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Floración sin Vernalización en Canola de Invierno (*Brassica napus*): uso del silenciamiento inducido por virus (VIGS) para acelerar la ganancia genética

Flowering Without Vernalization in Winter Canola (*Brassica napus*): use of Virus-Induced Gene Silencing (VIGS) to accelerate genetic gain

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Resumen

Ciclos de reproducción cortos y la oportunidad de incrementar la ganancia genética, junto con el estudio de las bases moleculares de la vernalización, son áreas esenciales de investigación dentro de la biología de plantas. Varios métodos se han empleado para lograr el silenciamiento génico en plantas, pero ninguno reportado a la fecha para canola (*Brassica napus),* y en particular para inducir la floración sin vernalización en líneas de invierno a través del uso de secuencias sentido de DNA en vectores diseñados para el silenciamiento génico inducido por virus (VIGS). La presente investigación provee los métodos para transitoriamente regular a la baja, por medio de VIGS, genes de la vernalización en plantas anuales de invierno, específicamente la familia de genes de *Flowering Locus C* (*FLC*) en canola de invierno *(BnFLC1* a *BnFLC5).* La regulación a la baja de estos genes permite a las plantas anuales de invierno florecer sin vernalización y, consecuentemente, provee los medios para acelerar la ganancia genética. El sistema de silenciamiento propuesto puede ser utilizado para regular a la baja familias de genes, para determinar la función génica, y para inducir la floración sin la vernalización en líneas de invierno tanto del género *Brassica* como de muchos cultivos importantes de invierno.

Palabras claves: vernalización, silenciamiento inducido por virus, *Flowering Locus C, Brassica*.

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Abstract

Shorter breeding cycles and the opportunity for enhanced genetic gains, together with the study of the molecular basis of vernalization, are essential areas of research in plant biology. Several approaches have been employed to achieve gene silencing in plants, but none so far reported in canola (*Brassica napus),* and particularly to induce flowering without vernalization in true winter lines by using sense DNA sequences in virus-induced gene silencing (VIGS) vectors. The present research provides the methods to transiently down-regulate, by VIGS technology, vernalization genes in winter annuals, specifically the family of *Flowering Locus C* (*FLC*) genes in winter canola *(BnFLC1 to BnFLC5).* Down-regulation of the *BnFLC* genes allows winter annuals to

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flower without vernalization and consequently provides the means for enhanced genetic gains. The proposed silencing system can be used to down-regulate gene families, to determine gene function, and to induce flowering without vernalization in winter *Brassica* lines as well as in many important winter crops.

Keywords: vernalization, virus-induced gene silencing, *Flowering Locus C*, *Brassica*.

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Introducción:

Vernalization is the subjection of seeds or seedlings to low temperature in order to hasten plant development and flowering. Vernalization is commonly required for winter annuals such as winter *Brassicas* and winter wheat. It is believed that seeds and buds of many plants require cold in order to break dormancy and switch from vegetative to reproductive growth (flowering). This mechanism ensures that plants flower during the warmer period of spring or summer. However, from a breeding perspective, the requirement for vernalization is a major impediment in accelerating the rate of genetic gain since the number of breeding cycles per year is restricted.

It has been reported that in winter annual ecotypes of *Arabidopsis thaliana*, the level of *FLOWERING LOCUS C* (*FLC*) activity is proportional to the lateness to flower, that is, loss of function or down-regulation of the *FLC* gene promotes flowering, while over-expression of *FLC* delays flowering (Michaels and Amasino, 1999; Sheldon et. al., 1999). Three additional genes of the vernalization pathway have been identified and cloned recently: *VIN3* (*VERNALIZATION-INSENSITIVE 3*), and *VRN1* and *VRN2* (*VERNALIZATION 1 and 2*) (Wood et al., 2006; Bastow et al., 2004; Sung and Amasino, 2004). *VIN3* is the first gene activated by vernalization. *VIN3* expression is necessary for de-acetylation of *FLC*, which in turn leads to histone methylation and the formation of mitotically stable heterochromatin at the *FLC* chromatin site by a process involving *VRN1* and *VRN2*. *VRN1* and *VRN2* are expressed constitutively before, during and after vernalization. It is believed that they function downstream of *VIN3* and maintain stability of the transiently acting complex that leads to *FLC* chromatin silencing and hence *FLC* repression (Sung and Amasino 2004).

In Arabidopsis, the difference between spring and winter growth habit is largely explained by molecular variation at the *FRIGIDA* (*FRI)* and *FLC* loci, while other genes are identified contributing to the annual and biennial behavior in this species (Werner et al. 2005). In winter canola, five *FLC* genes have been isolated, *BnFLC1 to BnFLC5* (Tadege et al., 2001). The five *FLC* genes from canola and the Arabidopsis *FLC* gene (*AtFLC*) have similar functions**.** Each of the five *BnFLC* genes can function to repress flowering in transgenic Arabidopsis. In addition, spring canola was delayed in flowering when transformed with the *AtFLC* gene (Tadege et al., 2001). All these *FLC* genes belong to a large multigene family of MADS-box transcription

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factors. Taken together, all scientific evidence has shown that the *FLC* gene is a key player for delayed flowering and vernalization requirement in winter annuals.

Gene down-regulation is known to occur in plants using anti-sense technology or co-suppression. More recently, virus-induced gene silencing (VIGS) has been used to down-regulate gene expression (Robertson, 2004). This technology utilizes plant viruses to express a small fragment of a host target gene in inoculated plants. The replication of the virus vector which includes the small fragment of the host target gene induces a host response that knocks down expression of the endogenous target gene. The fragment of the host target gene on the viral vector must share a certain degree of identity or complementarity to the target sequence in order for the silencing to occur. The target sequence may be native or transgenic (Turnage et al., 2002). It has been suggested that the mechanism involved is post-transcriptional and targets RNA molecules in a sequence-specific manner (Smith et al., 1994). Further, the fact that viruses can both cause and be the targets of gene silencing has suggested that the mechanism is associated with anti-viral plant defense mechanisms (Pruss et al., 1997). VIGS can be activated in virally infected plants when a gene, part of a gene, or its RNA is perceived as part of a virus genome or transcript. Additional, a portion of the viral genome can be sufficient to induce VIGS.

Geminiviruses are single-stranded DNA viruses that replicate through double-stranded DNA intermediates using the plant DNA replication machinery. Geminiviruses form a large family of plant viruses and are able to infect members of the *Brassicaceae* family. Cabbage Leaf Curl Virus (CaLCuV) is a bipartite geminivirus having single stranded DNA and infects *Arabidopsis* and *Brassica* species among others (Turnage et al., 2002). Geminiviruses replicate in the cell nucleus of the host, and foreign DNA can be stably integrated into the viral genome without significantly affecting replication or movement. The geminiviruses rely primarily on the machinery of the host to copy their genomes and express their genes. Most geminiviruses that infect dicot hosts are transmitted by the white fly and possess a bipartite genome comprising similarly sized DNA components (termed A and B). In bipartite genomes, the A component contains viral information necessary for the replication and encapsidation of viral DNA, while the B component encodes functions required for movement of the virus through the infected plant (Turnage et al., 2002).

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Brassica is an increasingly important crop. The most common cultivars of *Brassica* in the developed world are so-called "double-low" varieties: those varieties low in erucic acid in the oil and low in glucosinolates in the solid meal remaining after oil extraction. These higher quality forms of *Brassica*, first developed in Canada, are known as canola (Canola Council of Canada). There are primarily three commercial species of *Brassica*: *B. napus*, *B. rapa* and *B. juncea*. Within *B. napus* (canola), there are two sub-types: winter and spring varieties. The winter varieties are typically planted in the fall and undergo approximately 12 to 14 weeks of vernalization at approximately 4 to 10^{o}C prior to flowering.

We report here the use of VIGS technology to transiently down-regulate vernalization genes in winter genotypes to induce the transition to flowering without the vernalization requirement normally associated with winter lines. In particular, we show that flowering without vernalization is achieved in true winter genotypes of canola by sense down-regulation, through VIGS technology, of the family of vernalization *BnFLC* genes. The experiments described in this research were done by using DNA sense sequences that were cloned into the viral genome, to create the VIGS vectors. We are the first to show the successful use of VIGS technology in *Brassicas* as well as the first to demonstrate flowering without vernalization in true winter genotypes of canola. The ability to promote flowering by transiently silencing gene expression in canola has numerous implications in research and in agriculture.

Experimental Procedures

DNA isolation.

Isolation of genomic DNA from plant tissue was performed according to the CTAB protocol (Allen, et al. 2006).

RNA isolation.

Total RNA isolation was performed with the Trizol reagent (INVITROGEN), following the manufacturer's instructions.

Reverse Transcriptase (RT)-PCR reactions.

RT reactions were performed with 5 μg of total RNA in the presence of the MMLV-RT enzyme (INVITROGEN), following the manufacturer's instructions. To determine the optimum number

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of PCR cycles for semi-quantitative PCR, first we used a program with normal temperatures and times for the same primers that amplify the transcript of interest. We removed one set of tubes after 20, 25, and 30 cycles, then we ran 5ul of the different cycle samples on a 1% agarose gel and determined that the increase in the amount of amplicons stays exponential only for 25 cycles, after which the amplification rate reached a plateau. Thus, PCR conditions were as follow: 95^{o}C for 5', followed by 25 cycles for 30 secs at 95^{o}C, 30 secs at 58^{o}C, 30 secs at 72^{o}C, and a final cycle of 5' at 72^{o}C.

Cloning.

Plasmids containing the cloned CaLCuV viral DNA genome (A and B components, GenBank accession numbers U65529 and U65530) were provided by the University of Florida as pUC19 derivative vectors (Figure 1a) (Abouzid, et al, 1992). Specific primers were designed to amplify by RT-PCR the canola *FLC* cDNA's (GenBank accessions: *BnFLC1* AY036888, *BnFLC2* AY036889, *BnFLC3* AY036890, *BnFLC4* AY36891, *BnFLC5* AY036892), and to clone in a sense orientation only, the different *FLC* gene fragments from canola winter line "Express". The cloning primers used for each gene fragment are: *FLC1* F: gccagatggagaagagtaatctt, R: gagccggagagagagtatagattat; *FLC2* F: ctagccagatggagaagaataatc, R: gatatacaacgttcacccttatagg; *FLC3* F: gctgaaagaagagaatcaggc, R: ctcagccaagggagtattgag; *FLC4* F: ctagccagatggagaagaataatc, R: aagagagtgtgaagatatacaacgc; *FLC5* F: ccgaagctgataatatagagatgtc, R: gggttaaactgacataggttatttg. The primers cover the 3'-end and the 3'-UTR region of each gene. These amplified fragments, named *BnFLC1* to *BnFLC5*, were cloned into the pUC19 derivative vectors as described below. Also, a consensus sequence from the *BnFLC1 to 5* genes was identified, PCR amplified and cloned in the same vector (Figure 1b).

The cloning strategy was as follows: the *FLC1* gene fragment was PCR amplified and cloned into pBSIIKS+ at the EcoRI/HindIII sites. The SmaI/HincII fragment from this modified vector (named pBSIIKS+plus-*FLC1*), was removed and cloned into the vector provided by the U. of Florida, CaLCuV-A component vector, at the HincII/ScaI sites to generate CaLCuV-A+*FLC1*. This step replaced the coat protein with the FLC1 fragment (Figure 1c). Removing the coat protein gene reduced the likelihood that the virus could be transmitted by whiteflies (Robertson, 2004). Then, a PstI/HindIII fragment from the CaLCuV-A+*FLC1* vector was removed, and the

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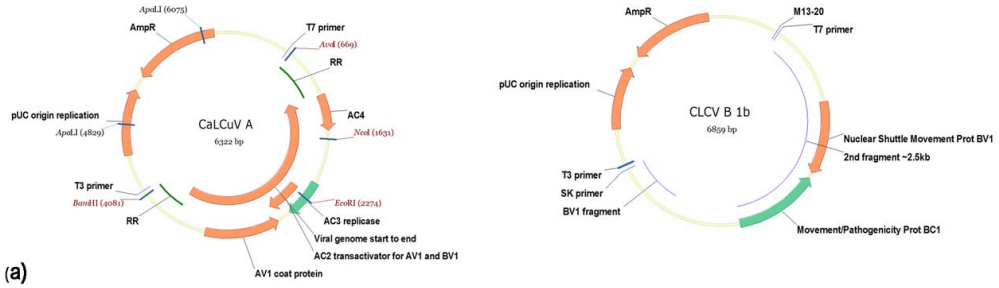
remaining PCR-amplified cDNA *BnFLC* fragments were inserted at the PstI/HindIII sites of the CaLCuV-A+*FLC1* vector (except for the *BnFLC3* fragment) in order to generate a total of four more different vectors. The plasmid containing *FLC5* is shown in vector map in Figure 1c. For the *BnFLC3* gene, the cDNA fragment was PCR amplified and cloned into the PstI/ClaI sites of the CaLCuV-A+*FLC1* vector.

For the *BnFLC-consensus*, the consensus sequence with certain degree of complementarity to all five *FLC* genes was recognized and cloned. This region was selected because it has no similarity or domains that are shared with any other known genes and because it was unique to the *Brassica FLC* gene family. In this way, silencing multiple *FLC* genes at once can be achieved (Figure 1b).

The consensus sequence was identified, divided and synthesized into two long oligonucleotides (*ConFLC*-LongF, 5':gccctctccgtaactagagctaggaagacagaactaatgttgaagcttgttgatagcc-3'; *ConFLC*-LongR, 5':tggttctcttctttcagcaaattctccttttctttgaggctatcaacaagcttcaacattagttctg-3'). A modified protocol described in Holowachuck and Ruhoff (1995) was used to clone the consensus sequence. The full consensus sequence was synthesized by an initial overlap annealing of single-stranded long oligonucleotides that span the length of the designed sequence. Then, the assembly/extension or 'fill-in' of the overlapping oligonucleotides was performed with the Taq Polymerase, as well as the selective amplification of full-sized gene product in the presence of short terminal oligonucleotide primers (Consensus FLC-F:5'-ctgcagGCCCTCTCCGTAACTAGAGC-3'; Consensus FLC-R: 5'-atcgatTGGTTCTCTTCTTTCAGCAA-3'). The short terminal oligonucleotide primers contain the PstI/ClaI restriction sites and the sequence was cloned at the PstI/ClaI sites of the CaLCuV-A+*FLC1* vector to generate the CaLCuV-A plus *FLC-Consensus* vector (Figure 1d).

The Phytoene Desaturase, *BnPDS*, gene (GenBank #CD827969) was used as an internal control to test the efficiency of infection and silencing. The PCR primers to clone the *PDS* fragment into the PstI/ClaI sites of the CaLCuV-A+*FLC1* vector were: (*PDS*F: 5'-gatataccaaggccagagctaga-3'; *PDS*R: 5'-tcccaagttctccaaataagttc-3') (Figure 1d).



(a)

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                                525                                 574
AY036888 BnFLC1  (446) GCCCTCTCCGTAACAAGAGCTAGGAAGACAGAACTAATGTTGAAGCTTGT
AY036889 BnFLC2  (409) GCCCTCTCCGTAACTAGAGCTAGGAAGACAGAACTAATGTTGAAGCTTGT
AY036891 BnFLC4  (522) GCCCTCTCCGTAACTAGAGCTAGGAAGACAGAACTATTGTTGAAGCTTGT
AY036890 BnFLC3  (424) GCCCTCTCTCTAACTAGAGCTAGGAAGACAGAACTAATGTTGAAGCTTGT
AY036892 BnFLC5  (438) GCCCTCTCTGTAACTAGAGCTCGGAAGGCAGAACTAATGTTAAAGCTTGT
Consensus        (525) GCCCTCTCCGTAACTAGAGCTAGGAAGACAGAACTAATGTTGAAGCTTGT
                                575                                 619
AY036888 BnFLC1  (496) CGAGAACCTTAAAGAAAAGGAGAAGTTGCTGGAAGAGGAGAACCA
AY036889 BnFLC2  (459) TGATAGCCTCAAAGAAAAGGAGAAATTGCTGAAAGAAGAGAACCA
AY036891 BnFLC4  (572) TGATAGCCTCAAAGAAAAGGAGAAATTGCTGAAAGAAGAGAACCA
AY036890 BnFLC3  (474) TGATAGCCTCAAAGAAAAGGAGAAGCTGCTGAAAGAAGAGAATCA
AY036892 BnFLC5  (488) TGAAAGTCTCAAAGAAAAGGAGAATCTGCTGAAAGAAGAGAACCA
Consensus        (575) TGATAGCCTCAAAGAAAAGGAGAAtTTGCTGAAAGAAGAGAACCA
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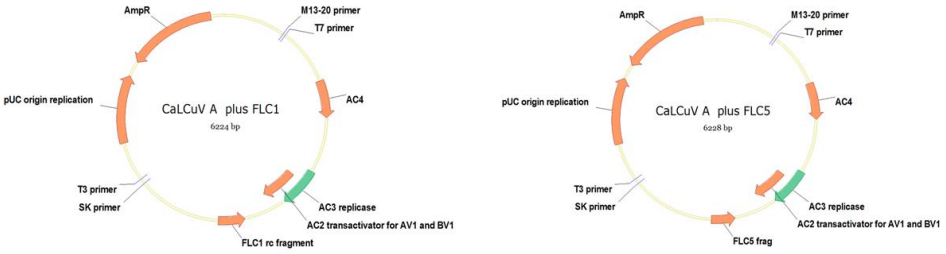
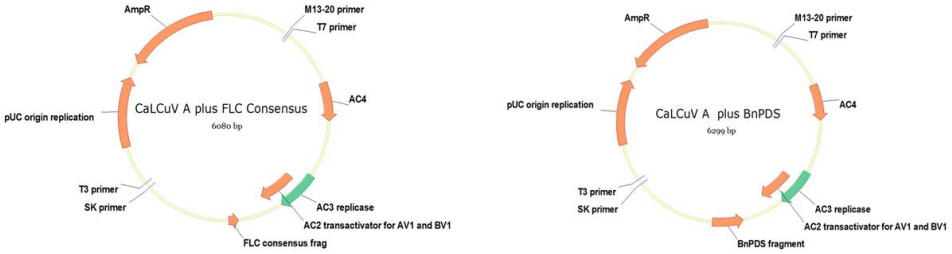
***BnFLC*-Consensus sequence**

GCCCTCTCCGTAACTAGAGCTAGGAAGACAGAACTAATGTTGAAGCTTGTTGATAGCCTCAAAG
AAAAGGAGAATTTGCTGAAAGAAGAGAACCA

(b)



(c)



(d)

Figure 1. Plasmid maps: (a) Plasmids containing the Cabbage Leaf Curl Virus DNA genome (A and B components). (b) Canola *FLC* consensus DNA sequence. The sequences in blue in the multiple sequence alignment correspond to the short terminal oligonucleotide primers. Numbers correspond to the position of the sequence inside the gene. (c) *CaLCuV-A+FLC1* and *CaLCuV-A+FLC5* VIGS vectors. (d) *CaLCuV-A+FLC-Consensus* and *CaLCuV-A+PDS* VIGS vectors.

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Plant Growth.

Canola seeds were surface sterilized for 1 minute in 70% ethanol, followed by 15 minutes in a 2 % sodium hypochlorite solution. Seeds were rinsed with de-ionized water and then plated onto solid germination B5+GA+2% SUCROSE media and placed in environmentally controlled growth chambers for 16 hours of light at 22 ^{o}C and 8 hours darkness at 18 ^{o}C. (Table 1). Plants were used for biolistic transformation with the VIGS vectors.

Recipe for B5 + GA + 2% Sucrose

B5 10x Stock (use 100ml/L of B5 10x stock to make 1L B5 media)

Stock Ingredients	**4 L**
Potassium Nitrate (KNO_3)	100.0 g
Magnesium Sulfate ($MgSO_4$-$7H_2O$)	10.00 g
Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$)	30.00g
Ammonium Sulphate ($(NH4)_2SO_4$	5.36 g
Sodium Phosphate Monobasic (NaH_2PO4-H_2O)	6.00 g
Sequestrene, 330 (10% Iron Chelate)	1.6 g

Bring up the volume to 4 L with filtered water.

B5 + GA + 2% SUCROSE

MEDIA INGREDIENTS	**2.5 L**
B5 10x stock	250ml
Sucrose (2 %)	50 g
Gibberellic Acid (0.1 mg/L)	250 ul (stock: 0.02g /20 ml EtOH = 1mg/ml)
Agar (Sigma # A1296)	15 g

pH sol'n to 5.8 and bring up the volume to 2.5 L with filtered water.

Autoclave and then pour 35 ml per plate.

Table 1. Recipe for B5 + GA + 2% Sucrose media.

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Plant transformation.

Winter canola was transformed by particle bombardment as described by Tulsieram et al., (2001). However, instead of bombarding microspores, seedlings were bombarded. Seeds from the winter line 'Express' were germinated on B5+GA+2% SUCROSE media. Approximately two weeks after germination, the seedlings were bombarded with the above mentioned vectors. The concentration of DNA used in the bombardment was 3 pg/bp/preparation. The actual concentration of DNA was dependant on the DNA fragment length. The height of the shelf was 20 cm. The distance between particles and plant tissue was 8 cm. The metal particles were gold (0.6 µm diameter) and the pressure was 900psi. After bombardment, the seedlings were kept one more week on the media plates, and then transferred to soil and placed in environmentally controlled growth chambers for 16 hours of light at 22 ^{o}C and 8 hours darkness at 18 ^{o}C. The seedlings were watered daily and fertilized every other day. Plants were never placed under vernalization conditions (at 4^{o}C). Vector combinations used for plant transformation were: (a) CaLCuV-A+*FLC1* plus CaLCuV-B component (CaLCuV-A+FLC1 comprises the FLC1 gene fragment); (b) CaLCuV-A+*FLC 2 to FLC5* plus CaLCuV-B (4 different combinations, one for each of *FLC2*, *FLC3*, *FLC4*, and *FLC5*); (c) CaLCuV-A+*FLC-Consensus* plus CaLCuV-B; (d) CaLCuV-A+*PDS* + CaLCuV-B.

Testing viral movement and gene down-regulation.

After biolistic transformation, and in order to determine the presence and dispersion of viral DNA A and B components, the following PCR primers were designed to test for viral movement: CLCV-BGenF: 5'-ggatctaccacgatatctaataggc-3'; CLCV-BGenR: 5'-acagagttagcgacacaaatgtg-3'; CLCV-AGenF: 5'-aataaagacgacgtctaccacaac-3'; CLCV-AGenR: 5'-tcttgtgctgtgctttgatagag-3'. The CLCV-BGenF and CLCV-BGenR primers target the B component, from the BV1 gene to the BC1 gene. That is, they span both genes. The CLCV-AGenF and CLCV-AGenR primers target the A component, from the AC4 gene to the AC3 gene. They span both genes. Also, in order to determine *BnFLC* gene down-regulation after plant transformation, the following primers were used: *BnFLC1F* 5'-gccagatggagaagagtaatctt-3'; *BnFLC1R* 5'-gagccggagagagagtatagattat-3' *BnFLC2F* 5'-ctagccagatggagaagaataatc-3'; *BnFLC2R* 5'-gatatacaacgttcacccttatagg-3'; *BnFLC3F* 5'-gctgaaagaagagaatcaggc-3'; *BnFLC3R* 5'-ctcagccaagggagtattgag-3'; *BnFLC4F* 5'-ctagccagatggagaagaataatc-3'; *BnFLC4R* 5'-

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aagagagtgtgaagatatacaacgc-3'; *BnFLC5F* 5'-ccgaagctgataatatagagatgtc-3'; *BnFLC5R* 5'-gggttaaactgacataggttatttg-3'; *PDSF* 5'-gataccaaggccagagctaga-3'; *PDSR* 5'-tcccaagttctccaaataagttc. The conditions for PCR were as follow: an initial cycle at 95°C for 5', followed by 32 cycles for 30 seconds at 95°C, 30 seconds at 58°C, 40 seconds at 72°C, and a final cycle of 5' at 72°C. Both, leaves and floral buds were assayed.

Densitometric analysis.

Band intensities from the agarose gels were quantified using ImageQuant™ software. Intensities were normalized versus gene expression of untransformed plants. Quantified band intensities plotted as percentage of the untransformed plants (taken as 100%) are shown as histograms. The *ACTIN* gene served as an internal control for RNA quantity in RT-PCR (data not shown).

Results.

Gene down-regulation and flowering phenotype in non-vernalized plants.

Several approaches have been employed to achieve gene silencing in *Brassica spp.*, but none using VIGS vectors. Here, with the use of biolistics and VIGS technology, we developed a silencing system in *Brassica* to determine gene function and to induce flowering without vernalization in winter *Brassica* lines. Canola seeds were surface sterilized and germinated on solid B5+GA media-containing plates in long day conditions (16 hours of light at 22°C and 8 hours darkness at 18°C). In a first biolistic transformation experiment, independent mixtures consisting of the Cabbage Leaf Curl Virus-B vector (viral DNA genome B component, CaLCuV-B) and each one of the rest of the VIGS constructs (viral DNA genome A component, CaLCuV-A) containing the different *BnFLC* genes (CaLCuV-A+*FLC1*, CaLCuV-A+*FLC2*, CaLCuV-A+*FLC3*, CaLCuV-A+*FLC4*, CaLCuV-A+*FLC5*), a consensus *BnFLC* sequence (CaLCuV-A+*FLC-Consensus*), and the *PDS* gene as control (CaLCuV-A+*PDS*), were used to transform 17-days old seedlings. Initially, 12 seedlings were transformed with each vector combination (seven combinations overall; 84 plants overall). Following transformation, the plants were maintained for 10 days on Petri dishes in long day conditions and then transferred to soil. After one week in soil, plants transformed with the control *PDS* containing-vector (CaLCuV-A+*PDS*) started to develop a visible phenotype. That is, forty percent of the PDS transformed plants developed yellow and/or chlorotic areas indicating *PDS* down-regulation (see Turnage et al., 2002).

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However, because down-regulation is not uniform, the phenotypes varied, as expected. Three plants transformed with the *BnFLC5* VIGS vector developed a mild phenotype as a result of the viral infection (Figure 2a). None of the other sets of transformed plants developed a visible phenotype.

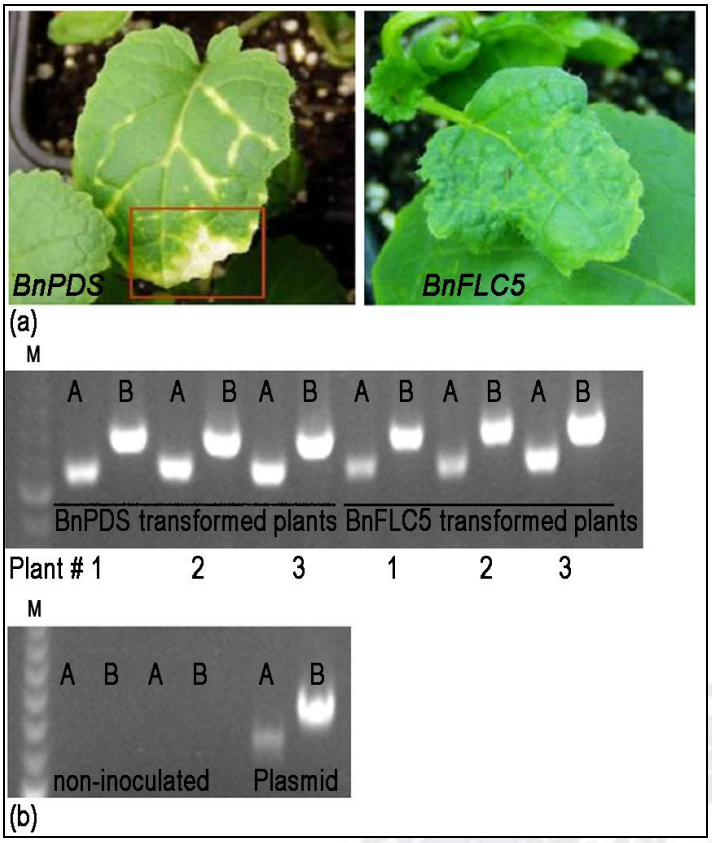

Figure 2. Test for viral movement. (a) Phenotypes of plants transformed with the *BnPDS* and *BnFLC5* VIGS vectors, one week post-transformation. (b) PCR reactions to determine systemic spreading of viral DNA in new tissue, 21 days post-transformation. A= viral DNA A component; B= viral DNA B component; M= 1kb DNA marker

In order to confirm the presence and spreading of the viral particles, primers designed to test for viral movement were used to determine if the viral DNA (A and B components) was present and spreading systemically. Samples were taken from new leaves of non-vernalized plants (three weeks post bombardment) and PCR reactions were performed. The results indicated that the viral DNA was present and that it was systemically spreading into new tissue (Figure 2b). The identity of all PCR products was confirmed by sequencing (not shown). Those two sets of plants having shown systemic spreading of the virus also showed gene down-regulation when tested by RT-PCR. In the case of the *BnPDS* transformed plants, the down-regulation of the endogenous

gene was 70% (by densitometry analysis). In the case of the *BnFLC5* transformed plants, the down-regulation of the endogenous *FLC5* was from 40 to 75% in 7.5-weeks old plants (5.5 weeks after transformation). No change in *BnFLC1-to-5* expression was detected in the *PDS* transformed plants. Down-regulation of *BnFLC1-to-4* was not significant in the *BnFLC5* transformed plants.

After 8 weeks post-transformation (10-weeks old plants), none of the plants had flowered (Figure 3a and 3b). The plants were left in the growth chambers (non-vernalization conditions) and the phenotype documented. After 10 weeks post-transformation (12-weeks old plants), a *BnFLC-Consensus* transformed plant began to flower (Figure 3c). In order to determine if the flowering phenotype was associated with the presence of the viral DNA and its systemic spreading, a series of PCR reactions (just as before) were performed in different plant tissues to detect the viral A and B components. Floral buds, rosette leaves, and cauline leaves were assayed. The results showed that the viral DNA was more abundant in leaves of the *BnFLC-Consensus* transformed plant than in floral buds, where it could not be detected. This is expected, as CaLCuV has been shown not to infect meristematic tissue (Peele, et al., 2001; Corbesier et al., 2007). This result indicated that the virus was present and that it was spreading throughout the *BnFLC-Consensus* transformed plant.

(a)

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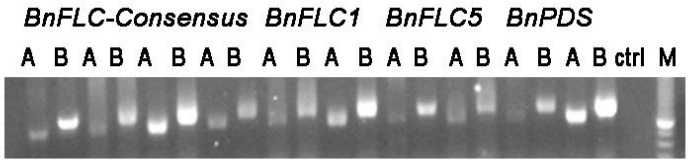
Figure 3. Phenotypes of non-vernalized plants post-transformation. (a) Phenotype of plants transformed with the VIGS *BnPDS* vector, 8 weeks post-transformation. (b) Phenotype of plants transformed with the VIGS *BnFLC5* vector, 8 weeks post-transformation. (c) Phenotype of VIGS *BnFLC-Consensus* transformed plant 10-weeks post-transformation.

Next, *BnFLC1-to-5* gene expression was determined by RT-PCR, in the *BnFLC-Consensus* transformed plant. *BnFLC2-5* gene expression was down-regulated in floral buds and rosette leaves of the *FLC-Consensus* transformed plant, when compared to control plants (from 77.5% to 9%). *BnFLC1-5* gene expression was down-regulated to a greater extent in leaves, having the greatest down-regulation the *BnFLC3* in cauline leaves (77.5%). These results suggest that in winter canola, flowering without vernalization is achieved by partial down-regulation of multiple *BnFLC* genes at once.

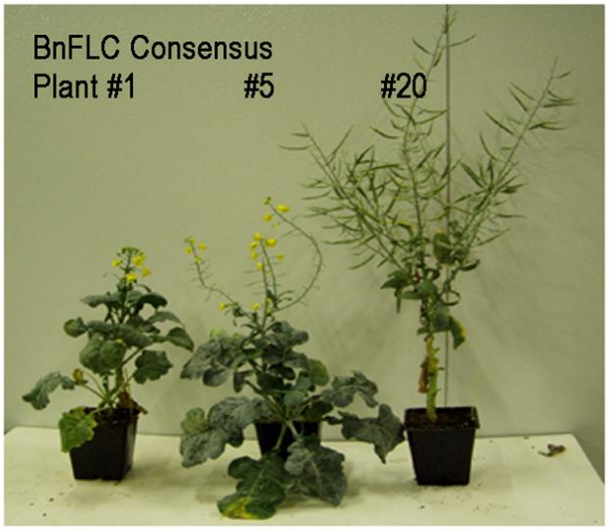
In order to corroborate all previous results, the biolistic transformation experiments were repeated with the same sense DNA vectors. Twenty-eight seedlings were transformed for each set of constructs (seven combinations overall) using the same biolistic method (Figure 4a). The plants were tested for the systemic spreading of the viral DNA in order to correlate flowering with gene down-regulation. The PCR results indicated that the virus was present in new tissue and replicating systemically (Figure 4b). After 9-weeks post-transformation (11-weeks old plants) with the *BnFLC-Consensus* vector, 20% of the plants (ConsB#1, ConsB#5, ConsB#16 and ConsB#20) flowered (Figure 4c), indicating that flowering in the canola winter line was achieved

by partial down-regulation of multiple *BnFLC* genes. Figure 4d shows the phenotype of a *BnFLC5* transformed plant and a *BnFLC-Consensus* transformed plant.

(a)



(b)



(c)

(d)

Figure 4. Second biolistic transformation. (a) Phenotypes of non-vernalized plants, one week post-transformation. (b) PCR reactions to determine spreading of viral DNA in new leaves from VIGS transformed plants; *BnFLC-Consensus*, *BnFLC1*, *BnFLC5*, *BnPDS vectors* (A = viral DNA, A component; B= viral DNA, B component; M= 1kb DNA marker; ctrl= control reaction in non-transformed plant). (c) Flowering phenotype of non-vernalized true winter lines, 9-weeks post-transformation with the *BnFLC-Consensus* VIGS vector. (d) Comparison in flowering of a *BnFLC5* transformed plant (plant on the left) and a *BnFLC-Consensus* transformed plant (plant on the right) 9-weeks post-transformation.

The timing for flowering is comparable to that of spring canola. None of the *BnFLC1-to-5* transformed plants flowered after 24 weeks post-transformation. Table 2 shows the results from the densitometric analysis of *FLC1-5* gene expression in *BnFLC Consensus* transformed plants as determined by semi-quantitative RT-PCR. Leaves (data not shown) and floral buds were assayed. Gene down-regulation in floral buds varies from, for example, 67% for the *FLC3* gene in plant #1, to 0% for the *FLC1* gene in plant #5. These results confirm that in winter canola, flowering without vernalization can only be achieved only by partial down-regulation of multiple *BnFLC* genes at once. Taken together, these findings demonstrated that winter lines were induced to flower without vernalization under similar conditions as those used for spring lines.

Gene / Plant#	*BnFLC1*	*BnFLC2*	*BnFLC3*	*BnFLC4*	*BnFLC5*
Cons#1	72	73	33	57	48
Cons#5	100	89	62	67	38
Cons#16	82	79	68	67	66
Cons#20	67	74	64	57	57

Table 2. Densitometric analysis in floral buds of *BnFLC1-5* gene expression in *BnFLCConsensus* transformed plants as determined by RT-PCR. Values are expressed as % of gene expression when compared to untransformed plants (100% expression). Only those plants that flowered after transformation (without vernalization) are shown.

Confirmation of no viral DNA integration into the plant genome.

To determine whether viral DNA was integrated into the plant genome during the biolistic transformation, sixteen T1 plants from the T0 winter *BnFLC-Consensus* transformed plants that flowered without vernalization were investigated. Plant material from four-week old seedlings was collected and analyzed. After isolating genomic DNA and performing PCR reactions, none of the T1 plants contained viral DNA (A or B components; Figure 5). In addition, none of these T1 plants flowered after six months, and no *FLC1-5* gene down-regulation was occurring in the T1 generation. These results confirm that the viral DNA was not integrated into the meristematic cells or germ line, and that it was not being transmitted to the next generation (in agreement with Peele et al., 2001).

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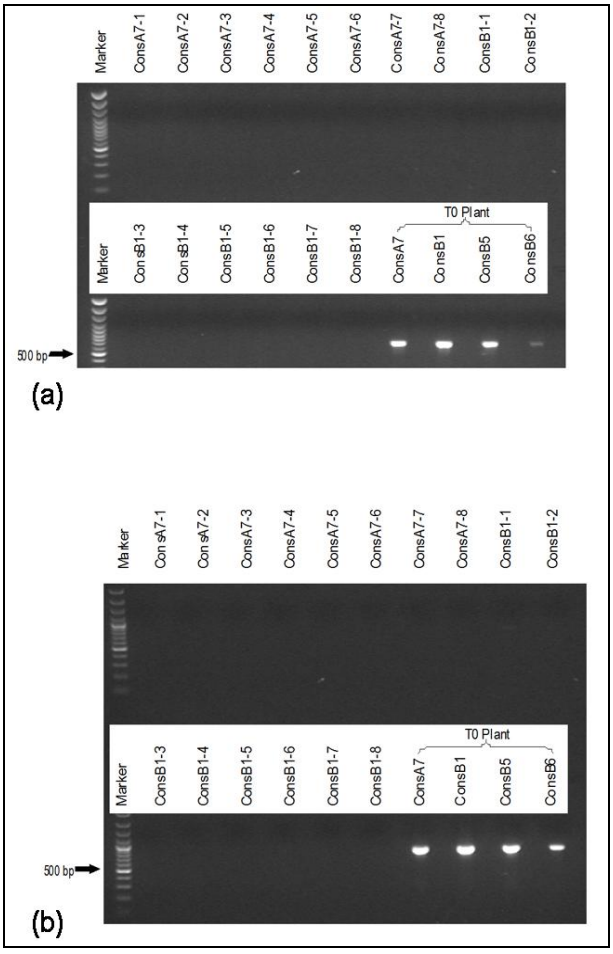

Figure 5. Absence of A and B Components integration in genome of T1 progeny of plants transformed with *BnFLC-Consensus* vector. (a) PCR reactions in T1 progeny to identify viral A component. (b) PCR reactions in T1 progeny to identify viral B component. Four-week old T1 seedlings were collected and analyzed.

Discussion.

Winter canola is the dominant oilseed in the European Union, where the market place is transitioning from open-pollinated to hybrid varieties. Thus, the application of new technologies that speed up the development of superior inbred and hybrids will provide a competitive advantage for farmers and biotechnology companies. As in most breeding programs, the biggest advantage in winter canola breeding is derived from increasing the rate of genetic gain for yield. To this end, efforts are being focused on reducing the length of the breeding cycle through the use of doubled haploids and marker-assisted selection. Non-vernalized crops such as spring canola, soybean and corn maximize annual genetic gain by using alternate season nurseries and greenhouses. Unfortunately, winter canola cannot take advantage of these opportunities because

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every generation requires a cold treatment (4^0C) for 12 -14 weeks to induce flowering. Reduction or elimination of the cold treatment requirement could cut the generation time in half, reducing cycle length and significantly increasing genetic gain over the long term. For example, in order to breed one cycle of a winter canola line, a full 9 -10 months are required. In comparison, a spring canola plant can complete a life cycle in 13-20 weeks, and it is not uncommon to grow three spring generations in one year, with the use of winter nurseries and greenhouses during the cold season.

VIGS has not been shown in *Brassica* prior to this research. Our study provides strong evidence that eliminating the requirement for vernalization in a winter crop can be achieved through VIGS by partial down-regulation at once of multiple genes. This might suggest the possibility of gene redundancy within the *BnFLC* gene family. VIGS technology is based on an RNA-mediated antiviral defense mechanism which makes use of the silencing machinery that regulates gene expression by the specific degradation of double stranded RNA into short RNA molecules (Ruiz et al., 1998). Using viral vectors to silence an endogenous plant gene, as we have shown here, involves cloning into the viral genome, without significantly compromising viral replication and movement, a nucleotide fragment sharing a certain percentage identity or complementarity to the endogenous plant gene(s) (Dinesh-Kumar et al. 2003; Lu et al., 2003). In the present research, the viral genome includes all genes except those encoding the coat protein.

VIGS technology, through the down-regulation of vernalization genes, was used herein to: (i) eliminate the long periods of vernalization, (ii) increase the number of breeding cycles per year to accelerate genetic recombination and (iii) increase the rate of genetic gain. This is important because it will reduce the length of time necessary to breed winter plants, which currently is a very lengthy and costly process. Thus, flowering in winter canola was achieved without vernalization by simultaneous down-regulation of multiple *FLC* genes (*BnFLC1-5*) through VIGS technology.

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