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Molecular Cloning and Characterization of Cold-Regulated Genes in Barley[1]

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ABSTRACT

Five different cDNA clones have been isolated which are homologous to cold-regulated mRNAs in barley (*Hordeum vulgare* L.). The analyses of their hybridizations indicate that the transcripts accumulate to different levels during cold-treatment. Hybridization experiments using RNAs isolated from different plant tissues indicate that several cold-regulated genes are expressed in a tissue-specific manner. The expression studies suggest that in barley several different genes are involved in the cold hardening process depending on developmental stages and tissues involved. Homology has been found between the isolated cDNAs and cold-induced transcripts of related cereals. DNA sequence analysis of the clones pT59 and pA086 reveals that the proteins deduced from the longest open reading frame contain arginine rich basic domains.

The modifications of gene expression due to different environmental conditions are a common response in the metabolism of plant cells (27). Gene activation due to environmental stimuli plays an extremely important role in the adaptation of plants to unfavorable conditions.

A common stress situation is exposure to low temperatures. It is already known that under low temperature conditions plant cells modify several physiological parameters leading to an improved cold resistance (14) and that cold-treatment leads to a modification in gene expression (10, 20, 21, 23). There is, for example, evidence from several plant species that low, nonfreezing temperatures promote the appearance of specific proteins (9, 16, 18, 20, 23). However, it is not understood how the transcription of a new set of genes can lead to an improved cold tolerance. Therefore, the isolation and analysis of cold-induced sequences is an interesting approach to studying the function of these newly induced proteins. In tomato, for instance, a cold-induced transcript has been identified that is highly homologous to thiol proteases (29).

In cereals cold resistance is an important agronomic character. Barley has been chosen as an experimental system to study molecular aspects of cold hardening. Previous results have demonstrated that in barley seedlings several new mRNAs are accumulated and translated during exposure to low temperatures (4, 11). These mRNAs accumulate with very different kinetics. To reveal the function of these cold-induced mRNAs several cDNA clones were isolated and characterized. The levels of the corresponding cold-induced transcripts were analyzed in different tissues and under different physiological conditions. The DNA sequences of two selected cDNA clones were determined.

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MATERIALS AND METHODS

Plant Material and Stress Conditions

Barley (*Hordeum vulgare* L., cv Georgie) seeds were surface-sterilized in 3.5% (v/v) NaOCl 0.1% (w/v) SDS for 10 min, rinsed with water, germinated on moist filter paper in sterile Petri dishes, and grown in the dark at 22°C for 4 d. The Petri dishes containing the seedlings were exposed to a temperature around +1°C by incubation on ice in a cold room for 4 d. After the treatment the shoots or roots were cut off, immediately frozen in liquid N_2, and stored at −80°C.

Adult Barley Plants

Barley plants (late tillering and preanthesis stages) grown in soil under greenhouse conditions (22°C for 14 h day and 10°C for 10 h night) were stressed in a cold room at 3°C for 4 d and the leaves harvested for total RNA isolation.

Other Cereals

Seedlings of other related cereals have been used to investigate the presence of cold-induced mRNAs homologous to barley cDNA clones. Seedlings were grown from wheat (*Triticum aestivum* L. winter cvs Ural and spring cvs Planet, Ajax, Kadett, Star), rye (*Secale cereale* L. cv Halo) and oats (*Avena sativa* L. cv Alfred), and the same cold treatment was applied as described for barley. The shoots were used for RNA extractions.

cDNA Library Construction and Screening

A cDNA library was prepared using 3 μg of poly(A) RNA isolated from 4 d stressed barley shoots and purified twice on oligo (dT) cellulose as described in Bartels and Thompson (2). The first strand synthesis was performed with 50 units of AMV reverse transcriptase (Life Science Inc.) in standard

reaction conditions (12). The second strand synthesis was carried out using RNase H and *Escherichia coli* DNA polymerase I. The cDNA was made blunt ended using T_4 DNA polymerase and size fractionated on an agarose gel. All molecules greater than 400 base pairs in length were eluted from the gel, tailed with dCTP, and annealed to dG-tailed Pst I cut pUC9 plasmid DNA (Pharmacia).

After transformation into the *E. coli* strain TG2 the recombinant plasmids were selected on ampicillin IPTG X-GAL plates. About 5000 recombinant colonies were screened by *in situ* colony hybridization as described in Maniatis *et al.* (15) using ^{32}P-labeled poly(A) RNA probes (2) from 4 d cold-stressed and from un-stressed barley shoots. The colonies which showed hybridization only with the cold-stressed RNA probe were selected.

All the recombinant DNA techniques were performed as described by Maniatis *et al.* (15).

Size Fractionation of RNA and Hybridization

RNA hybridization experiments: 2 μg of poly(A) RNA or 100 μg of total RNA were separated on a 1% agarose gel containing 2.2 M formaldehyde using 40 mM Mops (pH 7), 10 mM Na acetate, and 1 mM EDTA (1 × Mops buffer). Three volumes of loading buffer (0.2 mL 5 × Mops, 0.35 mL formaldehyde, 1 mL formamide) were added to the RNA. The samples were heated at 65°C, cooled in ice and loaded on the gel. RNA size markers (BRL) were used as mol wt standards. The size fractionated RNAs were transferred to nitrocellulose or nylon filters by blotting with 20 × SSC (3 M NaCl, 0.3 M Na citrate). The filters were prehybridized in 50% (v/v) formamide, 5 × SSC, 10 mM Pipes (pH 6.8), 0.1% (w/v) SDS, 1 × (v/v) Denhardt solution (15), at 45°C for 2 h. The prehybridization buffer was replaced by a fresh sample of the same buffer containing the ^{32}P-labeled probe (6). After hybridization overnight the filters were washed with several buffer changes of 1 × or 0.5 × SSC, 0.1% (w/v) SDS at 65°C.

To compare the amounts of poly(A) RNAs bound to the filters, in Northern analysis with poly(A) RNAs, the filters were hybridized with ^{32}P-labeled oligo (dT) end-labeled with T_4 kinase and [^{32}P]ATP. When total RNA was used, equal amounts of RNA were ensured by a hybridization with a wheat ribosomal RNA clone pTA71 (8).

Hybrid Release Translation

According to Bartels and Thompson (2), 10 μg of linearized plasmid DNA were loaded on nitrocellulose filters and hybridized with 15 μg of poly(A) RNA isolated from cold-treated shoots for 4 h at 52°C in 65% formamide, 10 mM Pipes (pH 6.4), 0.4 M NaCl. The filters were washed 10 times at 60°C with 0.15 M NaCl, 0.015 M Na citrate, 0.5% (w/v) SDS followed by two washes at room temperature with 2 mM EDTA (pH 8). The mRNA was eluted by boiling and used for *in vitro* translation assays (rabbit reticulocyte lysate, Promega) supplemented with [^{35}S]methionine (Amersham, 3.02 × 10^{13} Bq/mmol). The whole reaction mixture (18 μL) was used for analysis in two-dimensional acrylamide gels (1).

DNA Sequencing and Computer Analysis

The DNA sequence of the cDNA clones was determined on both strands by subcloning of restriction enzyme fragments into M13 mp 18 and mp 19 (17) followed by dideoxynucleotide sequencing with the T_7 polymerase kit (Pharmacia) according to Sanger *et al.* (28).

The program WISGEN of the University of Wisconsin Genetic Computer Group was used as described for nucleic acid and protein sequence analysis and comparison (5).

RESULTS

Isolation of cDNA Clones Encoding Cold-Regulated Transcripts

Poly(A) RNA from 4 d cold-stressed (1°C) barley shoots was used to construct a cDNA library. By differential hybridization, five different cDNA clones (pT59, pV60, pA029, pA086, pAF93) were selected. These clones did not cross-hybridize with each other and thus represent different genes.

In hybridization experiments with RNA from cold-treated shoots, it was confirmed that these five clones represent cold-regulated transcripts (Fig. 1, A–C). Only transcripts homologous to pA086 and pAF93 hybridized very weakly to mRNAs transcribed at 22°C, while no signal was detected by the other clones.

The lengths of the corresponding mRNAs were determined in comparison with RNA standards (BRL) and were around 0.9 kb for pT59, pV60, pA029, and pA086 and 1.5 kb for pAF93 (Table I).

Hybrid release experiments were performed to prove that the selected clones encode cold-induced proteins. A clear result was obtained for the clone pV60 (Fig. 2). The hybrid-released translation product of this clone correlated with a polypeptide of an apparent molecular mass of 22 kD detected only in *in vitro* translation products obtained from mRNAs of cold-stressed leaves (4). Direct proof was not obtained that the other clones specify protein products identified in *in vitro* translations as cold-induced polypeptides.

Accumulation Patterns of Cold-Regulated Transcripts

To evaluate the relation between the expression of cold-regulated mRNAs and the stress temperature applied to the seedlings, several RNA hybridization experiments were performed with total and poly(A) RNA extracted from shoots. The results are shown in Figure 1. All five cDNA clones detected homologous transcripts in barley shoots kept for 24 h at 1°C. The levels of the transcripts increased during longer cold-treatment (4 d).

When after 4 d at 1°C the temperature was raised to 22°C, the levels of the transcripts induced in low temperature conditions dropped appreciably after 2 h (Fig. 1, D–H). After 8 h at 22°C, no cold-induced mRNAs were measured. The five cDNA clones encode transcripts with different abundancies during the time courses tested at 1°C and the recovery at 22°C. All cold-related mRNAs accumulated upon cold stress but, for instance, could not be detected after a heat shock

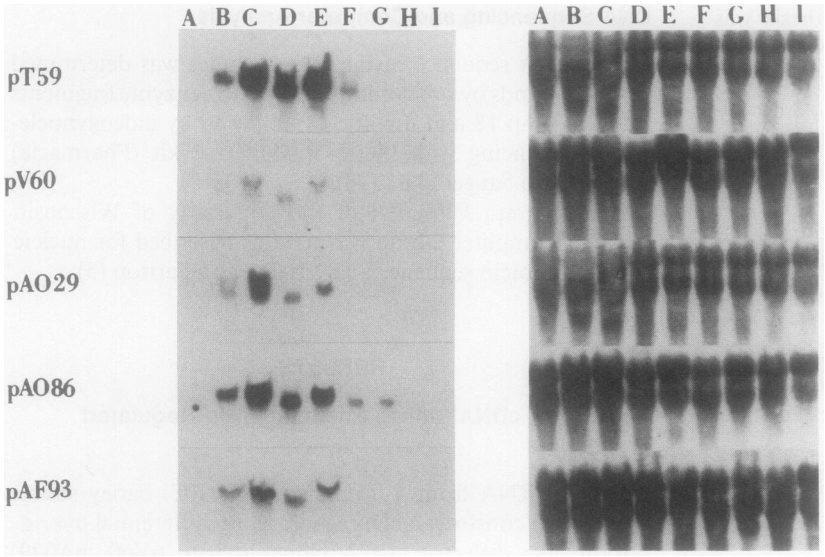

Figure 1. Left panel: Northern hybridization of the cold-regulated cDNA clones to total RNAs (80 μg per lane loaded) isolated from barley shoots after different temperature treatments. A, Control 22°C; B, 1 d 1°C; C, 4 d 1°C; D, 4 d 1°C then shift to 1 h at 22°C; F, 4 d 1°C then shift to 2 h at 22°C; F, 4 d 1°C then shift to 4 h at 22°C; G, 4 d 1°C then shift to 6 h at 22°C; H, 4 d 1°C then shift to 8 h at 22°C; I, heat shock (2 h 37°C). Right panel: As a control to ensure equal amounts of RNA the same filters as in left panel were hybridized with wheat ribosomal RNA clone pTA71.

treatment (Fig. 1I). As a control for the heat shock treatments, the filters carrying the heat shock RNAs were hybridized with a barley cDNA clone encoding a low mol wt heat shock protein. In all cases the clone hybridized to a specific RNA band.

When mRNAs from cold-stressed shoots and untreated shoots were hybridized with the ^{32}P-labeled insert of the barley ubiquitin cDNA clone pKG 3730 (7), no signal change was observed (Fig. 3). This result indicates that mRNAs exist that are not modulated by cold treatment and that specifically the level of ubiquitin mRNA is not increased by cold stress in barley, although in some other cases (22) ubiquitin has been reported to be stress-inducible.

Expression of the Cold-Regulated Transcripts in Different Plant Tissues

The expression of cold-regulated transcripts in cold-stressed tissues other than young shoots was investigated in RNA hybridization experiments (Fig. 4; Table I). For these hybridizations, RNAs were isolated from roots and mature leaves. Roots were derived from seedlings (one leaf stage) and mature leaves were harvested from two different developmental stages (late tillering and preanthesis). In hybridization experiments no differences between these two stages were detected; therefore, only the results derived from the preanthesis stage are shown in Figure 4. The clones pT59, pA029, pA086, and pAF93 hybridized clearly to mRNA of cold-treated mature leaves, but only pA029, pA086, and pAF93 gave a clear hybridization signal with mRNAs from cold stressed roots. The mRNA homologous to pA029 was more abundant in roots than in leaves. Cold treatment did not lead to an accumulation of the mRNA corresponding to the clone pV60 in roots or mature leaves. The results are summarized in Table I.

Homologous Transcripts are Expressed in Other Cereals

To establish whether homologies exist between cold-induced barley cDNAs and cold-induced mRNAs of other cereal species a specific experiment was performed. Total RNA of cold-stressed and nonstressed shoots of winter and spring wheat, rye, and oats were hybridized with the barley cDNAs using a stringency of 0.5 × SSC at 65°C. The results

Table I. *Characteristics of Described cDNA Clones*

Clone	Size of Corresponding mRNA	Tissue Specific Expression after Cold Treatment[a]				Homologous mRNAs in Other Cereals[a]		
		Shoots	Roots	leaves[b]		Wheat	Rye	Oats
				A	B			
	kb							
pT59	0.9	XXX		XXX	XXX	X	X	
pV60	0.9	XXX				X	X	
pA029	0.9	XXX	XXXX	XX	XX	X		
pA086	0.9	XX	X	XX	XX	X	X	
pAF93	1.5	XX	XX	X	X			

[a] The number of the crosses indicates the intensity of the hybridization signal. [b] A, Tillering stage; B, preanthesis stage.

presented in Figure 5 show that four barley cold-induced cDNAs hybridized with cold-induced mRNAs of wheat, three of them with cold-induced mRNAs of rye but none with those of oats at this hybridization stringency. Only the clone pAF93 was barley specific and did not cross-hybridize to the other cereals at this hybridization stringency.

DNA Sequence Analysis

The DNA sequences of the clones pT59 and pA086 were determined. The nucleotide and the amino acid sequence deduced from the longest open reading frame are shown in Figure 6. Both sequences are G-C rich (G-C content: pT59 = 63%, pA086 = 55%), but at the nucleotide level no homology between the two clones was apparent. At the amino acid level, however, both clones contain a region rich in the basic amino acids arginine in the longest open reading frame: pT59 contains a stretch of seven arginine residues between position 86 and 92 and four more arginines between position 93 and 100. In the amino acid sequence of pA086 two domains rich in basic residues are close to each other: seven arginine residues interspersed with five alanine residues between position 90 and 109 and eight arginine residues between position 116 and 125. Therefore, both putative polypeptides contain a domain with extremely basic properties.

A computer search revealed no significant homologies between the cold-induced sequences reported here and published DNA or protein sequences entered in Genbank and EMBL gene data bank. Therefore these selected cDNAs represent novel transcripts of unknown function.

DISCUSSION

Recently, we (4) reported the accumulation of cold-induced mRNAs in barley seedlings. To assess whether these cold-specific gene products are involved in the cold hardening process, the corresponding cDNA clones were isolated. The mRNAs encoded by the five cDNA clones described showed

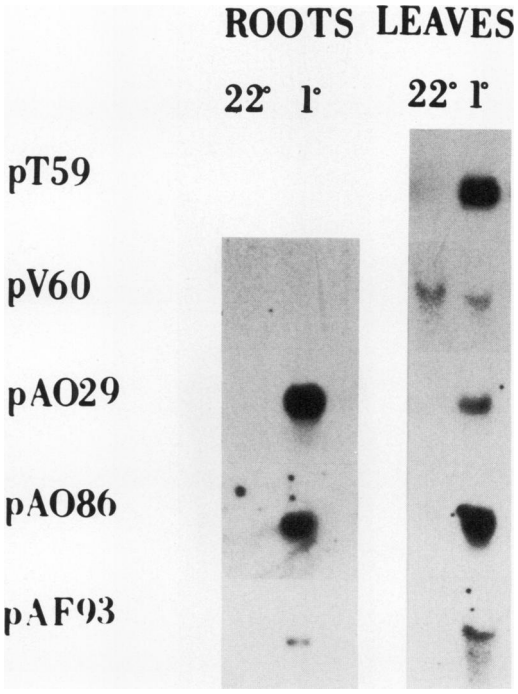

Figure 3. Northern hybridization of the cold-induced cDNA clones to total RNAs isolated from roots of seedlings (one leaf stage) and leaves (preanthesis stage) of barley plants before and after cold treatment for 4 d at 1°C.

a characteristic cold induction: they started to accumulate rapidly after the seedlings were transferred to the cold and decayed when the temperature was raised again to 22°C.

Expression of Cold Inducible mRNAs

The mRNA concentrations measured during the various cold stress temperature regimes could reflect either specific

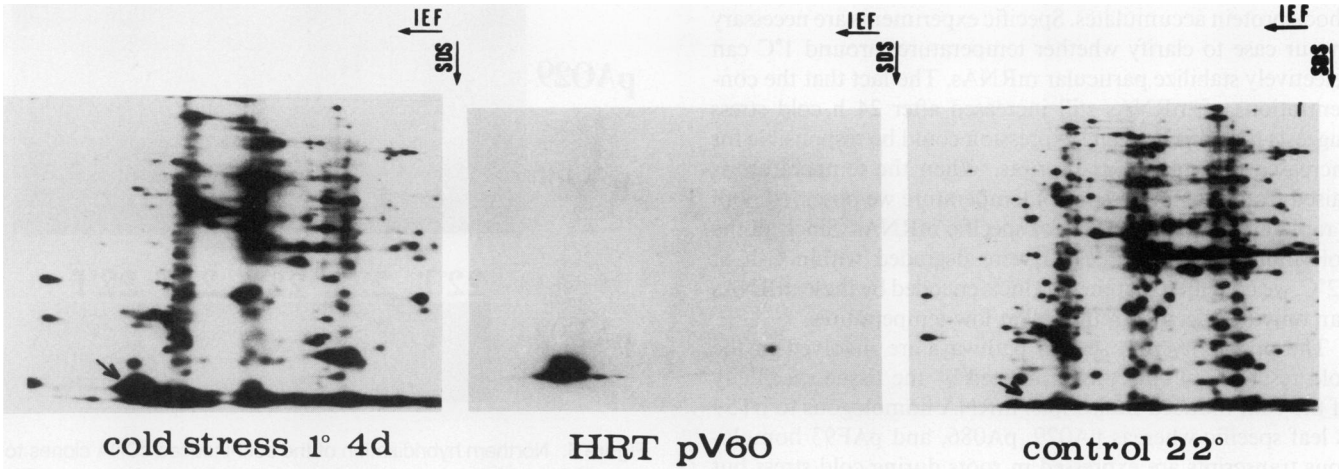

Figure 2. Hybrid release translation. Two-dimensional separation of *in vitro* translation products derived from: poly(A) RNA of cold-stressed shoots (left panel), RNA homologous to the clone pV60 (middle panel), poly(A) RNA of shoots grown at 22°C (right panel). The RNA homologous to pV60 encodes a polypeptide which can be correlated with a cold-specific protein indicated by an arrow in the left panel.

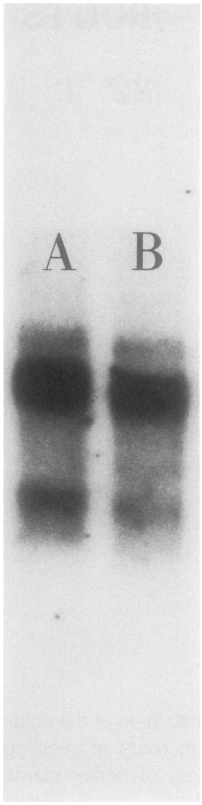

Figure 4. Northern hybridization of barley ubiquitin cDNA clone pKG 3730 to poly(A) RNAs isolated from (A) untreated and (B) cold-treated (4 d at 1°C) barley shoots.

gene transcription or selective thermostabilities of the mRNAs. A case of specific thermostability has, for instance, been reported for the mRNA encoding the heat shock protein HSP 70 from *Drosophila melanogaster* (24). The HSP 70 message is inherently unstable, but during heat shock the degradation of the message is suspended and the encoded heat shock protein accumulates. Specific experiments are necessary in our case to clarify whether temperatures around 1°C can selectively stabilize particular mRNAs. The fact that the concentrations of mRNAs still increased after 24 h cold stress suggests that *de novo* gene expression could be responsible for increased amounts of transcripts. When the temperature is raised from 1°C to the control temperature we observed both a rapid and a delayed decay of specific mRNAs. Since all the cold-induced mRNAs tested were degraded within 6 h at 22°C, we conclude that the products encoded by these mRNAs can only be effective in the cell at low temperatures.

The possibility that several pathways are involved in the cold response in barley is supported by the tissue specificity of the cold-induced transcripts. mRNA homologous to pT59 is leaf specific whereas pA029, pA086, and pAF93 homologous transcripts are expressed in roots during cold stress but at different levels in comparison with cold-stressed leaf tissue. As the plants show different cold-hardening capacities depending on the developmental stage (3) we examined the appearance of cold-induced transcripts in leaves from plants of three different ages. With the exception of the pV60 mRNA the other four cold-specific transcripts were induced after cold treatment also in mature leaves. The expression studies presented support the hypothesis that cold response in barley is a complex mechanism involving several pathways. This is in agreement with physiological studies where it has been reported that chilling increases enzyme activities in such different pathways like for instance phenylpropanoid (26) or sugar metabolism (25). The cross-hybridization data with the other cereals, on the other hand, suggest that the pathways have been conserved among closely related species.

DNA Sequence Analysis

In favorable cases, the comparison of DNA and protein sequences with known sequences may give a hint to the function of an isolated gene. We have not been able to find relevant homologies of the two cold-responsive genes described in this paper. This is not surprising, as up to now only the sequence for a cold-regulated cDNA from tomato has been reported to our knowledge (29). Although the sequences of pT59 and pA086 do not share homology to each other, their deduced amino acid sequences in the longest open reading frame exhibit common features: the presence of basic domains and evident hydrophilic properties. The presence of several arginine residues in close vicinity is particularly striking. An increasing list of proteins that contain functionally

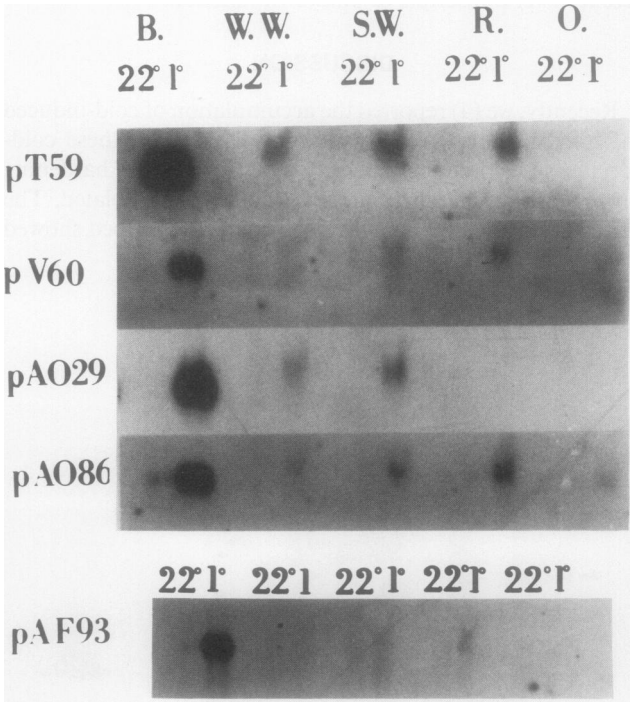

Figure 5. Northern hybridization of the cold-induced cDNA clones to total RNAs isolated from untreated and cold-treated (4 d at 1°C) shoots of barley (B.), winter wheat (W.W.), spring wheat (S.W.), rye (R.), and oats (O.). After the hybridization the filters were washed with 0.5 × SSC 0.1% SDS at 65°C.

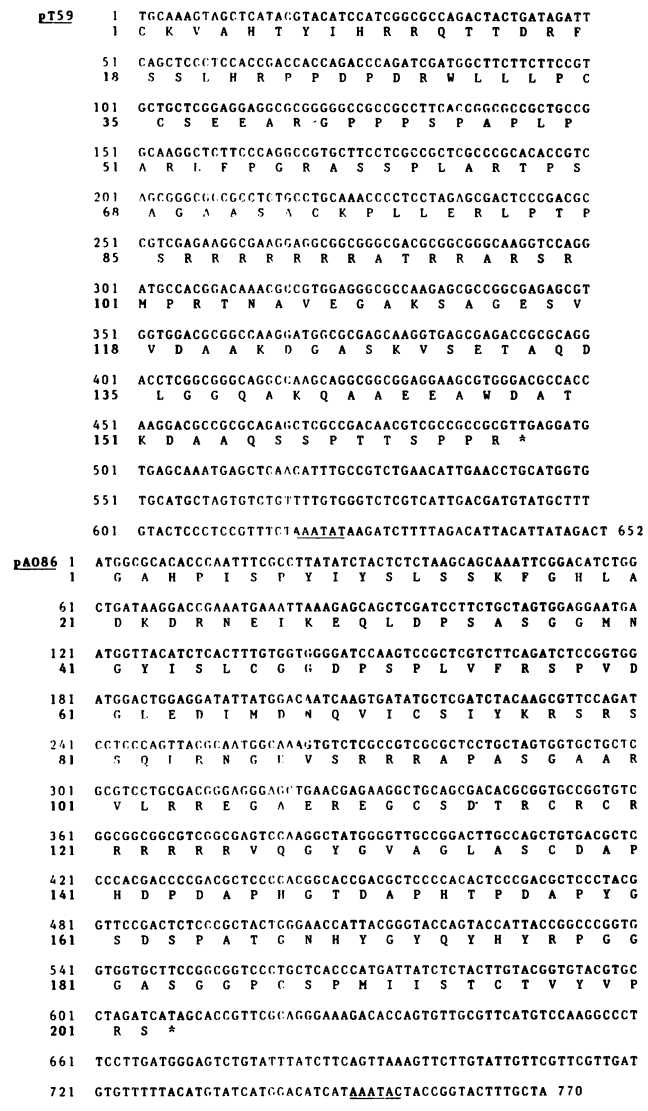

Figure 6. Nucleotide sequences of the clones pT59 and pA086. The amino acid sequences deduced from the longest open reading frame are shown. Possible polyadenylation signals at the 3′ end are underlined.

important arginine motifs is emerging. In these cases the arginine-rich sequences are involved in protein RNA interactions. Recently it has been demonstrated that sequence-specific recognition of RNA hairpins by bacteriophage antiterminators requires a conserved arginine-rich motif (13). Arginine repeats have also been observed in protein sequences for the coat proteins of several RNA viruses (19, 30). The suggestion was made that the arginine domain can interact with the virus RNA during the packaging of the viral particles. The role of the cold-induced mRNAs which encode arginine-rich proteins requires further investigations. The hypothesis should be tested whether the proteins encoded by pT59 and pA086 may stabilize specific mRNAs during cold stress.

Having cold-inducible cDNA clones available opens the way to studying the properties of the corresponding proteins *in vitro* and to the isolation of promoter sequences which would enable agronomically useful genes to be expressed during exposure to cold.

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