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Characterization of two barley genes that respond rapidly to dehydration stress

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Abstract

The present study investigates the barley cDNA clones *paf93* and *cdr29* which correspond to mRNAs regulated by low temperature and drought stress but not by exogenous ABA treatment. During the early stage of dehydration, these mRNAs are expressed before the induction of known ABA regulated genes such as dehydrins and when only a small increase occurs in ABA content. The cDNA clone *cdr29* cross-hybridizes with two classes of mRNAs differing both in size and expression patterns. The sequence analysis revealed that *paf93* encodes a protein homologous with the cold-regulated protein COR47 of *Arabidopsis* whereas *cdr29* represents a plant gene homologous to yeast and mammalian sequences coding for acyl-coenzyme A oxidase.

Keywords: Abscisic acid; Acyl-Coenzyme A oxidase; Barley (*Hordeum vulgare* L.); Cold-regulated genes; Dehydration; Drought response

1. Introduction

Plants can undergo reductions in moisture content during their life cycle in response to physiological needs (seed development) and to environmental stresses. Both conditions determine a rise in the endogenous content of ABA [1], which would thus seem to be involved in the ability of plants to tolerate drought. Numerous genes responsive to ABA have recently been isolated [2,3], and sequence motifs found in the promoter of such genes are reported to act as ABA-responsive elements [4–6].

Yet drought-induced genes whose expression is not controlled by ABA have also been isolated in pea [7], *Arabidopsis* [8] and barley [9]. An analysis of the promoter region of the *Arabidopsis* gene *rd29A* has made possible to identify a *cis*-acting element responsive to drought and low temperature but not to ABA [10].

The present study analyses the drought response of a class of ABA-independent barley genes. Two cDNA clones inducible by low temperature and

Abbreviations: ABA, abscisic acid; EDTA, ethylenediaminetetraacetic acid; TRIS, tris-hydroxymethylaminomethane; SDS, sodium dodecyl sulphate.

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drought stress were used to show that their rapid induction kinetic under dehydration conditions is markedly different from that of known ABA regulated genes such as dehydrins [11]. The mRNAs corresponding to the clones rapidly induced by drought, are already expressed at the initial onset of dehydration, when ABA content is still close to the background level, and they are not inducible by exogenous treatment with this hormone.

2. Materials and methods

2.1. Plant material and stress conditions

Barley (*H. vulgare* L.) seeds of the two-rowed cultivar 'Arda' were surface sterilized in 6.6% (v/v) NaOCl for 15 min and in ethanol for a few seconds, rinsed with distilled water, then germinated on moist filter paper in sterile Petri dishes and grown at 22°C for 5 days (9 h light and 15 h dark) until the first leaf stage. Moisture stress was induced by withholding water for 24 h until seedlings became wilted (water potential −1.2 MPa). ABA-treated shoots were sprayed with 1 mM ABA solution for 24 h. Cold-acclimated seedlings were exposed to 2°C for 3 days.

Four independent time-course experiments were performed with seedlings grown in 50% sand/50% soil in controlled environment growth chamber at 22°C 9 h light (180 $\mu E/m^2/s$) and 16°C 15 h dark, until the primary leaf was fully expanded. Leaf sections 3 cm long were cut from fully watered plants, dehydrated in standard conditions (24°C, 40% relative humidity) for 0, 0.5, 0.7, 1, 1.2, 1.5, 2, 5 and 24 h. Leaf samples were taken at each time point, weighed and frozen in liquid nitrogen; the loss of fresh weight was calculated.

2.2. cDNA cloning sequencing and computer analysis

Two cDNA clones were used: the previously isolated *paf93* [12] and the one denominated *cdr29* (cold-desiccation responsive), which was selected by differential hybridization from a cDNA library prepared in λgt10 by Clontech laboratories with poly(A)mRNAs isolated from shoots of barley seedlings exposed for 3 days at +3°C. The cDNA sequences were determined in both strands by dideoxy chain termination procedure [13] using the Multi-Pol DNA Sequencing System (Clontech). The program PC GENE was used for nucleic acid and protein sequence analysis and comparison.

2.3. RNA extraction and Northern blot analysis

The poly(A)RNAs were isolated by chromatography on oligo-dT-cellulose (Boehringer, Mannheim) according to published methods [14]. Equal amounts of poly(A)RNAs for each sample were separated in an agarose formaldehyde gel and transferred to nylon filter (MSI, Westborough, MA). The filters were hybridized with the cDNA fragments radioactively labelled with ^{32}P dCTP (Amersham) [15] in 6 × SSC (20 × SSC = 3 M NaCl, 0.3 M Na Citrate), 2 × Denhardt solution [14], 0.1% SDS and 100 μg/ml of denatured herring sperm DNA at 68°C. After hybridization, the filters were washed in 0.1 × SSC, 0.1% SDS at 68°C. The mRNA amounts were assessed by hybridization with the wheat ribosomal RNA clone *pTa71* [16]; the RNA ladder (Boehringer, Mannheim) was used to estimate mRNA size.

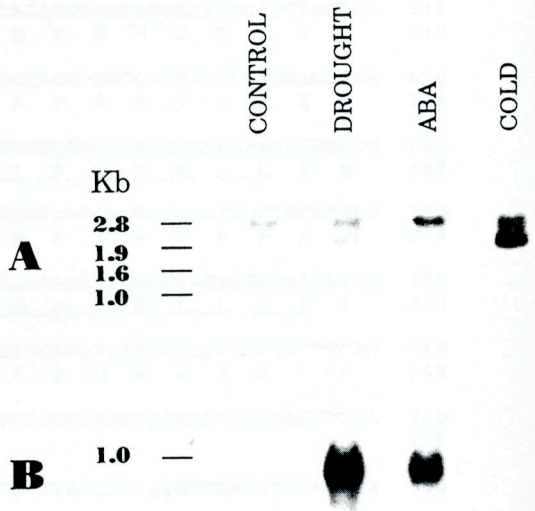

Fig. 1. (A) Northern blot analysis of the expression of mRNAs corresponding to *cdr29* cDNA clone. In each lane were loaded 3 μg of poly(A)RNA isolated from barley seedlings treated as indicated. The mRNA size markers are shown on the left. (B) To assay the effectiveness of exogenous ABA treatment, the same filter as in A was hybridized with *Dhn1* cDNA probe.

2.4. DNA isolation and Southern analysis

High molecular weight DNA was extracted from six barley cultivars (Arda, Onice, Mirco, Proctor, Nudinka and Thibaut) and purified by CsCl gradient [14]. A 10-μg quantity of genomic DNA was digested overnight with *Eco*RV restriction enzyme; the restriction fragments were separated in a 0.8% agarose gel and transferred to nylon filter (MSI, Westborough, MA). Hybridization conditions were the same as described for Northern analysis.

2.5. ABA extraction and immunoassay

Leaf tissues were weighed and frozen in liquid nitrogen for endogenous ABA determination, and the extraction was performed by shaking the tissues in distilled water (1:5, w/v) for 16 h at 4°C in the dark. ABA was measured using a radioimmunoassay with the monoclonal antibody of Quarrie et al. [17].

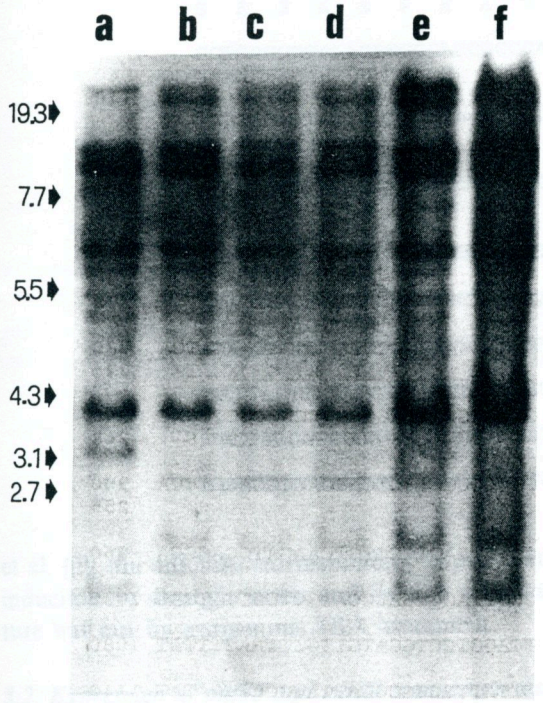

Fig. 2. Southern blot analysis of the *cdr29* gene family; 10 μg of DNA digested with *Eco*RV were loaded for each lane. a, cv. Onice; b, cv. Mirco; c, cv. Proctor; d, cv. Nudinka; e, cv. Thibaut; f, cv. Arda. Size markers are also indicated (kb).

3. Results

3.1. Stress-related expression of the cdr29 transcripts

Northern blot hybridization experiments were performed to study the expression pattern of the mRNAs corresponding to the *cdr29* clone under different stress conditions. A filter carrying 3 μg of poly(A)RNA isolated from control, drought-stressed, ABA-treated and cold-acclimated barley seedlings was hybridized with *cdr29* cDNA; the results are shown in Fig. 1A. Two mRNA classes differing in size and expression pattern cross-hybridized with *cdr29* cDNA at high stringency conditions (0.1 × SSC, 0.1% SDS, 68°C).

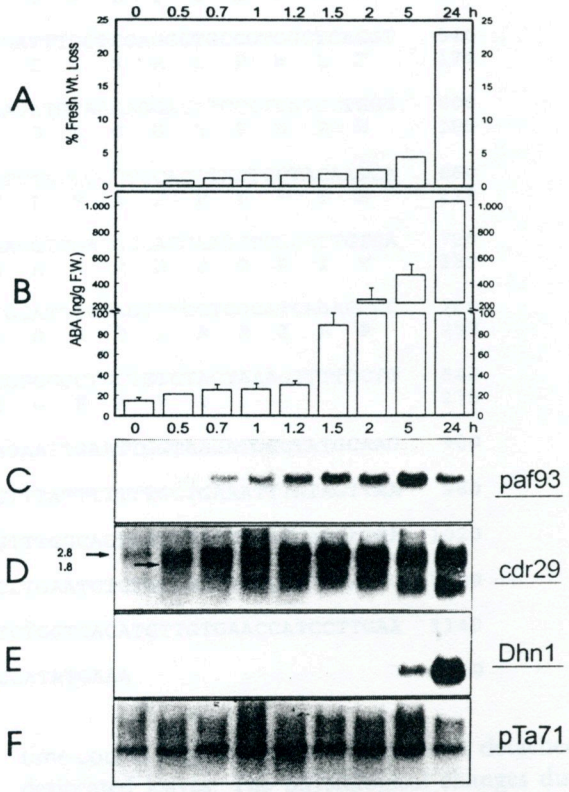

Fig. 3. Leaf segments were air-desiccated for the times indicated at the top. Fresh weight loss (A) and ABA content (B, the values ± S.E. are the average of three replications) were determined for each time point. Northern blot analysis of the expression of *paf93* (C), *cdr29* (D) and *Dhn1* (E) at different time points are shown. Arrows in D show the different transcripts detected using the *cdr29* probe. The amount of mRNA in each lane was assessed by hybridization with *pTa71* (F).

The longest mRNA class (2.8 kb), expressed in all samples, was slightly enhanced by ABA and cold treatments. In contrast, the 1.8-kb mRNA class was induced only in cold-treated and drought-stressed plants. Clear signals appeared in the lanes corresponding to drought-stressed and ABA-treated samples (Fig. 1B) when the same filter of Fig. 1A was hybridized with the *Dhn1* probe [11] to ascertain the effectiveness of the exogenous ABA treatment. Southern analysis run with six barley cultivars showed that *cdr29* is a member of a multicopy gene family (Fig. 2).

The expression pattern of *paf93* was already reported in Cattivelli and Bartels [12] and Grossi

Figure 4A

```
paf93 TTCAGAAAGCCACAAGCCAAGAACCAATAGTCTTTGCTGATCCGCTGTTTTCTCTAGCTC   60

      CCACGAGTCTTTAGCTGCACCGACCGATCTCGATCATGGAGGATGAGAGGAGCACCCAGT  120
                                          M  E  D  E  R  S  T  Q     8

      CATACCAGGGAGCTGAGGCCGATCAGGTGGAGGTGACGGACAGGGGCCTACTCGGCAACC  180
      S  Y  Q  G  A  E  A  D  Q  V  E  V  T  D  R  G  L  L  G  N    28

      TCCTCGGCAAGAAGAAGGAGGAGGAGGACAAGAAGAAGGAGGAAGAGCTGGTCACCGGCA  240
      L  L  G  K  K  K  E  E  E  D  K  K  K  E  E  E  L  V  T  G    48

      TGGAGAAGGTCTCCGTGGAAGAGCCCGAGGTTAAGGAGGATGGCGAGAAGAAGGAGACTC  300
      M  E  K  V  S  V  E  E  P  E  V  K  E  D  G  E  K  K  E  T    68

      TCTTCTCCAAGCTGCACCGATCCAGCTCCAGCTCCAGCTCGTCTAGTGACGAGGAGGAGG  360
      L  F  S  K  L  H  R  S  S  S  S  S  S  S  S  S  S  D  E  E  E    88

      AGGAGGAGGTCATCGATGAGAACGGTGAGGTGATCAAGAGGAAAAAGAAGAAGGGTCTCA  420
      E  E  E  V  I  D  E  N  G  E  V  I  K  R  K  K  K  K  G  L   108

      AGGAGAAGCTCAAGGAGAAGCTGCCCGGCCACAAGGACAACGAGGCTGAGCACGTGACGG  480
      K  E  K  L  K  E  K  L  P  G  H  K  D  N  E  A  E  H  V  T   128

      GCCTACCCGCACCGACGGCCCCCGCGTCTGTTCAGACTCACCATGACACCGACGTCGTCG  540
      G  L  P  A  P  T  A  P  A  S  V  Q  T  H  H  D  T  D  V  V   148

      TCGAGAAGATCGACGGCGACGCGAAGGCAGAGGCCACGCCGGCAGTGCCCGAGGAGGAGA  600
      V  E  K  I  D  G  D  A  K  A  E  A  T  P  A  V  P  E  E  E   168

      AGAAAGGCTTCTTGGAAAAGATCAAGGAGAAGCTGCCCGGCGGCCACAAGAAGCCGGAGG  660
      K  K  G  F  L  E  K  I  K  E  K  L  P  G  G  H  K  K  P  E   188

      ACGCTGCCGCGGTGCCCGTCACGCACGCTGCTCCAGCGCCAGTGCACGCGCCTGCGCCGG  720
      D  A  A  A  V  P  V  T  H  A  A  P  A  P  V  H  A  P  A  P   208

      CCGCCGAGGAGGTGAGCAGCCCGGACGCGAAGGAGAAGAAGGGCCTACTGGGCAAGATCA  780
      A  A  E  E  V  S  S  P  D  A  K  E  K  K  G  L  L  G  K  I   228

      TGGACAAGCTGCCCGGTTACCACAAGACAGGGGAGGAGGACAAGGCCGCCGCCCCTTCAG  840
      M  D  K  L  P  G  Y  H  K  T  G  E  E  D  K  A  A  A  P  S   248

      GCGAGCACAAGCCCAGAGCTTGATCGCCGCCGTGCCCCGAGACTCATCACCGGACCTCCA  900
      G  E  H  K  P  R  A                                          255

      TTAAATTGTTGGCGTGTGTCGTGTTTGCTTTACGTCTAAGTCCGTGTCAAGATGGAGGGA  960

      TTGATCGTCTTTGAAGGTCCGGTCTGTGAAGTCCGTTAGGTGACGGATGCTTGTGTTCAG 1020

      TTTGGGTATACGGGCTTCAGTTTGGTCCAGAGTCAGGTCCTGGATGTTGTTTAAGTTTAT 1080

      TTGCTTATTATTACGGGCACTTTGTGTATTGGTTTATTGCTGGGCATTATGCCTTGATAT 1140

      TAAAGATTTCCGCC                                               1154
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Fig. 4. (A) Nucleotide and deduced amino acid sequence of *paf93*. The lysine-rich domains are indicated by single underlining; the cluster of serine is indicated by double underlining. (B) Nucleotide and deduced amino acid sequence of *cdr29*.

Figure 4B

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cdr29 GGGCGAACCACAACTCCGGCTCATTCCTGGACCGCTTCACCGAGTCCCTGCACTACTACT   60
        A  N  H  N  S  G  S  F  L  D  R  F  T  E  S  L  H  Y  Y     19

      CCACCATGTTCTCACAGGACGCGATTGAAGAGTGCCGAAAGCTCTGCGGTGGACATGGTT  120
      S  T  M  F  S  Q  D  A  I  E  E  C  R  K  L  C  G  G  H  G     39

      ACCTGAACAGCAGTGGGCTCCCTGAACTGTTTGCGGTCTATGTTCCTGCCTGCACCTATG  180
      Y  L  N  S  S  G  L  P  E  L  F  A  V  Y  V  P  A  C  T  Y     59

      AAGGAGACAATGTCGTTTTGATGTTGCATGTTGCAAGGTTTTTGATGAAGACCGTCTCTC  240
      E  G  D  N  V  V  L  M  L  H  V  A  R  F  L  M  K  T  V  S     79

      AGGTGGCCACTAGGAACCAACCTGTCGGCACCGTAGCTTACATGGGCAACATACATCACT  300
      Q  V  A  T  R  N  Q  P  V  G  T  V  A  Y  M  G  N  I  H  H     99

      TGATGCAATGCAAAAGCGCTGTAAATACAGCCGAAGACTGGCTCAATCCTGCTACCGTAA  360
      L  M  Q  C  K  S  A  V  N  T  A  E  D  W  L  N  P  A  T  V    119

      AGAAGGTGTTTGAAGCTAGGGCCCTGAGGATGGCCGTGAACTGTGCCAGCAAGGCGCCAA  420
      K  K  V  F  E  A  R  A  L  R  M  A  V  N  C  A  S  K  A  P    139

      GCCAAGAAGAAGCATCTCTTAGTGAATTTCTTGGTGCACTCATTGACATGGATGATCTCG  480
      S  Q  E  E  A  S  L  S  E  F  L  G  A  L  I  D  M  D  D  L    159

      TTCCTTTGCTGGTTTCTCTGAGCTCTCACCTGATTTGCTCGAGGCTGCCGTGGCTCACGT  540
      V  P  L  L  V  S  L  S  S  H  L  I  C  S  R  L  P  W  L  T    179

      CAGCTGATCATTGTGACCAAATTCATCGAGAAGATGCAGGAGGACATCCCTGGCCCTGGG  600
      S  A  D  H  C  D  Q  I  H  R  E  D  A  G  G  H  P  W  P  W    199

      GTGAAGGAGCAGCTCCAGAAGCTGTGCAGCATCTACGCGCTCCACCTACTCCTACAAGCA  660
      G  E  G  A  A  P  E  A  V  Q  H  L  R  A  P  P  T  P  T  S    219

      CCTGGCCGGGGAGAGGCCACCGCCCACTTCGACGTCGACGCCATGAAGGTCGCATTGTCA  720
      T  W  P  G  R  G  H  R  P  L  R  R  R  R  H  E  G  R  I  V    239

      GGCTCCCGCCATGCCATCGAGGTCGCCGACTGCATGGCCAGGCTCGTCGCATCAGACCCC  780
      R  L  P  P  C  H  R  G  R  R  L  H  G  Q  A  R  R  I  R  P    259

      GTAAGCACTGCGGCACACCCCGTAAGCACACGGGGCCTGCTGTCTACTAAAGTTTTTCTG  840
      R  K  H  C  G  T  P  R  K  H  T  G  P  A  V  Y                275

      CAAGCATACTGCATTTTGTCGCGTGTCATCAGAATTGAATTGGTAAGATGCCTATGCAAG  900

      CAGTGCTTTGTGCAGGATGCTCCATATTACCTTTATTTTTTTGCTGAAATTTATAGTTAA  960

      GCATGTTTTTTTTTACCAGTGCTGCATGGAAGTTTGGCACTAGTGTAATTAAGCAGCATGA 1020

      TATTACTTAAATGTTAGGTTCCGGATGTTACCTGAATCTCTGCAGGTGTTGCTTATAGCC 1080

      CCCTTGCATGGAATCTGTGTCGCTATATGTTCTGGTCACATGTTGTGAACCATCCTTGAA 1140

      TATGTATGTGTGTTTTTGTGAAACTATGTTGGATATGAAA                      1180
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et al. [9], the mRNAs corresponding to *paf93* are inducible by drought stress and by low temperature but not by exogenous ABA treatment.

3.2. Expression of paf93 and cdr29 transcripts during progressive dehydration

The relationship of drought stress, ABA accumulation and gene expression was studied using time-course experiments performed with detached desiccated leaves. The physiological changes due to desiccation were monitored by measuring the fresh-weight loss and the endogenous ABA content at each time point. The data for fresh-weight loss (Fig. 3A) show that the slight drop (1.5%) registered after 1 h progressively increased thereafter, the value rising from 2.4% at 2 h to

4.3% at 5 h and to 23% at 24 h. The ABA content (Fig. 3B) after 1 h increases only to 1.8 times the background, whereas the greatest change being registered between 90 min (6.3 times) and 2 h (20 times).

The mRNAs corresponding to cDNA *paf93* were undetectable in the well watered leaves. While the signal was low after 30 min, the abundancy of mRNA increased after 75 min which rose to a peak level after 5 h of stress (Fig. 3C). The induction of the *cdr29*-corresponding mRNAs is shown in Fig. 3D. In fully hydrated leaves, only the signal of a transcript of 2.8 kDa was detectable with the *cdr29* cDNA probe. A second mRNA of 1.8-kb was induced after 30 min and its expression increased to a maximum level after 90 min.

The experimental conditions (i.e. percentage of fresh weight loss and ABA content) associated with the induction of genes known to be ABA-controlled were determined via the appearance of mRNAs homologous to the *Dhn1* probe [11]. The induction of the corresponding transcripts was detected after 5 h (Fig. 3E).

3.3. DNA sequence analysis

The nucleotide and the deduced amino acid sequences of the cDNA clones *paf93* and *cdr29* are shown in Fig. 4. The former revealed an insert of 1154 bp, with an open reading frame from position 75 to position 860 coding for 255 amino acids. The protein sequence deduced from *paf93* shows a serine cluster and four lysine-rich domains (see Fig. 4A). Analysis of the amino acid composition reveals a high content of glutamic acid and lysine while cysteine and tryptophan are absent. These characteristics make this sequence similar to other drought-induced proteins such as LEAs [18]. A comparison of the amino acid sequence of *paf93* with those in the Swiss-Prot data bank indicate that the most similar sequences are in the cotton

```
PAF93   M---------------------------------------EDERSTQSYQG     12
COR47   NHLKATTQVLKFSHIFYLLYKLLIKSRLTMAEEYKNNVPEHETPTVATEE     50
DHN3    MEHGHATNRV----------------------DEYGNPVAGHGVGTGMGAH     29
                                          ^  ^   *
PAF93   AEADQVEVTDRGLLGNLLGKKKEEEDKKKEEELVTGME-KVSVEEPEV--     59
COR47   SPATTTEVTDRGLF-DFLGKKEEEVKPQETTTLESEFDHKAQISEPELAA     99
DHN3    GGVGTGAAAG----GHFQPTREE---------------HKAG---------     52
          ^    ^^^^^^^     ^^^^ *^      ^       *     ^^^
PAF93   ---------------------KEDGEKKETLFSKLHRSSSSSSSSSDEEEE     89
COR47   EHEEVKENKITLLEELQEKTEEDEENKPSVIEKLHRSNSSSSSSSDEEGE    149
DHN3    -------------------------------GILQRSGSSSSSSSEDDG-     70
                                ^^  ^  ^     ^*^** *******^^^  ^
PAF93   EEVIDENGEVIKRKKKKGLKEKLKEKLPGHKDNEAEHVTGLPAPTAPASV    139
COR47   EKKEKKKKIVEGEEDKKGLVEKIKEKLPGHHDKTAEDDVPVST-TIPVPV    198
DHN3    ----------MGGRRKKGLKDKIKEKLPGGHGDQ-----------------     94
        ^          ^      **** ^* *******^  ^   ^^*         ^  ^   ^
PAF93   QTHHDTDVVVEKIDGDAKAEATPAVPEEEKKGFLEKIKEKLPGGHKKPED    189
COR47   -----SESVVEHDH-----------PEEEKKGLVEKIKEKLPGHHDEKAE    232
DHN3    --QQTGGTYGQHGHTGMTGTG--------------------------E     114
              ^^^                ^^^^^^^   ^^^^^^^^^^ ^
PAF93   AAAVPVTHAAPAPVHAPAPAAEEVSSPDAKEKKGLLGKIMDKLPGYHKTG    239
COR47   DSP-AVTSTPLVVTEHPVEPTTELPVEHPEEKKGILEKIKEKLPGYHAKT    281
DHN3    HGATATGGTYGQQGHTGMTGTGAHGTDGTGEKKGIMDKIKEKLPGQH---    161
          ^^          ^       ^        **** ^ **   ****^*
PAF93   EEDKAAAPSGEHKPRA    255
COR47   TEEEV---KKEKESDD    294
DHN3    ----------------    161
         ^         ^
```

Fig. 5. Comparison between the deduced amino acid sequence of *paf93* from barley, COR47 from *Arabidopsis* and DHN3 from barley. Amino acids conserved in all three sequences are indicated with *; amino acids conserved only between PAF93 and COR47 are shown with ^. Dashes were introduced to maximize sequence alignment.

```
CDR29       26  DAIEECRKLCGGHGYLNSSGLPELFAVYVPACTYEGDNVVLMLHVARFLMKTVSQVATRNQPVGTVAYMGNI   97
CAO1_RAT   389  --.....MA......SH...i.NiyVTfT....f..e.T.m..QT......IYD..RsGKLVG.M.s.LNDl  458
HSAOX15_1  389  --..A..MA......SHC....NiyVNfT.s..f..e.T.m..QT......sYD..HsGKLVC.M.s.lNDl  458
CAO_CAEEL  399  Qs.dQA.QA......SDa.Y..T.ytCS.G......e.m.ml.Qlsky...aAaKAEKGEEMAPL...---l  467
OXCKPM     422  e..dQ..QA......SSYN.FAKA.NDw.VQ..w....N..S.S.GkPii.QiI---------.IEDNGKTv  484
OXCKX4     422  e..dQ..QA......SSYN.FGKAyNDw.VQ..w....N..AmS.GkPiv.Q.I---------.IEDAGKTv  484
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Fig. 6. Homology of a CDR29 internal fragment (from amino acid 26 to amino acid 97) with several acyl-Coenzyme A oxidase. The amino acid sequences are indicate by the one-letter code. Residues identical to CDR29 sequence are marked by dots, homologous substitutions by lowercase letter, and non-homologous substitutions by uppercase letters. The acyl-Coenzyme A sequences compared were: CA01_RAT from rat [21], HSAOX 15_1 from human [22], CAO_CAEEL from *Caenorhabditis elegans* [23], OXCKPM from *Candida maltosa* [24] and OXCKX4 from *Candida tropicalis* [25]. The region of the proteins being compared are indicated (numbers indicate beginning and ending amino acid residues of published sequences). Dashes were introduced to maximize sequence alignment.

LEA protein D-11 [19] and the *Arabidopsis* COR47 protein. COR47, a protein homologous to the Group II LEA proteins, is inducibile by low temperature, drought and ABA [20]. The comparison between PAF93 and the most similar barley dehydrin protein (DHN3; [11]) confirms that PAF93 is more closely related to the *Arabidopsis* COR47 than to other barley drought and ABA induced proteins. These three amino acid sequences, PAF93, COR47 and DHN3, are compared in Fig. 5.

The sequence of clone *cdr29* proved to be 1189 bp (Fig. 4B). Comparison to the lengths of the corresponding messenger RNAs (1.8 kb and 2.8 kb) indicates that the isolated clone contains only part of the gene. The longest open reading frame stretched from the putative 5′ end of the cDNA to nucleotide position 830. A comparison with the sequences in the data bank showed both at the nucleotide and at the amino acid level a region of high homology with known sequences coding for peroxisomal acyl-Coenzyme A oxidase of rat [21], human [22], *Caenorhabditis elegans* [23] and *Candida* [24,25]. Because the similarity was concentrated in the *N*-terminal region of CDR29 protein, a multiple alignment was performed between a fragment of CDR29 (from residue 26 to residue 97) and the corresponding sequence of all other acyl-Coenzyme A oxidases. The results showed the existance of a highly conserved domain through all the sequences (Fig. 6). No other clear homologous domains were found.

Acyl-Coenzyme A oxidase, an enzyme involved in the oxidation of fatty acids, has been found in the peroxisomal and mitochondrial fractions of animal tissues and in peroxisomal fraction of plant tissues [26]. Interestingly, all sequences homologous to *cdr29* encode for the peroxisome localized enzyme.

4. Discussion

It has been widely demonstrated that drought and desiccation cause changes in gene expression, and many genes induced under these conditions have been isolated and characterized [2,3]. Most of these genes are ABA-regulated and can be induced by its exogenous application. Here we report the accumulation of mRNAs corresponding to two cDNA clones, *paf93* and *cdr29*, which respond rapidly to dehydration before a great increase in ABA content occurs. Only a few other examples of genes rapidly induced by dehydration have been reported to date [8,27,28].

In our time-course experiments, the induction of known ABA-regulated mRNAs, as detected by hybridization with the *Dhn1* probe, occurred after 5 h (4.3% loss of fresh weight), when the ABA level had already increased to 466 ng/g fresh weight, whereas the expression of the mRNAs corresponding to *paf93* was detected after 30 min (0.8% fresh weight loss), when the ABA level was only 20.5 ng/g fresh weight (1.5 times the background). An mRNA of 1.8 kb, homologous to clone *cdr29*, was induced in the same conditions. These findings, together with the impossibility of inducing the mRNAs corresponding to *paf93* [9] and the mRNA of 1.8 kb corresponding to *cdr29* (this paper) with an exogenous ABA treatment, suggest that two mechanisms are involved in the barley

molecular response to drought. Genes such as dehydrins are induced only after a great increase of ABA, whereas the genes rapidly induced by dehydration are either ABA-independent or triggered by very little variation in ABA content. It has been shown in other cases too that different pathways are involved in modulating the response to a given environmental stress. The *Arabidopsis* molecular response to low temperature involves ABA regulated as well as ABA independent genes. Nordin et al. [29] showed that two different signal transduction pathways are responsible for the expression of the two cold-regulated genes *lti65* and *lti78*. The gene *rd29A*, a sequence almost identical to *lti78* [30], was shown to have a *cis*-acting element responsible for its induction under low temperature as well as under drought conditions [10]. Indeed many genes rapidly induced during dehydration both in *Arabidopsis* [10,27] and barley are expressed even in low temperature conditions.

Since our time-course experiments have been carried out with detached leaves it could be argued that a wounding signal is also involved in gene expression. In some of our previous experiments, however, the expression of *paf93* and *cdr29* by wounding plant leaves was not observed (data not shown). Moreover many reports indicate that in plants such as potato, bean and tobacco, the wounding response requires more time than that necessary for the induction of *paf93* and *cdr29* during dehydration. For instance, the wounding-induced activity of the tyramine hydroxycinnamoyl transferase in potato tubers was detectable 3–4 h after wounding [31]. Wounded hypocotyl segments of beans accumulated chalcone isomerase mRNA after 2.5 h [32] and also the time for the maximal wounding expression of tobacco PR-genes was 2 days [33].

Sequence analysis of the cDNA clones revealed that *paf93* encodes a protein with similarity to Group II LEA proteins. These proteins accumulate in late embryogenesis and in response to water stress and ABA [2,11,18]. *paf93* represents the only example to date of a gene coding for a protein homologous to a class of ABA-regulated genes whose induction is ABA-independent. Another gene that is cold-induced and ABA-independent in wheat, *wcs120* [34], codes for a protein containing numerous domains homologous to the sequences of the dehydrin family, although the mRNAs corresponding to this sequence are not induced by drought [34]. By contrast, the protein sequence most similar to PAF93, COR47 of *Arabidopsis*, is encoded by a gene inducibile either by exogenous ABA treatment or by low temperature and drought [20].

The comparison of CDR29 with other known protein sequences revealed homologies with several peroxisome localized acyl-Coenzyme A oxidases. These results lead to the hypothesis that *cdr29* encodes for a potential acyl-Coenzyme A oxidase; however, several points should be considered. The length of the whole enzyme amino acid sequence, as deduced from cloned genes of rat, human, *C. elegans* and *Candida* range from 659 to 709 amino acids. Such sequence can be easily encoded by an mRNA of 2.8 kb, not by an mRNA of 1.8 kb. Although there is no evidence about the size of a plant acyl-Coenzyme A oxidase and several isoenzymes may exist, also in rat and in *Candida tropicalis* for instance, two isoenzymes have been found [21,25].

A study concerning the analysis of targeting sequences mediating the protein import to peroxisomes showed that the region from amino acid 402 to 464 of *Candida tropicalis* acyl-Coenzyme A oxidase (clone OXCKX4) is sufficient to direct efficient import into peroxisomes [35]. Because this protein domain overlaps extensively the region of homology between OXCKX4 (from residue 422 to 484) and CDR29 (from residue 26 to 97) (Fig. 6) it could be also that CDR29 has only a domain homologous to peroxisome targeting sequence.

Very recently we have isolated new *cdr29* corresponding clones and further studies now under way should enable us to elucidate this gene family via the sequencing of clones corresponding to single members.

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Somatic embryogenesis in *Citrus* from styles culture

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Abstract

Styles (including the stigma) of *Citrus aurantium* L. (cvs. 'AA 12', 'AA 30' and 'AA31'), *C. deliciosa* Tenore (cvs. 'Avana' and 'Tardivo di Ciaculli'), *C. paradisi* Macf. (cvs. 'Marsh seedless' and 'Star Ruby') and *C. sinensis* (L.) Osb. (cvs. 'Bonanza', 'Brasiliano 92', 'Sanguinello' and 'Valencia') were cultured for induction of somatic embryogenesis. Explants were excised from flower buds which were collected during full bloom, and cultured on Murashige and Skoog (MS) basal medium supplemented with 146 mM sucrose, 500 mg/l malt extract and 13.3 μM 6-benzylaminopurine (BAP) as well as the same medium without BAP. Callus development was observed from the style base 4 weeks after treatment initiation, and embryogenesis occurred 2–3 months later. Embryogenesis has been induced from the style-derived callus of all the cultivars tested except for the cultivars 'Avana' and 'Star Ruby'. The best results for callus growth and embryo regeneration was obtained from explants of 'Brasiliano 92' cultured on medium containing BAP. Somatic embryos were isolated from callus and placed on MS medium supplemented with 146 mM sucrose, 500 mg/l malt extract and 0.27 μM α-naphthaleneacetic acid (NAA) where they formed entire plants. Two months later plants were successfully established in soil.

Keywords: *Citrus*; Plant regeneration; Somatic embryogenesis; Style; Tissue culture

1. Introduction

The induction of somatic embryogenesis in vitro has been achieved in many *Citrus* species and cultivars. Since most *Citrus* species are polyembryonic and adventitious embryos arise in vivo from nucellar tissue, most attempts to induce embryogenesis have involved nucellar tissue, nucellar embryos and ovules. Only a few papers have indicated the embryogenic capacity of the somatic *Citrus* cells that are neither nucellar nor ovular in origin [1–3].

In *Citrus* species, propagation of proven genotypes is highly desirable. In these cases explant material should be restricted to tissues that can be reliably considered to express the known genotype. Unfortunately at the present time, somatic embryogenesis in many *Citrus* species can only be achieved from embryonic material. Since the style

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; GA, gibberellic acid; NAA, α-naphthaleneacetic acid; MS, Murashige and Skoog medium.

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