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# Expression Patterns and Promoter Activity of the Cold-Regulated Gene *ci21A* of Potato[1]

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**Storage of potato (*Solanum tuberosum*) tubers at 4°C is associated with the accumulation of several transcripts. DNA sequence analysis of cDNA clone CI21, which corresponds to one of the cold-induced transcripts, revealed high homology to transcripts of tomato (*Lycopersicon esculentum*) and wild potato (*Solanum chacoense*) induced by ripening and water stress. Two homologous, nonallelic genes, *ci21A* and *ci21B,* were isolated and sequenced. Northern blot analysis showed that CI21 transcripts were present at the highest levels in cold-stored tubers, at lower levels in stems and roots, and at the lowest levels in leaves and tubers stored at room temperature. Treatment with abscisic acid, heat, and a high concentration of salt had no marked effect on CI21 transcript levels in tubers and leaves. Drought was the only stress treatment that induced CI21 transcripts in leaves, but it did not do so in tubers. Western blot analysis detected CI21 protein only in tubers. Chimeric gene constructs between the putative *ci21A* promoter region and the *uidA* reporter gene were tested in transgenic potato plants for induction of β-glucuronidase activity by low temperature. A 2-fold increase of β-glucuronidase activity in response to tuber storage at 4°C was observed for fragments between 380 and 2000 bp of the *ci21A* promoter region.**

When exposed to low temperatures, plants respond with qualitative and quantitative changes in the observable patterns of gene transcripts and proteins (reviewed by Guy, 1990; Thomashow, 1990; Cattivelli and Bartels, 1992). A number of cDNA clones coding for cold-regulated transcripts and the corresponding genes have been isolated and characterized from a variety of plant species, including alfalfa, Arabidopsis, spinach, tomato, barley, and wheat (Cattivelli and Bartels, 1992, and refs. therein).

Many cold-induced transcripts are also induced by ABA and other abiotic stress treatments, such as drought (Cattivelli and Bartels, 1992; Thomashow, 1993). Both transcriptional and posttranscriptional control mechanisms have been reported for cold-stress-regulated gene expression (Hajela et al., 1990; Wolfraim et al., 1993; Dunn et al., 1994). Analysis of the promoters of cold-induced genes has provided further information regarding transcriptional regulation (Horvarth et al., 1993; Nordin et al., 1993; Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). For the Arabidopsis *cor15a* promoter (Baker et al., 1994) and the Arabidopsis *rd29a* promoter (Yamaguchi-Shinozaki and Shinozaki, 1994) fused to the *uidA* reporter gene, a clear induction after cold treatment was demonstrated in transgenic plants. A novel *cis*-acting element (the drought-responsive element) has been identified in the cold-inducible *rd29a* promoter and is responsive to low temperature, drought, and high salt stress (Yamaguchi-Shinozaki and Shinozaki, 1994). Variants of this motif have also been found in the promoter regions of *cor15a* (Baker et al., 1994) and other cold-induced genes (Kurkela and Borg-Franck, 1992; Nordin et al., 1993; White et al., 1994).

In potato (*Solanum tuberosum*) tubers stored at low temperatures, metabolic changes lead to the accumulation of the reducing sugars Glc and Fru, resulting from starch-sugar interconversion (Isherwood, 1973). The phenomenon of "cold-sweetening" during long-term storage of tubers at low temperature is negatively correlated with the quality of processed products (Schwimmer et al., 1957). When transgenes with inhibitory effects on the formation of reducing sugars can be specifically expressed in tubers stored at low temperatures, carbohydrate metabolism may be manipulated in such a way that cold-sweetening no longer occurs. For this reason, endogenous, tuber-specific, and cold-inducible (or at least cold-stable) promoters may be useful. We aimed, therefore, at the isolation of such potato promoters. As a first step we isolated cDNA clones encoding transcripts that accumulated during cold storage of potato tubers (van Berkel et al., 1994).

In this paper we report the further characterization of one of the cDNA clones, CI21, which encodes a transcript that is induced after 3 d of cold treatment of tubers and remains at elevated levels during the whole period of cold storage (van Berkel et al., 1994). We studied the expression of CI21 homologous transcript and protein in different tissues and in response to ABA and various other stress treatments. Two genes were isolated, and the promoter region of one was studied with respect to cold-inducible *cis* elements using chimeric constructs with the *uid*A reporter gene in transgenic potato plants.

## MATERIALS AND METHODS

### Plant Material and Stress Conditions

*Solanum tuberosum* ssp. *tuberosum* L. (cvs Desireé and Saturna) was grown under controlled environmental con-

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Abbreviation: X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.

ditions at 18°C and 75 to 80% RH, with a 14-h photoperiod. Whole plants were exposed to 4°C in the dark or with a 14-h photoperiod. Cold treatment of potato tubers was described previously (van Berkel et al., 1994). Heat treatment of whole plants was at 35°C for 3 and 5 h, respectively. For ABA treatment, sliced in vitro tubers (see below) or leaves were floated in Petri dishes on 100 $\mu$M ABA solution for 16 or 24 h, respectively. For salt treatment, sliced in vitro tubers or leaves were floated in Petri dishes on 50 mM NaCl for 8 h. Desiccation was induced by exposing sliced in vitro tubers and leaves on several sheets of filter paper to a continuous air flow at room temperature for 2 and 7 h, respectively. As a control, tissues were also incubated in Murashige-Skoog medium. Sampled plant tissues were frozen in liquid $N_2$ and stored at $-80$°C until further use.

### RNA Gel-Blot Analysis

Total RNA was extracted using the method described by Logemann et al. (1987). Total RNA (50 $\mu$g) was separated on 1.5% agarose gels in 0.66 M formaldehyde-containing buffer (Davis et al., 1986; Sambrook et al., 1989) and blotted onto nylon filters (Hybond-N, Amersham). Filters were hybridized to the CI21 cDNA insert (van Berkel et al., 1994) or to an rDNA fragment of wheat (Appels and Dvorak, 1982) labeled with [$\alpha$-$^{32}$P]dCTP by the random primer method of Feinberg and Vogelstein (1984). Hybridizations were performed for 16 h at 65°C in 2× Denhardt's solution, 6× SSC, 0.1% (w/v) SDS, and 20 $\mu$g mL$^{-1}$ denatured herring sperm DNA (Sambrook et al., 1989). After hybridization, filters were washed with several changes of buffer (2× or 0.5× SSC, 0.1% [w/v] SDS) at 65°C and exposed to film (XAR-5, Kodak) with intensifying screens (Trimax 3M, Neuss, Germany) at $-70$°C. For subsequent hybridizations, probes were removed by incubating the membrane for 1 h at 70°C in 0.1% (w/v) SDS. For gene-specific hybridization, filters were hybridized to the [$\gamma$-$^{32}$P]ATP end-labeled *ci21A* oligonucleotide (5'-TCTCCTCCTCCATCAGAT-3', position +101 to +84, Fig. 2B) and the *ci21B* oligonucleotide (5'-TCTCTTCAGCCATAATAT-3', position +822 to +805, Fig. 2C) according to the method of Wood et al. (1985). Hybridizations were performed for 16 h at 37°C in 0.9 M NaCl, 90 mM Tris-HCl, pH 7.6, 6 mM EDTA, 0.1% (w/v) SDS, 5× Denhardt's solution, and 200 $\mu$g mL$^{-1}$ tRNA. After hybridization, filters were washed once with 2× SSC at room temperature and three times with 3 M $(CH_3)_4NCl$, 50 mM Tris-HCl, pH 7.5, 0.05% (w/v) SDS, and 2 mM EDTA at 55°C.

### Western Gel-Blot Analysis

Overexpression and production of a CI21 fusion protein in *Escherichia coli* was carried out using the QIA express system (Qiagen, Hilden, Germany). The entire CI21 cDNA insert was subcloned as a *Bam*HI/*Xho*I fragment in-frame into the *Bam*HI/*Sal*I sites of the pQE-10 vector. Purification and affinity chromatography of the overexpressed CI21 fusion protein was performed under denaturing conditions according to the manufacturer's instructions. The purified CI21 fusion protein was used to raise a polyclonal antiserum by injecting three aliquots of 100 $\mu$g each into rabbits (Eurogentec, Seraing, Belgium). Total protein was extracted from potato tissues by grinding and precipitation with 10% (w/v) TCA in acetone. The protein pellets were resuspended in 5.6 mM $Na_2CO_3$, 56 mM DTT, 2% (w/v) SDS, 12% (w/v) Suc, and 0.005% (w/v) bromphenol blue and denatured for 5 min at 95°C. Proteins were separated by 15% (w/v) SDS-PAGE (Neville et al., 1971), transferred to a nitrocellulose membrane (Schleicher & Schuell) by "semidry" blotting (Kyhse-Anderson, 1984), or stained directly with Coomassie brilliant blue. The membrane was probed with antiserum (dilution 1:2500) raised against the CI21 fusion protein, followed by incubation with anti-rabbit IgG-conjugated horseradish peroxidase. The protein-antibody complex was detected with a chemoluminescense western blotting detection system (ECL, Amersham).

### Densitometry

The autoradiograms of northern and western gel-blot analyses were scanned (model GT-6000, Epson, Düsseldorf, Germany) and analyzed with the computer program ScanPack (version 14.1A24, copyright 1991, Biometra, Göttingen, Germany).

### Isolation of Genomic Clones

A genomic library of *S. tuberosum* cv Saturna constructed by Stratagene was kindly provided by DANISCO (Copenhagen, Denmark). The $\lambda$-EMBL3 library was screened by plaque hybridization ($10^6$ plaque-forming units) using the [$\alpha$-$^{32}$P]dCTP-labeled CI21 cDNA insert (van Berkel et al., 1994) as a probe (Sambrook et al., 1989). Hybridizing plaques were picked and purified by several rounds of plaque hybridization. $\lambda$-DNA was prepared according to the method of Sambrook et al. (1989) and the recognition sites of several restriction enzymes were mapped. DNA restriction fragments were subcloned into plasmid vector (pBluescript, Stratagene) using standard protocols.

### Primer Extension

The transcription start site of the *ci21A* gene was defined by primer extension analysis with 30 $\mu$g of total RNA from cold-treated (7d, 4°C) potato tubers annealed with 100 $\mu$g of an 18-mer oligonucleotide (5'-GTGGTGTTTCTCCT-CCTC-3') for 5 h at 42°C in 0.4 M NaCl, 10 mM Pipes, pH 6.8. The oligonucleotide was labeled with [$\gamma$-$^{32}$P]ATP using T4 kinase (Sambrook et al., 1989). The oligo-annealed RNA was then incubated in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 80 $\mu$M 2'-deoxynucleoside 5'-triphosphate, and 40 units of RNasin with 200 units of reverse transcriptase (BRL) for 1 h at 42°C. The reaction was stopped by ethanol precipitation. DNA extension products were resuspended in 12 $\mu$L of 80% (v/v) formamide, 10 mM NaOH, 1 mM EDTA, 0.25% (w/v) bromphenol blue, and 0.25% (w/v) xylenecyanol and analyzed on an 8% polyacrylamide gel (Garoff and Ansorge, 1981) using sequenced DNA as a reference.

### Construction of *ci21A* Promotor-*uidA* Gene Fusion Plasmids

The 5′ region of the *ci21A* gene (−4700 bp to +157 bp) was directionally inserted into the *Pst*I/*Sal*I site of pBI201.1 upstream of the *E. coli uidA* gene and the *nos* polyadenylation signal (Jefferson, 1988). Nested deletions of the 4.7-kb *Eco*RI/*Sal*I promoter fragment were generated using the exonuclease III procedure of Hemikoff (1984; constructs 1–8).

The −874/+83-bp 5′ region of the *ci21A* gene was inserted into the *Sma*I site of pBI201.1 upstream of the *E. coli uidA* gene and the *nos* polyadenylation signal (Jefferson, 1988; construct A). The promoter deletion mutants (nos. 1–8) and construct A were subcloned into the binary vector pBIN19 (Bevan, 1984) and transferred into *Agrobacterium tumefaciens* strain LBA4404 (Ooms et al., 1982) by electroporation (Wen-jun and Forde, 1989) using a gene pulser (Bio-Rad) according to the manufacturer's instructions.

### Transformation, Plant Regeneration, and in Vitro Tuberization

Leaf discs of *S. tuberosum* cv Desireé were transformed as described by Horsch et al. (1985). After inoculation with *A. tumefaciens* strains harboring the various promoter constructs, transformed cells were selected on Murashige-Skoog medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) Glc, 2 mL $L^{-1}$ zeatin riboside, 0.02 mg $L^{-1}$ NAA, 0.02 mg $L^{-1}$ GA, and the antibiotics cefataxime (250 mg $L^{-1}$) and kanamycin (100 mg $L^{-1}$). Kanamycin-resistant shoots were transferred to a root-promoting medium containing cefataxime (250 mg $L^{-1}$) and kanamycin (100 mg $L^{-1}$) and grown at 18 to 20°C for a 14-h photoperiod.

In vitro tuberization of stem explants to obtain in vitro tubers was as described by Perl et al. (1991). Single-node shoot cuttings from in vitro-grown plantlets were transferred to solidified, one-half-strength Murashige-Skoog medium (0.8% [w/v] agar) supplemented with 8% (w/v) Suc, 5 mg $L^{-1}$ kinetin, and 5 mg $L^{-1}$ ancymidol and cultured axenically in the dark at 20°C.

### PCR Analysis of Transgenic Plants

Kanamycin-resistant plants were analyzed for the integration of the *ci21A* promoter *uidA* gene constructs by PCR (Saiki et al., 1988) using a *ci21A* promoter sequence as 5′ primer (5′-GCCACCTCATGGCACAATAA-3′) and an *uidA* sequence as 3′ primer (5′-TTGCGCTGATACC-AGACGTT-3′). Genomic DNA was prepared from small leaf samples according to the method of Edwards et al. (1991). PCR was carried out with 2.5 $\mu$L of DNA, 0.25 $\mu$M each primer, 1 unit of *Taq*-polymerase (Pharmacia), and 100 $\mu$M 2′-deoxynucleoside 5′-triphosphate in a total volume of 50 $\mu$L under the following conditions: 1 cycle at 93°C for 2 min; 40 cycles at 93°C for 45 s, at 55°C for 45 s, at 72°C for 1 min 20 s; and 1 cycle at 72°C for 10 min. The expected PCR products of 460 bp were analyzed on 1% agarose gels.

### GUS Activity Assays

Crude extracts were prepared from small samples of tissue (leaves, stems, roots, and in vitro tubers) and assayed for GUS activity as described by Jefferson et al. (1987). Extracts were standardized based on protein concentration, as determined by the method of Bradford (1976). GUS activity is expressed as pmol 4-methylumbelliferyl-β-D-glucuronide $mg^{-1}$ protein $min^{-1}$. We used X-Gluc for histochemical staining for GUS activity in transgenic tissue sections, as described by Jefferson et al. (1987). Sliced tissues were stained with 3 mM X-Gluc in GUS extraction buffer at 37°C.

### Statistical Analysis of Transgenic Lines

GUS activities measured in four to five independent transgenic lines transformed with the same construct were analyzed according to the method of Nap et al. (1993). Median and median absolute deviation values were calculated. The Wilcoxon-Mann-Whitney test (Conover, 1980) was used to assess significance of differences between constructs and treatments.

### DNA Sequence Analysis

The nucleotide sequences of the cDNA clone CI21 and of fragments of the *ci21A* and *ci21B* genes in pBluescript vector were determined on both strands with sequence-specific oligonucleotide primers using an automated sequencer (model 373A, Applied Biosystems). The University of Wisconsin Genetics Computer Group (Madison, WI) program library was used for nucleic acid and protein sequence analysis (Devereux et al., 1984). The database search was performed using the BLAST algorithm from Altschul et al. (1990).

## RESULTS

### DNA Sequence Analysis of cDNA Clone CI21

The insert of cDNA clone CI21 was 675 bp long and contained an open reading frame coding for a polypeptide of 109 amino acids (12.4 kD). The cDNA sequence (CI21A in Fig. 1) was highly homologous to a transcript of tomato induced by water deficit and during ripening (Iusem et al., 1993) and to a water-stress-induced protein of *Solanum chacoense* (Silhavy et al., 1995). Figure 1 shows the comparison among the deduced amino acid sequences of CI21 cDNA (CI21A), ASR1 of tomato, and DS2 of *S. chacoense*. The highest homology (90% identity/93% similarity) was found between CI21A and ASR1. The homology between the CI21A and DS2 polypeptides was lower (60% identity, 72% similarity). Compared with CI21A and ASR1, the DS2 polypeptide contained at position 21 a stretch of 155 additional, unrelated amino acids. The putative CI21A protein is extremely hydrophilic because of its high content (>50 mol%) of charged amino acids, a characteristic it has in common with many other stress-induced proteins such as CI7, which is also induced in cold-stored potato tubers (van

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CI21A   MEEEK..HHHHLF.HHKDKAEE.GPV................DYE   25
ASR1    MEEEK..HHHHLF.HHKDKAEE.GPV................DYE   25
CI21B   MAEEK..HHHHLF.HHKNKEEEGGPV................DYE   26
ASR2    MAEEKHQHHHHLF.HHKNKEDEGGPV................DYE   28
DS2     ....KH.HFGGLFHHHKNEEED.TPV----------------DYE  178

CI21A   KETKHHKHLEQIGKLGTVAAGAYALHEKHEAKKDPEHAHKHKIEE   70
ASR1    KEIKHHKHLEQIGKLGTVAAGAYALHEKHEAKKDPEHAHKHKIEE   70
CI21B   KEVKHHSHLEKIGELGAVAAGAFALHEKHKAKKDPENAHKHKIEE   71
ASR2    KEVKHHSHLEKIGELGAVAAGALALHEKHKAKKDPEHAHKHKIEE   73
DS2     EEKKHHKHLEEIGGLGAVAAGAFALHEKHKAEKDPENAHKHKIEE  223

CI21A   EIAAAAAVGAGGFALHEHHEKKDAKKEQKKAEGGH.HH....LF.  109
ASR1    EIAAAAAVGAGGFAFHEHHEKKDAKKEEKKKLRGDTTISSKLLF.  114
CI21B   EIAAVAAVGAGGFAFHEHHQKKDSKKEKKEAEGG..HHY......  108
ASR2    EIAAVAAVGAGGFAFHEHHQKKDAKKEKKE...............  103
DS2     GIAAAAAIGAGGFAFHEHHEKKEAKEEEEEAEGKKKHHF....EN  264
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**Figure 1.** Comparison among the amino acid sequences predicted from the CI21 cDNA (CI21A), the coding sequence of gene *ci21B* (CI21B), the ripening-related and water-deficiency-induced transcript ASR1 from tomato (Rossi and Iusem, 1994), the coding sequence of tomato gene *asr2* (ASR2, Rossi and Iusem, 1994), and the water-stress-induced transcript DS2 from *S. chacoense* (Silhavy et al., 1995). Regions of homology (identity) are indicated in black. DS2 contains an additional 155 amino acids at position 20, which are indicated as bars (–).

Berkel et al., 1994). The predicted pI of the CI21A protein was 7.1.

### Isolation and Mapping of the Genes *ci21A* and *ci21B*

The cDNA insert of CI21 was used as a probe to isolate two independent genomic clones (*ci21A* and *ci21B*). Restriction endonuclease site mapping showed that the structure of the two clones was different (Fig. 2A). The 4.7-kb *Eco*RI/*Sal*I fragment, the 2.3-kb *Sal*I fragment of the genomic clone *ci21A*, and the 2.3-kb *Bam*HI/*Eco*RI fragment of the genomic clone *ci21B* were subcloned and subjected to DNA sequence analysis (GenBank accession nos. U76670 and U76677). The nucleotide and deduced amino acid sequence of a 3.2-kb fragment of clone *ci21A* containing the coding region and upstream and downstream sequences is shown in Figure 2B. The genomic sequence was identical with the CI21 cDNA sequence with the exception of 1 bp exchange: Ala for Glu at amino acid position 17. The nucleotide sequence of a 1.8-kb fragment of clone *ci21B* (Fig. 2C) revealed a coding region of 108 amino acids that was 77% homologous to the CI21 cDNA, whereas upstream and downstream sequences were unrelated to *ci21A*. This suggested the existence of at least two genes homologous to CI21 transcripts. The genomic clone *ci21B* was over a 1100-bp region 90% homologous to the genomic sequence of the *asr2* gene of tomato (Rossi and Iusem, 1994), a second member of the tomato *asr* gene family. The polypeptides deduced from the sequences of genes *ci21B* and *asr2* were more similar to each other than to the CI21A and ASR1 polypeptides (Fig. 1).

Gene-specific fragments of the unrelated promoter regions of genes *ci21A* and *ci21B* were used as marker probes for restriction fragment length polymorphism mapping in potato (Gebhardt et al., 1994). Both genes mapped to the same position on chromosome IV and were, therefore, tightly linked (data not shown).

The coding regions of *ci21A* and *ci21B* were interrupted by one intron at identical positions. The intron of the *ci21A* gene was 930 bp long and not sequence related to the 133-bp intron of the *ci21B* gene. Both introns were bordered by GT..AG (Fig. 2, B and C). The transcription start site of the *ci21A* gene that corresponded to the CI21 cDNA was identified by primer extension analysis to be 89 bp upstream of the translation start codon. Putative polyadenylation signals (Joshi, 1987) were present downstream of the translation stop codon at positions +1360 (AATAA), +1547 (AATAA), +1623 (AATAAA), and +1776 (AATAA). Several motifs corresponding to known regulatory signals of eukaryotic gene expression were found upstream of the transcription start site: A putative TATA box motif at position −28 bp and a putative CAAT box at position −68 bp. In addition to these ubiquitous motifs, the *ci21A* promoter region contained sequences similar to regulatory promoter elements found in other plant genes. A G-box-like element (TGACGTGG) was located at position −259 bp (Williams et al., 1992) and a D-box-like element (TAAAAGTTAGATTA) was found further upstream at position −387 bp. The D-box is reported to play a role in the regulation of the patatin class I gene (Kim et al., 1994). In the *ci21A* promoter region a 110-bp gene shared high homology (80% identity) with intron 5 of a patatin class I gene (Rosahl et al., 1986) from position −830 to −720 bp.

In the *ci21B* gene, putative polyadenylation signals could be identified at positions 1319 (AATAAAA) and 1473 (AATTAAA). Upstream of the translation initiation codon, with the exception of four putative TATA box motifs (at positions 611, 647, 711, and 727, Fig. 2C), no homology was found to regulatory promoter elements of other plant genes.

### Expression of *ci21* Homologous Transcripts

As reported earlier (van Berkel et al., 1994), CI21 transcripts were induced in potato tubers after 3 d of exposure to 4°C. The level of expression after induction remained the same during the whole period of cold treatment. Aside from a major transcript of approximately 800 bp, two minor transcripts of approximately 740 and 670 bp were also detected on northern gel blots (Fig. 3A). Two of the three transcripts were also detectable in untreated stems and roots but not in leaves (Fig. 3A). Two oligonucleotides complementary to the region around the translation start codon of the *ci21A* and *ci21B* gene were designed to allow discrimination between the two transcripts on northern gel blots. The same filters shown in Figure 3A were hybridized to the labeled oligonucleotides. The *ci21A*-specific oligonucleotide detected the same transcripts as the CI21 cDNA probe, whereas the *ci21B*-specific oligonucleotide did not detect any transcript (data not shown), indicating that all three transcripts were preferentially derived from the *ci21A* gene. We cannot rule out, however, that a fraction of the transcripts detected by the CI21 cDNA probe in the samples shown in Figure 3A originated from the *ci21B* gene.

We tested whether exposure of plants to 4°C in the light or in the dark had any influence on CI21 transcript levels in

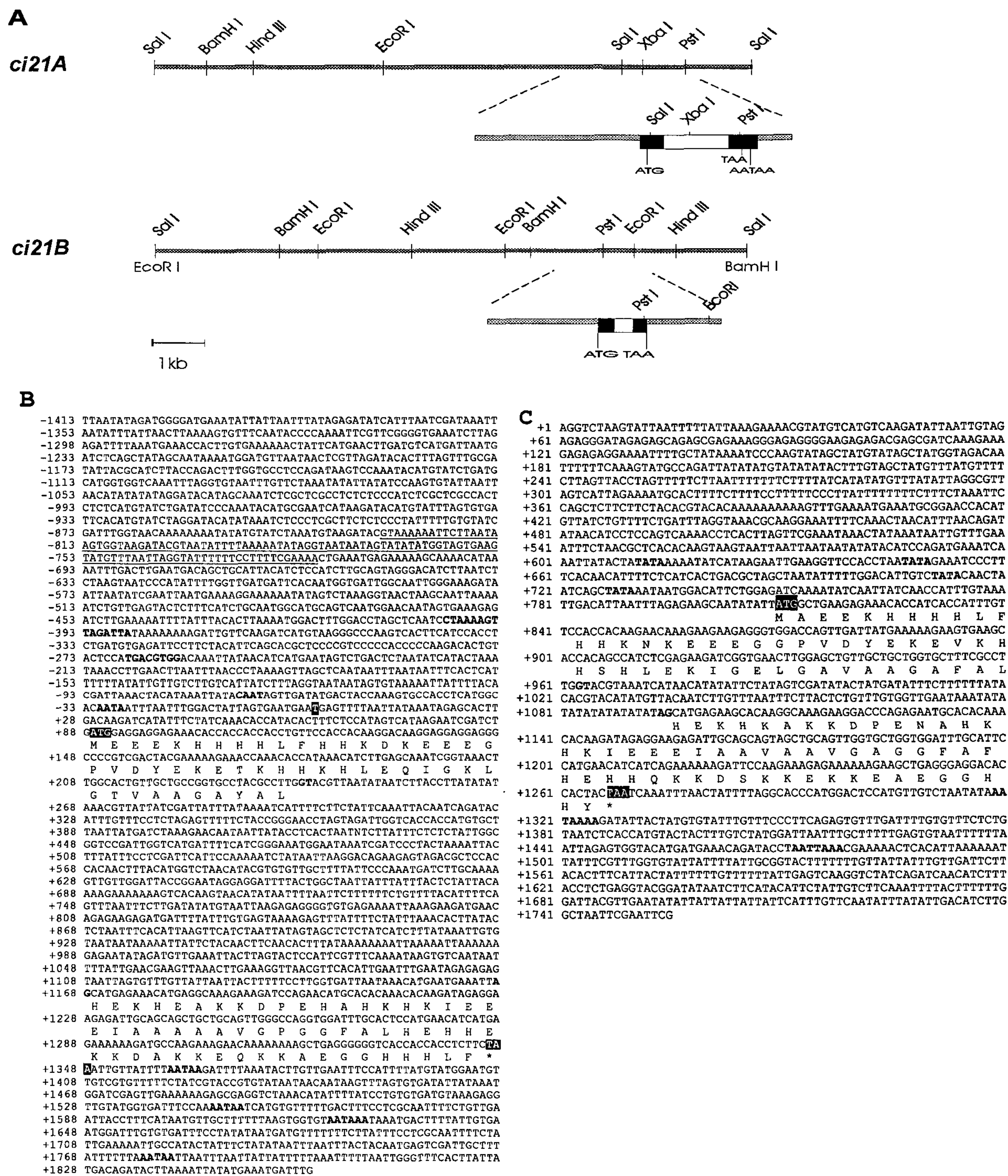


**Figure 2.** Structural analysis of genes *ci21A* and *ci21B*. A, Schematic representation of genomic clones *ci21A* and *ci21B*. The upper parts show the restriction map of the genomic clones and the lower parts indicate a magnification of approximately 3.2 kb of *ci21A* and approximately 1.8 kb of *ci21B*, containing the putative transcribed region (black boxes) with one intron (white box). B, Nucleotide sequence of the *ci21A* gene and predicted amino acid sequence of the encoded polypeptide corresponding to the CI21 cDNA. The sequence represents 3273 bp of the subcloned *Eco*RI/*Sal*I and *Sal*I/*Sal*I fragments isolated from genomic clone *ci21A*. Numbering starts at the transcriptional start site T, which is underlined in black. The translation start and stop codons are also underlined in black. Putative upstream regulatory elements, putative polyadenylation signals, and the intron boundaries are marked in bold letters. The region homologous to intron 5 of a patatin gene (Rosahl et al., 1986) is underlined. C, Nucleotide sequence of the *ci21B* gene and predicted amino acid sequence of the encoded polypeptide. The sequence represents 1755 bp of the subcloned *Bam*HI/*Eco*RI fragment of genomic clone *ci21B*. Start and stop codon, putative regulatory motifs, and intron boundaries are highlighted as in B.

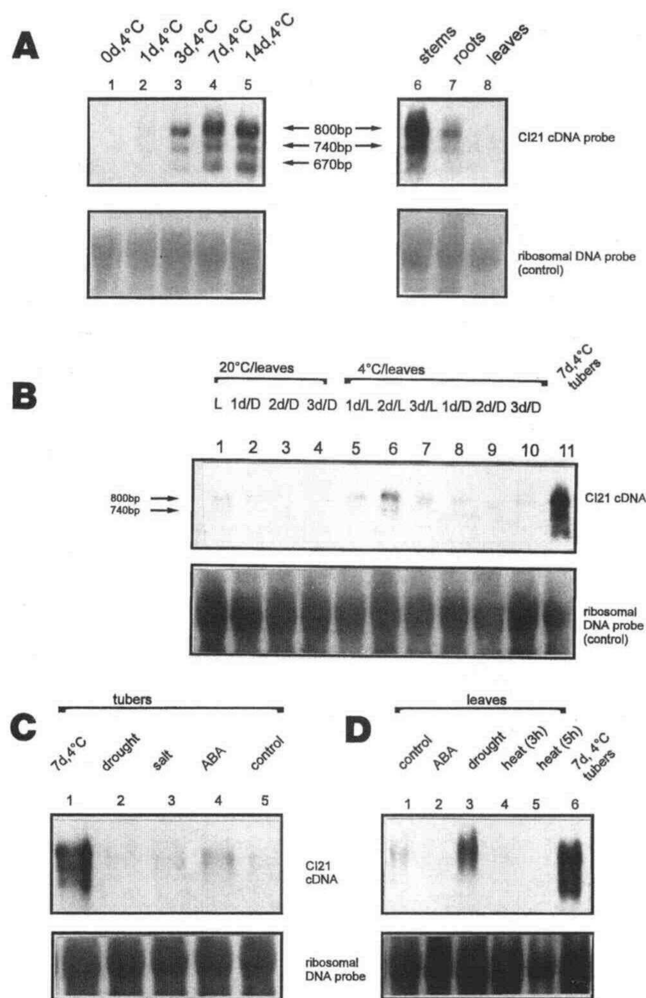


**Figure 3.** Expression studies of CI21 transcripts. A, Northern gel-blot analysis of potato tubers stored at 4°C for the number of days indicated (lanes 1–5) and in untreated stems, roots, and leaves (lanes 6–8). B, Northern gel-blot analysis of potato leaves during cold treatment. Leaves cut at the petiole were kept in water at 20 or 4°C in the light (L) or in the dark (D) between 1 and 3 d. C, Northern gel-blot analysis of potato tubers treated with ABA (76 h), high salt (8 h), drought (2 h), or liquid medium (control). D, Northern gel-blot analysis of potato leaves untreated or treated with ABA (24 h), drought (7 h), or heat. Tubers stored at 4°C for 7 d were used as a positive control in B, C, and D.

leaves. In leaves of plants grown under normal light conditions at 20°C, CI21 transcripts were just detectable and disappeared completely when the plants were transferred to the dark (Fig. 3B, lanes 1–4). Low-temperature treatment of leaves in the light resulted in a slight and transient induction of CI21 transcripts, which was not observed when leaves were kept at 4°C in the dark (Fig. 3B, lanes 5–10).

It was shown previously that most transcripts induced by cold stress also respond to desiccation, ABA, or high-salt treatments (Hajela et al., 1990; Yamaguchi-Shinozaki and Shinozaki, 1994). Therefore, we determined the effect of various stimuli on CI21 transcript levels. Treatment with ABA had no effect on CI21 transcript levels in tubers or in leaves (Fig. 3C, lane 4, and 3D, lane 2). Exposure to heat, high salt, or drought also did not elevate the expression of CI21 except in desiccated leaves (Fig. 3C, lanes 2 and 3, and 3D, lanes 3–5). The increase of the CI21 mRNA level in leaves during desiccation was, however, clearly smaller when compared with cold-stored potato tubers (Fig. 3D, lane 6). Whether the response seen in desiccated leaves results from CI21A or CI21B transcripts was not determined. The presence of equal amounts of total RNA on the northern blots was confirmed by control hybridization using as a probe rDNA of wheat (Appels and Dvorak, 1982).

## Expression of the CI21 Protein

A polyclonal antibody was raised against the 17-kD CI21 fusion protein synthesized in *E. coli* via the QIA express system (Qiagen; Fig. 4). The antiserum detected a single protein of 13 kD in extracts of potato tubers. The molecular mass of 13 kD was in agreement with the molecular mass calculated from the deduced amino acid sequence of the cDNA clone CI21 (12.4 kD). The accumulation of CI21 protein during low-temperature treatment of potato tubers was monitored by western gel-blot analysis (Fig. 5). Relative to the untreated control, the accumulation of CI21 protein was less pronounced than the accumulation of CI21 transcript (Figs. 5 and 6). Whereas the transcript level increased about 7-fold, only a 2-fold increase was observed at the protein level. No protein was detectable in stem, root, or leaf tissue (Fig. 5); however, the presence of protein in the extracts from stems, roots, and leaves was confirmed by Coomassie blue staining of the polyacrylamide gels (Fig. 5).

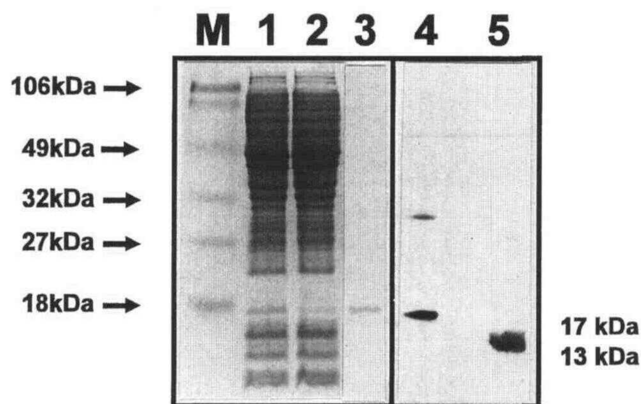


**Figure 4.** Purification of CI21 fusion protein and western blot analysis. A 17-kD protein appears in *E. coli* cells induced with isopropylthio-$\beta$-galactoside (lane 1), which is absent in noninduced cells (lane 2). The purified protein is shown in lane 3. M, Molecular mass marker. Proteins were stained with Coomassie brilliant blue. An antiserum raised against the purified CI21 fusion protein was used in western blot analysis. The antiserum reacts with the CI21 fusion protein (lane 4) and with an approximately 13-kD protein from potato tubers stored at 4°C for 7 d (lane 5).

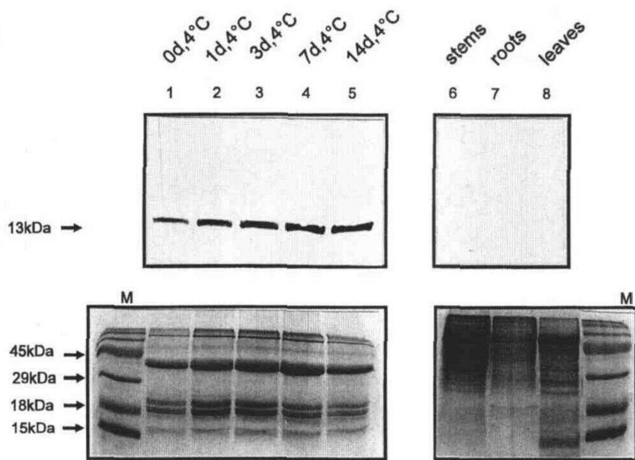


**Figure 5.** Western gel-blot analysis of total protein of potato tubers stored at 4°C for the number of days indicated (lanes 1–5) and in untreated stems, roots, and leaves (lanes 6–8). Tissue samples were identical with the ones used for northern blot analysis in Figure 3A. Hybridization was carried out with antiserum raised against the CI21 fusion protein (dilution 1:2500). As a control, the protein gels were stained with Coomassie brilliant blue.

## Analysis of the *ci21A* Promoter in Stably Transformed Potato Plants

The *ci21A* gene was chosen for further studies of the promoter region because it was the one that corresponded to the CI21 cDNA and was expressed in cold-stored tubers. To identify sequence elements possibly involved in the cold-induced expression of the *ci21A* gene, chimeric genes were constructed and tested in transgenic potato plants. Five prime flanking sequences of the *ci21A* genomic clone were fused with the *uidA* gene either at position +83 bp in the untranslated leader (construct A) or at position +157 downstream of the ATG codon (constructs 1–8). A series of eight promoter-deletion mutants of the directionally fused chimeric gene were prepared. The promoterless *uidA* gene served as a negative control (construct 0). The constructs were introduced into *S. tuberosum* cv Desireé by *A. tumefaciens*-mediated transformation. Four to five kanamycin-resistant plants originating from independent transformation events were regenerated per construct. Transgenic plants were identified by PCR using a construct-specific primer combination (data not shown). GUS enzyme activity was measured in in vitro tubers, roots, stems, and leaves of transformed plants.

Figure 7 shows the 10 constructs examined, as well as the GUS activities measured and averaged (Nap et al., 1993) over four to five independent transgenic plants per construct (except construct A, for which only one transgenic plant was available). No significant GUS activity was observed in any tissue of control plants transformed with the promoterless *uidA* gene (no. 0). Plants carrying *uidA* controlled by promoter fragments of 136 bp (no. 1) and 238 bp (no. 2) expressed very low GUS activities in all tissues examined. The GUS activities in plants with construct 2 were, however, significantly lower (P = 0.001) than in plants with construct 1. This suggests that sequence motifs inhibitory for *uidA* expression may be located between positions −136 and −238. In all other transgenic lines (constructs 3–8 and A), GUS activity was detected in all tissues examined. In tubers stored at 4°C for 7 d, the highest GUS activity was consistently measured for all constructs with promoter fragments between 387 bp (no. 3) and 2700 bp (no. 7). When compared with tubers stored at 20°C, the GUS activities increased to about 1.7-fold. The increase was significant at P = 0.05 or less for constructs 3, 4, 6, and 7 but not for construct 5 (P = 0.1). This increase corresponded to the approximately 2-fold increase observed for the endogenous CI21 protein (Fig. 5). Approximately 900 bp of the promoter region was sufficient for conferring maximum cold induction. No difference was observed between construct A and the directional chimeric gene fusions 3 to 7. The lowest level of GUS activity was consistently found in leaves. Compared with the leaves, GUS activity was 4 to 5 times higher in stems and roots and 6- to 7-fold higher in tubers stored at 20°C. This finding contrasts with the absence of CI21 protein in stems, roots, and leaves as determined by western gel-blot analysis (Fig. 5). Similar results were obtained when slices of in vitro tubers of transformed plants were histochemically stained for GUS with and without cold treatment (Fig. 8). Tubers from plants grown under normal conditions in the greenhouse gave essentially the same results (data not shown).

## DISCUSSION

In this study we characterized two genes of potato, *ci21A* and *ci21B*, which have been discovered based on elevated levels of CI21 transcripts in tubers exposed to low temperatures (van Berkel et al., 1994). The *ci21* genes encode homologous, highly hydrophilic polypeptides with a molecular mass of approximately 12 kD. The sequences upstream and downstream of the coding regions and the sequences of the single intron were, however, unrelated. This indicates that *ci21A* and *ci21B* are nonallelic and that

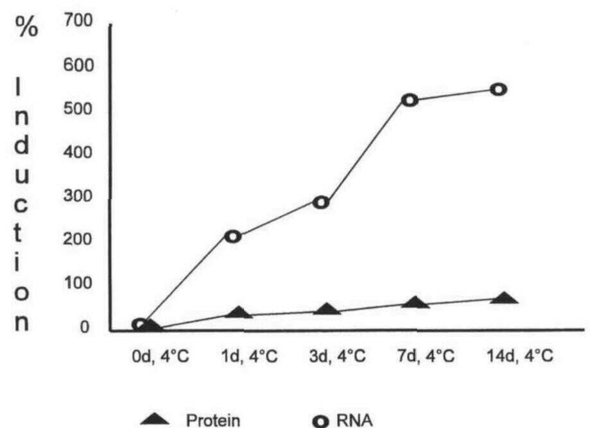


**Figure 6.** Induction of RNA and protein during cold storage of potato tubers. Autoradiograms of northern blots (○) and western blots (▲) were analyzed by densitometry. Induction is shown as the percentage increase of band intensity relative to band intensity at the beginning of cold storage (0 d, 4°C).

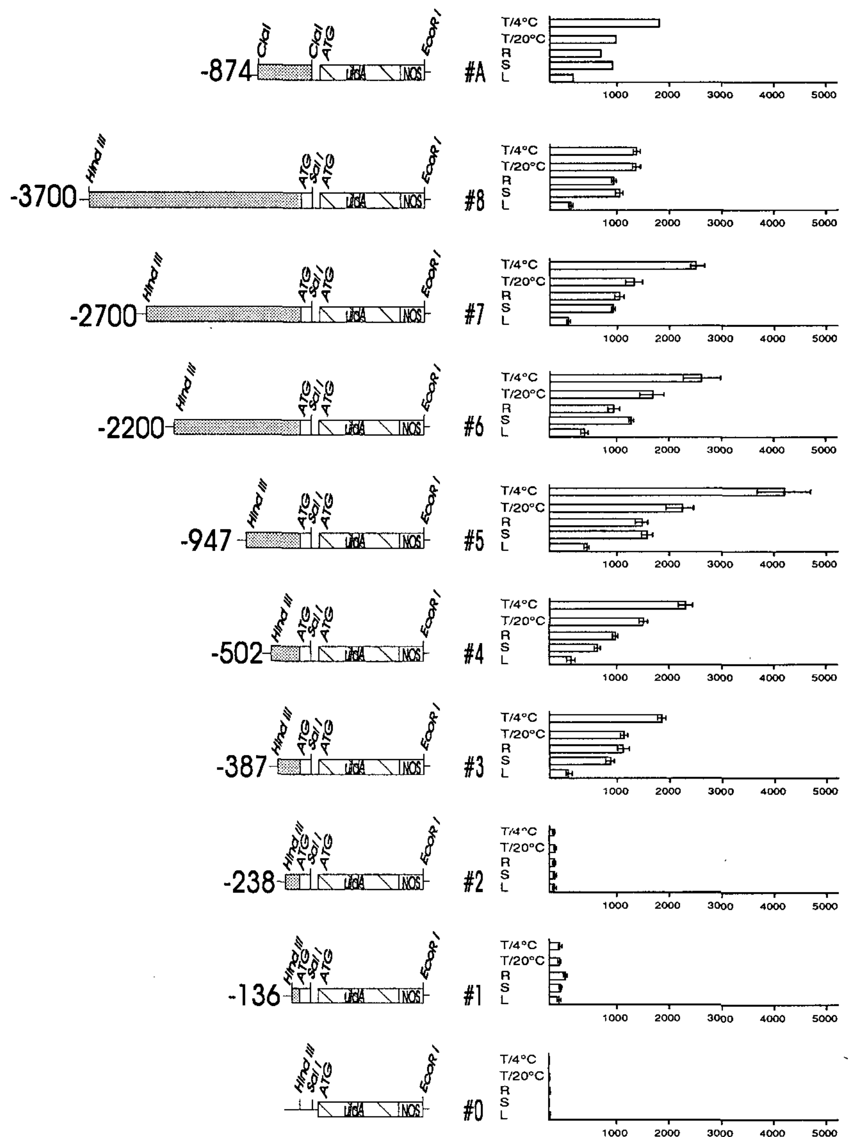


**Figure 7.** Schematic representation of the chimeric *ci21A/uidA* gene constructs (left) and GUS activities measured in tubers stored 7 d at 4°C, in tubers stored at 20°C, in roots (R), stems (S), and leaves (L) of four to five independent transgenic potato plants (right). Constructs 1 to 8 were derived from the directional fusion of deletions of the 4.7-kb *Eco*RI/*Sal*I promoter fragment to the *uidA* reporter gene. Construct A contains a *Cla*I/*Cla*I fragment fused to the *uidA* reporter gene. Only one transgenic line was available for this construct. Construct 0 represents the promoterless *uidA* reporter gene (Jefferson, 1988). Specific GUS activity is expressed as pmol 4-methylumbelliferyl-$\beta$-D-glucuronide $mg^{-1}$ protein $min^{-1}$. The bars represent the median and median absolute deviation values of four to five independent transgenic lines per construct.

the expression of both genes may be controlled differently. Restriction fragment length polymorphism-mapping studies indicated that the genes *ci21A* and *ci21B* are tightly linked and located on potato chromosome IV. Linked pairs of homologous, stress-responsive genes have also been described in the Arabidopsis genes *cor15a* and *cor15b* (Wilhelm and Thomashow, 1993), *kin1* and *kin2* (Kurkela and Borg-Franck, 1992), and *rd29A* and *rd29B* (Yamaguchi-Shinozaki and Shinozaki, 1993). Different regulation of expression has been demonstrated for *rd29A* and *rd29B* (Yamaguchi-Shinozaki and Shinozaki, 1994).

*ci21A* and *ci21B* were sequence-related to two genes of tomato that were discovered based on their responsiveness to osmotic stress (Iusem et al., 1993; Rossi and Iusem, 1994). Whereas the sequence of the CI21 cDNA corresponding to the coding region of the *ci21A* gene was most closely related to the ASR1 transcript of tomato induced by water deficit and ripening (Iusem et al., 1993), the sequence of *ci21B* was more related to the sequence of the tomato *asr2* gene (Rossi and Iusem, 1994) than to *ci21A*. *ci21A* and *ci21B* were both also homologous to the DS2 transcript of the wild potato species *S. chacoense*, which is induced by water deficit (Silhavy et al., 1995). These sequence homologies are paralleled by the observation that, aside from cold temperature in tubers, water stress in leaves was the only stimulus for which an increase in CI21 transcripts was observed. Whether the CI21 transcripts in desiccated leaves originate from gene *ci21A* or *ci21B* has not been determined. The responsiveness of the *asr1/asr2* genes of tomato to stimuli other than water deficit, such as cold temperature, high salt, or ABA, is as yet unknown.

Sequence similarity between transcripts and proteins regulated by cold and dehydration has been observed previously in various plant species, e.g. the cold-regulated CI7 protein of potato tubers and the dehydration-induced proteins ("dehydrins") from several other plant species (van Berkel et al., 1994). The CI21 protein, which is not sequence-related to CI7 or to any other dehydrin, is there-

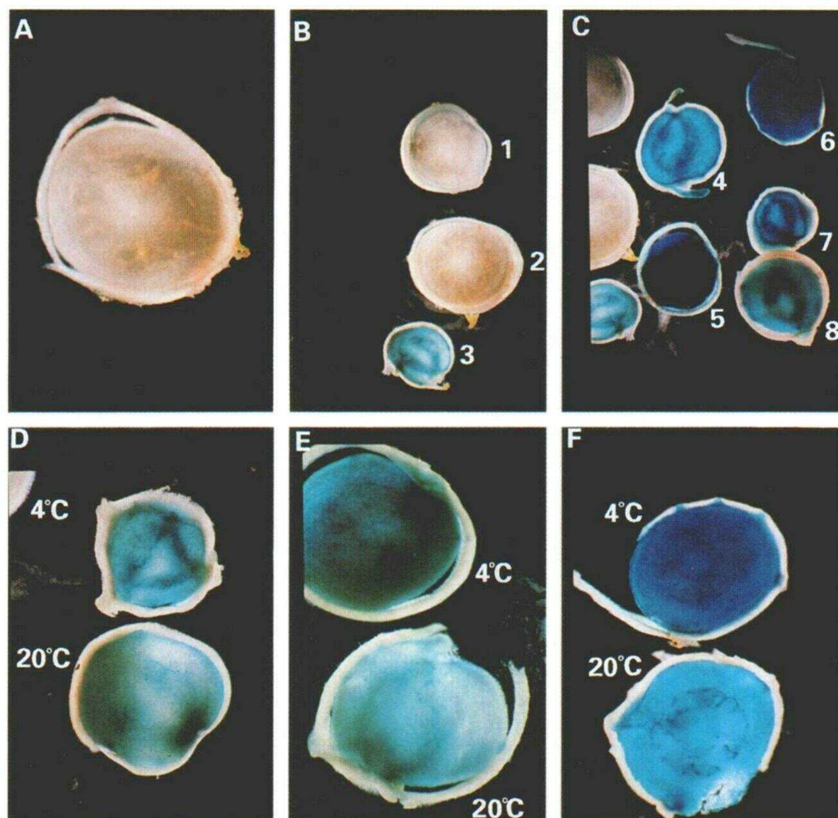


**Figure 8.** Tissue-specific expression of GUS in transgenic potato plants stained with X-Gluc. A, Section of an in vitro tuber from a transgenic plant carrying the promoterless *uidA* gene (no. 0). B, Sections of in vitro tubers from transgenic plants carrying constructs 1 to 3, which were stored at 4°C for 7 d. C, Sections of in vitro tubers from transgenic plants carrying constructs 4 to 8, which were stored at 4°C for 7 d. D, E, and F, Sections of in vitro tubers from transgenic plants carrying construct 3 (D), 4 (E), or 6 (F), which were stored either at 20 or 4°C for 7 d.

fore a member of a new class of proteins synthesized in response to osmotic stress connected with both exposure to low temperatures and drought. The possible functional role of CI21 in adaptation to low temperatures is unknown. The highly homologous protein ASR1 of tomato has been localized in the nucleus and was suggested to have DNA binding and protective properties (Iusem et al., 1993).

Expression patterns differed considerably between CI21 transcripts and protein. CI21 protein was detectable only in tubers, whereas CI21 transcript was also detected in stems and roots and at very low levels in leaves. Cold induction was observed only in tubers and was more pronounced in the transcript than on the protein level. Aside from expression at low temperatures, tuber specificity is certainly a desirable attribute with respect to the intended use of *ci21* regulatory sequences in chimeric gene constructs. It remains to be examined whether the tuber-specific expression of CI21 protein is an intrinsic property of the structural part of the *ci21* gene that may determine, for example, the stability of the protein in a particular cellular environment, or of regulatory sequence motifs present in the untranslated regions of the gene.

Sequence analysis of the *ci21A* and *ci21B* promoter regions did not reveal homology to regulatory motifs that have been shown to be required for induction by ABA (Guiltinan et al., 1990; Mundy et al., 1990) or by drought or low temperature (Yamaguchi-Shinozaki and Shinozaki, 1994). The analysis of transgenic plants containing the *uidA* reporter gene fused to *ci21A* promoter deletion fragments from −387 to −2700 bp revealed a small but significant induction of GUS enzyme activity in tubers stored at 4°C compared with tubers stored at 20°C. Promoter fragments between approximately 380 and 1000 bp were sufficient to confer cold inducibility. The approximately 2-fold induction of GUS activity in cold-stored tubers was of the same order of magnitude as the induction of the endogenous CI21 protein. In contrast to the CI21 protein, GUS activity was also detectable in root, stem, and leaf tissue of transgenic plants. This observation may be explained by the high stability of the GUS protein in plant tissues (Jefferson et al., 1887).

The results obtained with chimeric gene constructs suggest that transcription of the *ci21A* gene may be controlled to some extent by temperature. However, compared with the 10-fold transcriptional activation observed at low temperatures in the *cor15a* and *rd29a* promoters of *A. thaliana* (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994), the response of the *ci21A* promoter is small. The discrepancy between steady-state levels of CI21 transcript and protein at different temperatures and in different tissues indicates that posttranscriptional and/or posttranslational control mechanisms may be more important. The cold induction observed for CI21 transcripts could also be the consequence of increased stability of the mRNA at low temperatures. Rapid turnover of CI21 protein in leaves, stems, and roots compared with tubers may be responsible for the tissue specificity observed for the protein but not for the transcript. Other cold-regulated genes have also been reported to be controlled at both the transcriptional and

posttranscriptional level (Hajela et al., 1990; Horvarth et al., 1993).

The number of characterized, endogenous promoters that can be used to control the expression of transgenes in potato in a nonconstitutive manner is rather limited. The *ci21A* promoter is the first one described for the potato that confers some degree of cold inducibility combined with preferential expression in tubers. When genes are placed under control of the *ci21A* promoter, the accumulation of reducing sugars in cold-stored tubers may be reversibly suppressed without affecting the general agronomic performance of transgenic cultivars.

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