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Characterization of Five Abscisic Acid-Responsive cDNA Clones Isolated from the Desiccation-Tolerant Plant *Craterostigma plantagineum* and Their Relationship to Other Water-Stress Genes[1]

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ABSTRACT

Leaves of resurrection plants tolerate desiccation as do embryos of many higher plants. From the resurrection plant *Craterostigma plantagineum* a number of desiccation-related transcripts have recently been cloned; they are abundantly expressed in dried leaves and abscisic acid-treated dried callus (D Bartels, K Schneider, G Terstappen, D Piatkowski, F Salamini [1990] Planta 18: 27–34). Five distinct cDNA clones representing low copy number genes were selected for further characterization. Their nucleotide sequences were determined and proteins were predicted with a molecular mass between 16 and 34 kilodaltons. Three of these proteins have unusual amino acid compositions and extreme hydrophilic characters. Two of them contain a cluster of contiguous serine residues and lysine-rich repeats. These sequence motifs display homologies to desiccation-related genes expressed in embryos or dehydrated seedlings of several plants. A third cDNA clone contains tracts of sequences which are related to a cotton Lea (late embryogenesis abundant) gene (JC Baker, C Steele, L Dure III [1988] Plant Mol Biol II: 277–291). Secondary structure predictions are discussed and suggest that the deduced proteins could play a role in protecting core cell structures in a dehydrated cell. It is concluded that at least in part the gene products involved in the desiccation-induced pathways are common to leaves of resurrection plants and embryos. Two cDNA clones appear to code for *Craterostigma*-specific mRNAs. The expression patterns of all five transcripts were studied in comparison to desiccated leaves in dehydrated roots, in wound-stressed leaves and in salt-stressed callus. The data obtained point to the possibility that not only specificity of induction but also the expression level of specific gene products may be of importance for osmoprotection.

To isolate gene products involved in osmoprotection of plant cells two experimental systems are under investigation: the developing embryos of higher plants and leaves of poikilohydric or resurrection plants (4, 11). In the majority of higher plants, only the embryo acquires the ability to withstand protoplastic dehydration during seed maturation, and genes have been isolated that encode transcripts accumulating in this tissue as seeds approach maturity and begin to desiccate (1, 14, 15, 17, 19, 21, 24, 26, 28). Characteristic for these genes is that they are abundantly expressed during late embryogenesis and are responsive to the plant hormone ABA. Based on their features, it is suggested that the corresponding gene products are involved in osmoprotection during the desiccation phase of the maturing seed (8).

Resurrection plants possess mature foliage that displays tolerance to extreme desiccation (4, 11). Plants can survive in a dry state for long periods and resume full physiological activity after rehydration within several hours. Recently, desiccation-related, ABA-responsive cDNAs have been cloned from the resurrection species *Craterostigma plantagineum* (fam. Scrophulariaceae) (4, 10). The most abundant cDNA clones were grouped into 10 major hybridization groups and representative clones were correlated with proteins specifically expressed in desiccated leaves and ABA-treated dried callus. Five cDNA clones derived from independent hybridization groups were selected for further molecular analysis.

Here we report on the DNA and deduced protein sequences of these cDNA clones, on their genomic organization and on the expression of their transcripts in different tissues and different stress situations. Sequence comparisons revealed that three of the deduced proteins contain conserved amino acid sequence motifs reported from several ABA-responsive genes isolated from mature embryos of other plant species (1, 8, 21, 26).

MATERIALS AND METHODS

Isolation of Plant DNA and Southern Blot Analysis

Genomic DNA from Craterostigma plantagineum was isolated from young leaves by CsCl centrifugation (18). For genomic Southern blot analysis (18) high mol wt DNA (5 μg) was digested with the restriction enzymes *Eco*RI or *Hin*dIII, separated on 0.8% agarose gels and transferred to nylon filters (Hybond N; Amersham) which were reused. The filters were hybridized in 1 × Denhardt's solution, 5 × SSC, and 20 mM Na-phosphate buffer for at least 16 h at 68°C and then washed subsequently with 2 × SSC, 0.2% SDS, 25°C, 0.5 × SSC, 0.2% SDS, 25°C, and one final wash with 0.2 × SSC, 0.2% SDS, 55°C.

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Extraction of RNA and Northern Hybridizations

Extraction of poly(A)$^+$ RNA was done as described (2). Total RNA was extracted in a similar way except that after the phenol-chloroform step total RNA was precipitated with 2 M LiCl. The conditions for the Northern analysis of size fractionated RNAs are given in Bartels *et al.* (4). To compare the amounts of poly(A)$^+$ RNA or total RNA bound to the filters, the filters were hybridized with ^{32}P-labeled oligo (dT) (4) or with a ^{32}P-labeled ribosomal RNA clone pTA 71 (12), respectively.

Plant Material

The origin, propagation, and stress treatments of the *Craterostigma plantagineum* Hochst. plants as well as the conditions of callus culture were as described previously (4). For the wounding stress, one-half of the leaves were cut into 1 to 2 mm strips and incubated for 14 h in 10 mM K-phosphate buffer containing 100 μg rifampicin; the other half of the leaves were immediately frozen as control tissue. For salt treatments, the callus was kept on medium containing 150 or 300 mM NaCl for 3 d.

Recombinant DNA Techniques

Recombinant DNA procedures were essentially as described by Maniatis *et al.* (18). ^{32}P-Labeled DNA probes were obtained by random primer labeling (9). The construction, isolation, and classification of the cDNA clones were described previously (4).

DNA Sequencing and Computer Analysis

The DNA sequence of the cDNA clones was determined on both strands by subcloning of restriction enzyme fragments into pUC19 or M13mp18 and M13mp19 (20) followed by dideoxynucleotide sequencing with the T7 polymerase kit (Pharmacia LKB Freiburg, FRG). Plasmid DNAs for sequencing were prepared using the alkaline lysis method (18) and subsequent treatment with pancreatic RNAse (1 μg/μL). Where problems with the sequencing reactions arose due to GC tails, sequence specific oligonucleotides were synthesized and used as primers. The program WISGEN of the University of Wisconsin genetic computer group was used for nucleic acid and protein sequence analysis (7). The programs TFASTA and MALI (22, 27) were used for amino acid comparisons. Hydrophilicity was predicted by the method of Kyte and Doolittle (16).

In Vitro Transcription Translation Assays and Hybrid-Selected Translations

For *in vitro* transcription translation assays, the cDNA inserts were subcloned into pGEM 3Zf+; from this vector single-stranded DNAs were propagated. The single-stranded DNA was transcribed in the presence of all four nucleotides with SP6 polymerase (TransProbe SP kit, Pharmacia LKB Freiburg, FRG), and the resulting RNA was translated in a rabbit reticulocyte lysate system (Promega Biotec, Madison, USA) incorporating ^{35}S-methionine (4). The proteins were separated by two-dimensional electrophoresis as described (3). The hybrid-arrested and hybrid-released translations were also performed with single-stranded DNA subclones in pGEM (2, 4).

RESULTS

For our studies the following five cDNA clones representing independent hybridization groups were selected: pcC27–04, pcC6–19, pcC3–06, pcC27–45, pcC13–62 (4).

Organization of the Desiccation-Related Genes

Total DNA from *Craterostigma plantagineum* was digested to completion with *Eco*RI and *Hin*dIII and analysed by Southern blot hybridization using the cDNA inserts as probes. With each cDNA clone specific, simple hybridization patterns were obtained showing a low number of strongly hybridizing fragments (Fig. 1). This indicates that the genes are present in the genome at low copy numbers and are possibly members of small gene families.

Characterization of cDNA Clones and Deduced Proteins

The DNA sequences of the five cDNA clones and the corresponding predicted amino acid sequences are shown in Figure 2; the main features are summarized in Table I. For all the sequences, the most likely translation initiating ATG codon and a 3′ poly(A) tract were identified. Possible polyadenylation signals in the 3′ part of the sequence are underlined. For the clones pcC27–04, pcC6–19, and pcC3–06, the chosen open reading frames were the only possibility to yield proteins of the appropriate molecular mass and with the relative isoelectric point as determined by hybrid released translation and predicted from calculations (Table I) (4). For pcC6–19 and pcC27–04, the given reading frames are also

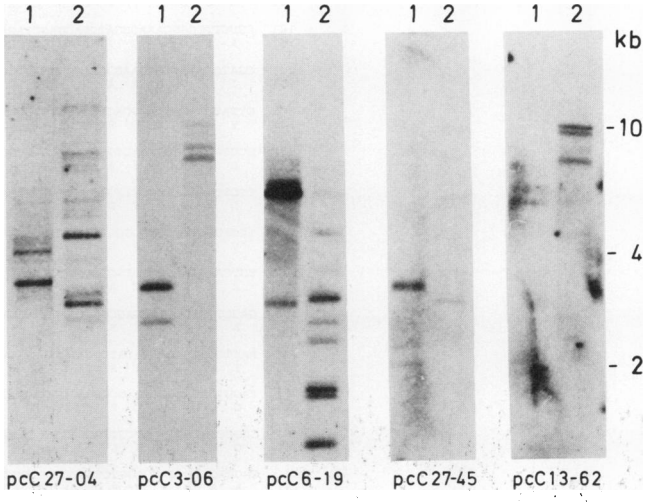

Figure 1. Southern analysis of genomic DNA from *C. plantagineum*. DNA was cut with (lane 1) *Eco*RI and (lane 2) *Hin*dIII, separated on an agarose gel transferred to a nylon filter, and probed with ^{32}P-labeled inserts of the cDNA clones as indicated. DNA size markers (1 kb ladder BRL) were run on all gels.

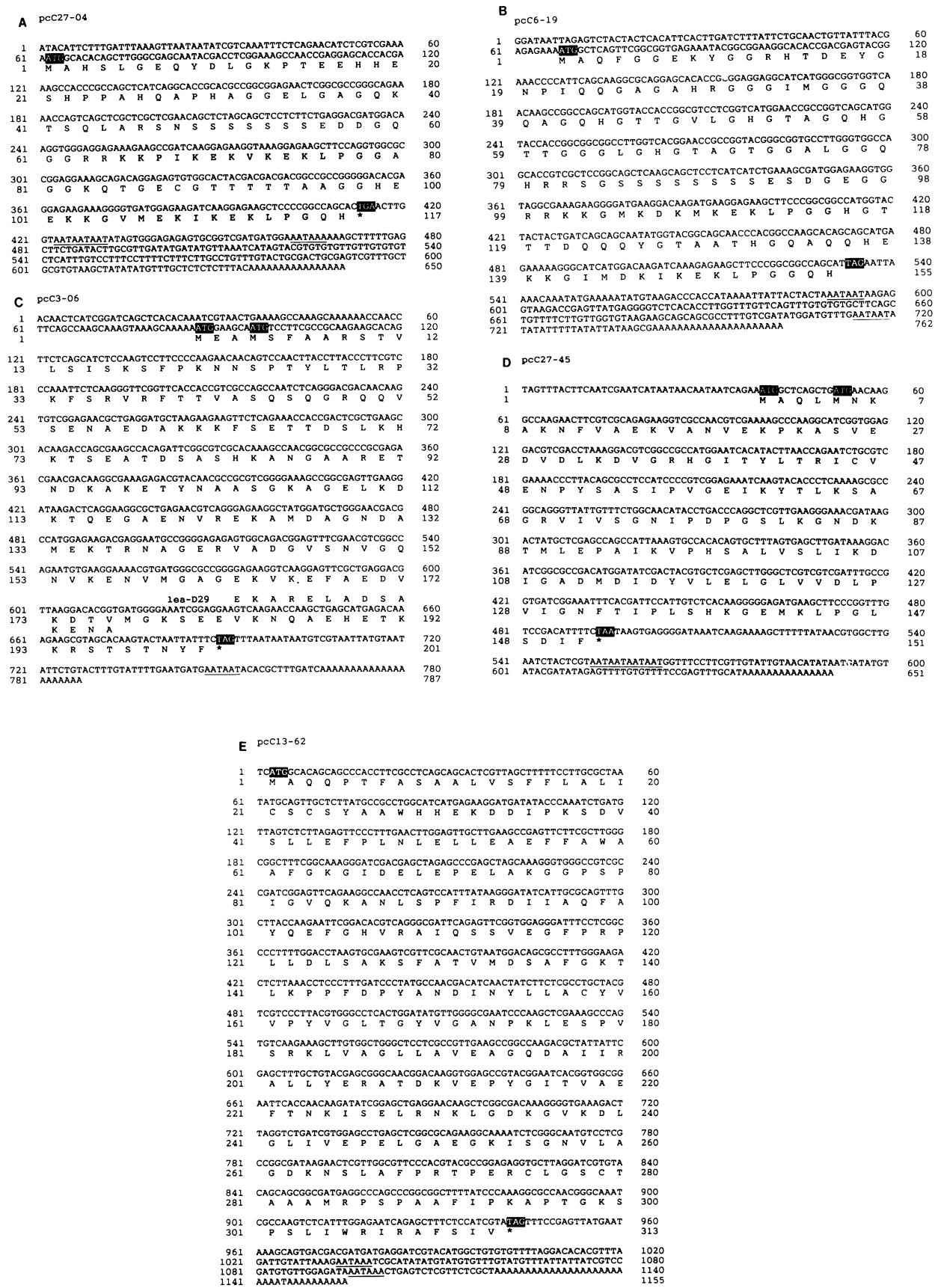
A
pcC27-04
B
pcC6-19
C
pcC3-06
lea-D29
D
pcC27-45
E
pcC13-62

Table I. *Features of cDNA Clones*

cDNA Clone	bp Determined	Coding Region	Molecular Mass of Predicted Proteins[a]	Isoelectric Point[b]
		bp	*kD*	
pcC27-04	650	351	14.3 (17-20)	8.5
pcC6-19	764	468	16.3 (17-18.5)	10.5
pcC3-06	787	603	21.9 (26-28)	9.7
pcC27-45	651	456	16.3 (15)	6.3
pcC13-62	1155	939	33.8 (34)[c]	5.9

[a] The first number gives the mol wt calculated from the amino acid composition and the number in brackets gives the relative molecular mass estimated from hybrid released translation experiments. [b] Calculated from the amino acid composition. [c] Estimated from *in vitro* transcription/translation experiments.

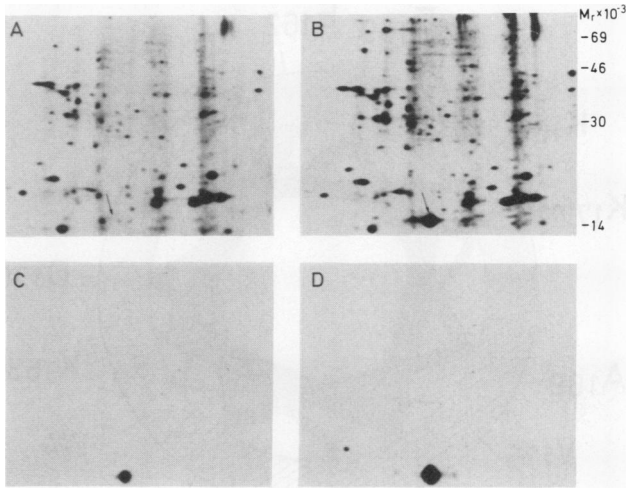

Figure 3. Fluorographs of *in vitro*-synthesized proteins: the proteins were first separated by isoelectric focusing and then in a 12% SDS polyacrylamide gel. A, Results from a hybrid arrested translation using pcC27-45, the arrow points to the missing protein; B, control experiment in which the hybrid had been melted and the RNA subsequently translated; the arrow points to the pcC27-45 related protein; C, product derived from an *in vitro* transcription/translation experiment with pcC27-45; D, hybrid release translation product of pcC27-45.

supported by amino acid homology noted with sequences from other plants (see later). For pcC27-45, an *in vitro* transcription/translation experiment resulted in an *in vitro*-synthesized protein that had mobilities identical with the hybrid selected translation product when separated in a two-dimensional gel electrophoresis (Fig. 3).

For pcC27-45 and pcC3-06, two ATG codons are in close proximity to the putative translation start points (Fig. 2). At present it cannot be decided which one is used.

The cDNA clones (pcC27-45, pcC27-04, pcC6-19, and pcC3-06) encode proteins with some properties in common with a number of proteins occurring abundantly during late embryogenesis in the seeds of higher plants (8). They lack features of a signal sequence at the N-terminus. The amino acid composition is unusual (Fig. 2): many lysine residues, tryptophan and cysteine residues are absent (the exception is one cysteine residue per molecule coded by pcC27-45 and pcC27-04) and a high number of glycines in pcC27-04 and pcC6-19 derived proteins. In pcC3-06, 40% of the whole protein comprises alanine, lysine, glutamine, and serine. It has been noted that this protein has some regions of homology with the Lea protein D-29 from cotton (1). The amino acid sequences of the cotton protein where homologies have been found are indicated in Figure 2C. This homology spans amino acid sequences which have the potential to form amphiphilic helices based on a periodic occurrence of charged and unpolar amino acid sequences. The longest fragment of pcC3-06 (amino acid 162-175) which could form an amphiphilic helix is shown in Figure 4.

pcC6-19 and pcC27-04 share sequence homology on the nucleotide and protein level. The most noticeable highly conserved sequence blocks are a serine cluster and two lysine-rich repeat motifs (indicated in gray in Fig. 2, A and B). Most of the differences between the coding regions of pcC27-04 and pcC6-19 can be assigned for by the addition of a fragment between amino acid residue 46 and 83 (pcC6-19, Fig. 2B). The central core of this region confers some hydrophobic features to the otherwise extremely hydrophilic protein predicted from pcC6-19. The protein deduced from pcC27-04 is exclusively hydrophilic.

Genes with structures similar to those of pcC6-19 and pcC27-04 have recently been discovered in several plant species (1, 6, 8, 21, 26, 28). A comparison of the amino acid sequences of the genes derived from the different plants is shown in Figure 5. The conserved sequence features are highlighted. From rice and barley, four structural genes have been sequenced (6, 28), but only one from each species was chosen for the comparison. Besides the conservations of the lysine and serine and of the carboxy terminus, another conserved fragment at the amino terminal end of the genes was observed, which is present in all genes except in pcC27-04. Within one species (rice, barley, *Craterostigma*) the genes are more similar to each other in barley and rice than they are in *Craterostigma*.

In coupled transcription translation assays, the cDNA clone pcC13-62 encodes a protein of 34 kD; this agrees with the estimated length of the RNA transcript (1.3 kb) (4). The N-terminal part of the protein sequence (amino acid 1-26) (Fig. 2E) appears to possess features of an N-terminal signal sequence (13); but at present there is no experimental evidence for its function as a signal sequence. A potential glycosylation site (NLS) is recognized at the amino acid positions 87-89.

Figure 2. Nucleotide sequences (mRNA strand) and predicted amino acid sequences of the five *Craterostigma* cDNA clones: A, pcC27-04; B, pcC6-19; C, pcC3-06; D, pcC27-45; E, pcC13-52. The putative translation start codons and the stop codons are marked, the possible poly(A) addition signals are underlined. The serine clusters and lysine-rich motifs in pcC27-04 (A) and pcC6-19 (B) are marked in gray. In C, the amino acid sequences of the cotton lea D-29 protein (1) displaying homology are aligned with the corresponding pcC3-06 amino acids.

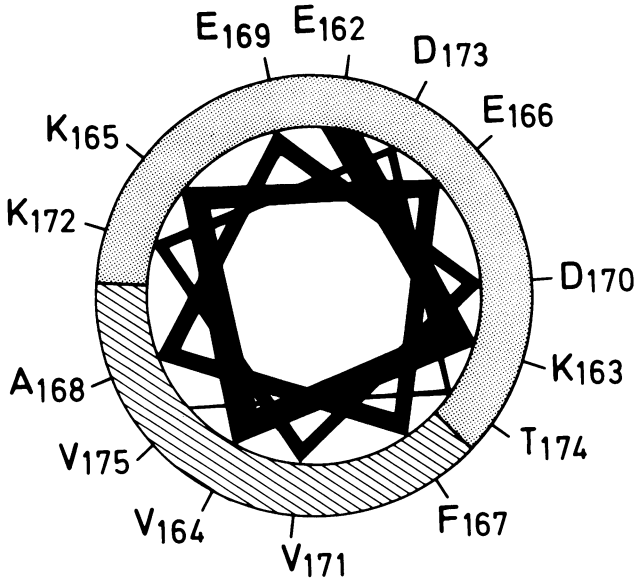

Figure 4. Diagram of a potential amphiphilic helix of the protein deduced from the clone pcC3-06. The amino acids are numbered according to Figure 2C. Hydrophobic residues are hatched and hydrophilic residues are stippled.

Expression of the *Craterostigma* Genes

As reported, transcripts homologous to the five *Craterostigma* cDNA clones accumulate rapidly in leaves during dehydration or in callus and leaves upon ABA treatment (4). It was investigated whether these transcripts can be detected in other organs and under different stress situations. The results are summarized in Figure 6. Hybridization was found in untreated roots to pcC27-04. After drying of roots, transcripts were detected with pcC6-19, pcC27-04, and pcC27-45, and at a lower level for pcC3-06 and pcC13-62. When callus was treated with 150 or 300 mM NaCl for 3 d, all described clones detected RNAs although at different levels of abundancy. No differences in hybridization signal were observed for tissue samples treated with 150 or 300 mM NaCl.

Wounding stress is reported to result in increased levels of ABA (23); therefore, we examined wounding stress in *Craterostigma*. Poly(A)$^+$ RNA extracted from wound-stress and control leaves did not hybridize with any of the *Craterostigma* cDNA clones tested (Fig. 6).

DISCUSSION

The dehydration of leaves and ABA-treated callus of the resurrection plant *Craterostigma plantagineum* leads to the rapid accumulation of a number of transcripts and proteins. The study reported here was designed to investigate the nature and distribution of the desiccation-related products coded by genes belonging to five different gene families.

Distribution of Transcripts

Besides in ABA-treated leaves or callus (4) the transcripts examined were neither found abundantly in other organs nor were they induced to high levels by other stresses. Yet, where tested, several of the homologous mRNAs could be detected in root and seed tissues (not shown) or in callus after salt stress (salt-stressed plants were not tested) (Fig. 6). However, inducibility by ABA was not mediated by wounding stress which was shown to lead to increased ABA levels and subsequent expression of specific genes (23). Vice versa, the wound-induced proteinase inhibitor II gene from potato (23) is ABA responsive but not inducible by water stress. This supports the hypothesis (23) that more than one mechanism must exist for ABA induction of gene activity and that there must be factors allowing the plant cells to discriminate specific stress forms.

From the different RNA hybridization experiments the conclusion can be drawn that the five ABA-responsive *Craterostigma* cDNA clones encode transcripts predominantly stimulated by osmotic stress. Nevertheless, the cDNA clones can be distinguished by their hybridization behavior and

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  1 MAQFGGEKYGGRHT----DEYGNPLQQGAGAHR-GGGIMG--GGQQAGQHGTT pcC6-19
  1 MEHQGQHGHVTS----RVDEYGNPVGTGAGHGQMGTAGMGTHGTAGTGRQFQP rab21
  1 MEH-G---HATN----RVDEYGNPVAGHGVGTGMGA--HGGVGTGAAAGGHFQ barleyB17
  1 MEY-GQQGQHGHGATGHVDQYGNPVGGVEHGTGGMRHGTGTGG-MGQLGEHGG maizeM3
  1 MAHFQNQYSAPEV--TQTDAYGNPTRRTDEYGNPIPYQETGRGILGIGGHHHG cottonD11
  1 MAHSLGEQYDLGKPTEEHHESHPPAHQAPHAGGELGAGQKTSQLAR------- pcC27-04

 47 GVLGHGTAGQHGTTGGGLGHGTAGTGGALGGQHRRSG-SSSSSSSSESDGLGG pcC6-19
 50 M----------------------REEHKTGGVLQRSG--SSSSSSSEDDGMGG rab21
 44 PT---------------------REEHKAGGILQRSG--SSSSSSSEDDGMGG barleyB17
 52 AGMGGGQFQP------------AREEHKTGGILHRSG--SSSSSSSEDDGMGG maizeM3
 52 -------------------------GHHGLH-RTGSSSSSSSSSEDEGT-G cottonD11
 47 ----------------------------------SNSSSSSSSEDDGQGG pcC27-04

 99 RRKKGMKDKMKEKLPGGHG-TTTDQQQY----GTAATHGQA---------- pcC6-19
 79 RRKKGIKEKIKEKLPGGNK---GEQQHAMGGTGTGTGGTTGTGGA------- rab21
 74 RRKKGLKDKIKEKLPGGHG----DQQQTG---GTYGQHGHTGMTGTGEHGATA barleyB17
 91 RRKKGIKEKIKEKLPGGHK---DDQHATATTGGAYGQQGHTGSA--------- maizeM3
 76 KKKKGLKERLKEKIPGNKE----HQSQATSTTTPGQGPTYHQHHREERSD--- cottonD11
 63 RRKKPIKEKVKEKLPGGAG---GKQTGECGTTTTTAAGGHEEKKGVM------ pcC27-04

136 ------QQ-H----------------EKKGIMDKIKEKLPGGQH      pcC6-19
121 ----YGQQGHGTGMTTGTTGAHGTTTTDTGEKKGIMDKIKEKLPG-QH    rab21
120 TGGTYGQQGHTGMTGTGAHGTDG-----TGEKKGIMDKIKEKLPG-QH    barleyB17
132 ----YGQQGHTGGAY--ATGTEG-----TGEKKGIMDKIKEKLPG-QH    maizeM3
122 ----GQDQGEAPWSPQPLISCLWSAISY                        cottonD11
107 ----------------------------------EKIKEKLPG-QH     pcC27-04
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Figure 5. Comparisons of the amino acid sequences of two *Craterostigma* proteins (pcC6-19 and pcC27-04), rab 21 from rice (21), dehydrins from barley (6) and maize (M3) (6), and cotton D11 (1). Boxes show the conserved motifs.

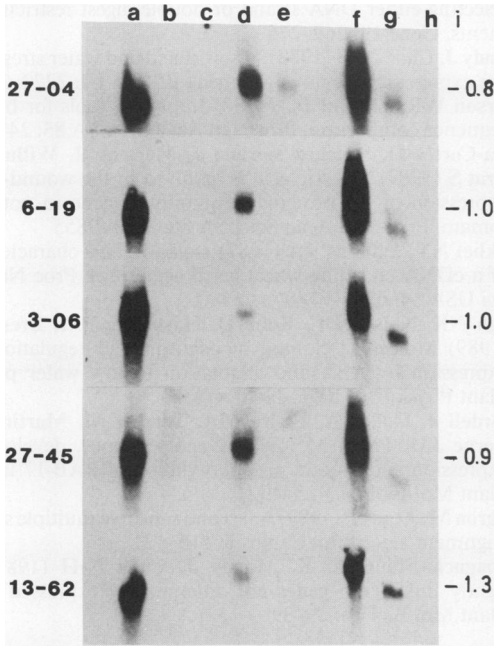

Figure 6. Northern hybridizations of the desiccation-induced *Craterostigma* cDNA clones as indicated to total RNA extracted from *Craterostigma* tissues: (a) desiccated leaves, (b) untreated leaves, (c) wound-stressed leaves, (d) desiccated roots, (e) untreated roots, (f) ABA-treated callus, (g) NaCl-treated (300 mM) callus, (h) untreated callus. (i) Lane i is size of transcripts ($N \times 10^{-3}$); RNA size markers (BRL) were used as standards. (Hybridization was at 42°C, and washes were at 2 × SSC, 65°C.)

signal strengths—pcC27–45 and pcC13–62 hybridize mainly to mRNAs specifically induced during dehydration in *Craterostigma* leaves. The expression of pcC6–19, pcC27–04, and pcC3–06 homologous mRNAs can be triggered in other tissues under dehydration stress or ABA treatment and thus possibly code for more generally occurring water stress proteins.

Sequence Analysis and Occurrence of Common Amino Acid Domains

The sequence analysis of pcC6–19 and pcC27–04 revealed the presence of characteristic sequence motifs conserved in genes from several plant species which are abundantly expressed in dehydrated embryos and are related to water stress (1, 6, 21, 26, 28). Thus, pcC6–19 and pcC27–04 belong to a class of genes present in an array of monocotyledonous and dicotyledonous plants. A comparison of the gene structures derived from five different species (Fig. 5) reveals regions which have been conserved during evolution, probably due to their functional significance. Other regions of the N-terminal half of these proteins tolerate a broad sequence diversity both in sequence length and sequence composition. Also, the protein predicted from pcC3–06 shows a short region of homology to the embryo protein D-29 from cotton (1) (Fig. 2C).

The sequence homologies reported here may be of considerable importance as these findings demonstrate that a common set of genes is induced during desiccation of mature embryos of higher plants and leaves of the resurrection plant *Craterostigma plantagineum*. This indicates that metabolic pathways leading to desiccation tolerance share common components in embryos and in leaves of resurrection plants.

It has recently been reported that some of the gene products abundantly expressed in dehydrated embryos are also detected in seedlings of desiccation-intolerant plants undergoing dehydration (6, 14, 21, 26). The observation implies that for the survival of cells after dehydration, as in resurrection plants or in the embryos, additional factors are required. Possibly, the cellular amounts of specific gene products or their proper subcellular locations are important for a protection during desiccation.

Although a number of genes have been described that are responsive to osmotic stress (5, 25) or which are abundantly expressed during the desiccation phase of embryos (1, 8, 14, 24, 26, 28) no sequence homologies were found for the genes related to pcC27–45 and pcC13–62. The sequence data available and the results of the RNA hybridizations point to the possibility that the transcripts of pcC27–45 and pcC13–62 may be characteristic for the resurrection-type of plants and could encode proteins missing in desiccation-intolerant plants.

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