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MINIREVIEW OF

RESEARCH ACTIVITY

BIENNIALISM AND VERNALIZATION-PROMOTED FLOWERING IN *HYOSCYAMUS NIGER*: A COMPARISON WITH *ARABIDOPSIS*

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There are genetic similarities between the biennial growth habit of *Hyoscyamus niger* (*H. niger*) and winter annual ecotypes of *Arabidopsis thaliana*, including the response to demethylating agents. One focus of our research group at Marquette is to determine whether *FLOWERING LOCUS C* (*FLC*) homologs or *FLC*-related MADS-box genes are involved in biennialism of *H. niger*. This review also summarizes our initial characterization of expression profiles of 4 groups of *H. niger* MADS-box genes. Our results suggest that B-class floral homeotic gene homologs of *H. niger* are differentially expressed in flowers of annual and vernalized, biennial plants.

Introduction

Angiosperms have adapted different mechanisms that allow each species to flower at a specific time of year. Both environment and developmental age influence the transition from vegetative to reproductive development (4). An environmental cue that promotes flowering in many plants consists of a prolonged exposure to cold temperatures, a process called vernalization. The requirement for vernalization can be either facultative (quantitative) or obligate (qualitative). Winter annual races of *Arabidopsis thaliana* (*Arabidopsis*) have a facultative requirement for cold and are late-flowering in the absence of vernalization. By contrast, biennial races of *Hyoscyamus niger* (*H. niger*) have an obligate requirement for cold, continue to grow vegetatively, and will eventually senesce in the absence of vernalization (15).

Very little is known about the molecular mechanisms of vernalization and how it promotes flowering. However, quite a bit of information about mechanisms that confer a facultative vernalization requirement has recently been accumulated from elegant work done in *Arabidopsis* (reviewed in 19). The following

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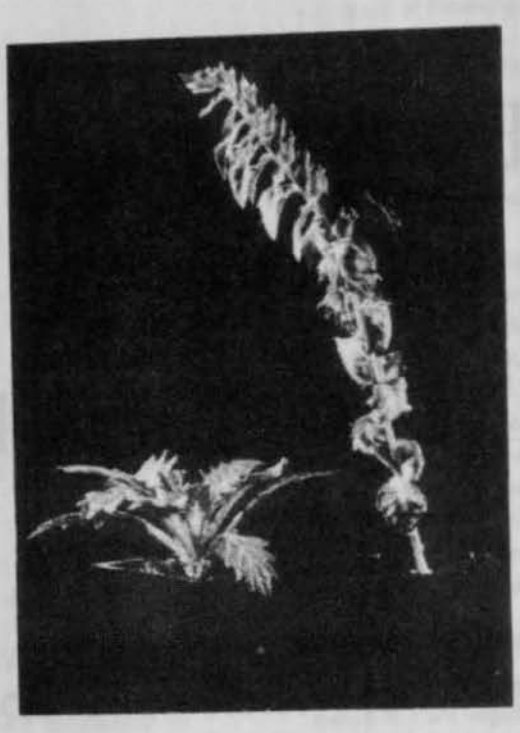

Figure 1. Annual (right) and biennial (left) growth habit of *Hyoscyamus niger* in long-day photoperiods.

minireview is a progress report on our laboratory's effort to determine whether similar mechanisms confer an obligate vernalization requirement in biennial races of *H. niger*.

Genetics of flowering in *H. niger*

Annual races of *H. niger* flower rapidly after 30 to 40 days in controlled growth chamber conditions (22-25 °C, 16-h day/8-h night cycles, ~200-300 µM light flux from fluorescent tubes supplemented with ~5% incandescent light), whereas biennial races remain vegetative (Fig. 1). This was observed in our laboratory with 2 different sources of *H. niger* seeds (M. Patel and M. Schläppi, unpubl. results). One source was obtained commercially from Richters (Goodwood, Ontario, Canada), while the other consisted of the original strains described by G. Melchers and A. Lang (reviewed in 15). Early genetic crosses between annual and biennial varieties demonstrated almost 100 years ago that biennialism segregates as a dominant trait in *H. niger* (8; reviewed in 15). We have likewise found that the unvernalized F_1 generation of crosses between annual and biennial *H. niger* remained vegetative for more than 80 days while the annual parent began to flower after less than 30 days under controlled growth conditions (M. Schläppi, unpubl. results). This situation is thus similar to that of naturally occurring flowering time variants of *Arabidopsis*, where the facultative vernalization requirement of winter annualism is a dominant trait. Interestingly, however, an extended analysis of flowering time in *H. niger* showed that annual/biennial F_1 hybrids actually did flower after about 180 days without vernalization while the biennial parent remained vegetative (22). Thus, whereas late flowering was clearly dominant in F_1 hybrids of *H. niger*, the true biennial growth habit with an obligate vernalization requirement of the biennial parent was converted into a winter annual habit with a facultative vernalization requirement in the hybrid. This again resembles the situation in *Arabidopsis*, where crosses between naturally occurring early- and late-flowering ecotypes can have intermediate phenotypes (13, 17, 23).

Potential relationship between biennialism and *FLOWERING LOCUS C*

Crosses between naturally occurring early- and late-flowering races of *Arabidopsis* determined that a vernalization requirement is caused by the synergistic interaction of *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) (6, 7, 11, 13, 16, 17). *Arabidopsis* lines with functional copies of *FRI* have high levels of *FLC* mRNA, suggesting that *FRI* activates *FLC*

and that *FLC* represses flowering (18, 24). By contrast, vernalization downregulates *FLC* expression in a quantitative manner (25, 26), and lower levels of *FLC* mRNA result in earlier flowering. Levels of *FLC* mRNA remain low for the remainder of the plant's life, however, *FLC* is reactivated in the progeny of vernalized plants. Vernalization-promoted downregulation of *FLC* is thus restricted to a single generation.

Since *FLC* mRNA levels remain low in vernalized plants even in the presence of active *FRI* and since high levels of *FLC* mRNA reappear in their progenies, it was suggested that vernalization may be an epigenetic phenomenon involving reversible cytosine methylation (5, 9). Consistent with this view, 5-azacytidine (5-azaC)-promoted demethylation and decreased levels of cytosine methylation in transgenic plants containing antisense constructs against the methyltransferase gene *MET1* can indeed partially substitute for vernalization (5, 10). It was also reported that antisense *MET1*-promoted reduction of cytosine methylation in transgenic plants may correlate with decreased levels of *FLC* mRNA (24).

Other winter annuals besides *Arabidopsis* were shown to flower earlier after incubation with 5-azaC (5, 9), but to our knowledge it was never reported whether demethylating agents could convert a true biennial into an annual plant. We thus used the observations gained from *Arabidopsis* as a starting point for a comparative analysis of the obligate vernalization requirement for flowering in biennial *H. niger*. To determine whether demethylation partially substitutes for vernalization, we germinated annual and biennial *H. niger* seeds on filter paper soaked with 100 µM 5-azaC, which was added fresh daily for one week. Annual and biennial control plants were germinated in pure water or 100 µM cytidine. Both 5-azaC- and control-treated annual plants flowered after 30 days, indicating that the demethylating agent had no adverse effect on flowering. In one out of 3 experiments with biennial plants, 15% (2/13) of unvernalized seedlings that were germinated in the presence of 5-azaC flowered after 35 days while, significantly, 100% (120/120) of the control plants remained vegetative for more than 80 days (M. Schläppi, unpubl. results). Overall, 4% (2/47) of unvernalized, 5-azaC-treated, biennial plants flowered as early as annual plants. In addition, 8% (2/24) of 5-azaC-treated annual/biennial F_1 hybrids also flowered after 35 days while 100% (51/51) of the control plants remained vegetative for more than 80 days. These results indicated that, at least in a few cases, treatment with a demethylating agent is indeed enough to convert a true biennial into an annual plant. It is even more noteworthy to emphasize that the 5-azaC treatment of biennial *H. niger* was effective at the young seedling stage, because biennial *H. niger* responds to vernalization only after a juvenile-to-adult phase transition, which is reached after 20 to 30 days of development (22). Taken together, the results suggested that *H. niger* and *Arabidopsis* may have some molecular mechanisms of vernalization-promoted flowering in common.

A hypothetical mechanism shared between *H. niger* and *Arabidopsis* could involve *FLC*. In *Arabidopsis*, downregulation of *FLC* mRNA is a major consequence of vernalization and a prerequisite for early flowering. The importance of low levels of *FLC* mRNA can be demonstrated in transgenic *Arabidopsis* that contain extra genomic copies of *FLC*. Such plants have higher levels of *FLC* mRNA than wild type and have now acquired an obligate vernalization requirement for flowering (19). Thus, overexpression of *FLC* in *Arabidopsis* transforms a facultative cold-requiring annual into a true biennial plant. Differences in the levels of *FLC* mRNA could, therefore, explain why biennials have an obligate vernalization requirement while winter annuals have a facultative requirement. It is thus conceivable that very high RNA levels of an *FLC* homolog are responsible, at least in part, for the biennial growth habit of *H. niger*. This hypothesis would also explain why F_1 hybrids between annual and biennial *H. niger* are converted to winter annuals. That is, analogous to the situation in *Arabidopsis* where weak alleles of *FLC* are semidominant in certain genetic backgrounds (17, 23), annual strains of *H. niger* may have weak alleles of an *FLC* homolog that act semidominantly in F_1 hybrids to convert the biennial to a winter annual growth habit. To test these hypotheses at the molecular level, we decided to attempt the cloning of a putative *FLC* homolog from biennial *H. niger*.

Is there an *FLC* homolog in biennial *H. niger*?

Several *FLC* homologs have been cloned from different plant species since the gene was first isolated from *Arabidopsis* (9, 12, 19). However, all of the species belonged to the family of *Brassicaceae* and were thus related to *Arabidopsis*. As our first approach to isolate a

putative *FLC* homolog from a member of the *Solanaceae* family, we used *FLC* cDNA probes in DNA gel blot analyses to determine whether a crosshybridizing sequence could be detected in annual or biennial *H. niger*. To address genome complexity, we first determined the size of the nuclear genome of our *H. niger* lines. The flow cytometry analysis was done in the laboratory of E. Earle (Cornell University, Ithaca, NY, U.S.A.) and the results indicated that the Richters lines of *H. niger* have a 2C nuclear DNA content of 2.63 pg (E. Earle and M. Schläppi, unpubl. results). This is about 9 times larger than the 2C nuclear DNA content of *Arabidopsis* (0.3 pg) and about the same value as that of *Petunia hybrida* (3). Thus, because *Arabidopsis* DNA was included as hybridization controls in the DNA gel blot analyses, about 8-10 µg of *H. niger* DNA was analyzed per 1 µg of *Arabidopsis* DNA. To potentially allow some degree of crosshybridization between the *Arabidopsis* probe and the *H. niger* template, all DNA blots were manipulated under low stringency conditions. In several experiments we determined, however, that whereas *FLC* cDNA probes hybridized well to expected fragments of genomic *Arabidopsis* DNA, the same probes did not bind to DNA from either annual or biennial *H. niger* (M. Patel and M. Schläppi, unpubl. results). The results thus indicated that *FLC* cDNA probes specifically recognize *Arabidopsis* DNA and do not crosshybridize to genomic DNA of *H. niger*, even under low stringency conditions.

Based on the premise that a putative *FLC* homolog should be more highly expressed in tissues of unvernalized, biennial, than annual *H. niger*, we also determined whether *FLC* probes could crosshybridize to *H. niger* RNA. About 25 µg total RNA of root, apex, and young seedling tissues from annual and biennial plants was probed and washed under low stringency conditions with *FLC* cDNA in RNA gel blot analyses. The 3 tissue types were chosen because they had the highest levels of *FLC* mRNA in *Arabidopsis* (18, 24). However, as in DNA gel blots, whereas *FLC* cDNA probes hybridized well to RNA from *FRI*-containing *Arabidopsis* but not to RNA from early-flowering control plants, no significant hybridization was detected to RNA from any annual or biennial *H. niger* tissue (M. Patel and M. Schläppi, unpubl. results), that is, no differences in background hybridization was seen between annual and biennial samples. Perhaps even more significantly, the same results were obtained with full-length *FLC* cDNA and with probes made exclusively to the conserved MADS-box region of the gene.

Taken together, we concluded the following from these results. First, that the nucleotide sequence of a putative *FLC* homolog in *H. niger* is too divergent to be easily recognized by *Arabidopsis*-specific *FLC* probes. Second, that even a MADS-box region-derived *FLC* probe is highly specific for *FLC* itself and that it does not significantly crosshybridize to other MADS-box-containing sequences in *Arabidopsis*. And third, that these hybridization experiments neither confirm nor rule out the existence of a closely related *FLC* homolog in *H. niger*.

Isolation of MADS-box transcripts from biennial *H. niger*

Based on the above results and the premise that an *FLC*-like MADS-box gene may be involved in biennialism, we began to isolate MADS genes from biennial *H. niger*. We also argued that the systematic analysis of expression patterns of biennial *H. niger*-derived transcripts with MADS-box homology will allow us to isolate even distantly related *FLC*-like genes. We predicted that an *FLC*-like MADS box gene involved in biennialism would be more highly expressed in biennial than annual *H. niger*, and/or that its transcript would be more abundant in unvernalized than vernalized plants.

We used a conservative RT-PCR approach to isolate MADS-box transcripts from 10-day-old seedling tissue of biennial *H. niger*. The method was based on a previously published protocol that was successfully employed to amplify MADS genes from a variety of species, including non-flowering plants (14). We designed a degenerate primer corresponding to the octapeptide sequence NRQVTYSK, a highly conserved region at the N-terminus of many MADS domain proteins, and used the primer mix together with a 3' RACE adapter primer in RT-PCR reactions with RNA templates from biennial *H. niger*. Amplified cDNA fragments of different length increments (500-1,000 bp and 1000-1,500 bp) were ligated into plasmid vectors and unique clones were sequenced. This showed that almost 90% (7/8) of the cDNA clones analyzed thus far were similar to MADS-box genes (M. Patel and M Schläppi, unpubl. results). By contrast, RT-PCR reactions using degenerate primers designed to sequences more specific for FLC and FLC-like MADS domains (M. Schläppi, unpubl. results), have not yet

Table 1. Characterization of MADS-box genes isolated from biennial *H. niger*

H. niger MADS box clones	Sequence similarity to	Approximate transcript size (kb)
HnMADS1	*NMH7*; *TDR8*; *AGL30* (MADS domain)	N.D.
HnMADS2	*TDR6*; *AP3*	1.3
HnMADS3	*pMADS2*; *PI*	0.95
HnMADS4	*DEFH125*; *AGL17*	2.0
HnMADS5	*AGL17*	N.D.
HnMADS6	*TDR6*; *AP3*	1.3
HnMADS7	*pMADS2*; *PI*	0.95

N.D.: Not Determined

produced any cDNA fragments with similarity to MADS-box genes. We thus proceeded with the analysis of the first 7 MADS-box genes so far obtained from biennial *H. niger*.

The isolated sequences were analyzed with the BLASTx program and similarities to known MADS-box genes are summarized in Table 1 (M. Patel and M. Schläppi, unpubl. results). The results indicated that 4 different groups of cDNAs were obtained. *HnMADS1* was the only member of the first group and had overall homology to an *Arabidopsis* sequence with similarity to *HMN7* from alfalfa and MADS domain similarity to *AGL30* (1); *HnMADS2* and *HnMADS6* were members of the second group

Table 2. Expression of MADS-box genes in vernalized[a] biennial *H. niger*

	Root	Shoot Apex	Leaf	Bract[b]	Flower[b]	Seed pods[b]
HnMADS1	-	-	-	-	-	-
HnMADS2/6	+	+	+	+	+++	++
HnMADS3/7	-	-	-	-	+++	+
HnMADS4	-	-	++	++	-	-
HnMADS5	-	-	-	-	-	-

[a] Vernalization of 4-wks old plants; 8 wks at 4 °C at constant low light intensity

[b] Tissues produced after vernalization

Table 3. Expression of MADS-box genes in annual *H. niger*

	Root	Shoot Apex	Leaf	Bract	Flower	Seed pods
HnMADS1	-	-	-	-	-	-
HnMADS2/6	+	+	+	+	+	+
HnMADS3/7	-	-	-	-	++	+
HnMADS4	-	-	++	++	-	-
HnMADS5	-	-	-	-	-	-

with high similarity to an *APETALA3* (*AP3*) homolog from tomato (20); *HnMADS3* and *HnMADS7* were members of the third group with high similarity to a *PISTILLATA* (*PI*) homolog from Petunia (2); and *HnMADS4* and *HnMADS5* were members of the fourth group and had similarity to *AGL17* from *Arabidopsis* (21). Members of the same cDNA group had very similar but not identical nucleotide sequences, indicating that they probably originated from different genes. However, none of the MADS-box genes isolated from biennial *H. niger* had significant sequence similarity to *FLC*. To determine whether any of the genes were differentially expressed, gel blot analyses with RNA from different tissues of annual and biennial plants were done.

B-class floral homeotic genes appear overexpressed in flowers of vernalized *H. niger*

To determine whether one of the 7 MADS-box genes was more highly expressed in biennial *H. niger*, we compared their expression profile in different tissues of annual and biennial plants. Based on the premise that a MADS-box gene with analogous function to *FLC* may act as floral repressor in *H. niger*, we expected that transcripts of such a gene would be more abundant in biennial than annual or vernalized biennial plants. As summarized in Tables 2 and 3, none of the 7 *HnMADS* genes fit such a profile (M. Patel and M. Schläppi, unpubl. results). We concluded from this limited analysis that the systematic search for a functional homolog of *FLC* in *H. niger* needs to continue. However, transcripts from 2 of the 7 *HnMADS* genes were not detectable in standard RNA gel blot analyses (Tables 2 and 3), suggesting that their expression profile needs to be determined with more sensitive techniques such as quantitative RT-PCR.

Surprisingly, however, we have preliminary evidence that compared to unvernalized, annual plants, putative *H. niger* homologs of *Arabidopsis* B-class homeotic genes (*AP3* and *PI*) are overexpressed in flowers of vernalized, biennial plants (Tables 2 and 3). Unlike in *Arabidopsis*, *H. niger* homologs of *AP3* also hybridize to RNA from vegetative tissue such as leaf and bract. For instance, *HnMADS2* is expressed at comparable levels in bract tissue of annual and biennial plants, but is clearly overexpressed in flowers of biennial plants (Fig. 2; M. Patel and M. Schläppi, unpubl. results). The vernalized flower-specific overexpression was observed for both *AP3* and *PI* homologs in different blots and with different RNA preparations. We are currently analyzing different flower stages from annual and vernalized, biennial plants, and are using a *H. niger*-specific ubiquitin probe to normalize more accurately for RNA loading.

Summary and future perspectives

We have shown that unvernalized biennial *H. niger* can be converted into an annual plant when germinated in the presence of a demethylating agent. Since this was an indication that similar mechanisms may confer a vernalization requirement in *H. niger* as in *Arabidopsis*, we analyzed *H. niger* nucleic acid sequences and isolated MADS-box genes from biennial plants in search for a functional homolog of *FLC*. In preliminary studies we could neither confirm nor clearly rule out the existence of such a homolog in *H. niger*, because the gene must have diverged enough from *FLC* to be easily detectable by standard hybridization and RT-PCR techniques. So far we have also isolated 7 MADS-box-containing cDNA sequences from biennial *H. niger* and made the surprising observation that relative to annual flowers, B-class floral homeotic genes are overexpressed in flowers of vernalized, biennial plants. A mechanistic connection between overexpression of *AP3*/*PI* homologs in biennial flowers and vernalization remains to be determined and is a current focus in our laboratory. To investigate the vernalization-specific regulation of those homologs we will isolate genomic sequences for manipulation in *H. niger* and heterologous plants.

We have recently isolated a vernalization-responsive gene from *Arabidopsis* using a traditional subtractive hybridization technique (26). Based on the success of this approach, we are currently establishing annual and biennial *H. niger* cDNA libraries for specific subtractive hybridization and general screening purposes. In future work we will use *H. niger*-specific MADS sequences and various molecular methods such as reverse-RNA gel blot analyses to screen both cDNA libraries for MADS-box genes that are differentially expressed in either annual or biennial plants. We have also produced plasmid libraries of reciprocally-subtracted cDNA fragments from RNA of annual and biennial plants and are screening them for differentially-expressed genes. In collaboration with Moshe

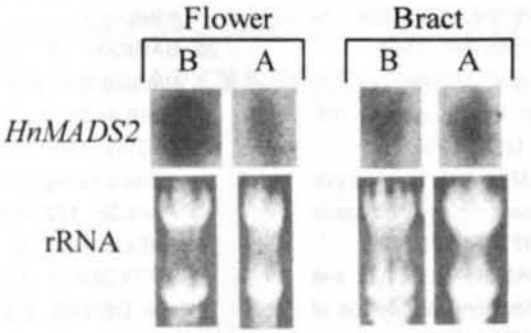

Figure 2. RNA gel blot analysis of expression profiles of the *AP3*-like gene *HnMADS2* in flowers and bracts of annual (A) and vernalized biennial (B) *H. niger*. RNA samples were hybridized with the same probe in one gel blot. rRNA: Ethidium bromide-stained ribosomal RNA.

Reuveni (ARO, Bet Dagan, Israel) we are also developing tissue culture protocols for the regeneration and genetic transformation of *H. niger*. This will allow us in the future to analyze putative *H. niger* flowering time genes and B-class homologs by reverse-genetic approaches.

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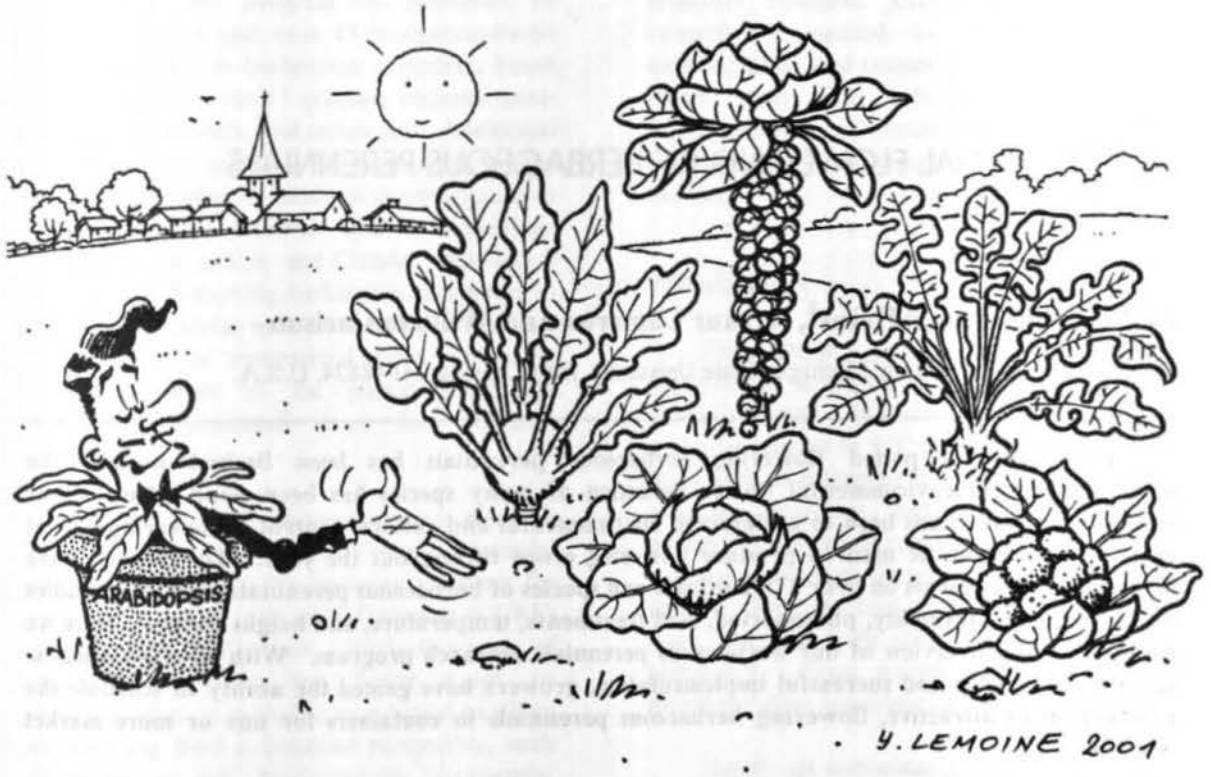
REFERRING TO THE PAPER OF KOLE *ET AL.* IN *THEORETICAL AND APPLIED GENETICS* (102: 425-430, 2001) SHOWING THAT GENES HOMOLOGOUS TO *ARABIDOPSIS FLC* MAY CONTROL FLOWERING TIME IN VARIOUS *BRASSICAS*



Arabidopsis : - Look at these members of my family. Despite we share important genes, these illustrious countryfolks considered me as a villain because I was only known in the past as a tiny weed. But now that I became the world star of the plant kingdom, they are full of respect for me.