect the attractor reached and those which initial states do no affect the attractor reached. The former are *EMF*1, *LFY*, *AG*, *AP*3, *PI* and *SUP*, and those that do not affect the final network state are *TFL*1, *AP*1, *CAL*, *LUG*, *UFO* and the AP3/PI complex (*BFU*). This could imply that a perturbation of one of the elements of the first group might be sufficient to change the fate of the cells in the developing organ by altering the identity of the attractor. On the other hand, a perturbation in any of the elements of the second group would not alter the cell differentiation commitment. Moreover, if our model turns out to be experimentally robust, it is possible to predict that floral genes still to be discovered most probably will either belong to the second group or connect to elements in that group. In any case, the inclusion of such new genes in the NET model would alter the topology of the network but would not modify its final attractors. Hence the capacity of a network of absorbing perturbations due to the inclusion or deletion of elements depends on the connectivity of the whole network. Previous theoretical statistical analyses (Wagner, 1996) have also shown that the final activation pattern in a network might remain unchanged, or very slightly modified, despite large deletions.

Genes belonging to each of these two groups might be prone to different evolutionary forces. We may speculate that network elements that are less critical

for the establishment of adequate activation patterns, might be more prone to neutral mutations, than those that are critical for maintaining the correct pattern. For example, if a mutation permanently "turns off" a gene member of those genes that do not alter the attractors of the network, then the transient activation pattern of the network might be altered, but the final activation state would remain unchanged. The reverse hypothesis can also be postulated. Genes belonging to the group that affect the attractors might be more sensible to mutations, and therefore the ratio of non-neutral to neutral mutations would be higher in these genes than in those not involved in establishing the network attractors. This bold hypothesis could be tested by making comparative analyses of sequence data of genes belonging to each of these two groups from different species

There is another way in which the addition of a newly discovered gene would not alter our results. Suppose that the inhibition of *AG* by *LUG* is not a direct one as depicted in Fig. 2 but is indirectly mediated by a still undiscovered gene *X*. Adding this new gene to the NET model would certainly modify the topology of the network and might also alter the transient dynamic behavior. However, since *LUG* belongs to the group of genes that are not critical to reach the attained attractors, the activation state of any gene that is exclusively controlled by *LUG* cannot affect the attractors, because this imaginary gene *X* would be only a relay between *LUG* and *AG*. Therefore, we do not postulate that all the genes directly connected in our model have direct interactions *in vivo*. This has to be tested experimentally for most cases. We state, however, that many intermediary genes might act only as relays and would not affect the stable states reached. Nevertheless, not all possible alterations are of this kind, some gene inclusions might actually add new attractors to the dynamics of the network. These new attractors would not invalidate our model, however. In this first model we are dealing only with four major regions of gene activation as stated by the ABC model, but each floral whorl contains different cell types. Therefore, future experimental results may be incorporated into larger networks, ideally yielding as many attractors as cell types are in the entire flower.

We want to make clear that the dynamic approach used here has clear advantages over a static analysis. This point can be illustrated by comparing, for example, the activation state of *AP*3, *PI* and *SUP* in the basins of attraction (last three values in every vector, Fig. 4) to their activation in the corresponding attractors. Notice that those attractors in which the B activity is present (AP3 and PI active) come from initial states in which *AP*3 and *PI* are active but *SUP* is inactive. This means that the activity of *SUP* determines if the B activity will be present or not: if during the initial state *SUP* is active, the B activity will not be established. This result indicates that a gene inhibiting the B activity genes is absolutely necessary to obtain a result compatible with the ABC model. However, *SUP* is probably acting at the cellular level regulating cell division rate rather than directly regulating the transcription of B genes. If this proves to be the case, our dynamic model predicts that there would be a still undiscovered gene that inhibits *AP*3 and *PI* at the transcriptional level, because a negative transcriptional regulator of the B genes is required to yield some regions with and other regions without the B activity.

One of the objectives of this study was to ask if the network model could reach as stable states the four patterns of gene activation predicted for *AP*1, *AP*3, *PI* and *AG* in the ABC model. Although other genes may be involved in determining the A and C functions (Bowman, 1997), the ABC model is the best global description of the activities of flower organ identity genes. Therefore, the numerical values of network parameters that could not be estimated based on current experimental results were obtained with a genetic algorithm using a fitness function defined according to the gene activation patterns proposed in the ABC model. However, the topology of the NET model included more genes and more interactions than those included in the ABC model. Hence, there is no *a priori* reason to expect that the dynamic model proposed here should reach the same gene activation patterns predicted by the combinatorial static ABC model. Furthermore, the NET model constitutes a new hypothesis regarding the molecular mechanisms underlying the establishment of the ABC functions.

Although the dynamics of the NET model heavily depends on the non-zero values of $\mathbf{W}$ and $\boldsymbol{\theta}$, the topology of the network imposes restrictions. For example, it was impossible to obtain values of $\mathbf{W}$ and $\boldsymbol{\theta}$ that yielded only the four floral stable states plus a unique non-floral state. The sixth stable pattern of Fig. 4, that does not have a biological interpretation, is therefore a restriction imposed by the topology of the network on its dynamic behavior. On the other hand, certain network topologies never attain the ABC activation patterns as stable states, no matter which numerical values of $\mathbf{W}$ and $\boldsymbol{\theta}$ are used (including those used here, results not shown). Therefore, the coincidence between the NET and ABC models validates some of the assumptions incorporated in both models. Simulations in progress

(Mendoza & Alvarez-Buylla, in prep.) and additional theoretical (Thieffry *et al*., 1995; Thomas, 1991; Thomas & D'Ari, 1990, Thomas *et al*., 1995) and experimental work should help us elucidate which of these assumptions are valid and which are not.

How can we reconcile the use of the network approach with the temporal pattern of flower development in *Arabidopsis*? In early flower primordia (stages 1 and 2, Smyth *et al*., 1990) before floral organ primordia are apparent, the regulatory genetic network of each cell forming the bud would be in the same state, namely 110000000000 according to the NET model. As cell division proceeds and mediated by still unknown cell–cell signaling cues and mechanisms, the stable activation pattern of the network is changed only by turning off *EMF*1, for example. This putative signal acting only on one gene would be enough to ensure commitment of cells to differentiate into sepals, the organs of the outermost whorl of floral organs. Such a perturbation would take the network to the state 010000000000, that is part of the attractor that eventually leads to the state in which only *AP*1 is on (000100000000) and determines sepal differentiation according to a simplification of the ABC model. As flower development proceeds, the same or other putative signals might prompt the genetic network to each of the other attractors for petals, stamens and carpels, thus completing flower development. This account is largely speculation, but it might be useful for thinking about the role that genetic regulatory networks play in the process of cellular differentiation and development of flowers.

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