Minireview of research activity: analysis of floral repression process in Arabidopsis

Sylvie Pouteau, Valérie Gaudin, V. Ferret, D. Lefebvre, M. Libault, F. Prunus, M. Sabar, Gang Zhao

MINIREVIEW OF RESEARCH ACTIVITY

ANALYSIS OF THE FLORAL REPRESSION PROCESS IN *ARABIDOPSIS*

Sylvie Pouteau[1], Valérie Gaudin, Valérie Ferret, Delphine Lefebvre, Marc Libault, Franck Prunus, Mohammed Sabar and Gengchun Zhao

Laboratoire de Biologie Cellulaire, INRA, Route de Saint-Cyr, F78026 Versailles cedex, France

We have isolated about 80 early flowering mutants of *Arabidopsis* under short days (SDs), mostly from the T-DNA insertion collection of Versailles. For 13 mutants, the early flowering phenotype is genetically linked to a T-DNA insertion. For one of them, the corresponding locus, *LHP1*, has been characterised and encodes a chromo domain protein showing similarity with proteins involved in heterochromatin formation. An integrated description of the floral repression process has been undertaken based on morphological and physiological analyses. The mutants exhibit a wide range of flowering time under SDs and most of them are characterised by highly diverse morphology, photoresponse, and response to florigenic substances. Several classes of response have been determined based on a number of criteria: sensitivity to high sucrose concentration, response to the light and to the dark, and response to photoperiod. The characterisation of the quantitative response to photoperiod suggests that most mutants are affected in environmental control of phase change whilst few mutants may correspond to endogenous phase change mutants. The determination of the mutant norms of reaction in response to various factors is expected to help to identify the different levels of regulation of the floral repression process. This also constitutes an opportunity to investigate how genetic homeostasis of flowering is achieved and how phenotypic plasticity contributes to this regulation.

Introduction

In a previous issue of the *Flowering Newsletter* (May 2001; # 31), the interest and the conceptual context of floral repression in *Arabidopsis* was presented (14). A study of this process was initiated about 5 years ago in our Department in Versailles by screening for early flowering mutants. The genetic, molecular, and physiological analyses in progress for these mutants is reviewed in this paper. The results suggest that the investigation of floral repression can provide new insights into the interpretation of the complexity of the floral transition. Potential perspectives for this work can lead to a better understanding of the role of phenotypic plasticity and genetic homeostasis in the regulation of flowering.

[1] Author for correspondence: Sylvie.Pouteau@versailles.inra.fr

Identification of new floral repression mutants

Constitution of a collection of early flowering mutants

This programme was initiated by screening early flowering mutants in short day (SD) conditions. The material used for the screen was mostly the T-DNA insertion collection from Versailles (Wassilewskija ecotype : Ws) as a collaboration of our Department with G. Pelletier and N. Bechtold of the Department of Genetics (1, 2). We also used an EMS-mutagenised collection provided by C. Bellini in our Department (Columbia ecotype: Col-0). A large scale screen was started in the fall of 1997 and was completed by the beginning of 2000. In a primary screen performed under natural SD conditions during the winter in a glasshouse, we screened about 8000 lines and identified 549 independent early flowering candidates (Table 1).

A secondary screen was performed in growth chambers on individual progenies of the primary candidates grown under SDs in individual pots. Flowering time was assessed by determining the number of leaves and the bolting time. This led to the confirmation of about 80 early flowering mutants (Table 1). Three additional mutants bolt early although their number of leaves is not reduced and possibly have a modified plastochron; one of these mutants displays abnormal meristem behaviour that could be responsible for the apparent reduction of the plastochron. Early flowering is also observed for another 60 mutants but the segregation of this phenotype is abnormal, possibly due to complex rearrangements resulting from T-DNA insertion. Dominance tests for 64 mutants showed that 11 mutants are semi-dominant and 53 are recessive. This indicates that the mutations involved may affect activation as well as repression mechanisms, or dosage effect repression processes. Complementation tests initiated for about 30 mutants suggest that the number of different loci affected is probably very high in the collection. So far, only 5 complementation groups display several alleles. However, this corresponds to only 14 mutants in the collection and the number of alleles in each group is no higher than 3. Therefore, many mutants in the collection are probably unique members of the corresponding complementation groups. Because of this lack of redundancy, it is likely that the collection is not saturated and that the number of loci involved in floral repression is very high. Comparisons with mutants described in the literature or provided by colleagues have been started on a case by case basis. So far, we found that one mutant is allelic to *early flowering 3* (*elf3*) (19). Apparently no allele of *spindly* (*spy*) (10) or *curly leaf* (*clf*) (6) has been recovered in our screen.

Gene tagging and molecular analyses

The study of the genetic linkage of the early flowering mutations with a T-DNA insertion (bearing a kanamycin resistance gene) shows that about 20 % of the mutations are tagged. This corresponds to the average of tagged mutations observed for various genetic screens in the Versailles collection as well as in other T-DNA

Table 1. Screen for early flowering mutants of *Arabidopsis* in SDs

Lines (Ecotype)	T-DNA (Ws)		EMS (Col-0)		Total	
Primary Screen (mostly under natural SDs during the winter in the glasshouse)						
Lines screened under SDs	7653		384		8037	
Earliness detected in SDs	357	405	64	144	421	549
in LDs*	48		80		128	
no progeny	29		15		44	
Secondary Screen (on individual progenies grown under SDs in growth chambers)						
Confirmed earliness	65		18		83	
% confirmed earliness	0.9%		>3.1%		-	
Plastochron possibly shorter	3		nd		nd	
Abnormal segregation	49		13		62	

nd : not determined

* From screens performed by INRA colleagues in other programmes.

insertion collections. So far, genetic linkage has been found for 13 mutants (less than 3% genetic distance) and for 8 of them, the genetic distance is below 1 %.

The molecular analysis of the T-DNA insertions is in progress for the 13 mutants displaying genetic linkage with a T-DNA insertion. Southern blot analysis shows that 2 mutants (including the *lhp1-1* mutant, see below) exhibit one single, simple T-DNA insertion whilst the other mutants bear complex, multiple insertions. Amplification and analyses of the flanking sequences of the T-DNA (FST) are in progress. So far, several FSTs have been identified in the FlagDB database generated by the Department of Research in Plant Genomics (URGV) at INRA in Evry, France. Confirmation of the correlation between these FSTs and their putative function in floral repression is awaiting further characterisation.

The *LHP1* locus was isolated by molecular analysis of the *lhp1-1* tagged mutant and maps to the top region of chromosome 5 (4). Sequence analysis shows that LHP1 belongs to the superfamily of chromo domain proteins and is the homologue of the drosophila Hetrochromatin Protein 1 (HP1). *LHP1* is the first example of a functional HP1-like protein identified in plants and was named after this specificity (*LIKE HP1*). Two *lhp1* mutant alleles were recovered in the T-DNA insertion collection. In *lhp1-1*, a single T-DNA copy is inserted in the promoter region of *LHP1* leading to transcription deregulation (see below). In *lhp1-2*, a single point mutation creating a stop codon is detected and probably results in a truncated LHP1 protein.

The probably ubiquitous function of LHP1 in chromatin dynamics is reflected by the highly pleiotropic phenotype of *lhp1* mutants. *lhp1-1* and *lhp1-2* show extremely early flowering under SD conditions and are more or less insensitive to photoperiod. In addition, other developmental regulations are disrupted as shown by dramatic changes in plant architecture and leaf morphology, as well as dwarfism. Transcription analysis by RT-PCR revealed that *LHP1* is ubiquitously transcribed in all tested organs (root, leaf, floral bud, and silique) at various developmental stages and under different photoperiodic conditions. These results suggest that *LHP1* is constitutively transcribed and that its expression is regulated at a post-transcriptional level. Transcription is not abolished in *lhp1-1* although it is weaker than in the wild type (WT). This may indicate that the level of transcription and/or translation is involved in a dosage effect regulation of LHP1 function (4).

Integrated description of the floral repression process

Induced mutants are expected to reflect the range of possible genotypic and phenotypic variations within a given ecotype, including extreme phenotypes that are not found in nature. This range of variation probably not only points to the function of individual genes but also to complex arrays of interactions which are integrated in global patterns of phenotypic response within and across environments. The phenotype is thus the outcome of complex synergistic developmental systems and the repertoire of environmentally contingent phenotypic possibilities of a genotype is referred to as its 'norm of reaction'. Mutant phenotypes and behaviours can be studied to approach the floral repression process at a global level and to understand how phenotypic plasticity and genetic homeostasis are involved in this process (14). A functional classification of the early flowering mutants was thus initiated based on morphological and physiological analyses and on the study of genetic interactions between mutants.

Morphological diversity

The early flowering mutants isolated display a large range of additional phenotypes such as changes in plant architecture, modified leaf morphology, or altered pigmentation. Flowering time in SDs varies from extremely early (about 10 leaves) to moderately early (30 leaves) compared to 40 leaves for the WT. A correlation between flowering time and the level of morphological pleiotropy is observed in most mutants, extremely early flowering mutants being often more strongly affected. The characterisation of flowering time in SDs reveals that a proportion of the mutants exhibit late bolting compared to their total number of leaves and could thus have a modified plastochron. A number of examples show that early flowering mutations can result in opposite phenotypes, such as paler/darker green pigmentation, increased/decreased apical dominance, enlarged/slender leaf shape, longer/shorter petiole, etc. This may constitute evidence that multiple developmental pathways contribute to

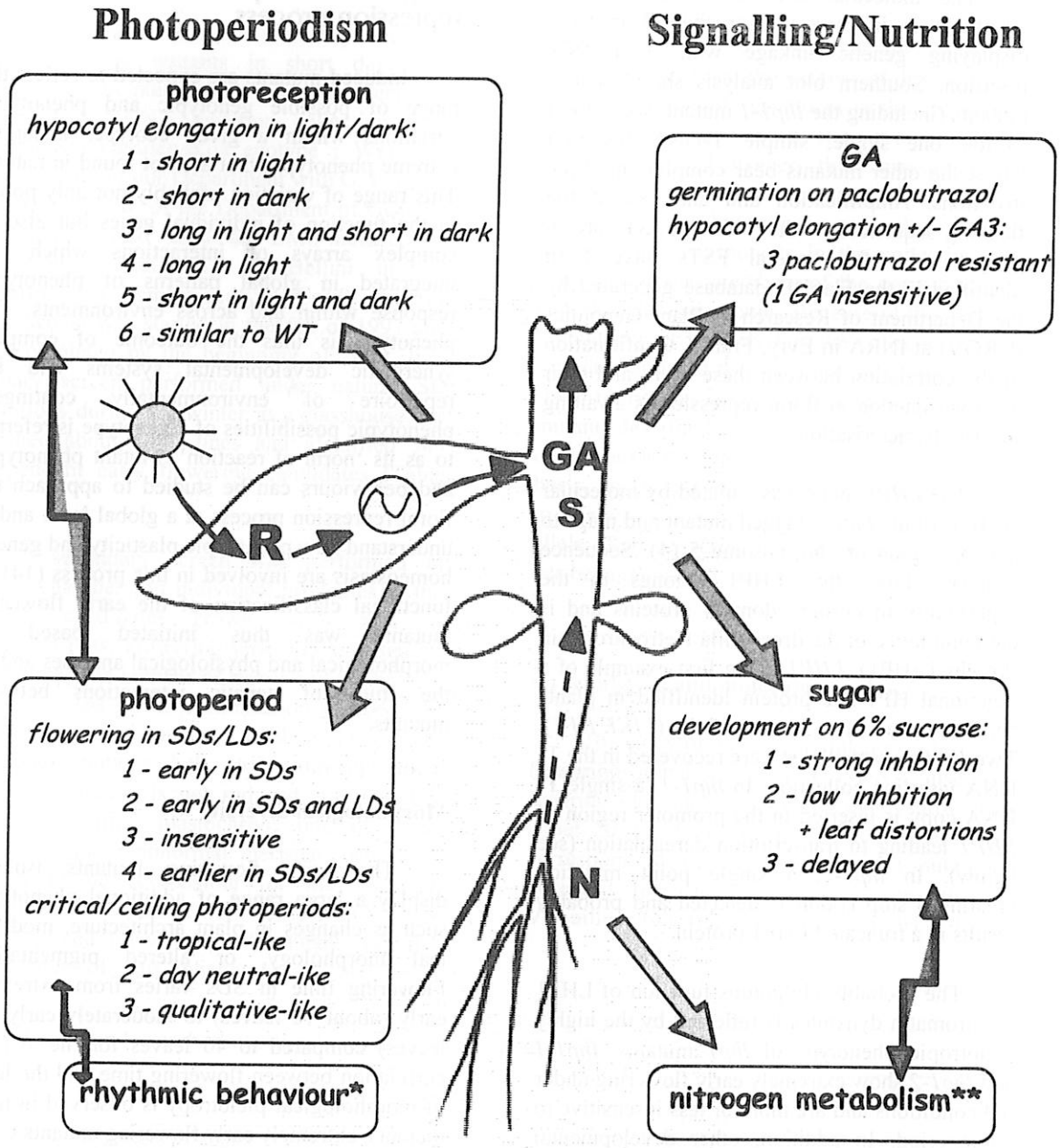

Figure 1. Summary of the functional characterisation of early flowering mutants. Analyses of the mutant norms of reaction in response to environmental factors and florigenic substances known to contribute to the regulation of flowering homeostasis. The main steps of the light signalling pathways, photoreceptors (R) and the circadian clock (sinusoid curve), are represented. The florigenic substances, gibberellins (GA) and sugars (S) used in the characterisation, as well as nitrogen (N), are indicated.

*In collaboration with I. Carré, University of Warwick, UK.

**In collaboration with A. Krapp, INRA, Versailles, France.

the global homeostasis of flowering time control. Alternatively, this may reflect a breaking down of global genetic canalisation and buffering processes that oppose developmental perturbation by the environment or mutations rather than specific effects on regulatory pathways (14, see Discussion and perspectives). To explore further these possibilities, the response to florigenic substances and photoperiod was analysed (Fig. 1).

Responses to florigenic substances

To study the hormonal and metabolic signalling of flowering in the mutants, indirect screens were performed. These included resistance to paclobutrazol, an inhibitor of gibberellin (GA) biosynthesis, and sensitivity to high concentration of sucrose. Three mutants were found to be resistant to paclobutrazol, one of which is highly resistant to up to 10^{-3}M paclobutrazol. This mutant is not allelic to *spy* (10) and genetic analysis showed that the 2 phenotypes are additive. In addition, this mutant shows a decreased sensitivity to exogenous addition of GAs suggesting that it may represent a new GA signalling mutant. Four classes of response to high concentration of sucrose were observed. Classes 1 to 3 are characterised by various degrees of developmental inhibition by sucrose whilst class 4 was not significantly different from the WT (Fig. 1). Some of the mutants most sensitive to sucrose also display early senescence or produce more or less distorted leaves and are slow bolting. It is thus possible that these mutants are affected in carbon and/or nitrogen metabolism rather than in specific sugar signalling of flowering.

Photoresponse

The photoresponse of the mutants was investigated by analysing the responses to light and dark and to photoperiod. To characterise the perception of light and dark by the mutants, hypocotyl elongation of seedlings grown *in vitro* was measured under different light conditions. Six groups have been identified so far (Fig. 1). Groups 1 to 4 exhibit a response that is modified specifically in the light or in the dark and may include mutants affected in photomorphogenesis or skotomorphogenesis. Group 5 has shortened hypocotyls in both conditions and may comprise mutants affected in cell expansion. Finally, group 6 shows no modification compared to the WT.

The response to photoperiod was determined by direct measurement of flowering time in SDs and in long days (LDs) and compared to the WT. Four classes of response to photoperiod have been identified: 1) early flowering specifically under SDs, 2) early flowering under both SDs and LDs, 3) photoperiod insensitive early flowering, and 4) flowering earlier in SDs than in LDs (Fig. 1). Groups 3 and 4 may be expected to include arrhythmic mutants. Indeed, one mutant is allelic to *elf3* (8, 19) and 2 other mutants, which belong to the same complementation group, are also arrhythmic (I. Carré, personal communication). The latter 2 mutants complement *elf3* (8, 19) and the *late elongated hypocotyl* (*lhy*) (16) mutant and may potentially correspond to new circadian clock mutations. In addition to the arrhythmic mutants, group 3 also includes the 2 *lhp1* alleles as well as a third mutant having phenotypic similarity with *lhp1* (4) and *clf* (6) mutants and possibly affected in similar functions.

The study of genetic interactions between the 'arrhythmic' subgroup and the 'chromatin' subgroup of mutants and a number of known mutants of the photoperiodic pathway and circadian clock function was initiated. Preliminary results show that only the crosses between the chromatin subgroup of mutants and *elf3* or *lhy* result in an additive phenotype, whilst other crosses with mutants such as *constans* (*co*) (15) and *luminidependens* (*ld*) (11) reveal epistatic interactions.

Quantitative response to photoperiod

The quantitative response to photoperiod was further investigated in the mutants responsive to photoperiod (i.e. groups 1 and 2) and compared to the WT. Only few mutants exhibit a response curve with some overlap with the WT curve around the critical photoperiod (Pc). Most mutants display a response curve below that of the WT. About one third of the mutants analysed so far have a longer ceiling photoperiod (Pce) and about 50% of them also have a shorter Pc. Two thirds of the mutants show a shorter Pc and for 50% of them the Pce is also shorter or possibly absent.

A preliminary classification in 3 groups can tentatively be proposed (Fig. 1). Although there is some degree of modification in the response to the environment in all mutants, the mutants with an increased Pce may result from endogenous reduction in the capacity to initiate leaves. When

only the Pce is increased, the mutants may correspond to true endogenous phase change mutants. These mutants having a narrower window of response to photoperiod may be compared to tropical species that exhibit a sharp quantitative response to photoperiod (Group 1). But the vast majority of the mutants is affected in photoperiodic perception as shown by a reduction in Pc and a decrease in the slope of the response curve for several of them, suggesting a tendency toward day-neutrality (Group 2). The mutants with a shift in Pc and Pce toward lower photoperiod may reveal a tendency toward a qualitative response to photoperiod (Group 3). These interpretations require further investigation through assays under short photoperiods and analysis of the correlation with the response to light and dark and the rhythmic behaviour.

Discussion and perspectives

One outcome of this work is the production of the first general description of early flowering mutants in *Arabidopsis*. The integration of the various mutant norms of reaction should help to identify the different levels of regulation of the floral repression process. This could be used as a basis for comparison in a database. The characterisation of tagged mutations will allow the identification of new loci involved in the regulation of flowering at the molecular level. So far, the first locus analysed, *LHP1*, points to the role of chromatin structure in this regulation (4). This result and other reports (5, 6, 7, 13, 17, 18) on epigenetic regulation of flowering may indicate that the role of individual gene functions is not sufficient to account for the complexity of the flowering process. McClintock was the first to put forward the idea of interactions between the environment and the genome referring to mobile genetic elements. Thereby, she introduced the notion of 'genomic stress' forcing the genome to restructure itself in order to survive (12). Besides the activity of diverse mobile genetic elements, chromatin dynamics contributes also to genomic plasticity and may constitute another level of more global regulation, at the interface between the environment and individual gene functions.

The current interpretations of flowering mutations have led to the integration of individual regulatory players in sequential models, either activation or repression models (14). In these models, gene functions are viewed as specific rulers of flowering. However, gene functions are highly dependent on the context in which they are placed at the organism and the environment levels (3, 9). It is thus possible to propose another, complementary interpretation in which a flowering balance needs to be achieved through multiple, dynamic interrelations between different processes. This can lead to a 'cross-talk' or 'homeostasis' model, in which genetic canalisation or buffering and phenotypic plasticity are essential components to explain the regulation of flowering (14). This model postulates that: i) the vegetative and the floral states are present at all stages of the plant life; ii) below a set threshold of the vegetative to floral (V/F) balance, the vegetative state is maintained; iii) the ontogenic stage interacts with the V/F balance.

In contrast to most late flowering mutants, early flowering mutants are often very pleiotropic and display a large range of different phenotypes, as reviewed in this paper. This can be due to homeostasis disruptions due to a V/F balance that is not adapted to precocious ontogenic stages. A change in the V/F balance at a later ontogenic stage is less likely to affect dramatically the general homeostasis, hence the less pleiotropic effects of late flowering mutations. This interpretation opens up new perspectives for the understanding of the regulation of flowering. Our analysis of early flowering mutants, in addition to providing new information for the classical sequential models, can potentially constitute an original approach to the regulation of genetic stability and phenotypic plasticity of a complex process.

The identification of mutant norms of reaction in response to various factors such as sugar supply, light condition, and day-length, provides an opportunity to investigate how genetic and phenotypic homeostasis is achieved. The analysis of multivariate correlation is expected to reveal how these factors are correlated and how stability is achieved through multiple interactions. Furthering the analysis of the quantitative response to photoperiod, it would be interesting to analyse the contribution of phenotypic plasticity to the regulation of flowering. A combination with other factors such as temperature and light intensity should allow the determination of parameters of plasticity and stability, synergy or compensation effects, and hierarchy of the parameters of homeostasis. Another perspective is the analysis of the interplay between ontogeny, plasticity, and stability. The development of a homeostatic model of regulation of flowering may prove

useful for the prediction of multivariate combinations and constitute a basis to investigate flowering behaviour in changing environment, especially for predictions in response to climatic changes. This may provide new insights into the agronomic implications of phenotypic plasticity.

Acknowledgements

We are grateful to Hervé Ferry, Jean-Marie Pollien, and Joël Talbotec for technical assistance in the mutant screen and plant culture. We thank Vincent Cudo, Benoît Lacroix, Virginie Léon, Cécile Marchenay, and Sophie Peyramaure for their help as trainee students. We also thank Anne Krapp for comments on the manuscript.

This work was supported by a CNRS-INRA grant (GDR *Arabidopsis*) and by the EC Biotechnology Programme DG XII (contract BIO4-CT97-2340).

References

1. BECHTOLD, N., ELLIS, J. and PELLETIER, G. *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C.R. Acad. Sci. Paris* **316:** 1194-1199, 1993.
2. BOUCHEZ, D., CAMILLERI, C. and CABOCHE, M. A binary vector based on basta resistance for *in planta* transformation of *Arabidopsis thaliana*. *C.R. Acad. Sci. Paris* **316:** 1183-1193, 1993.
3. DUBOULE, D. and WILKINS, A.S. The evolution of 'bricolage'. *Trends Genet.* **14:** 54-59, 1998.
4. GAUDIN, V., LIBAULT, M., POUTEAU, S., JUUL, T., ZHAO, G., LEFEBVRE, D. and GRANDJEAN, O. Mutations in *LIKE HETEROCHROMATIN PROTEIN 1* affect flowering time and plant architecture in *Arabidopsis*. *Development*, 2001 (in press).
5. GENDALL, A.R., LEVY, Y.Y., WILSON, A. and DEAN C. The *VERNALISATION 2* gene mediates the epigenetic regulation of vernalisation in *Arabidopsis*. *EMBO Worshop, The Molecular Basis of theTransition to Flowering*, John Innes Centre, Norwich, U.K., S9, July 2001.
6. GOODRICH, J., PUANGSOMLEE, P., MARTIN, M., LONG, D., MEYEROWITZ, E.M. and COUPLAND, G. A polycomb group gene regulates homeotic gene expression in *Arabidopsis*. *Nature* **386:** 44-51, 1997.
7. GOTO, K., KOTABE, T., OHTO, M., KAINOU, T. and KURODA, N. *Arabidopsis TFL2* gene encoding a novel polycomb protein, makes a complex with CO-like protein and represses FT expression during floral transition. *EMBO Worshop, The Molecular Basis of theTransition to Flowering*, John Innes Centre, Norwich, U.K., S42, July 2001.
8. HICKS, K.A., MILLAR, A.J., CARRÉ, I.A., SOMERS, D.E., STRAUME, M., MEEKS-WAGNER, D.R. and KAY, S.A. Conditonal circadian dysfunction of the *Arabidopsis early flowering 3* mutant. *Science* **274:** 790-792, 1996.
9. HOLDREGE, C. *Genetics and the Manipulation of Life: The Forgotten Factor of Context*. Lindisfarne Press, Hudson, 1996.
10. JACOBSEN, S.E and OLSZEWSKI, N.E. Mutations at the *SPINDLY* locus of *Arabidopsis* alter gibberellin signal transduction. *Plant Cell* **5:** 887-896, 1993.
11. LEE, I., AUKERMAN, M.J., GORE, S.L., LOHMAN, K.N., MICHAELS, S.D., WEAVER, L.M., JOHN, M.C., FELDMANN, K.A. and AMASINO, R.M. Isolation of *LUMINIDEPENDENS*: a gene involved in the control of flowering time in *Arabidopsis*. *Plant Cell* **6:** 75-83, 1994.
12. McCLINTOCK, B. The significance of responses of the genome to challenge. *Science* **226:** 792-801, 1984.
13. PINEIRO, M., GÓMEZ-MENA, C., MARTÍNEZ-ZAPATER, J. and COUPLAND, G. The role of *EBS* in the repression of flowering in *Arabidopsis*. *EMBO Worshop, The Molecular Basis of the Transition to Flowering*, John Innes Centre, Norwich, U.K., S15, July 2001.
14. POUTEAU, S. Conceptual context of floral repression in *Arabidopsis*. *Flowering Newslett.* **31:** 12-18, 2001.
15. PUTTERILL, J., ROBSON, F., LEE, K., SIMON, R. and COUPLAND, G. The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell* **80:** 847-857, 1995.
16. SCHAFFER, R., RAMSAY, N., SAMACH, A., CORDEN, S., PUTTERILL, J., CARRÉ, I.A. and COUPLAND, G. The *late elongated hypocotyl* mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* **93**: 1219-1229, 1998.
17. SHELDON, C.C., FINNEGAN, E.J., ROUSE, D.T., TADEGE, L., BAGNALL, D.J., HELLIWELL, C.A., PEACOCK, W.J. and DENNIS, E.S. The control of flowering by vernalisation. *Curr. Opin. Plant Biol.* **3:** 418-422, 2000.

18. SOPPE, W.J.J., JACOBSEN, S.E., ALONSO-BLANCO, C., JACKSON, J.P., KAKUTANI, T., KOORNNEEF, M. and PEETERS, A.J.M. The late flowering phenotype of *fwa* mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. *Mol. Cell* **6:** 791-802, 2000.

19. ZAGOTTA, M.T., HICKS, K.A., JACOBS, C.I., YOUNG, J.C., HANGARTER, R.P. and MEEKS-WAGNER, D.R. The *Arabidopsis ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. *Plant J.* **10:** 691-702, 1996.