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Cold Induction of EARLI1, a Putative Arabidopsis Lipid Transfer Protein, Is Light and Calcium Dependent

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By J. Bubier and M. Schläppi

As sessile organisms, plants must adapt to their environment. One approach toward understanding this adaptation is to investigate environmental regulation of gene expression. Our focus is on the environmental regulation of EARLI1, which is activated by cold and long-day photoperiods. Cold activation of EARLI1 in short-day photoperiods is slow, requiring several hours at 4ºC to detect an increase in mRNA abundance. EARLI1 is not efficiently cold-activated in etiolated seedlings, suggesting that photomorphogenesis is necessary for its cold activation. Cold activation of EARLI1 is inhibited in the presence of the calcium channel blocker lanthanum chloride or the calcium chelator EGTA. Addition of the calcium ionophore Bay K8644 results in cold-independent activation of EARLI1. These data suggest that EARLI1 is not an immediate target of the cold response, and that calcium flux affects its expression. EARLI1 is a putative secreted protein and has motifs found in lipid transfer proteins. Over-expression of EARLI1 in transgenic plants results in reduced electrolyte leakage during freezing damage, suggesting that EARLI1 may affect membrane or cell wall stability in response to low temperature stress.

Introduction

The transition of a flowering plant from the vegetative to the reproductive growth phase is a highly regulated developmental event. Vernalization is the promotion of flowering from the sustained exposure of plants to cold temperatures that occurs during a typical winter thus permitting vernalization-requiring plants to flower in the spring. Since plants are sessile in nature and can be considered poikilothermic, during the process of vernalization plants must become concurrently cold-acclimated and freezing-tolerant. We have previously shown that the *early Arabidopsis aluminium-induced gene1* (*EARLI1*; Richards & Gardner 1995; Richards *et al*. 1998) is stably activated by vernalization (Wilkosz & Schläppi 2000). In certain late-flowering ecotypes grown in short-day photoperiods, *EARLI1* RNA abundance is higher after vernalization and remains high for at least 20 d after the cold treatment. We also showed that *EARLI1* RNA levels are even higher when vernalized plants are grown in long days, another condition that promotes flowering. Plants experience low temperature stress associated with vernalization as dehydration or osmotic stress. Changes in osmotic pressure affect the turgor of plants, which induces the biosynthesis of the plant stress hormone abscisic acid (ABA; Wang *et al*. 1995). Interestingly, we

showed that although *EARLI1* is transiently induced by an overnight low-temperature treatment, it does not respond to ABA.

An immediate effect of cold stress on plants is a change in membrane fluidity, which is considered a primary sensor of the cold stimulus (Örvar *et al*. 2000; Sangwan *et al*. 2002; Henriksson & Trewavas 2003). Changes in membrane fluidity are thought to occur in distinct microdomains of the plasma membrane. Local membrane changes may lead to cytoskeletal rearrangements, specifically affecting the reorganization of actin microfilaments (Sangwan *et al*. 2001). This is believed to result in the opening of mechanosensitive calcium channels and in an increase of cytosolic calcium, triggering cold-induced gene expression and cold acclimation (Ding & Pickard 1993; Örvar *et al*. 2000; Sangwan *et al*. 2002; Henriksson & Trewavas 2003). One focus of the present study was thus to determine whether calcium is involved in cold regulation of *EARLI1*. Because *EARLI1* has such a unique expression profile (Wilkosz & Schläppi 2000), we further investigated the cross-talk between light and cold in the regulation of *EARLI1* expression. We also determined the effect of *EARLI1* over-expression on flowering time and freezing-induced membrane damage.

Materials and Methods

Plant material and growth conditions

The late-flowering line Col-*FRI*-Sf2 contains the dominant San Feliu (Sf2) allele of *FRIGIDA* (*FRI*) and a dominant Columbia (Col) allele of *FLOWERING LOCUS C* (*FLC*) in the Columbia background (Lee & Amasino 1995; Michaels & Amasino 2001). One hundred surface-sterilized seeds were grown in each sterile Petri dish (9 cm diameter) on agar-solidified half-strength Murashige and Skoog (MS) medium without sucrose (Murashige & Skoog 1962). The MS plates were incubated at 4 ºC for 1 to 2 d to break seed dormancy, then grown for 10 d in a short-day photoperiod (8 h light/16 h dark cycles) with approximately 65 µmol m^{-2} s^{-1} photon flux and 25 ºC day/22 ºC night temperatures or in a long-day photoperiod (16 h light/8 h dark) under the same light intensity and temperature conditions. Some long-day treatments were performed with approximately 115 µmol m^{-2} s^{-1} photon flux of cool fluorescent light at 22 ºC day/20 ºC night temperatures. For transient exposure to cold, seeds were grown after breakage of dormancy on agar medium for 10 d at 25 ºC day/22 ºC night temperatures in either long- or short-day photoperiods, then transferred to 4 ºC for the indicated amount of time after which RNA was isolated immediately, as described below. For the abscisic acid (ABA) assay, plants grown in short-day photoperiods were flooded with 10 mL of 100 *µ*M ABA in 1% dimethylsulphoxide

(DMSO) for 5 min, then washed three times with sterile water. RNA was isolated immediately or 8 h after the treatment. For the L-type Ca^{2+} channel ionophore assay, 10 mL of 10 mM Bay K8644 (1,4 dihydro-2,6dimethyl-5-nitro-4-[trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester) in 1% DMSO was applied for 1 h, then washed with sterile water. For the Ca^{2+} channel blocker assay, 10 mL of 10 mM LaCl3 (pH 5.2) was applied for 1 h, then washed with sterile water. For the Ca^{2+} chela-tor assay, 10 mL of 10 mM ethylene glycol-bis-(b-aminoethylether)-*N*,*N*,*N*',*N*',-tetraacetic acid (EGTA) (pH 4.0) was applied for 1 h, then washed with sterile water. RNA was isolated 16 h after each treatment, unless otherwise stated. For cold acclimation assays, Columbia wild-type and transgenic plants, grown in short-day photoperiods that were either cold-acclimated for 3 d at 4 ºC or kept non-acclimated at 22 ºC, were frozen at -5 or -10 ºC for 3 d, then returned to normal growth temperature (22 ºC) for 1 week and assayed for survival.

RNA isolation

Unless otherwise stated, whole-seedling tissue was harvested by quick freezing in liquid N2 and stored at -80 ºC. Seedlings were harvested at approximately the same time (5–7 h after subjective dawn), unless otherwise stated. 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 using a mini-prep procedure described previously (Wilkosz & Schläppi 2000).

RNA gel blot analysis and probes

About 20 µg of total RNA was separated by electrophoresis in 1.2% formaldehyde/3-(*N*-Morpholino) propanesulfonic acid (MOPS) gels containing ethidium bromide (Sambrook, Maniatis & Fritsch 1989). RNA was transferred to Protran nitrocellulose (Schleicher & Schuell, Keene, NH, USA) membranes and cross-linked with a Stratalinker (Stratagene, La Jolla, CA, USA). Pre-hybridization and hybridization were performed at 65 ºC in 5 x Denhardt's solution, 6 x sodium chloride and sodium citrate (SSC), 0.5% sodium dodecyl sulphate (SDS) and 0.1 µg mL^{-1} denatured salmon sperm DNA (Roche, Indianapolis, IN, USA). All probes were α^{32}P-dATP-labelled (Perkin-Elmer, Boston, MA, USA) by the random primer method using the Megaprimer labelling kit scaled down to two-fifths the recommend size reactions (Amersham Biosciences, Piscataway, NJ, USA). Probes were purified by G-50 spin columns, heat-denatured and hybridized at a concentration of at least 1×10^6 cpm mL^{-1} onto nitrocellulose membranes in a Hybaid oven (Labnet, Edison, NJ, USA) at 65 ºC for at least 16 h. Membranes were washed at 65 ºC twice in 2 x SSC/0.1% SDS for at least 10 min and briefly rinsed with 0.2 x SSC before exposure to a phosphorimager screen (Molecular Dynamics/Amersham

Biosciences) or to X-ray film (Kodak, Rochester, NY, USA).

EARLI1 probes were made from the 208 bp cDNA fragment isolated by subtractive hybridization (Wilkosz & Schläppi 2000). *COR15a* and *ACT2* probes were made from their 3´-UTRs using genomic DNA as template and PCR primers 5´-AGATTTCGTGACGGATAAAA-3´ and 5´TGTGACGGTGACTGGGATA-3´ for *COR15a* and 5´ATGAAGATTAAGGTCGTGGCA-3´ and 5´-TCCGAG TTTGAAGAGGCTAC-3´ for *ACT2*. For the 18S probe, genomic DNA was used as a template in polymerase chain reaction (PCR) using primers 5´-CTCGTCCTTTCTCT CTTTCC-3´ and 5´-GAAACCTTGTTACGACTTCTCC3´. The *KIN2* probe was made from genomic DNA using PCR primers 5´-ATGTCAGAGACCAACAAGAATG CC-3´ and 5´-CCGAATCGCTACTTGTTCAGGC-3´.

EARLI1 over-expression construction

The full length *EARLI1* coding sequence was obtained from wild type Columbia plants by PCR using primers which introduced a 5´ *Bam*HI site and a 3´-*Eco*RI site. The PCR product was ligated into a pBlueScript vector containing a manopine synthase (mas) transcriptional terminator creating pBS*EARLI*-mas (Comai, Moran & Maslyar 1990). The *EARLI1*-mas fragment was then inserted into binary vector pPZP211 downstream of the MAC promoter (Comai *et al*. 1990; Gleave 1992). This was introduced into *Agrobacterium* strain ABI and transformed into the Columbia ecotype of *Arabidopsis* using the floral dip procedure (Clough & Bent 1998). T1 seedlings were selected on half-strength MS medium with 50 μg mL^{-1} kanamycin, then transferred to soil (2 : 1 : 1 mix of peatmoss : vermiculite : perlite) and allowed to mature at room temperature under long-day photoperiods.

Electrolyte leakage assays

The assay is based on the protocol of Sukumaran & Weiser (1972).
Six-week-old-seedlings were grown under short-day photoperiods. A young leaf was harvested and placed in a 15-mL Falcon tube on ice for 1 h. Tubes were then nucleated with an ice chip and frozen to different subzero temperatures (-2.5 ºC, -4 ºC, -7 ºC) in a refrigerated circulating bath (NesLab RTE-5B; Neslab/Thermo Electron, Waltham, MA, USA) for 1 h. Samples were removed and thawed at 4 ºC overnight and 5 mL of deionized water was then added to each tube and the tubes were gently shaken at room temperature for 3 h. The liquid was then removed and its conductivity measured (Corning 441; Corning, Acton, MA, USA). To induce 100% leakage, plant-containing tubes were then incubated at -80 ºC for 1 h. The original liquid was then returned to the each tube and shaken vigorously at room temperature for 3 h before conductivity measurements. The percentage freezing-induced leakage was calculated by dividing the first reading by the second (total leakage).

Results

Brief cold treatment causes transient expression of *EARLI1*

EARLI1 is transiently expressed after an overnight cold treatment in a mixed Columbia/Niederzenz genetic background (Wilkosz & Schläppi 2000; Schläppi 2001).To investigate the maintenance of cold temperature activation of *EARLI1* in the homogeneous Col-*FRI*-Sf2 background, seedlings were grown in short-day photoperiods for 10 d at room temperature, then incubated for 16 h at 4 ºC. *EARLI1* expression levels were determined by RNA gel blot analyses immediately after the cold period or 4, 8 and 24 h after the cold treatment. As shown in Fig. 1, *EARLI1* RNA abundance was highest immediately after 16 h of cold treatment and returned to basal levels 24 h after transfer to room temperature. Thus, a 16-h cold treatment resulted in robust activation of *EARLI1*; however, steady-state mRNA levels were not maintained after transfer to room temperature.

To determine whether *EARLI1* RNA levels increased immediately after exposure of plants to cold, RNA was isolated from Col-*FRI*-Sf2 plants after 1, 3, 4, 8, 12 and 16 h of incubation at 4 ºC and analysed. As shown in Fig. 2, *EARLI1* levels slowly increased after several hours of cold treatment. This suggests that *EARLI1* was not an immediate target of the cold stimulus, but was rather a downstream target of a cold-responsive regulatory factor.

Cold activation of *EARLI1* is light dependent

Vernalized Columbia/Niederzenz plants grown in long-day photoperiods have higher levels of *EARLI1* RNA than vernalized plants grown in short days (Wilkosz & Schläppi 2000). For Col-*FRI*-Sf2, as shown in Fig. 3a the basal level of *EARLI1* RNA was also higher in unvernalized plants grown at room temperature in long-day than in plants grown in short-day photoperiods. This indicates that long-day photoperiods enhanced *EARLI1* expression in the absence of cold. Moreover, as shown in Fig. 3b, overnight-cold-treated plants grown in long days or continuous light had even higher *EARLI1* RNA levels than their untreated counterparts. To determine whether cold temperature activation of *EARLI1* required light, RNA levels of *EARLI1* in 10-day-old Col-*FRI*-Sf2 seedlings grown in complete darkness at 25 ºC or in complete darkness at 4 ºC were analysed. As shown in Fig. 3c, no *EARLI1* RNA was detectable in etiolated seedlings whereas cold treatment resulted in the accumulation of a detectable amount of *EARLI1* RNA. However, the total RNA accumulation after light- and cold-treatment (Fig. 3b) was higher than the sum of the individual treatments (Fig. 3a & c). Thus there is synergy between light and cold activation. Moreover, in comparison with untreated counterparts, *EARLI1* RNA levels increased even more in plants that were cold treated in complete darkness when they were first grown for 10 d in continuous light, then kept in continuous darkness for 2 d before the

cold treatment (data not shown). This suggests that photomorphogenesis was necessary for maximum cold temperature activation of *EARLI1*.

Cold temperature induction of *EARLI1* is ABA independent, but calcium dependent

It was previously shown that *EARLI1* in the Columbia/ Niederzenz mixed genetic background was not activated by exogenous application of ABA (Wilkosz & Schläppi 2000). To determine whether *EARLI1* in the Col-*FRI*-Sf2 background was activated by ABA, 10-day-old seedlings were incubated with a 100-mM solution of ABA for 5 min, then washed three times with sterile water. RNA was isolated immediately after rinsing with water or 8 h later and analysed. Although RNA abundance of *COR15a*, a cold-regulated gene previously shown to respond to ABA, increased, no increase in *EARLI1* RNA level was observed (data not shown). This indicates that *EARLI1* in the Col-*FRI*-Sf2 background was not activated by exogenous application of the stress hormone ABA.

To determine whether calcium acted as second messenger in the cold temperature induction of *EARLI1*, plants were cold-treated after application of 10 mM EGTA, a calcium chelator, or 10 mM LaCl3, a calcium channel blocker. As shown in Fig. 4, in comparison with untreated controls there was no cold-mediated increase in *EARLI1* RNA abundance in plants treated with the calcium antagonists. To determine whether the antagonists had a general effect on gene expression, RNA levels of constitutively expressed *UBIQUITIN10* were analysed, indicating that EGTA and LaCl3 had no effect on its expression (Fig. 4). In addition, the L-type calcium channel ionophore Bay K8644 was applied to plants at room temperature to determine whether an influx of calcium could mimic the effect of cold treatment. As shown in Fig. 5, the calcium ionophore was indeed able to increase *EARLI1* RNA abundance to levels similar as those seen in cold temperature-treated plants. By contrast, the cold-regulated gene *KIN2* was strongly induced by cold, but only weakly by Bay K8644. This suggests that under the experimental conditions the Bay K8644-mediated increase in intracellular calcium levels activated *EARLI1*, but not *KIN2.* A similar result was obtained with *KIN1 (*data not shown). DMSO alone induced *EARLI1* expression (Fig. 5b), which explains why the low-temperature induction appears less robust compared with other control cold treatments (e.g. Fig. 4).

Over-expression of *EARLI1* neither induces early flowering nor enhances survival of frozen plants

EARLI1 was previously shown to be stably activated by vernalization (Wilkosz & Schläppi 2000). To determine whether this gene affects flowering time, transgenic Columbia plants over-expressing *EARLI1* from a strong constitutive promoter were analysed. Although *EARLI1* RNA levels were constitutively high, none of the transgenic lines analysed flowered earlier than

untransformed control plants under short-day photoperiods (data not shown).This indicates that over-expression of *EARLI1* was not sufficient to affect flowering time.

The cold-specific expression profile of *EARLI1* suggested that it could be involved in the cold acclimation process of Arabidopsis. To determine whether constitutive expression of *EARLI1* enhances freezing tolerance, non-acclimated plants were frozen at -5 and -10 ºC and survival was analysed. Over-expression of *EARLI1* did not enhance freezing tolerance compared with untransformed Columbia controls at either temperature (data not shown). This suggests that over-expression of *EARLI1* did not produce constitutively cold-acclimated plants when assayed at these temperatures.

Over-expression of *EARLI1* protects Arabidopsis cells from freezing damage

It was previously suggested that *EARLI1* might be a membrane protein (Wilkosz & Schläppi 2000). However, as shown in Fig. 6, the putative membrane domain of *EARLI1* also has strong similarity to motifs found in plant lipid transfer proteins (LTPs). Cryoprotectin, a special LTP homologue isolated from cold-acclimated cabbage leaves was shown to protect isolated chloroplast thylakoid membranes from freeze–thaw damage (Hincha *et al*. 2001). To determine whether *EARLI1* protects plant cells from freezing damage, electrolyte leakage assays were performed on both wild-type Columbia plants and *EARLI1*-over-expressing lines. Electrolyte leakage is proportional to the degree of cellular damage sustained after exposure of plants to freezing temperatures. The assay involves the controlled freezing of leaves or whole seedlings followed by conductivity measurement to determine the amount of leached electrolytes. As shown in Fig. 7, plants over-expressing *EARLI1* RNA overall leached fewer electrolytes after freezing-induced cellular damage than wild-type controls, especially at temperatures of -2 and -4 ºC. This suggests that a high and sustained expression of *EARLI1* protected Arabidopsis cells from freezing-induced damage.

Discussion

It was previously shown that RNA levels of *EARLI1* increase in response to aluminium, light, long-day photo-period, transient cold, and vernalization (Richards *et al*. 1998; Wilkosz & Schläppi 2000). Here we report that RNA levels of *EARLI1* increase slowly after several hours of cold treatment. The response to cold thus appears to be distinct from the rapid response to aluminium, which was shown to lead to a significant increase in *EARLI1* RNA levels 15 min after aluminium treatment (Richards *et al*. 1998). This suggests that *EARLI1* is not a direct target of the cold stimulus but rather a downstream target of a cold response regulator such as the cold binding factor (CBF) regulon (Medina *et al*. 1999; Thomashow 2001; Viswanathan & Zhu 2002).

It also suggests that *EARLI1* is regulated by a different factor in response to aluminium than to cold. The cold response factor may require protein synthesis whereas the aluminium factor may already be present and rapidly activated through post-translational modification or nucleo-cytoplasmic partitioning. *EARLI1* does not have a typical CRT/DRE *cis* element in its promoter region (Wilkosz & Schläppi 2000) and is therefore most likely not under direct control of the CBF regulon. This notion is supported by results of an Arabidopsis transcriptome profiling project, which indicated that *EARLI1* is cold-activated independently of the CBF response pathway (Fowler & Thomashow 2002).

The role of light in the environmental regulation of *EARLI1* gene expression appears to be complex. It was previously shown that levels of *EARLI1* RNA are higher in long-day-grown plants compared with short-day-grown plants (Wilkosz & Schläppi 2000). Here we show that initial photoperiodic growth is necessary for *EARLI1* to react robustly to the cold stimulus. This suggests that a developmental programme associated with photomorphogenesis is necessary for maximum cold-mediated activation of *EARLI1* gene expression. It is therefore possible that *EARLI1* is also under tissue-specific control and that etiolated tissue is both incompetent to express the gene at a detectable baseline level and to respond robustly to the cold stimulus without light. Interestingly, light was also shown to be important for the regulation of cold-induced genes containing the CRT/DRE element (Kim *et al*. 2002). Moreover, cold acclimation of Arabidopsis plants appears to be light dependent (Wanner & Junttila 1999). Furthermore, it was recently shown that Arabidopsis has two circadian oscillators with different temperature sensitivities, which allow plants to integrate light and temperature signals in response to a changing environment (Michael, Salome & McClung 2003). Taken together, these data suggest that there is significant cross-talk between the two environmental stimuli of light and cold in the regulation of gene expression and adaptation of plants to their environment.

Calcium is increasingly recognized as an important second messenger for the low-temperature signal transduction pathway (Thomashow 2001). We have likewise found that cold-mediated activation of *EARLI1* is calcium dependent. This conclusion is based on the following pharmacological evidence. First, pre-treatment of plants with lanthanum, a calcium channel blocker, abolished cold-mediated activation of *EARLI1*. Second, pre-treatment of plants with EGTA, a calcium chelator, abolished cold-mediated activation of *EARLI1* and third, *EARLI1* expression was induced at 25 ºC when plants were treated with the calcium ionophore Bay K8644, which opens L-type channels and causes an influx of calcium from extracellular stores (Nowycky, Fox & Tsien 1985; Monroy & Dhindsa 1995). Taken together, these results indicate that pharmacological blocking or promoting of calcium influx into Arabidopsis cells affects cold

temperature-mediated activation of *EARLI1* gene expression. Since Bay K8644 does not open stretch-sensitive calcium channels, as suggested for activation of many cold-responsive genes (Örvar *et al*. 2000; Sangwan *et al*. 2002; Henriksson & Trewavas 2003), it appears that a non-cold-specific increase in cytosolic calcium levels suffices to activate *EARLI1* at room temperature.

The conceptual EARLI1 protein is a small, 168 amino acids-long, hydrophobic, and proline-rich protein containing a putative N-terminal signal sequence of 25 amino acids (Wilkosz & Schläppi 2000). Interestingly, the C-terminus contains an 82-amino-acid-long region with similarity to an a-amylase inhibitor domain. This domain might form a four-helix bundle with an internal cavity and is found in putative plant lipid transfer proteins (LTPs; Molina *et al*. 1996; Wirtz 1997). Plant LTPs are mostly soluble, small basic proteins with eight conserved cysteine residues (Fig. 6). Plant LTPs were found in the cytoplasm, but also in microsomal membranes or as secreted proteins in the cell wall (Thoma, Kaneko & Somerville 1993). Consistent with this, EARLI1 appears to localize to the plasma membrane or cell wall (Bubier *et al.* unpublished). Lipid transfer proteins have been shown to be involved in environmental stress responses ranging from pathogen defence to drought (Wirtz 1997). Low-temperature-regulated lipid transfer proteins have previously been identified in several plant species such as *Hordeum vulgare* (barley) and *Brassica oleracea* (cabbage; Molina *et al*. 1996; Dunn *et al*. 1998; Hincha *et al*. 2001). It was hypothesized that plant LTPs might deposit extracellular lipophilic molecules, such as cutin or wax, into the cell wall (Sterk *et al*. 1991). Moreover, cabbage cryoprotectin, which does not have *in vitro* lipid transfer activity, was shown to protect thylakoid membranes from freezing damage (Hincha *et al*. 2001). It is therefore possible that EARLI1 somehow modifies Arabidopsis cell wall or membrane composition in response to light and cold. In agreement with this hypothesis, over-expression of *EARLI1* protects Arabidopsis cells from freezing-induced cellular damage at temperatures of -2 to -4 ºC. Thus, a simple model for the function of *EARLI1* in Arabidopsis would be that cold and light, two determinants critical for cold acclimation, activate its expression in a calcium-dependent manner, resulting in some protection against freezing-induced cellular damage, possibly through modification of either the cell membrane or the plant cell wall.

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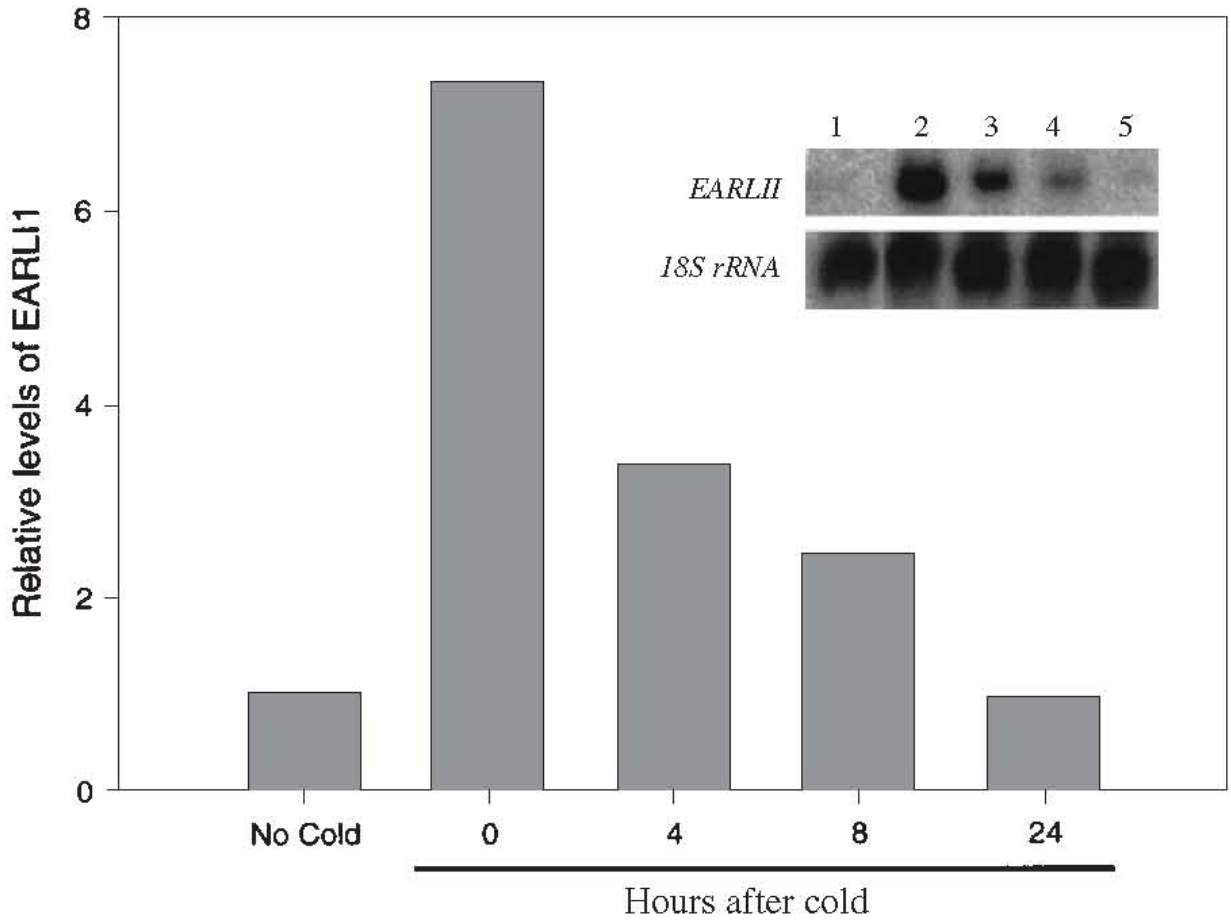
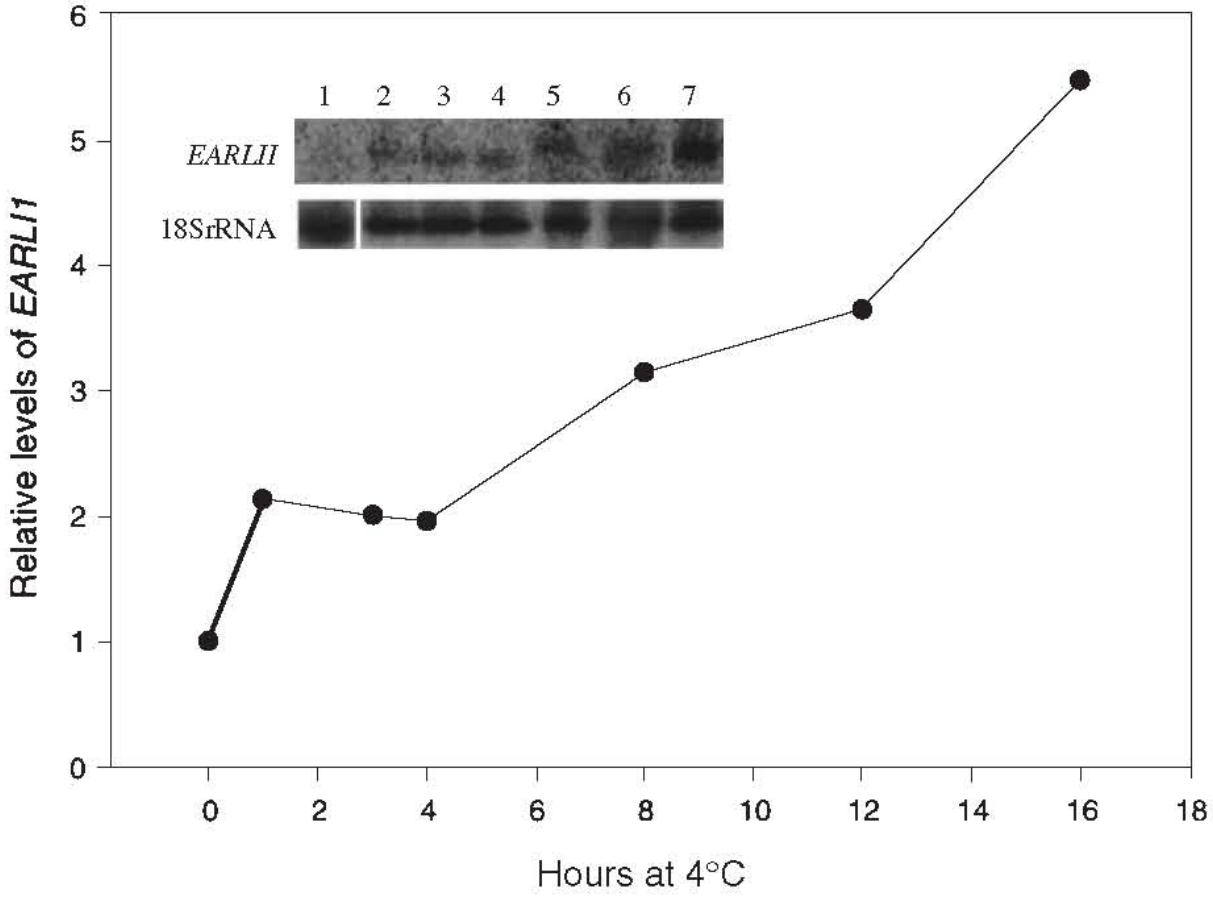
Appendix

Figure 1



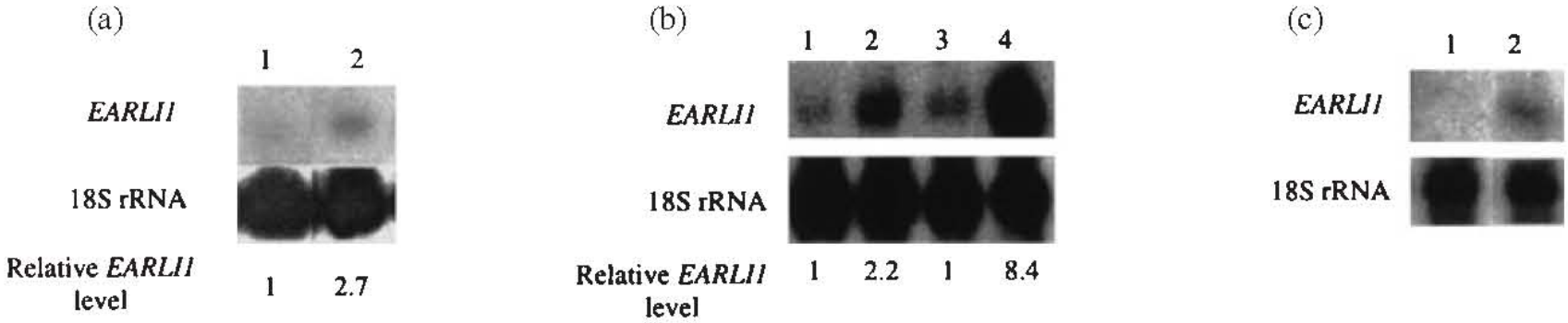
Transient induction of *EARLI1* by low temperature–total RNA gel blot analysis. RNA was isolated from 10-day-old Col-*FRI*-Sf2 seedlings grown at room temperature in short day photoperiods (SD) then incubated 16 h at 4ºC in SD. RNA from untreated control plants (1); RNA isolated immediately after 4ºC treatment (2); RNA isolated 4, 8 and 24 h after 4ºC treatment, (3), (4) and (5), respectively. The RNA blot was probed with a specific 208 bp fragment to detect *EARLI1*; the same blot was reprobed with a 1.8-kb 18S rDNA fragment to normalize RNA loading; band intensities were quantified using a phosphorimager and IMAGEQUANT software (Molecular Dynamics/Amersham Biosciences).

Figure 2



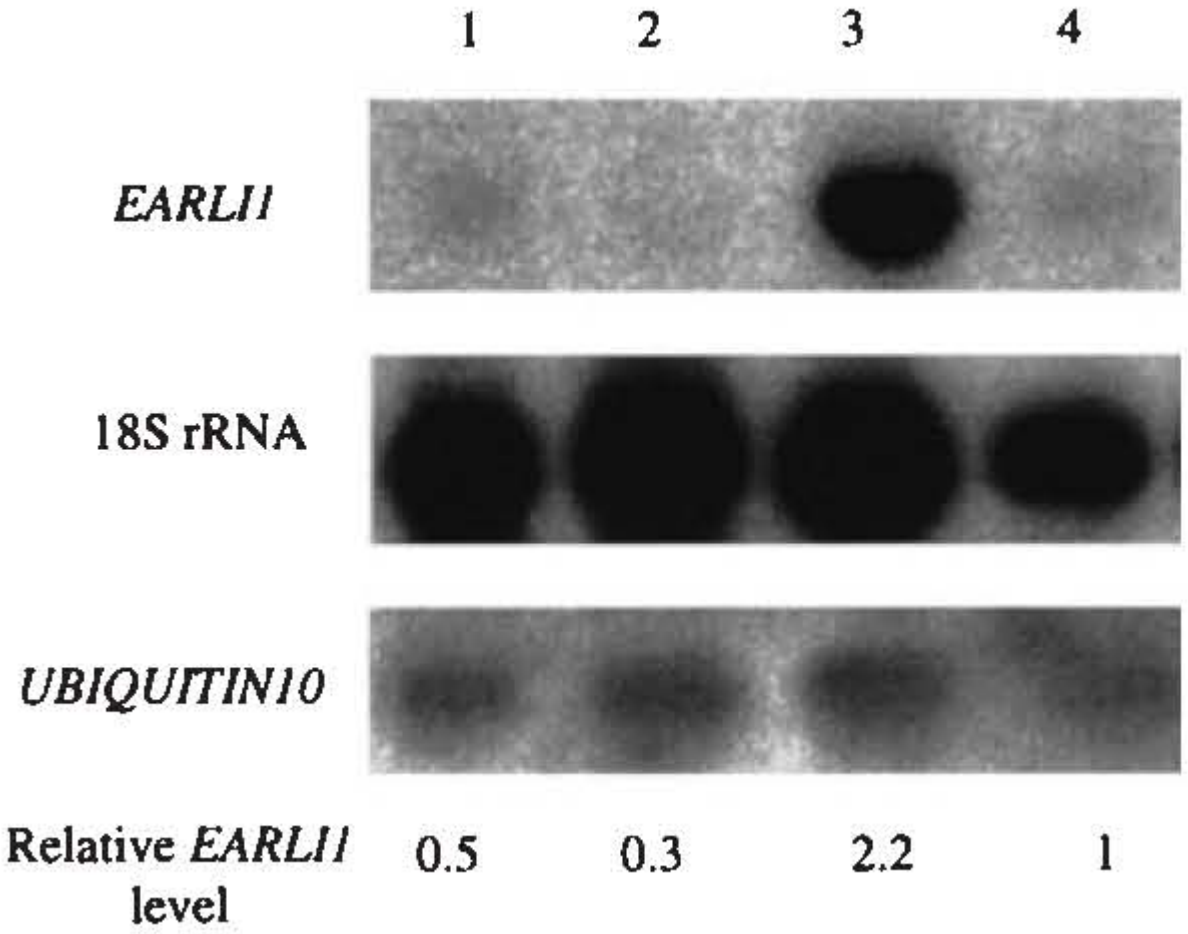
Kinetics of *EARLI1* cold induction–total RNA gel blot analysis. RNA was isolated from 10-day-old Col-*FRI*-Sf2 seedlings grown in short-day photoperiods for different amounts of time at 4ºC. Room temperature control (1); 1 h cold (1); 3 h cold (3); 4 h cold (4); 8 h cold (5); 12 h cold (6); 16 h cold (7). The RNA blot was probed with a specific 208 bp fragment to detect *EARLI1*; the same blot was reprobed with a 1.8-kb 18S rDNA fragment to normalize RNA loading; band intensities were quantified using a phosphorimager and IMAGEQUANT software.

Figure 3



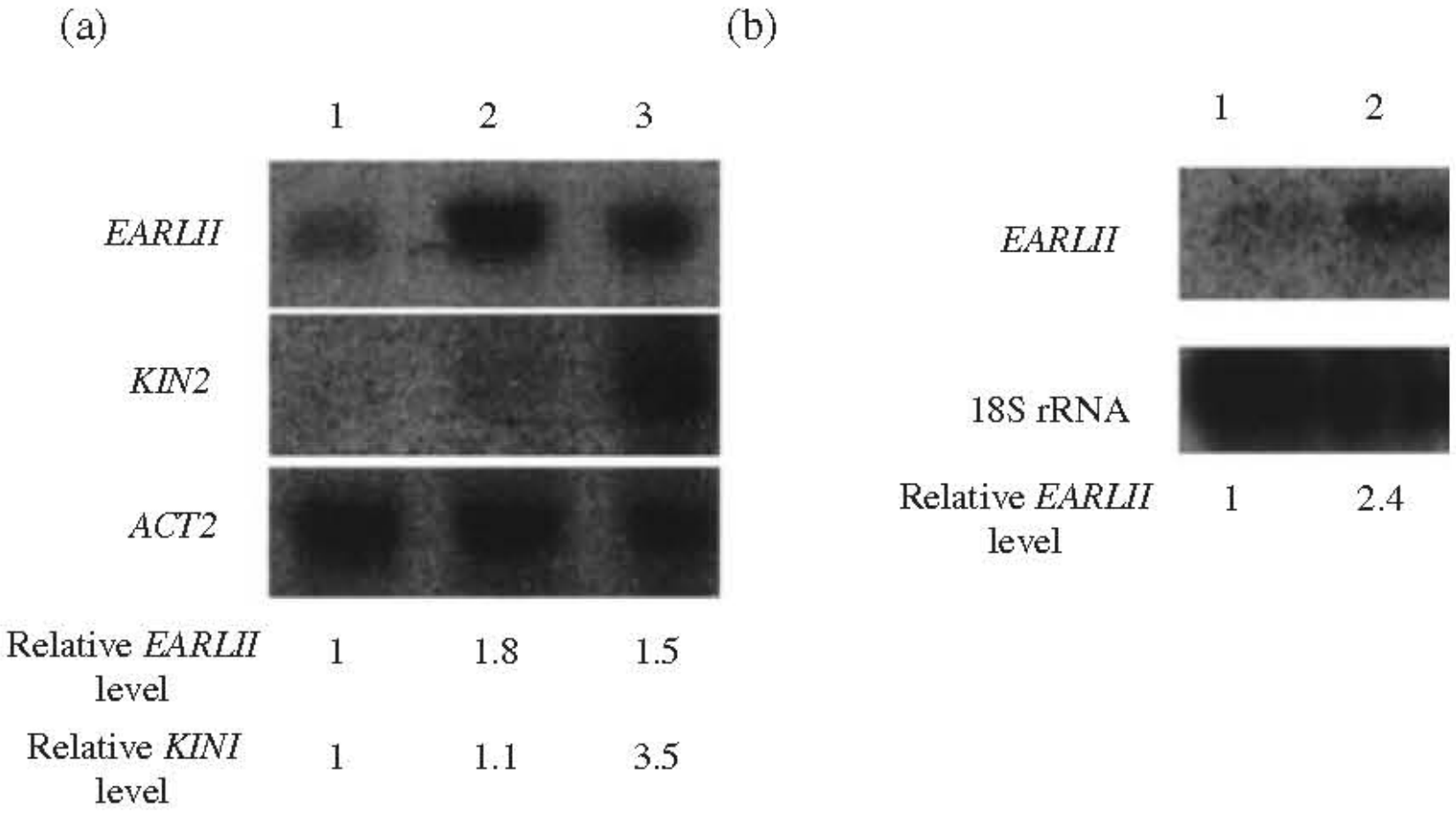
Effect of light and photoperiod on cold-mediated activation of *EARLI1*–total RNA gel blot analysis. (a) short-day (SD) photoperiods (1); long-day (LD) photoperiods (2); RNA was isolated from 10-day-old Col-*FRI*-Sf2 seedlings 5 h after subjective dawn. (b) LD photoperiods at 25ºC (1); LD photoperiods at 25ºC and cold-treated at 4ºC for 16 h (2); continuous light (CL) at 25ºC (3); CL at 25ºC and cold-treated at 4ºC for 16 h (4); RNA was isolated from 10-day-old Col-*FRI*-Sf2 seedlings. (c) continuous darkness (CD) at 25ºC (1); CD at 25ºC and cold-treated at 4ºC for 16 h (2); RNA was isolated from etiolated, 10-day-old Col-*FRI*-Sf2 seedlings. The RNA blots were probed with a specific 208 bp fragment to detect *EARLI1*(a)–(c); the same blots were reprobed with 1.8 kb 18S rDNA to normalize RNA loading (a)–(c); similar results were obtained when normalizing with *ACT2* (not shown); band intensities were quantified using a phosphorimager and IMAGEQUANT software; relative amount of *EARLI1* RNA in control plants (A-1; B-1; B-3) were set as 1.

Figure 4



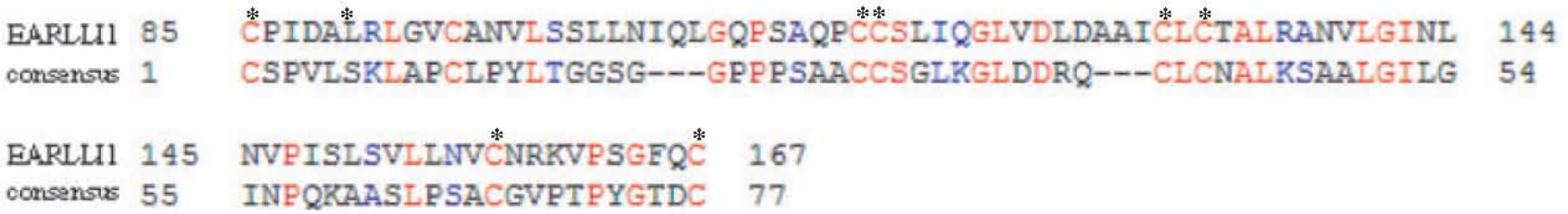
Effect of calcium antagonists on cold temperature expression of *EARLI1*–total RNA gel blot analysis. RNA was isolated from 10-day-old Col-*FRI*-Sf2 seedlings grown in short-day photoperiods and treated for 16 h at 4ºC (1–3) after application of calcium inhibitors for 1 h at room temperature. 10 mM EGTA (1); 10 mM LaCl3 (2); mock treated with water (3); control plants at room temperature (4). The RNA blot was probed with a specific 208 bp fragment to detect *EARLI1*; the same blot was reprobed with a 1.8-kb 18S rDNA fragment to normalize RNA loading; same RNA was reprobed with a 500-bp fragment to detect *UBIQUITIN10*; band intensities were quantified using a phosphorimager and IMAGEQUANT software; relative amount of *EARLI1* RNA in control plants (4) was set as 1.

Figure 5



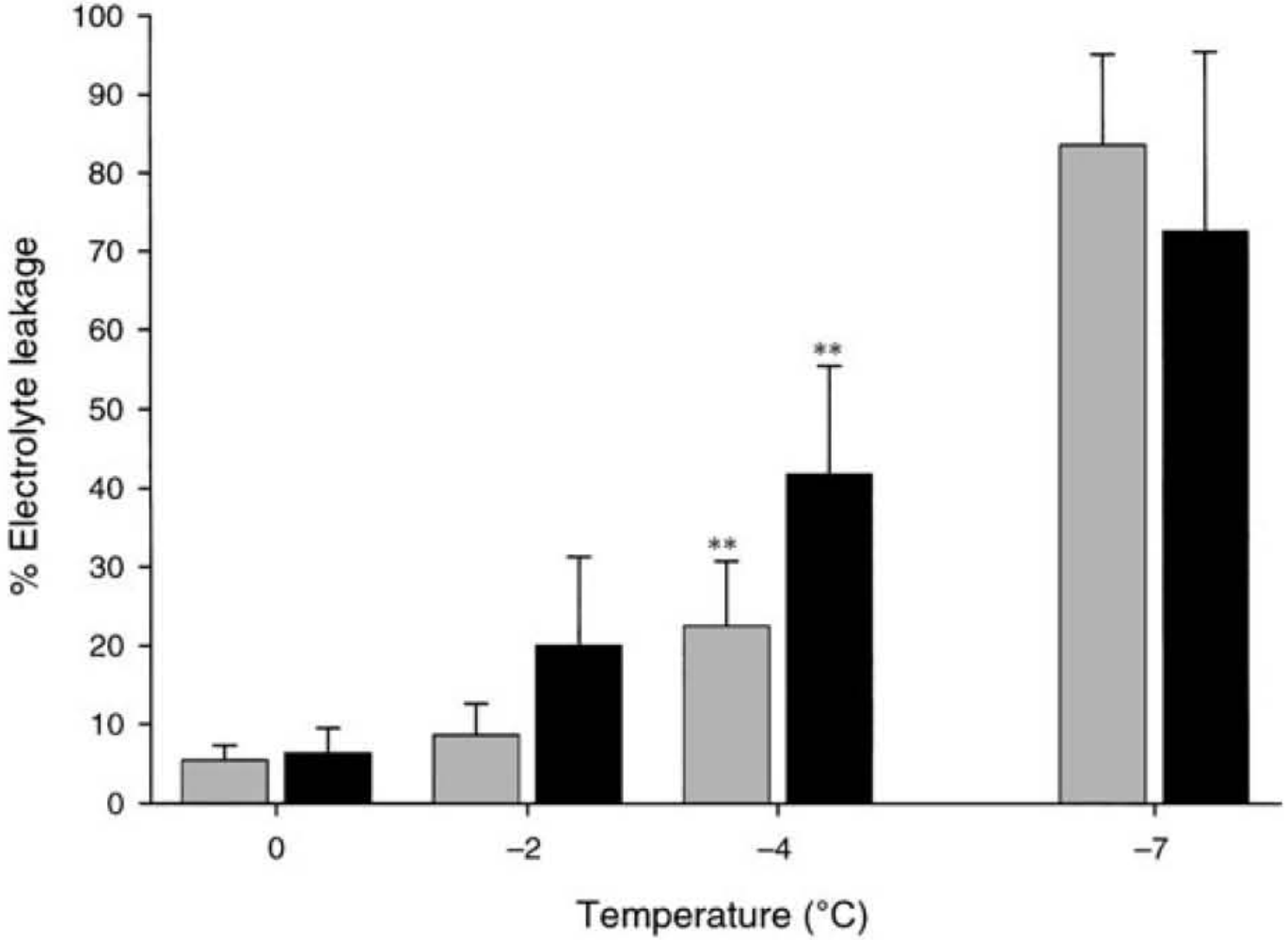
Effect of calcium ionophore Bay K8644 on *EARLI1* expression–total RNA gel blot analysis. Plants at room temperature were treated with DMSO or Bay K8644 for 1 h before rinsing with water; RNA was isolated 6 h after the treatment. (a) Mock-treated with 1% DMSO at 25ºC (1); Bay K8644/1% DMSO-treated at 25ºC (2); cold-treated at 4ºC for 16 h (3). (b) Mock-treated with H2O at 25ºC (1); 1% DMSO-treated at 25ºC (2). The RNA blot was probed with a specific 208 bp fragment to detect *EARLI1*; the same blot was reprobed with specific fragments to detect cold-responsive *KIN2* and *ACT2* or 18S for treatment control; band intensities were quantified using a phosphorimager and IMAGEQUANT software; relative amount of *EARLI1* or *KIN2* RNA in control plants (1) were set as 1. Similar results were obtained when normalizing with 18S rRNA (not shown).

Figure 6



Alignment of EARLI1 protein residues 85–167 with consensus sequence of putative plant lipid transfer proteins (LTPs). Conserved cysteine residues characteristic of hydrophobic cavity-forming domains of LTPs marked with asterisks (*).

Figure 7



Effect of over-expression of *EARLI1* on freezing-induced cellular damages–electrolyte leakage assay. Results are representative of three experiments with two different transgenic lines; leaves from adult nonacclimated control and transgenic plants were frozen side-by-side at indicated temperatures; the extent of cellular damage was estimated by measuring electrolyte leakage (see Materials and methods); error bars indicate standard deviations; black bars are control Columbia (Col) plants; grey bars are transgenic Col plants over-expressing *EARLI1* RNA. $P < 0.03$ (**).