



A gene expression screen identifies *EARLII* as a novel vernalization-responsive gene in *Arabidopsis thaliana*

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Abstract

Vernalization promotes early flowering in late ecotypes of *Arabidopsis thaliana*. The mechanisms of vernalization are poorly understood. A subtractive hybridization approach was used to isolate vernalization-responsive genes from a late-flowering ecotype of *Arabidopsis thaliana* based on the premise that transcript levels of such genes would increase with cold treatment and remain high even after removal of the vernalization stimulus. *EARLII* is the first *Arabidopsis* gene shown to be stably activated by vernalization. The abundance of its RNA is progressively elevated by vernalization and remains high for at least 20 days at room temperature. The basal level of *EARLII* RNA is higher in early-flowering ecotypes, but is increased also after vernalization. Vernalization and subsequent growth in long-day photoperiods have an additive or synergistic effect on *EARLII* activation. *EARLII* RNA levels are also transiently induced by brief exposures to cold, but not to abscisic acid. *EARLII* is thus a novel vernalization-responsive gene in *Arabidopsis thaliana* that can be used to investigate vernalization-specific transcriptional regulation.

Introduction

The transition from vegetative to reproductive development is a critical stage in the life cycle of plants. The timing of this transition is carefully regulated to synchronize flowering with environmental conditions that ensure maximum survival of the next generation. Vernalization (prolonged exposure to low temperatures) during winter months followed by long-day photoperiods in the spring promote early flowering in many naturally occurring ecotypes of *Arabidopsis thaliana* (Martínez-Zapater and Somerville, 1990; Bagnall, 1993; Lee and Amasino, 1995). Seeds then germinate during the summer or fall, but plants remain vegetative until vernalization occurs again over the winter and induces synchronized flowering in the spring. Such *Arabidopsis* ecotypes are late-flowering without vernalization, because they contain dominant alleles of *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), which synergistically confer the strong requirement for vernalization to induce early flowering (Lee *et al.*,

1993, 1994; Clark and Dean, 1994; Koornneef *et al.*, 1994; Sanda *et al.*, 1997).

The transition to flowering in *Arabidopsis* is regulated by four interacting genetic pathways (Koornneef *et al.*, 1998; Levy and Dean, 1998; Piñeiro and Coupland, 1998; Simpson *et al.*, 1999). A floral repressor pathway is proposed to maintain the vegetative phase until the flowering promotion pathways initiate reproductive development (Martínez-Zapater *et al.*, 1994). These include the long-day, autonomous, and vernalization promotion pathways of flowering (Koornneef *et al.*, 1998; Levy and Dean, 1998; Simpson *et al.*, 1999). In the absence of dominant alleles of *FRI*, plants do not require vernalization and flower early due to activities of the autonomous and long-day promotion pathways. However, both dominant *FRI* and recessive mutations in genes of the autonomous promotion pathway appear to effect late flowering by increasing the level of *FLC* RNA, suggesting that *FLC*, a MADS box protein, may be a major repressor of early flowering in *Arabidopsis thaliana* (Sanda *et al.*,

1996; Michaels and Amasino, 1999; Sheldon *et al.*, 1999, 2000). Conversely, vernalization decreases the level of *FLC* RNA in late-flowering plants, which correlates with early flowering. We thus define *FLC* as the first member of a group of *bona fide* vernalization-responsive genes in *Arabidopsis thaliana*, whose expression responds negatively to vernalization. That is, *FLC* is silenced by vernalization and remains off even after the vernalization stimulus is removed.

This study describes the first example of another group of vernalization-responsive genes in *Arabidopsis thaliana*, those whose expression responds positively to vernalization. Because a drug- or transgene-mediated reduction of cytosine methylation partially substituted for vernalization in late-flowering plants, it was proposed that vernalization may activate genes by de-methylation of specific DNA sequences (Burn *et al.*, 1993; Dennis *et al.*, 1996, 1997; Finnegan *et al.*, 1998). Using a traditional subtractive hybridization approach, we were able to identify *EARLII* as a *bona fide* vernalization-responsive gene in *Arabidopsis thaliana*, because the gene is activated by vernalization and its RNA abundance remains high even after the vernalization stimulus is removed. We show here that the level of *EARLII* RNA is progressively elevated with increased length of vernalization and that it remains high for at least 20 days after vernalization. *EARLII* transcript levels are further increased when plants are grown in long-day photoperiods after vernalization. *EARLII* is also transiently induced by a short period of cold, but not by abscisic acid. Hence we define *EARLII* as a novel vernalization-responsive gene.

Materials and methods

Plant material and treatment

Early-flowering *Arabidopsis* ecotypes Columbia (Col-0) and Nossen (No-0) were obtained from ABRC (Ohio State University, OH) and Landsberg *erecta* (Ler) was kindly provided by T.-P. Sun (Duke University, NC). Late-flowering ecotypes containing dominant alleles of *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) in the Col-0 background were kindly provided by E. Himelblau and R. Amasino (University of Wisconsin-Madison, WI; Lee *et al.*, 1993). About 500 surface-sterilized seeds were grown *in vitro* in a sterile petri dish (9 cm diameter) on agar-solidified half-strength MS medium without sucrose (Murashige

and Skoog, 1962). Unless otherwise stated, all plates containing unvernallized control seeds were placed at 4 °C for 2 to 3 days to break seed dormancy, then grown for 7 days with a 8 h short-day photoperiod under cool fluorescent light and ca. 20 °C day/night temperature. Unless otherwise stated, seeds were vernalized on agar plates at 4 °C for 14 to 35 days in 8 h short-day photoperiods under cool fluorescent light, then grown for 7 days with a short-day photoperiod and 20 °C day/night temperature. For transient exposure to cold, seeds were grown after breakage of dormancy on agar medium for 7 days at 20 °C in an 8 h short-day photoperiod, then transferred overnight to 4 °C and then grown for 0 to 8 h at 20 °C. For transient exposure to abscisic acid (ABA), 10 ml of a 100 μM solution of ABA (Sigma) in 1% DMSO was added to plates with 7-day old seedlings grown in short-day photoperiods. Seedlings were incubated for 5 min, then the ABA was washed away. Plates were then left in the light for 2 to 8 h at 20 °C. Control plates were treated the same way with 1% DMSO lacking ABA.

RNA isolation

Unless otherwise stated, whole-seedling tissue was harvested by quick freezing in liquid N₂ and stored at -70 °C. RNA from vernalized and unvernallized tissues was isolated at the same time. The frozen tissue was ground to a fine powder on dry ice in a mortar and pestle with added liquid N₂. Total RNA was isolated by a modified miniprep procedure as described previously (Yeh *et al.*, 1990). Briefly, 100–500 mg of crushed tissue was resuspended in 1 ml of extraction buffer (7.5 M guanidine hydrochloride (Sigma), 25 mM sodium citrate (Sigma), 0.5% w/v sodium lauryl sarcosine (Sigma), 0.1 M 2-mercaptoethanol) and spun for 10 min at maximum speed in an Eppendorf centrifuge. The supernatant was extracted three times with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1), followed by one extraction with chloroform/isoamyl alcohol (24:1), and total RNA was precipitated at -20 °C in an equal volume of isopropanol and 0.1 volume of 3 M sodium acetate (Sigma). The pellet was washed in 75% v/v cold ethanol, vacuum-dried, and resuspended in 30–50 μl DEPC-treated water. Total RNA was quantified spectrophotometrically and stored in aliquots (20 μg) at -70 °C.

Subtractive hybridization and colony screening

Three rounds of reciprocal subtractive hybridization were performed as described previously (Wang and Brown, 1991; Cook *et al.*, 1995). Briefly, poly(A)⁺ mRNA from vernalized and unvernallized plants was selected by passage of total RNA through an oligo(dT) cellulose column from the Oligotex mRNA Mini Kit (Qiagen) and cDNA was synthesized with a cDNA synthesis kit as recommended by the supplier (Amersham). Double-stranded cDNA was dissolved in 20 μ l water and 8 μ l aliquots were digested with either *AluI* or *AluI* plus *RsaI* (American Allied Biochemical) and ligated to a duplex oligonucleotide containing one blunt end and a 4 base protruding 3' end. The duplex oligonucleotide was made by mixing the kinased 25-mer oligonucleotide 5'-TAGTCCGAATTCCAAGCAAGAGCACACA-3' with the 21-mer oligonucleotide 5'-CTCTTGCTTGAA-TTCGGACTA-3' (*EcoRI* sites in bold). The ligated cDNA was purified from unligated linker by electrophoresis through a 1.4% low-melting agarose gel. The region containing fragments of 150 bp to 1.5 kb was cut from the gel and stored at 4 °C. The gel slices were dissolved at 65 °C and 1 μ l was used for 100 μ l standard PCR reactions in 1 \times buffer (Promega) containing 1.25 mM MgCl₂, 1 μ g of the 21-mer primer, 200 μ M dNTPs, and 2.5 units of *Taq* polymerase (Promega). PCR-amplified driver cDNA (55 μ g) was cut with *EcoRI* and biotinylated twice with 50 μ g Photobiotin (Vector Labs) in an ice bath under a 275 W sunlamp (Vector Labs). For each round of subtractive hybridization, 1.25 μ g of tracer cDNA was denatured together with 25 μ g of biotinylated driver cDNA at 100 °C and hybridized for 20 h (long hybridization) in 1.5 M NaCl, 50 mM Hepes pH 7.3, 10 mM EDTA, 0.2% SDS in a 68 °C water bath. Biotinylated driver and tracer cDNA that was hybridized to driver cDNA was removed by repeated addition of 10–15 μ g streptavidin and phenol/chloroform extractions, then incubated for 2 h (short hybridization) with 12.5 μ g of biotinylated driver cDNA, followed by streptavidin/phenol/chloroform extractions. After each round, subtracted cDNA was PCR-amplified as described and used in the next round of subtractive hybridization. After three rounds, cDNA fragments were cloned into *EcoRI*-digested pBlueScript II plasmid (Stratagene).

A reverse northern hybridization method was used to specifically screen for cDNA fragments of genes that were induced or up-regulated by vernalization. In-

dividually cloned, subtracted cDNA fragments from either vernalized or unvernallized plants were PCR-amplified with pBlueScript plasmid vector-specific primers and slot-blotted (1 μ l) in duplicate onto nitrocellulose membranes using the Bio-Dot SF Microfiltration Apparatus (BioRad). Individual membranes containing subtracted cDNA fragments from vernalized and unvernallized plants were hybridized with first-strand cDNA made from RNA of either vernalized or unvernallized plants. This reverse northern first-strand cDNA probe was prepared in the presence of 10 μ Ci of α^{32} P-dATP using a cDNA synthesis kit (Amersham) as recommended by the supplier. Colonies that only hybridized to the cDNA probe made from RNA of vernalized plants were selected and used in RNA gel blot analyses.

RNA gel blot analysis and probes

About 20 μ g of total RNA was separated by electrophoresis in 1.2% formaldehyde gels containing ethidium bromide (Sambrook *et al.*, 1989). RNA was transferred onto nitrocellulose membranes and cross-linked with a Stratalinker (Stratagene). Prehybridization and hybridization was performed at 65 °C in 5 \times Denhardt's solution, 6 \times SSC, 0.5% SDS and 0.1 mg/ml denatured salmon sperm DNA. All probes were ³²P-labeled by the random primer method using the MegaPrime labeling kit as recommended by the supplier (Amersham), purified by G-50 spin columns, heat-denatured and hybridized at a concentration of 1 \times 10⁶ cpm/ml onto nitrocellulose membranes in a Hybaid oven (Labnet) at 65 °C for at least 16 h. Membranes were washed twice in 2 \times SSC/1% SDS for 5 min at room temperature, then in 0.2 \times SSC/0.1% SDS for at least 30 min at 65 °C. Membranes were briefly rinsed in 0.2 \times SSC before autoradiography.

Unless otherwise stated, *EARLII* probes were made from the 208 bp cDNA fragment isolated by subtractive hybridization (see Figure 2). A full-length *EARLII* probe was made from cDNA generated by RT-PCR with Superscript II (Gibco-BRL) and *Taq* polymerase (Promega) using primers 5'-ACTGAATGGATCCTTAAAACAAAC-3' and 5'-TAGCTGAATTCCTTCAAGCACATT-3'. *FLC* probes were made from cDNA lacking the conserved MADS box region (kindly provided by S. Michaels and R. Amasino, University of Wisconsin-Madison). *COR15a* (Lin and Thomashow, 1992) and *ACT2/ACT8* (An *et al.*, 1996) probes were made from 3'-UTR regions of cDNA isolated by RT-PCR with *COR15a*

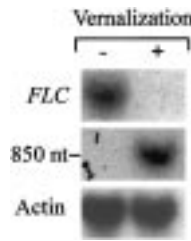


Figure 1. Vernalization-responsive expression of a gene identified by subtractive hybridization. Upper panel: steady-state mRNA abundance of *FLC* (Michaels and Amasino, 1999) is stably decreased in the late-flowering, *FRIGIDA*-containing Columbia ecotype of *Arabidopsis thaliana*. RNA was prepared from unvernallized (–) and 35-day vernalized (+) seedlings grown *in vitro* for 7 days at room temperature in short-day photoperiods. The 3′ end of *FLC* cDNA lacking the conserved MADS box sequence was used as a probe to detect *FLC* mRNA. Middle panel: steady-state mRNA abundance of the identified gene is stably elevated in the *FRIGIDA*-containing Columbia ecotype after 35 days of vernalization. The same RNA blot shown in the upper panel was reprobbed with a 208 bp cDNA fragment isolated by subtractive hybridization to detect a ca. 850 nt vernalization-responsive transcript. Lower panel: the RNA blot was reprobbed a second time with the actin gene *ACT2/ACT8* (An *et al.*, 1996) to determine the relative amount of RNA loaded in each lane.

primers 5′-AGATTTTCGTGACGGATAAAA-3′ and 5′-TGTGACGGTACTGTGGATA-3′ and *ACT2/ACT8* primers 5′-GCTTCGTATTCTGA-3′ and 5′-TAGCTGAATTCCTCAAGCACATT-3′.

Results

EARL11 is a vernalization-responsive gene that is activated in all Arabidopsis tissues

A sensitive PCR-based gene expression screen was used to identify vernalization-responsive genes in late-flowering *Arabidopsis thaliana* containing dominant alleles of *FRI* and *FLC* (see Materials and methods). By definition, the RNA level of vernalization-responsive genes is either stably increased or decreased by vernalization, which is distinct from the transient activation of cold acclimation or stress-responsive genes (Thomashow, 1994). To identify *bona fide* vernalization-responsive genes in *Arabidopsis*, seeds imbibed on agar medium were vernalized for 35 days at 4 °C, then grown at room temperature for seven days prior to RNA isolation. The seedlings had two cotyledons and two leaves at this stage. Unvernallized, 2-day cold-treated, control plants were grown in parallel and were phenotypically identical to vernalized plants after 7 days at room temperature. All plants were grown in short-day photoperiods

to reduce differences in long-day photoperiod-specific gene expression between vernalized and unvernallized plants.

The MADS box gene *FLC* is the first known vernalization-responsive gene whose RNA level is decreased by vernalization in *Arabidopsis thaliana* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Therefore, *FLC* served as positive control in repeated rounds of reciprocal subtractive hybridization between cDNA from vernalized and unvernallized *Arabidopsis* plants. *FLC* was indeed selectively and progressively enriched in cDNA from unvernallized plants during three rounds of subtractive hybridization, but was absent in cDNA from 35-day vernalized plants (data not shown). Conversely, the amount of the constitutively expressed actin gene *ACT2/ACT8* was progressively reduced in cDNA from both vernalized and unvernallized plants during three rounds of subtractive hybridization (data not shown). Hence it was concluded that both the cDNA material and the experimental approach were suitable for the identification of novel, vernalization-responsive genes.

After three rounds of subtraction against cDNA from unvernallized control plants, PCR-amplified cDNA fragments from vernalized plants were ligated to plasmid vectors and cloned. About 100 individual clones were hybridized in duplicate with radiolabeled probes made from the two original, unsubtracting cDNA populations. Two clones were identified that only hybridized to cDNA from vernalized plants. Both clones had ca. 200 bp identical inserts and were used in RNA gel blot analysis with total RNA from vernalized and unvernallized plants grown for seven days at room temperature in short-day photoperiods. As shown in Figure 1, the *FLC* control hybridized strongly to RNA from only unvernallized, but not vernalized, plants. When the same blot was stripped and re-probed with the 200 bp cDNA fragment, hybridization signal was only seen in RNA from vernalized plants. Thus, the gene expression screen identified a novel vernalization-responsive gene that is strongly activated by 35 days of vernalization, and remains on after 7 days of growth in short-day photoperiods.

The ca. 200 bp cDNA fragment was sequenced and analyzed. As shown in Figure 2A, database searches determined that the 208 bp cDNA fragment was identical to *EARL11* (accession number L43080), originally identified as a gene transiently activated in response to toxic levels of aluminum (Richards and Gardner, 1995). *EARL11* belongs to a group of small (ca. 18.7 kDa), hydrophobic, and proline-rich proteins that

A.

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atg gct tca aag aac tca gcc tct att gct ctt ttc ttc gcc ctt aac atc ata ttc ttc
M  A  S  K  N  S  A  S  I  A  L  F  F  A  L  N  I  I  F  F
acc tta acc gct gca aca gat tgt ggt tgc aac cca agt cct aag cac aag cct gtc cca
T  L  T  A  A  T  D  C  G  C  N  P  S  P  K  H  K  P  V  P
agt cct aaa ccc aag ccg gtc cca agt ccc aaa ccc aag ccg gtc cca agt cct tca gta
S  P  K  P  K  P  V  P  S  P  K  P  K  P  V  P  S  P  S  V
cca agt cct tcg gtc cca agt cct aac cct aag ccg gtc acg cct ccg aga acc cct ggc
P  S  P  S  V  P  S  P  N  P  R  P  V  T  P  P  R  T  P  G
tca tct gga aac tgt cct atc gat gct ctc aga ctc ggt gta tgt gcg aac gtt tta agc
S  S  G  N  C  P  I  D  A  L  R  L  G  V  C  A  N  V  L  S
agt cta ctc aac att caa ttg ggt cag cca tca gct caa cca tgt tgc tcg ctc atc caa
S  L  L  N  I  Q  L  G  Q  P  S  A  Q  P  C  C  S  L  I  Q
ggg ttg gtt gac ctc gac gct gcc att tgt ctt tgc act gcg ctt agg gct aac gtt ctt
G  L  V  D  L  D  A  A  I  C  L  C  T  A  L  R  A  N  V  L
ggg atc aac ctt aac gtc ccg ata tct ctc agt gtt ctt ctc aac gtt tgt aac aga aag
G  I  N  L  N  V  P  I  S  L  S  V  L  L  N  V  C  N  R  K
ggt ccg tct ggc ttc caa tgt gct
V  P  S  G  F  Q  C  A

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B.

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TIP17.80      1  ATGSCCTCAAAAGAACTCAGCCTCTCTGCTCTTTCCTTTCCTCAACATCCCTTTTTC
TIP17.90      1  ATGSCCTCAAAAGAACTCAGCCTCTCTGCTCTTTCCTTTCCTCAACATCCCTTTTTC
TIP17.70      1  ATGSCCTCAAAAGAACTCAGCCTCTCTGCTCTTTCCTTTCCTCAACATCCCTTTTTC
TIP17.60      1  ATGSCCTCAAAAGAACTCAGCCTCTCTGCTCTTTCCTTTCCTCAACATCCCTTTTTC
TIP17.100     1  ATGSCCTCAAAAGAACTCAGCCTCTCTGCTCTTTCCTTTCCTCAACATCCCTTTTTC

TIP17.80      61  ACCTTAACCGCTGCTACTAATTGTAGATGCAACCCGAGTCCTAAGCCTAGSCCGCTCCCA
TIP17.90      61  ACCTTAACCGCTGCTACTAATTGTAGATGCAACCCGAGTCCTAAGCCTAGSCCGCTCCCA
TIP17.70      61  ACCTTAACCGCTGCTACTAATTGTAGATGCAACCCGAGTCCTAAGCCTAGSCCGCTCCCA
TIP17.60      61  ACCTTAACCGCTGCTACTAATTGTAGATGCAACCCGAGTCCTAAGCCTAGSCCGCTCCCA
TIP17.100     61  ACCTTAACCGCTGCTACTAATTGTAGATGCAACCCGAGTCCTAAGCCTAGSCCGCTCCCA

TIP17.80      121  AATCCTAAAGGTCCCAAGTCTTAAGGTCCCAACTCCTTCGCTCCCAAGTCCATATSTTCCA
TIP17.90      121  AATCCTAAAGGTCCCAAGTCTTAAGGTCCCAACTCCTTCGCTCCCAAGTCCATATSTTCCA
TIP17.70      121  AATCCTAAAGGTCCCAAGTCTTAAGGTCCCAACTCCTTCGCTCCCAAGTCCATATSTTCCA
TIP17.60      121  AATCCTAAAGGTCCCAAGTCTTAAGGTCCCAACTCCTTCGCTCCCAAGTCCATATSTTCCA
TIP17.100     107  AATCCTAAAGGTCCCAAGTCTTAAGGTCCCAACTCCTTCGCTCCCAAGTCCATATSTTCCA

TIP17.80      181  ACTCCTTCGCTCCCAAGTCTTTCCTGCTTACCAACTCCTTCGCTCCCAAGTCCATATSTTCCA
TIP17.90      171  ACTCCTTCGCTCCCAAGTCTTTCCTGCTTACCAACTCCTTCGCTCCCAAGTCCATATSTTCCA
TIP17.70      162  ACTCCTTCGCTCCCAAGTCTTTCCTGCTTACCAACTCCTTCGCTCCCAAGTCCATATSTTCCA
TIP17.60      150  ACTCCTTCGCTCCCAAGTCTTTCCTGCTTACCAACTCCTTCGCTCCCAAGTCCATATSTTCCA
TIP17.100     118  ACTCCTTCGCTCCCAAGTCTTTCCTGCTTACCAACTCCTTCGCTCCCAAGTCCATATSTTCCA

TIP17.80      241  AGTCCTAAGCCTACGCGAGGATTCCTCCGAGAACCCCTGCTGATCCGGAAGC---TGT
TIP17.90      226  AGTCCTAAGCCTACGCGAGGATTCCTCCGAGAACCCCTGCTGATCCGGAAGC---TGT
TIP17.70      199  AGTCCTAAGCCTACGCGAGGATTCCTCCGAGAACCCCTGCTGATCCGGAAGC---TGT
TIP17.60      175  AGTCCTAAGCCTACGCGAGGATTCCTCCGAGAACCCCTGCTGATCCGGAAGC---TGT
TIP17.100     122  AGTCCTAAGCCTACGCGAGGATTCCTCCGAGAACCCCTGCTGATCCGGAAGC---TGT

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Figure 2. Nucleotide sequence, deduced amino acid sequence, and partial nucleotide sequence alignment of the vernalization-responsive gene identified by subtractive hybridization. A. The gene corresponds to *EARLII* (Richards and Gardner, 1995). *EARLII* is intron-less and the genomic sequence is shown. The 208 bp cDNA sequence isolated by subtractive hybridization is underlined. A putative N-terminal signal sequence of the EARLII protein is shown in *italics*. The proline-rich domain of the protein is shown in bold. B. Partial nucleotide sequence alignment of *EARLII* with four of its most related family members. TIP17.70 corresponds to *EARLII*. The cDNA sequence isolated by subtractive hybridization is underlined.

are developmentally and environmentally regulated in a wide variety of plant species (Richards and Gardner, 1995). As shown in Figure 2A, *EARLII* contains a putative signal sequence at the N-terminus, followed by a proline-rich hydrophilic domain, and a hydrophobic C-terminus with a putative transmembrane domain. *EARLII* maps to *Arabidopsis* chromosome 4, between RFLP markers g4108 and mi456, and is located on BAC T1P17 (accession number AL049730). Database analysis of BAC T1P17 showed that *EARLII* is flanked

by five related *EARLII*-like genes, which are tandemly repeated over a region of 20 kb. The genes contain intron-less coding regions and they are separated by 2 to 3 kb of 5'- and 3'-flanking sequences. Another related protein was found on BAC T3P18 that maps to chromosome 1. A protein alignment (not shown) indicated that the highest degree of similarity between the related proteins exists at the C-terminus. As shown in the nucleotide alignment in Figure 2B, the 208 bp *EARLII* cDNA fragment maps to a region of low

similarity between the most related genes. When the 208 bp fragment was used in a genomic Southern hybridization experiment, only bands corresponding to *EARLII*-specific restriction fragments were detected (not shown). We thus concluded that the hybridization signal seen in RNA gel blot analyses was specific for *EARLII*.

The expression of *EARLII* shown in Figure 1 was analyzed in RNA isolated from whole seedlings. To determine whether *EARLII* activation was tissue-specific in vernalized plants, total RNA was isolated from individual tissues and subjected to gel blot analysis. *EARLII* transcripts were detected at approximately equal levels in the shoot apex, cotyledons, the hypocotyl, and in roots (data not shown). This indicated that *EARLII* is activated in all tissues tested and suggested that the gene is under developmental, but not spatial, control.

Extended periods of vernalization quantitatively activate stable EARLII expression

The vernalization response is quantitative. That is, the longer plants are vernalized, the earlier they flower (Lee and Amasino, 1995). One prediction from this observation is that *bona fide* vernalization-responsive genes are also quantitatively activated or silenced during extended periods of vernalization. To test this prediction, the level of *EARLII* and *FLC* transcripts was measured in plants exposed to varying periods of vernalization treatment. As before, all plants were grown for seven days at room temperature and in short-day photoperiods prior to RNA isolation. As shown in Figure 3A, *EARLII* RNA levels rapidly and stably increased with every week of vernalization, reaching the highest level in 35-day vernalized plants. In contrast, *FLC* RNA levels were highest in unvernallized plants, but rapidly and stably decreased with every additional week of vernalization to almost undetectable levels (Figure 3A). Thus, both *EARLII* and *FLC* satisfy the conditions for *bona fide* vernalization-responsive genes, whose transcripts are quantitatively induced and repressed, respectively.

To determine how long *EARLII* expression levels remain high after vernalization, 35-day vernalized plants were grown for 7, 10, 15, and 20 days at room temperature. No decline in level of *EARLII* transcripts was seen over a 10-day growth period and mRNA levels were still high after 20 days (Figure 3B). Thus, the activated state of *EARLII* gene expression was

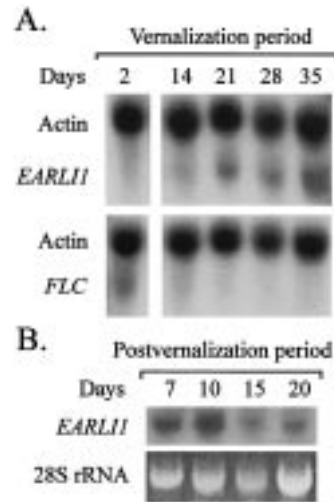


Figure 3. Quantitative effect of vernalization on *EARLII* and *FLC* steady-state mRNA levels and postvernalization-sustained expression of *EARLII*. **A.** Upper panel: *EARLII* is progressively activated with increased time of vernalization. RNA was prepared from *FRIGIDA*-containing late-flowering Columbia seedlings vernalized *in vitro* for the indicated time periods, then grown for 7 days in short-day photoperiods. The RNA gel blot was simultaneously probed with the 208 bp subtracted cDNA fragment (Figure 2) to detect *EARLII* mRNA and with the actin gene *ACT2/ACT8* (An *et al.*, 1996) to determine the relative amount of RNA loaded in each lane. Lower panel: *FLC* is progressively silenced with increased time of vernalization. RNA was from the same isolation as in the blot shown in the upper panel. The RNA gel blot was simultaneously probed with *FLC* cDNA (Michaels and Amasino, 1999) lacking the conserved MADS box to detect *FLC* mRNA and with *ACT2/ACT8* to determine the relative amount of RNA loaded in each lane. **B.** RNA gel blot showing sustained levels of *EARLII* mRNA. Upper panel: RNA was prepared from 35-day vernalized *FRI*-containing late-flowering *Arabidopsis* seedlings grown after vernalization for the indicated periods of days at room temperature and in short-day photoperiods. Lower panel: ethidium bromide-stained rRNA (28S) as loading control.

maintained for at least 20 days of growth at room temperature.

Basal EARLII expression is higher in early-flowering ecotypes

We observed an inverse correlation between RNA levels of *EARLII* and *FLC* as a function of vernalization (Figure 3A). This suggested that *FLC* may repress *EARLII*. If so, it might be expected that the basal level of *EARLII* RNA is higher in early-flowering plants, which contain a very low basal level of *FLC* RNA. *EARLII* mRNA abundance was thus analyzed in three early-flowering ecotypes of *Arabidopsis*, Col-0, No-0, and Ler. To measure basal or elevated transcript levels, seeds were kept for two days in the cold or

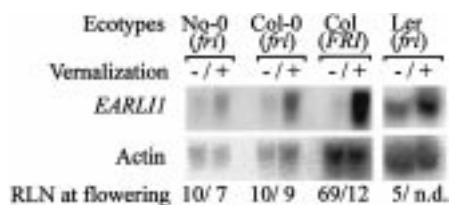


Figure 4. The basal level of *EARLII* steady-state mRNA is higher in unvernallized, early-flowering ecotypes of *Arabidopsis thaliana*. RNA was prepared from unvernallized (-) or 28-day vernalized (+) seedlings grown *in vitro* for 7 days at room temperature in short-day photoperiods. The 208 bp subtracted cDNA fragment (Figure 2) was used as a probe to detect *EARLII* mRNA. The RNA gel blots were reprobbed with the actin gene *ACT2/ACT8* (An *et al.*, 1996) to determine the relative amount of RNA in each lane. Flowering time of ecotypes in long-day photoperiods is indicated as the number of rosette leaves (RLN) made at the time of bolting. No-0, early-flowering ecotype Nossen; Col-0; early-flowering ecotype Columbia; Col(*FRI*), late-flowering ecotype containing the Sf-2 allele of *FRIGIDA* in the Col-0 background (Lee *et al.* 1993); Ler, early-flowering ecotype Landsberg *erecta*; n.d., not determined. All early-flowering ecotypes contain recessive *frigida* (*fri*) alleles.

vernallized for 28 days, then grown for seven days at room temperature in short-day photoperiods. The result of RNA gel blot analyses is shown in Figure 4. As in late-flowering plants, *EARLII* is activated and remains on after 28 days of vernalization in early-flowering plants. However, compared to unvernallized late-flowering plants, the basal level of *EARLII* RNA is clearly higher in early-flowering ecotypes, particularly in Ler, which contains a weak allele of *FLC* (Lee *et al.*, 1994; Koornneef *et al.*, 1994). Thus, *FLC* may indeed directly or indirectly repress *EARLII* expression.

Vernalization coupled with long-day photoperiods have an additive or synergistic effect on *EARLII* expression

EARLII is efficiently activated when plants are both vernalized and grown in short-day photoperiods (Figure 1). To determine whether *EARLII* expression is responsive to differences in photoperiod, four pools of seeds were vernalized for either 14 or 28 days in either long- or short-day photoperiods. Subsequently, each pool of vernalized seeds was transferred to room temperature, further subdivided, and grown separately in either long- or short-day photoperiods. Hence, some plants were vernalized in short days, then grown in either long- or short-day photoperiods for one week before RNA isolation. Conversely, other plants were vernalized in long days, then grown in long- or short-day photoperiods before RNA isolation. As shown in

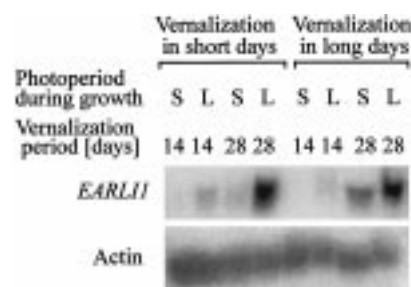


Figure 5. Growth in long-day photoperiods after vernalization increases the level of *EARLII* steady-state mRNA. Late-flowering *FRIGIDA*-containing Columbia seedlings were vernalized *in vitro* for 14 or 28 days in either short days or long days, then subdivided and grown at room temperature as indicated in either short-day (S) or long-day (L) photoperiods. The 208 bp subtracted cDNA fragment (Figure 2) was used as a probe to detect *EARLII* mRNA. The RNA gel blots were reprobbed with the actin gene *ACT2/ACT8* (An *et al.*, 1996) to determine the relative amount of RNA in each lane.

Figure 5, the level of *EARLII* RNA was always higher in plants that were grown in long-day photoperiods after vernalization, regardless of the photoperiod during vernalization. Although *EARLII* was activated after vernalization and growth in short days, vernalization followed by growth in long-day photoperiods had an additive or synergistic effect on gene activation. Thus, vernalization may make *EARLII* competent to respond to long-day photoperiods.

EARLII is not a general stress-responsive gene

EARLII was previously identified as a gene whose RNA was transiently induced by exposure to toxic levels of aluminum (Richards and Gardner, 1995). This suggests that *EARLII* may be transiently activated by abiotic stress, as shown for other proline-rich proteins (Kurkela and Franck, 1990). To test this prediction, unvernallized 7-day old plants were exposed only overnight to cold, then grown for 0 to 8 h at room temperature prior to RNA isolation. In another experiment, unvernallized 7-day old plants were exposed for 5 min to 100 μ M of the stress hormone abscisic acid (ABA) to determine whether ABA was able to transiently activate *EARLII*. The level of *EARLII* expression was measured at each time point by RNA gel blot analysis and compared to the level of *COR15a* expression. *COR15a* served as positive control, because its RNA level is transiently elevated by both cold and ABA (Hajela *et al.*, 1990; Thomashow, 1994). As shown in Figure 6, *EARLII* was transiently activated by cold, but not by exposure to ABA. By contrast, *COR15a* was activated by both cold and

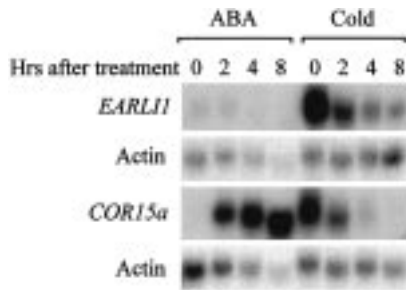


Figure 6. Effect of transient cold and exogenous application of abscisic acid (ABA) on the levels of *EARLII* and *COR15a* steady-state mRNA. Upper two panels: *EARLII* is transiently induced by cold, but not by ABA. ABA treatment: late-flowering *FRIGIDA*-containing Columbia seedlings (Col[*FRI*]) grown *in vitro* for 7 days at room temperature in short-day photoperiods were flooded with 10 ml of 100 μ M ABA for 5 min, then washed with sterile water. RNA was prepared from seedlings grown for the indicated length of time in hours (Hrs) after the ABA treatment. Transient cold treatment: late-flowering Col(*FRI*) seedlings were grown *in vitro* for 7 days at room temperature in short-day photoperiods, then transferred overnight to 4 °C. RNA was prepared from seedlings grown for the indicated length of time in hours (Hrs) at room temperature after the cold treatment. A full-length cDNA probe was used to detect *EARLII* mRNA and a 3'-UTR-specific cDNA fragment of the actin gene *ACT2/ACT8* (An *et al.*, 1996) to determine the relative amount of RNA in each lane. Lower two panels: the cold-regulated gene *COR15a* is transiently induced by both cold and ABA. Col(*FRI*) seedlings were treated as described above. A 3'-UTR-specific cDNA fragment were used to detect *COR15a* mRNA (Baker *et al.*, 1994). The actin gene *ACT2/ACT8* was used to determine the relative amount of RNA in each lane. It may be worth noticing that ABA lowers the level of actin steady-state mRNA.

ABA. Both *EARLII* and *COR15a* transcripts returned to basal levels with similar kinetics after removal of transient cold, suggesting that the stable activation of *EARLII* by vernalization is promoted by a different mechanism. Thus, the regulation of *EARLII* is distinct from that of *COR15a*, a cold acclimation-responsive gene, in three ways. First, *EARLII* is stably activated by vernalization and *COR15a* is not (Thomashow, 1994). Second, *EARLII* is transiently activated only by cold and not by ABA, whereas both treatments induce *COR15a*. And third, *EARLII* does not have a *COR15a*-like low-temperature-responsive element (LTRE) in its promoter sequence (data not shown), suggesting that another control element may respond to transient cold. We hence conclude that *EARLII* is a novel vernalization- and cold-responsive gene with a unique expression pattern distinct from general stress-responsive genes.

Discussion

Identification of EARLII as a vernalization responsive gene in Arabidopsis thaliana

The paucity of vernalization-responsive genes has been a major obstacle to analyzing vernalization-specific regulation of gene expression in *Arabidopsis thaliana*. Previous to this study, the only cloned *Arabidopsis* gene that fits the definition of *bona fide* vernalization-responsive gene was *FLOWERING LOCUS C (FLC)* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). The level of *FLC* RNA is high in un-vernallized, *FRIGIDA (FRI)*-containing late-flowering plants, but is decreased after vernalization and remains low even after removal of the vernalization stimulus (Michaels and Amasino, 1999; Sheldon *et al.*, 1999; this study). The observed expression profile fits *FLC*'s proposed function as a MADS box gene encoding a transcription factor that represses flowering (Simpson *et al.*, 1999). We report here on the identification of *EARLII*, the first vernalization-responsive gene from *Arabidopsis* that is activated by vernalization and remains on after removal of the cold stimulus. The abundance of *EARLII* mRNA is very low in un-vernallized, late-flowering *Arabidopsis* plants, but is robustly increased and remains high after vernalization in all vegetative tissues tested. This up-regulation is quantitative, thus the longer plants are vernalized, the higher the abundance of *EARLII* mRNA. Conversely, we found that the level of *FLC* mRNA is quantitatively decreased to very low levels over a 35-day period of vernalization (Figure 3). The inverse RNA profiles of *EARLII* and *FLC* may also correlate with the quantitative effect of vernalization on flowering time (Lee and Amasino, 1995). That is, the longer late ecotypes of *Arabidopsis* are vernalized, the earlier they flower, which correlates with higher and lower levels of *EARLII* and *FLC* RNA, respectively (Schläppi, unpublished; Sheldon *et al.*, 2000). It is thus possible that *FLC* directly or indirectly represses *EARLII* expression. Consistent with this idea, we found that, compared to late-flowering plants, *EARLII* is more highly expressed in un-vernallized, early-flowering ecotypes of *Arabidopsis* such as Col-0 or Ler (Figure 4). This would suggest that *EARLII* is under negative control by *FLC*. However, we consider it equally or more likely that *EARLII* is mainly under positive control by other factors. This is because vernalization activates the gene even in the Ler ecotype, which has undetectable levels of *FLC* RNA (Figure 4; Sheldon

et al., 1999). Moreover, typical consensus sequence binding sites for MADS-box proteins (Riechmann and Meyerowitz, 1997) are not found in the 5'-upstream region of *EARLII* (not shown), which may suggest more likely an indirect effect of *FLC*.

In this initial characterization we present evidence that *EARLII* is a *bona fide* vernalization-responsive gene in *Arabidopsis* that is specifically activated by vernalization. The only vernalization-related (*ver*) genes other than *EARLII* that have been reported to date were found in winter wheat (Chong *et al.*, 1994, 1998). Like *EARLII*, vernalization-related winter wheat genes were induced after 26 to 30 days of vernalization, but it was not reported as to whether transcript levels remain high after removal of the vernalization stimulus.

EARLII is regulated by both vernalization and long-day photoperiods

The level of *EARLII* transcript is higher in vernalized plants grown in long days than in vernalized plants grown in short days (Figure 5). In contrast, the basal level of *EARLII* transcript is similar in unvernallized, late-flowering ecotypes grown in short- or long-day photoperiods (data not shown). Taken together, this suggests that vernalization makes *EARLII* competent to respond to long-day photoperiods. It is thus possible that the *EARLII* promoter is modular, containing both novel vernalization and long-day-responsive elements that act additively or synergistically to increase RNA levels. Since *EARLII* is intron-less (Figure 2), it seems likely that the gene is mainly regulated by its 5' control region. The 5'-upstream region of *EARLII* contains several myb core elements and different myb consensus sites (not shown), which may be candidate sequences for photoperiodic regulation (Wang *et al.*, 1997; Schaffer *et al.*, 1999). In addition, several GT-1 consensus binding sites involved in light regulation (Terzaghi and Cashmore, 1995) are also found in the same 5'-upstream regions (not shown). Vernalization response elements are unknown and most likely represented by novel DNA sequences. Analysis of changes in the DNA methylation state of *EARLII* promoter elements before and after vernalization may help to identify vernalization-responsive elements. Alternatively, different response elements could be identified by traditional promoter deletion analysis.

DNA sequence motifs CCGAC for the low-temperature response elements (LTRE; Baker *et al.*, 1994) are not present in the 5'-upstream region of

EARLII (not shown). However, *EARLII* RNA is transiently induced by overnight exposure to cold, but not by the stress hormone abscisic acid (ABA), which is distinct from LTRE-mediated regulation (Figure 6). It is thus possible that the *EARLII* promoter contains both vernalization and novel low-temperature-responsive elements, the latter only responding to transient cold and not to ABA-mediated, general stresses. However, the possibility that *EARLII* is also responsive to other types of abiotic stresses such as high salt or wounding needs to be addressed in further experiments.

Possible function of EARLII

In this study we provide evidence that *EARLII* is activated by vernalization, but we do not know whether the gene is required for vernalization-promoted early flowering. The *EARLII* protein belongs to a class of small proline-rich proteins found in a variety of plant species (Richards and Gardner, 1995) with similarity to extensin-like or putative cell wall plasma membrane-disconnecting CLCT proteins in *Arabidopsis* (e.g. accession number AAC22151 and AF098630, respectively). The protein may thus be plasma membrane- or cell wall-anchored and could be involved in signal transduction pathways. Alternatively, it could affect the developmental potential of certain cells by modifying plasma membrane or cell wall properties. In addition to testing whether *EARLII* is involved in the regulation of flowering time, it will be interesting to determine whether *EARLII* is regulated by known genes of the vernalization promotion pathway (Chandler *et al.*, 1996).

In summary, we have identified a vernalization-responsive gene in *Arabidopsis thaliana* that responds positively to vernalization and long-day photoperiods, to transient cold, but not to ABA. It should now be possible to identify *cis*-acting control elements that respond positively to the different environmental stimuli. This may ultimately allow us to identify vernalization-specific *trans*-acting factors and to advance the understanding of the relatively obscure process of vernalization-promoted flowering.

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