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Novel Drought-Inducible Genes in the Highly Drought-Tolerant Cowpea: Cloning of cDNAs and Analysis of the Expression of the Corresponding Genes

Satoshi Iuchi [2,3], Kazuko Yamaguchi-Shinozaki [1,4], Takeshi Urao [1], Tomio Terao [1] and Kazuo Shinozaki [2,3]

[1] *Biological Resources Division, Japan International Research Center for Agricultural Sciences (JIRCAS), Ministry of Agriculture, Forestry, and Fisheries, 2-1 Ohwashi, Tsukuba, Ibaraki, 305 Japan*
[2] *Laboratory of Plant Molecular Biology, The Institute of Physical and Chemical Research (RIKEN), Tsukuba Life Science Center, 3-1-1 Koyadai, Tsukuba, Ibaraki, 305 Japan*
[3] *Institute of Biological Science, Tsukuba University, Tennohdai, Tsukuba, Ibaraki, 305 Japan*

Ten cDNAs of genes that were induced by dehydration stress were cloned by differential screening from the highly drought-tolerant legume, cowpea (*Vigna unguiculata*), a major crop in West Africa. The clones were collectively named CPRD (cowpea clones responsive to dehydration). Northern blot analysis revealed that nine of the CPRD genes were induced by dehydration stress, but the timing of induction of mRNA synthesis varied among the CPRD genes. We analyzed the effects of other environmental stresses on the expression of the CPRD8, CPRD14 and CPRD22 genes, and we found that these genes were strongly induced by high-salinity stress but not by cold or heat stress. Drought-stressed cowpea plants accumulated abscisic acid (ABA) to a level that was 160 times higher than that in unstressed plants. The CPRD8 and CPRD22 genes were induced to a significant extent by the application of exogenous ABA but the CPRD14 gene was not. These results indicate the existence of at least two signal-transduction pathways between the detection of water stress and the expression of CPRD genes in cowpea. Sequence analysis of CPRD8 and CPRD22 cDNAs revealed that they encoded putative proteins that were related to old yellow enzyme and group 2 LEA proteins, respectively. The protein encoded by CPRD14 exhibited sequence homology to dihydroflavonol-4-reductase (DFR) and vestitone reductase (VR). Old yellow enzyme, DFR and VR have not been identified as drought-inducible proteins in other plants, whereas LEA genes have been well characterized as drought-inducible genes. The various gene products might function to protect cells from environmental stress.

Key words: Abscisic acid — Cowpea (*Vigna unguiculata*) — Drought — Gene expression — Stress response.

Abbreviations: DFR, dihydroflavonol-4-reductase; LEA protein, Late Embryogenesis Abundant protein; RW, relative weight; TLC, thin-layer chromatography; VR, vestitone reductase.

The nucleotide sequences of CPRD8, CPRD14 and CPRD22 cDNAs reported in this paper have been submitted to EMBL, GenBank, and DDBJ with accession numbers D83970, D83971 and D83972, respectively.

[4] To whom correspondence should be addressed.

Plants respond to environmental stresses by initiating a number of physiological and developmental changes. Drought is one of the most severe environmental stresses that plants encounter and it affects almost all plant functions, including photosynthesis, growth, and development. Several genes have been described that respond to dehydration at the transcriptional level in a variety of plant species (Skriver and Mundy 1990, Bray 1993, Bartels and Nelson 1994, Shinozaki and Yamaguchi-Shinozaki 1996). Analysis of these genes has indicated that their products might function cooperatively to protect cells from dehydration (Bray 1993, Bartels and Nelson 1994, Bohnert et al. 1995, Shinozaki and Yamaguchi-Shinozaki 1996). The gene products are thought to function in the control of water potential; the accumulation of osmoprotectants, such as sugars, proline and betaine; protein turnover; the protection of macromolecules; signaling pathways; transcriptional control, and so on (Bray 1993, Bohnert et al. 1995, Shinozaki and Yamaguchi-Shinozaki 1996). The existence of complicated mechanisms of drought tolerance in plants is indicated from the results of analysis of the functions of the various gene products.

Abscisic acid (ABA) plays an important role in the responses of plants to drought stress. Many drought-inducible genes are also induced by the application of exogenous ABA (Skriver and Mundy 1990, Bray 1993). It appears that drought stress triggers the production of endogenous ABA, which, in turn, induces the transcription of several genes. Many genes that respond to ABA are also expressed at the late stages of embryogenesis during the development of seeds, and that are thought to function in the protection of cells from dehydration (Skriver and Mundy 1990). The expression of ABA-inducible genes has been analyzed extensively, and *cis*- and *trans*-acting factors involved in ABA-inducible gene expression have been reported (Bray 1993, Giraudat et al. 1994). There are, however, several genes

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that are induced by dehydration but not by exogenous ABA (Bray 1993, Shinozaki and Yamaguchi-Shinozaki 1996). Analysis of these genes has revealed that ABA-independent, as well as ABA-dependent, signal-transduction cascades operate between the initial signal of drought stress and the expression of specific genes. The ABA-independent expression of drought-inducible genes has been analyzed extensively and multiple signal-transduction cascades have been proposed between the initial signal of drought stress and the expression of the genes (Bray 1993, Yamaguchi-Shinozaki et al. 1994, Shinozaki and Yamaguchi-Shinozaki 1996).

Drought-inducible genes have been analyzed extensively in the resurrection plant *Craterostigma plantagineum* (Bartels and Nelson 1994) and in the model plant *Arabidopsis thaliana* (Shinozaki and Yamaguchi-Shinozaki 1996). By contrast, drought-inducible genes in drought-tolerant crops have not been studied. It is obviously important to analyze drought-inducible genes and their expression in drought-tolerant crops if we are to understand the molecular mechanisms of drought tolerance for development of technologies for the molecular breeding of drought-tolerant crops. Cowpea is a leguminous crop that is widely grown in semi-arid regions of the tropics because of its tolerance to drought and its superiority as a source of protein (Summerfield et al. 1985). Cowpea is well adapted to a wide range of growth conditions and can be cultivated from semi-arid regions to humid regions in the tropics. Moreover, many cultivars with different levels of tolerance to drought conditions have been isolated. The ability of cowpea to tolerate severe drought conditions makes it ideal for the study of the molecular mechanisms of drought tolerance in crops. However, there have been no reports on drought-inducible genes in cowpea. In order to analyze the responses to dehydrationstress of such a drought-tolerant plant at the molecular level, we isolated 24 cDNA clones that corresponded to dehydration-induced genes from cowpea by a differential screening method. The cDNA clones were classified into ten groups and collectively named CPRD (cowpea clones responsive to dehydration). We report the cloning and initial characterization of these 10 CPRD cDNAs and the expression of the 10 corresponding CPRD genes in response to dehydration and rehydration. Sequence analysis of CPRD8 and CPRD22 cDNAs revealed that they encoded putative proteins that were related to old yellow enzyme and group 2 LEA (Late Embryogenesis Abundant) proteins, respectively. The protein encoded by CPRD14 cDNA exhibited homology to dihydroflavonol-4-reductase (DFR) and vestitone reductase (VR). We also analyzed the expression of these three CPRD genes in response to other environmental stresses and to exogenous abscisic acid.

Materials and Methods

Plant growth—Cowpea plants (IT84S-2246-4) were grown in pots of soil, and watered regularly, with continuous illumination at approximately 2,500 lux at 25°C, for 4 weeks. Four-week-old plants were used for the preparation of mRNA for construction of cDNA libraries and Northern blot analysis.

Dehydration—For quantification of ABA and for Northern blot analysis, leaves were cut off at the petiole close to the stem from four-week-old cowpea plants, weighed, and then dehydrated on Whatman 3MM paper (Whatman International Ltd, Maidstone, England) at room temperature and approximately 60% humidity under dim light. The leaves were then weighed again and frozen in liquid nitrogen.

For construction of a cDNA library, whole plants were subjected to dehydration as described below for the preparation and screening of the cDNA library.

For the progressive-dehydration treatment, cowpea plants were grown in soil for 4 weeks and then drought conditions were induced by withholding water from pots for 0, 1, 2 ,5, 10, 15 and 20 days. The leaves were cut off at the petiole from each plant and frozen in liquid nitrogen.

Extraction and quantification of ABA—ABA was extracted as described by Kiyosue et al. (1994). ABA in the extract was purified by thin-layer chromatography (TLC) as described by Zeevaart and Milborrow (1976). After purification by TLC, ABA was quantified by high-performance liquid chromatography (HPLC). The sample was injected onto a C_{18} column (Zorbax ODS; 150 mm × 4.6 mm i.d.; Rockland Technologies, Inc., PA, U.S.A.) that had been set up with a precolumn (Zorbax ODS). The columns were maintained at 40°C and the sample was eluted with 50 mM potassium phosphate buffer (pH 7.0) that contained 5% methanol at a flow rate of 0.8 ml min^{-1}. The eluate was monitored at 244 nm and the eluted ABA was quantified by analysis of the peak area with reference to a standard curve.

Preparation and screening of a cDNA library from dehydrated plants—Whole plants were harvested, washed gently to remove soil from the roots, and then left to dry on Whatman 3MM paper at room temperature and approximately 60% humidity under dim light. Twenty grams of whole plants, which either had been or had not been dehydrated, were frozen in liquid nitrogen and ground to a powder with a mortar and pestle. Total RNA was prepared as described previously (Nagy et al. 1988). Poly(A)$^+$RNA was isolated by two passages of the total preparation of RNA over a column of oligo-dT cellulose, as described by Sambrook et al. (1989). About 2% of the RNA that was initially applied to the column was recovered in the poly(A)$^+$RNA fraction. Double-stranded cDNA was synthesized from poly(A)$^+$RNA with cDNA Synthesis System Plus (Amersham, Buckinghamshire, England). Using a cDNA Cloning System from Amersham, we prepared a cDNA library from the cDNA. We screened 2.4×10^4 plaques of the primary cDNA library by the plaque hybridization method of Sambrook et al. (1989).

Stress treatments and Northern blot analysis—Cowpea leaves were cut off plants at the petiole and then dehydrated on Whatman 3MM paper at room temperature and approximately 60% humidity under dim light. Plant leaves subjected to salt stress or treatment with ABA were cut off plants at the petiole. The base of the petiole was quickly submerged in water and the lower portion of petiole was cut off. Each leaf was then placed in a tube that contained 250 mM NaCl or 100 μM ABA. For heat- or cold-stress treatment, plant leaves in tubes that contained water were transferred to incubators that had been set at either 40°C or 4°C. In

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each case, the leaves were subjected to the stress treatment for various periods and then frozen in liquid nitrogen. Total RNA was isolated as described by Nagy et al. (1988). Ten micrograms of total RNA were subjected to electrophoresis in a 1% agarose gel and blotted onto a nylon filter. RNA gel blot hybridization was performed as described previously by Yamaguchi-Shinozaki et al. (1990).

Analysis of DNA sequences—Plasmid DNA templates were prepared with the Automatic Plasmid Isolation System (model PI-100; KURABO, Osaka, Japan), and sequenced with a DNA sequencer (model 373A; ABI, San Jose, CA, U.S.A.). Nucleotide sequences and amino acid sequences were analyzed with the GeneWorks software system (IntelliGenetics, Inc., Mountain View, CA, U.S.A.) and the University of Wisconsin Genetics Computer Group (GCG) program.

Results

Decrease in relative weight and accumulation of ABA during dehydration stress in cowpea leaves—We first measured the decrease in weight of cowpea leaves, defined as the ratio to that of their original weight, and the accumulation of endogenous ABA level under dehydration conditions. As shown Figure 1, cowpea leaves lost weight gradually during dehydration treatment, whereas accumulation of ABA began to increase 2 h after dehydration started and reached a maximum at 10 h. The level of ABA in 10 h-dehydrated leaves was 160 times higher than that in unstressed control leaves.

Isolation of cDNA clones that corresponded to genes induced by dehydration—A cDNA library was constructed with poly(A)$^+$RNA that had been isolated from plants after dehydration for 10 h, and it was differentially screened with cDNA prepared from poly(A)$^+$RNA that had been isolated from unstressed plants and with cDNA prepared from poly(A)$^+$RNA that had been isolated from plants after dehydration stress for 10 h. Twenty-four plaques gave a stronger hybridization signal with [^{32}P]-labeled cDNA from dehydrated plants. The plasmid regions of the 24 phage clones were excised in vivo and used to transform *Escherichia coli* cells. The cDNA fragments from the resultant plasmids were analyzed by Southern and Northern hybridization. From the hybridization analysis, we classified these 24 cDNA clones into 10 CPRD groups (Table 1).

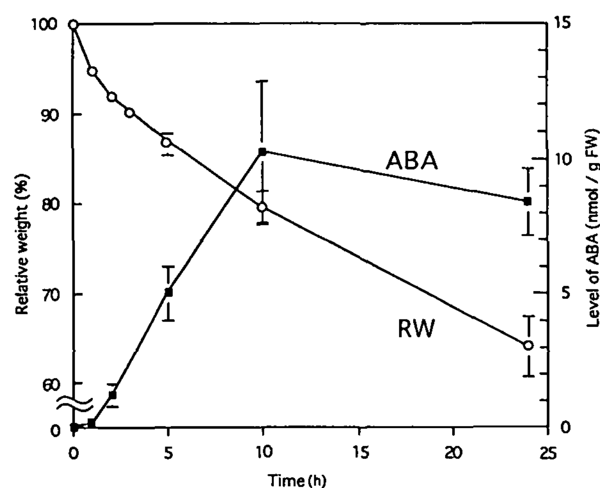

Fig. 1 Decreases in relative weight (RW; open circles) and the accumulation of endogenous abscisic acid (ABA; filled squares) in cowpea leaves during dehydration treatment. Dehydrated cowpea leaves were collected at the times indicated. The procedure for quantification of ABA is described in Materials and Methods. Error bars show standard errors. The experiment was repeated three times.

Dehydration-induced expression of the genes that corresponded to the 10 groups of CPRD clones was analyzed by Northern blot hybridization. Sets of a petiole with three leaflets were cut off plants and dehydrated for various periods of time up to 24 h. As controls, similar cowpea leaves were subjected to water treatment with the base of the petiole submerged in water. Total RNA was then isolated from dehydrated or water-treated leaflets for Northern blot hybridization. Figure 2 shows the time course of induction of the genes that corresponded to the CPRD clones in response to dehydration. All the CPRD genes were induced by dehydration stress, but the timing of induction varied among the ten classes of CPRD genes. The mRNAs corresponding to CPRD8, CPRD14, CPRD22, CPRD47 and CPRD48 began to accumulate within 2 h of the start of dehydration and those corresponding to CPRD2, CPRD12 and CPRD49 were detected after 5 h of dehydration. The levels of accumulated mRNAs were low in the case of the CPRD47, CPRD48, and CPRD49 genes. However, the level of CPRD49 mRNA was higher than those of CPRD47 and CPRD48 mRNAs at 24 h. The mRNA corresponding to CPRD46 accumulated only transiently. The mRNA corresponding to CPRD29 accumulated slightly during water treatment, as well as during dehydration, although the other CPRD mRNAs did not (data not shown).

Table 1 General characteristics of the CPRD clones

CPRD	Insert (kb)	Isolated clones [a]
CPRD2	1.7	2, 4, 5, 20, 18, 19, 33, 36
CPRD8	1.4	8
CPRD12	1.1	12
CPRD14	1.2	3, 14, 15, 30, 31, 32, 37
CPRD22	1.1	21, 22
CPRD29	2.3	29
CPRD46	2.6	46
CPRD47	1.1	47
CPRD48	0.7	48
CPRD49	0.7	49

[a] Serial numbers of isolated clones obtained by differential screening.

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When cowpea leaves were dehydrated for 10 h, they wilted. Subsequently wilted leaves were rehydrated by transfer to water, then they seemed to recover within 4 h. After rehydration started, the levels of the accumulated CPRD mRNAs decreased and these mRNAs became undetectable within 24 h, with the exception of CPRD29 and CPRD46 mRNAs (Fig. 2). The CPRD2, CPRD8, CPRD12, CPRD14, and CPRD22 genes exhibited typical and significant responses to water stress, namely, induction by dehydration and reduction in the level of the transcript upon rehydration. Since the induction of the CPRD29 gene was transient, we were unable to detect a decrease in the level of its transcript in rehydrated plants. Conversely, the level of CPRD46 mRNA decreased for the first 2 h but then it accumulated at a high level from 5 to 24 h after the start of rehydration.

Sequence analysis of the CPRD8, CPRD14 and CPRD22 cDNAs—To examine the structure of the CPRD8, CPRD14, and CPRD22 cDNA clones, we sequenced the inserted DNA fragments of 1.4 kbp, 1.2 kbp and 1.1 kbp, respectively. The nucleotide sequence of CPRD8 cDNA contained a single open reading frame that encoded 384 amino acids, a 5′-flanking region of 15 bp and a 3′-flanking region of 345 bp (Fig. 3A). The amino acid sequence of the putative CPRD8 protein was deduced from the nucleotide sequence (Fig. 3A), and the calculated molecular mass of this putative protein was 42.1 kDa. We searched the nucleotide and protein sequence databases for sequences homologous to that of CPRD8 protein and found the putative protein to be homologous to morphinone reductase (*morB*) of *Pseudomonas putida* and old yellow enzyme of *saccharomyces cerevisiae* with 47% and 30% homology, respectively (Fig. 3B; French and Bruce 1994, Stott et al. 1993). The sequence of the putative CPRD8 protein was also similar to that of the estrogen-binding protein of *Candida albicans* (Madani et al. 1994) and bile acid-inducible protein C of *Eubacterium* sp. VPI 12708 (Mallonee et al. 1990). The putative CPRD8 protein contained conserved amino acids that are involved in the active site (His191-Asn194) and flavin mononucleotide-binding (FMN-binding) site (Thr37, Gln114 and Arg243, but not Arg348) or old yellow enzyme (Fig. 3B; Fox and Karplus 1995).

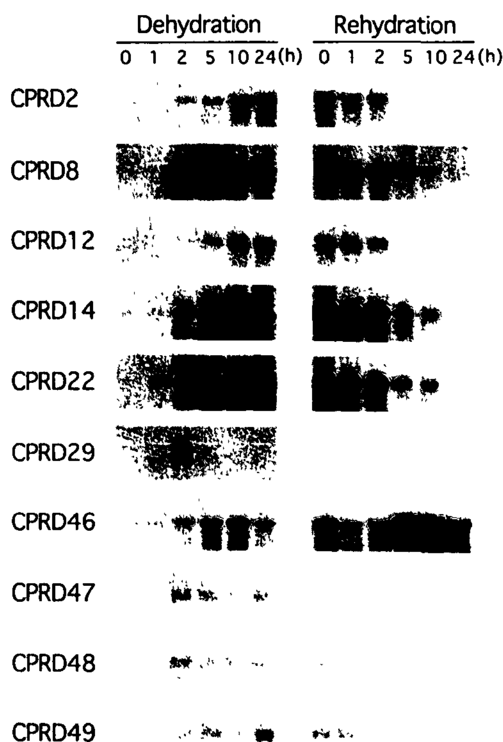

Fig. 2 Northern blot analysis of the expression of CPRD genes upon dehydration or rehydration. Total RNA was prepared from 1-month-old cowpea leaves that had been dehydrated for 0, 1, 2, 5, 10, and 24 h or rehydrated for 0, 1, 2, 5, 10, and 24 h after dehydration for 10 h. Each lane was loaded with 10 μg of total RNA. The RNA was fractionated on 1% agarose gels, blotted onto nylon membranes and probed with [^{32}P]-labeled cDNA inserts of the CPRD clones.

The nucleotide sequence of the CPRD14 cDNA included a single open reading frame of 325 amino acids, a 5′-flanking region of 41 bp and a 3′-flanking region of 208 bp (Fig. 4A). The amino acid sequence of the putative CPRD14 protein was deduced from the nucleotide sequence (Fig. 4A), and the calculated molecular mass of the putative protein was 35.6 kDa. The CPRD14 protein exhibited sequence homology to dihydroflavonol-4-reductase (DFR). Comparison of the deduced amino acid sequence of the CPRD14 protein revealed homology to DFR from *Vitis vinifera* (39%; Sparvoli et al. 1994) and *Gerbera hybrida* (35%; Helariutta et al. 1993) and to vestitone reductase from alfalfa (36%; Guo and Paiva 1995), as shown in Figure 4B.

The CPRD22 cDNA included an open reading frame of 256 amino acids, a 5′-flanking region of 45 bp, and a 3′-flanking region of 313 bp (Fig. 5A). The amino acid sequence of the putative CPRD22 protein is shown in Figure 5A, and its calculated molecular mass was 25.9 kDa. The CPRD22 protein was homologous to group 2 LEA proteins (Fig. 5B). Group 2 LEA proteins usually contain a conserved sequence, DEYGN, at the amino-terminus, a cluster of serine residues, and a lysine-rich conserved sequence, KIKEKLPG. The putative CPRD22 protein contained both the DEYGN and the KIKEKLPG sequences, but there was no cluster of serine residues. A stretch of 69 amino acids with five tandemly repeated Gly-rich sequences was found in the amino-terminal half of the polypeptide. The consensus sequence of these repeats was GTTGGFTGDTGRQ, and the function of such sequences remains to be elucidated.

Northern blot analysis of the expression of the CPRD8, CPRD14 and CPRD22 genes—We analyzed the

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A

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   1 CCTAACACCACCACAATGGTCACCGCCACCGCCACCGCCGCCGCCACCGCTCCTTCTCACCAGGCAATTCCTCTTCTCACTCCCTACAAGATGGGCAAAT  100
                      M  V  T  A  T  A  T  A  A  A  T  A  P  S  H  Q  A  I  P  L  L  T  P  Y  K  M  G  K  F
 101 TCAATCTCTCTCACAGAGTTGTCTTGGCACCGCTCTCCAGAGAGAGGTCCTACAACAACGTTCCGCAACCCCACGCCGTCGTCTATTATTCTCAGCGAAC  200
      N  L  S  H  R  V  V  L  A  P  L  S  R  E  R  S  Y  N  N  V  P  Q  P  H  A  V  V  Y  Y  S  Q  R  T
 201 CTCCAACGGTGGCCTTCTCATCGCCGAAGCTACCGGAGTTTCCGACACCGCTCAAGGGTACCCCAACACGCCTGGCATATGGACGAAAGAGCAGGTGGAA  300
     S  N  G  G  L  L  I  A  E  A  T  G  V  S  D  T  A  Q  G  Y  P  N  T  P  G  I  W  T  K  E  Q  V  E
 301 GCATGGAAACCCATCGTCGATGCCGTTCATGCCAAAGGCGGTATCTTCTTTTGCCAGATTTGGCATGTAGGAAGGGTTTCAGATTCAAGTTATCAGCCGA  400
     A  W  K  P  I  V  D  A  V  H  A  K  G  G  I  F  F  C  Q  I  W  H  V  G  R  V  S  D  S  S  Y  Q  P  N
 401 ATGGGCAAGCACCCATTTCTTCTACCGACAAGCCACTGCCACCAACGCCTCGAGCTAACGGCCTTGGATATATCGAACACACGCCACCACGGCGCCTAAC  500
      G  Q  A  P  I  S  S  T  D  K  P  L  P  P  T  P  R  A  N  G  L  G  Y  I  E  H  T  P  P  R  R  L  T
 501 GACCGAAGAACTTCCTGGTATCGTCAATGACTTCAGAATTGCTGCAAGGAATGCCATCGAAGCTGGTTTTGATGGGGTTGAGGTCCATGGGGCACATGGG  600
      T  E  E  L  P  G  I  V  N  D  F  R  I  A  A  R  N  A  I  E  A  G  F  D  G  V  E  V  H  G  A  H  G
 601 TACCTACTTGATCAATTTTTGAAAGACCAGGCTAATGACAGAACAGATCAATACGGTGGATCCCTCGAGAACCGGTGCAGACTTCCTCTGGAGGTTGTTG  700
     Y  L  L  D  Q  F  L  K  D  Q  A  N  D  R  T  D  Q  Y  G  G  S  L  E  N  R  C  R  L  P  L  E  V  V  E
 701 AAGCTGTTGTGAATGAGGTTGGGGCTGATAGAGTTGGAATTAGGTTATCACCTTTTGCAGACTTTAACGATTGTGGTGACTCAAATCCCCTGCAACTGGG  800
      A  V  V  N  E  V  G  A  D  R  V  G  I  R  L  S  P  F  A  D  F  N  D  C  G  D  S  N  P  L  Q  L  G
 801 GCTTTACATGGTCAATGCACTCAATAAGTATAACATTCTCTACTGTCACATGGTGGAACCCAGAATGGGAAGTGTTGGAGGTCCAGATGAAAACCTGACG  900
      L  Y  M  V  N  A  L  N  K  Y  N  I  L  Y  C  H  M  V  E  P  R  M  G  S  V  G  G  P  D  E  N  L  T
 901 CCAATGAGAAAGGTCTTCAATGGCACTTTTATAGTTGCCGGAGGTTATGACCGGGAAGATGGGATCAAAGCCATTGCTGAAGACAGGGCTGATCTTGTTG  1000
     P  M  R  K  V  F  N  G  T  F  I  V  A  G  G  Y  D  R  E  D  G  I  K  A  I  A  E  D  R  A  D  L  V  A
1001 CCTATGGCCGTTTGTTCTTGGCCAATCCAGATTTGCCAAAGAGATTTGCTCTCAATGCTCCTCTCAACCAGTATAATCGCAAGACATTCTACCACGAGGA  1100
      Y  G  R  L  F  L  A  N  P  D  L  P  K  R  F  A  L  N  A  P  L  N  Q  Y  N  R  K  T  F  Y  H  E  D
1101 TCCTGATCCGCTTGTTGGGTACATCGATTATCCGTTTCTTGATGAAGAATCGAACGGTGTAGCATCCTAGGGTTCTTACTAACCTGTTTTCCCCTGCAAA  1200
     P  D  P  L  V  G  Y  I  D  Y  P  F  L  D  E  E  S  N  G  V  A  S
1201 TTCCTTGCCATTTTCTATCTTGTTGGTAAGATTTTCATTCACATAATATTCTTGAGATTCTCTGCCAAGGCTTTGGATCCAGTTAACACATGTTCTAAAT  1300
1301 TGTTAGGGCATTTAGTTTGTGGAACTATTATTGTTGTGATGATGTCGCTGGAACCCCTGCATGTTTTGGGCAAAAGCGACGGCACCACCTGTTTTTGTTG  1400
1401 CAGTACTCTGCTTTGTTCTTTGAACCGAGACATGTCTCCTCTTACTTTTGTAATCGTACTTCAAAAGTTTGATTAGTGGTTTAAATAAACCTGACAATCA  1500
1501 TTACTTAAAAAAAAA                                                                                       1515
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B

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CPRD8  VTATA--------TAAATAPSHQAIP LT  MGKFN   LAPL RER --YN  PQPH-AVV S   SNGG-       GVSDT    78
morB   ---P--------DTSFSNPG-----   LGSLS   APL RSR --PD PGRL-QQI G  S-AG-       NISPT    68
OYE2   --PF--------VKDFKPQALGDTN  IGNNE   IPPL  RW  HPG PNRDWAVE A  RPGT       FPSPQ    79
OYE1   --SF--------VKDFKPQALGDTN  IGNNE   IPPL  RW  HPG PNRDWAVE Y  RPGT       ISPQ    79
ebp    IESTNSFVVPSDTKLIDVTPLGSTK  VGNNV   YVPT RFR -SKD PSDL-QLN N  YPGT       FASER    88
baiC   ---------------SYEA-------   VRGLE    LPGM TKM KNKH GEDM--IA HV  KAGCA      ALCPA    66

CPRD8  AQGYPNTP  W KE VEAWKP V   AKG IFFC   HV RVSDSSYQPNGQAPIS TD-KPLPP---TPRA GL YIEHTP-PRR  T   163
morB   ARGYVYTP  W DA EAGWKG VE  AKG RIAL   HV RVSHELVQPDGQQPVA SALKAEGAECFVEFE GT GLHPTSTPRA  T   158
OYE2   SGGYDNAP  W EE IKEWTK FK  ENK FAWV   L  WAAFPDTLARDGLRYD ASDNVYMN----AEQ EK KKANNP-QHS  K   164
OYE1   AGGYDNAP  W EE MVEWTK FN  EKK FVWV   L  WAAFPDNLARDGLRYD ASDNVFMD----AEQ AK KKANNP-QHS  K   164
ebp    GGIDLHVP  Y DA AKSWKK NE  GNG FSSV   L  RVANAKDLKDSGLPLI PS-AVYWD----ENS KL KEAGNE-LRA  E   172
baiC   PHAYMYM-  Y DH VEQLKK TD  EAG KMGI   HG -------------FS --QMF--------FD TN LETPD----T  V   127

CPRD8  PGI N- RI RN                      KDQA              LPLE VV EVGA R GIR S FADFN   251
morB   PGI E- RQ QR KR                   ATGT              FPLE VA VFGP R GIR T FLELF   246
OYE2   KQY K- VQ KN IA                   DPHS              FTLE VV AIGP K GLR S YGVFN   252
OYE1   KQY K- VQ KN IA                   DPHS              FTLE  V AIGH K GLR S YGVFN   252
ebp    DHI EV PN KH LE                   NLAS              LLLR  I VGA R ALR S WASFQ   262
baiC   HEI E- GR RM VQ                   SPGM              FCYE  IR NI-P D PFF R DCIDE   214

CPRD8  DC-GDS PLQLG-L-YMVN NKYN-----ILYCHMV P-RMGS-VGGPDENLTPMRKVF----- GTF VA GYD-REDGIKAIAE --   323
morB   GL-TDD PEAMA-F-YLAG DRRG-----LAYLHFN PDWIGGDITYPEGFREQMRQRF----- GGL YC YD-AGRAQARLDD --   320
OYE2   SMSGGA TGIVAQYAYVLG ERRAKAGKRLAFVHLV PRVTNPFLTEGEGEYNGGSNKFAYSIW GPI RA NFALHPEVVREEVK --   340
OYE1   SMSGGA TGIVAQYAYVAG EKRAKAGKRLAFVHLV PRVTNPFLTEGEGEYEGGSNDFVYSIW GPV RA NFALHPEVVREEVK --   340
ebp    GM--EI GEEI--HSYILQ QRADNGQQLAYISLV PRVTGIY-DVSLKDQQGRSNEFAYKIW GNF RA YTYDAPEFKTLIN LK   347
baiC   LMEQTM EEEIVTFINKCA GVDVADLSRGNATSFA VYEVPPF-NLAHGFNIENIYNIKKQI- IPV GV RINTGEMANKVIEE --   300

CPRD8  -RADL   FL   PK FAL A-P Q---------Y -------RKTFYHEDPDPLVG DYPFL E-ESNGVASA   385
morB   -TADA   FI   PE RL A-A E---------P -------PSTFY-GGAE--VG TDYPFL N-GHDRLG--   377
OYE2   -PRTL   FI   VD EK -P K---------Y -------RDTFYKMSAEGYID PTYE-- A-LKLGWDKN   400
OYE1   -KRTL   FI   VD EK -P K---------Y -------RDTFYQMSAHGYID PTYE-- A-LKLGWDKK   400
ebp    NDRSI   FF   VE KL -P Y---------Y -------REEFYKYYN---YG NSYDES K-QVIGKPLA   407
baiC   -KFDL   QL   IT RE KED RHCIGCDQGCY AVINPKMKHITCTHNPGLCLE QGMPKT APKKVMIVGG   380
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Fig. 3 (A) The nucleotide and deduced amino acid sequences of CPRD8 cDNA. The nucleotide sequence includes a putative coding region and 5′- and 3′-noncoding regions. The amino acid sequence of the putative coding region is shown below the nucleotide sequence in the single-letter code. (B) Comparison of the deduced amino acid sequences of CPRD8, morB (morphinone reductase of *Pseudomonas putida*; French and Bruce 1994), OYE2 (old yellow enzyme of *Saccharomyces* spp.; Stott et al. 1993), OYE1 (old yellow enzyme of *Saccharomyces carlsbergensis*; Saito et al. 1991), ebp (estrogen-binding protein of *Candida albicans*; Madani et al. 1994) and baiC (amino-terminal region, namely, residues 1 to 380, of bile acids-inducible protein C of *Eubacterium* sp. VPI12708; Mallonee et al. 1990). Dashes indicate gaps that were introduced to maximize alignment. Boxes and shadowing indicate identical and similar amino acids, respectively. The underlined sequence and arrows indicate amino acids that are involved in the active site and FMN-binding site of OYE1, respectively.

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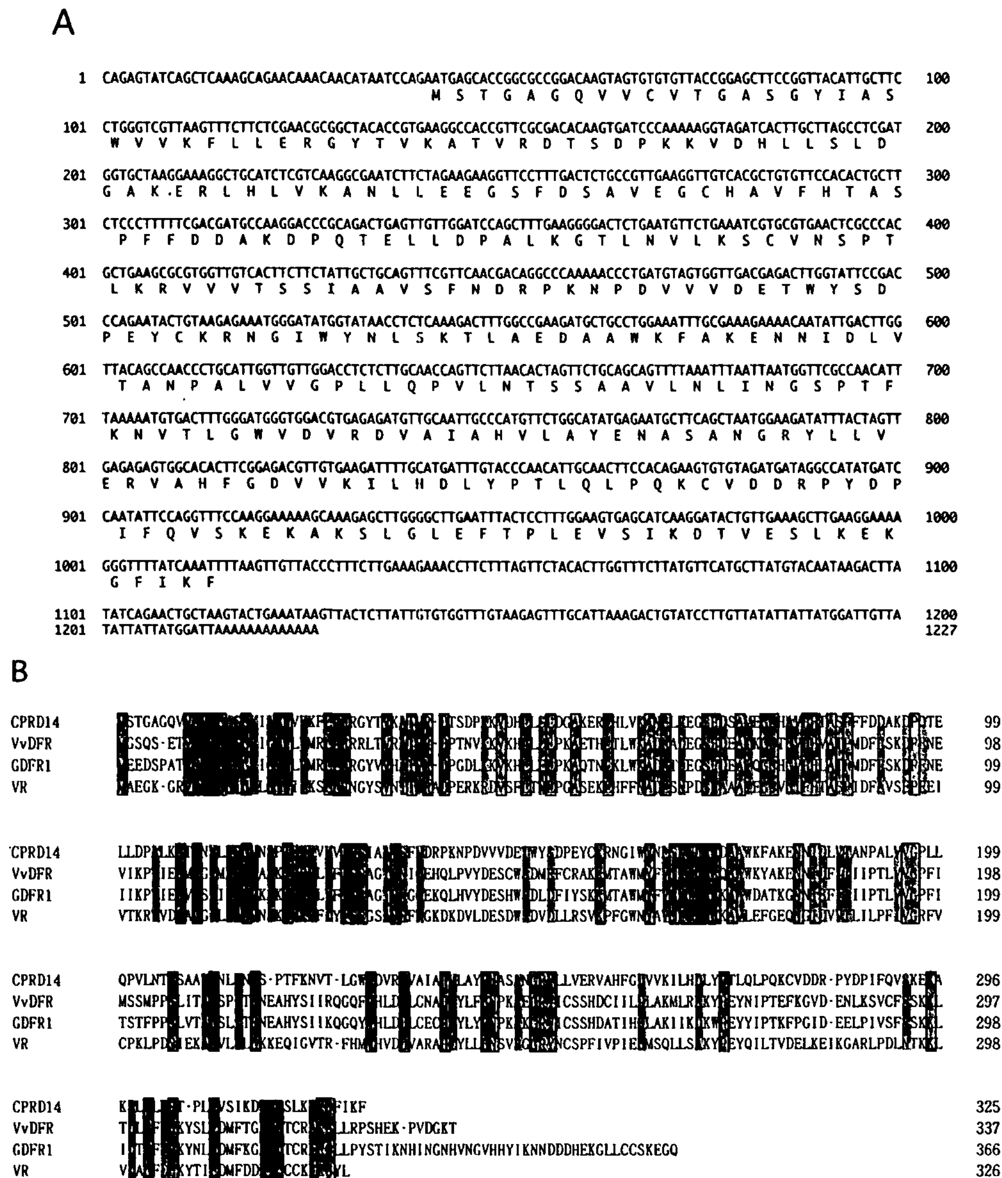

Fig. 4 (A) The nucleotide sequence and deduced amino acid sequence of CPRD14 cDNA. The amino acid sequence of the putative coding region is shown below the nucleotide sequence. (B) Comparison of the deduced amino acid sequences of CPRD14, VvDFR (DFR of grape, Sparvoli 1994), GDFR1 (DFR of *Gerbera*, Helariutta 1993) and VR (vestitone reductase of alfalfa; Guo and Paiva 1995). Dashes indicate gaps that were introduced to maximize alignment. Gray enclosed boxes indicate identical amino acids. Shadowing indicates similar amino acids.

effects of various environmental stresses on the expression of the CPRD8, CPRD14, and CPRD22 genes, and we found that all three genes were strongly induced under high-salt conditions (250 mM NaCl) but none was induced by cold stress (4°C) or heat stress (40°C, Fig. 6). Various environmental stresses, such as high salinity, water deficiency, and low temperature, trigger the synthesis of ABA, which induces the expression of many genes. To determine whether or not ABA is involved in the induction of CPRD genes, we performed Northern hybridization using RNAs isolated from leaves that had been treated with ABA or with water (Fig. 6). The transcription of the CPRD8 and CPRD22 genes was induced to a significant extent by the application of exogenous ABA, but that of the CPRD14 gene was not.

Northern blot analysis was performed to determine the kinetics of induction of the CPRD8, CPRD14 and CPRD22 genes in cowpea plants that were grown in soil under drought conditions (Fig. 7). We found that all three genes were induced gradually by withholding water for 5 to

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A

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   1 CAAAAGTTCTTAAAATCACAACCTATCACAACAACAAGAACAACCATGGCAAGTTACCAGAAGCAGTACGAGGATCAGGGTCGCAAGATCGACGAGTATG  100
                                                 M  A  S  Y  Q  K  Q  Y  E  D  Q  G  R  K  I  D  E  Y  G

 101 GCAACGTTGTACAGGAAACTGATGAATATGGCAACCCGGTTCATGCAGCAAGTGTCACCTACATAACCTCCACCACCGGTGGTCTTGGGGATGACTCTAA  200
       N  V  V  Q  E  T  D  E  Y  G  N  P  V  H  A  A  S  V  T  Y  I  T  S  T  T  G  G  L  G  D  D  S  N

 201 CAAGCAACATGATACCAGTAATGTCTACGGTGCAGACACCCGTAGACAACACGGAACTATAGGTGACACCGGTAGACAGCACGGAACTACCGGTGGTTTT  300
      K  Q  H  D  T  S  N  V  Y  G  A  D  T  R  R  Q  H  G  T  I  G  D  T  G  R  Q  H  G  T  T  G  G  F

 301 ACTGGTGACACCGGTAGACAATATGGCACTACCGGAGGTTTTACCGGTGACACCGGGAGACAACATGGGACTACCGGTGGGTTTACCGGTGACACCGGGA  400
     T  G  D  T  G  R  Q  Y  G  T  T  G  G  F  T  G  D  T  G  R  Q  H  G  T  T  G  G  F  T  G  D  T  G  R

 401 GACAACATGGGACTACCGGTGGTTTTACCGGTGACACTGGGAGACAACATGGGACTACCGGTGGGTTTACCGGTGACACTGGGAGACAACATGGGACTAC  500
       Q  H  G  T  T  G  G  F  T  G  D  T  G  R  Q  H  G  T  T  G  G  F  T  G  D  T  G  R  Q  H  G  T  T

 501 CGGCGGCTTTACTGGTGGTGACACTGGTCTGGGAGGCCCTTACGTTGGAGCCAACACCGCTGATACAGGGACTGGTCCTAGAAGTGGAACCACAGGTGGC  600
      G  G  F  T  G  G  D  T  G  L  G  G  P  Y  V  G  A  N  T  A  D  T  G  T  G  P  R  S  G  T  T  G  G

 601 AGCGCCTATGGATCGGGTGGCGCTGGTGGTGGTTATGGAAGTGGAGCTGGAGCTGGTGGAGCTGGGTATGGTATGAACACAGGGGGAGCACACAGTGATG  700
     S  A  Y  G  S  G  G  A  G  G  G  Y  G  S  G  A  G  A  G  G  A  G  Y  G  M  N  T  G  G  A  H  S  D  E

 701 AAAGGTATGGAAGGGAGTATCGTGAGCATGATCAGTCTCGTGGAGATCATGACAAGAAAGGGATAGTGGACAAGATTAAGGAGAAGCTTCCCGGAGGACA  800
       R  Y  G  R  E  Y  R  E  H  D  Q  S  R  G  D  H  D  K  K  G  I  V  D  K  I  K  E  K  L  P  G  G  H

 801 CAGTGACAACAAGTAGAGACATCATGGGTGTGCATGCATGCGTATATATACATAGTATAATTAAAGATGTTATATTGTTGTGTTTTTGAATAAGTTTGCT  900
      S  D  N  K

 901 GCATATATACGTACTCGTACAGTACTGTCTTGTCGTGTAAATGTGGTGGATCTTGTATATGGTTATAGTGTATGCAAGAGGAATAAAAGGTGCATGCATG  1000
1001 CAAGGGAAACAAAGAAGGCATAGATGTTCTGCCAATGGATATGTAAAACCTACCCTGTTGTAGAGCAGGGTGCTTCAGTTGTGTGTTTTGAATTTTTCAA  1100
1101 TGAAGCAACTTTTTCTTTCAAAAAAAAAA                                                                        1129
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B

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CPRD22  MASYQKQYEDQGRKIDEYGNVVQETDEYGNPVHAASVTYI-TSTTGGLGDDSNKQHDTSNVYGADTRRQHGTIGDTGRQH   79
 MAT1   MASYQKHYDDQGRKVDEYGNVERQTDEYGNPVHATSVTYVATKSVGGYNDDTNKQHDTTGVYPEK---------DTGRHH   71
 DHN1   MSQYQNQYGAQTGMTDEYGNPVNQVDQYGNPI----------------------------------------------   32
RAB16A  -----------MEHQGQHGHVTSRVDEYGNPV----------------------------------------------   21
 TAS14  -----------MAQYGNQDQM-RKTDEYGNHV----------------------------------------------   20

CPRD22  GTTGGFTGDTGR-QYGTTGGFTG-DTGRQHGTTGGFTGDTGRQHGTTGGFTG--DTGRQHG---TTGGFTGDTGRQHGTT  152
 MAT1   -FGRGYDGDTNKQQHDAIGVYPGIDIGRDHETTGVYGLNTDRHHGTTGVNPGIDTHNQQHG---TTGGYAGDTGRQHGNT  147
 DHN1   -SGGGFTGEAGRQHFGTTGGATDHGHGHGQQHRGV---DQTTGYGTHTGGVGGYGTNPEYGNTNTGSGYGTGTGYGGSGT  108
RAB16A  ------GTGAGHGQMGTAG-MGTHG-------T----AGTGRQFQPM---REEHKTGGVLQRSGSSSSSSSEDDGMGGR-   79
 TAS14  ------QETGVYQGTGTGGMMGGTG-------TGGMMGGTGGEYGTQGMGTGTHHHEGQQQLRRSDSSSSSEDDGEGGR-   86

CPRD22  GGFTGGDTGLGGPYVGANTAD--TGTGPRSGTTGGSAYGSGGAGGGYGSGAGAG---GAGYGMNTGGAHSDERYGREYRE  227
 MAT1   GGL----------YYGTDTAD--TGTGPRSGNTGGTGYG-GTGGTDYGTAGGTGYGSGTGYGINTGGAHTEAGYGKEHRQ  214
 DHN1   NEYVREDHHGDKKGVMDKIKEKIPGTEQSRTNTDGAGYGS----TGYGASGG-------------GIGNTGQEYVREEHR  171
RAB16A  ----------RKKGIKEKIKEKLPGGNKGEQQHAMGGTGTGTGTGTGGAYGQQGHGTGMTTGTTGAHGTTTTDTG---  146
 TAS14  ----------RKKGLKEKIMEKMPGQHEGE---------------------YGQ-------------------TTGE---  113

CPRD22  HDQSR-GDHDKKGIVDKIKEKLPGGHSDNK     256
 MAT1   HEQSHGGQHEKKGILDKIKEKLPGGHSDK      243
 DHN1   VDHG-----EKKGIMDKIKEKLPGTGGCTGH    197
RAB16A  ---------EKKGIMDKIKEKLPGQH         163
 TAS14  ---------EKKGMMDKIKDKIPGMH         130
```

Fig. 5 (A) The nucleotide sequence and deduced amino acid sequence of CPRD22 cDNA. The amino acid sequence of the putative coding region is shown below the nucleotide sequence in the single-letter code. (B) Comparison of the deduced amino acid sequences of CPRD22, MAT1 (soybean; Chyan et al. 1992), DHN1 (pea; Robertson and Chandler 1992), RAB16A (rice; Mundy and Chua 1988) and TAS14 (tomato; Godoy et al. 1990). Dashes indicate gaps that were introduced to maximize alignment. Boxes indicate conserved regions. Arrows indicate the direct repeats of the sequence GTTGGFTGDTGRQ.

10 days and, moreover, that high levels of their transcripts were detectable at 20 days. These observations indicate that these genes responded naturally to a water deficit. Even after 20 days of drought treatment, the cowpea plants were able to grow normally upon rehydration.

Discussion

As a first step towards full characterization of the responses of drought-tolerant plants to dehydration stress at the molecular level, we cloned cDNAs for genes in the drought-tolerant cowpea plant that are induced by dehydration stress, and we analyzed their structure and the expres-

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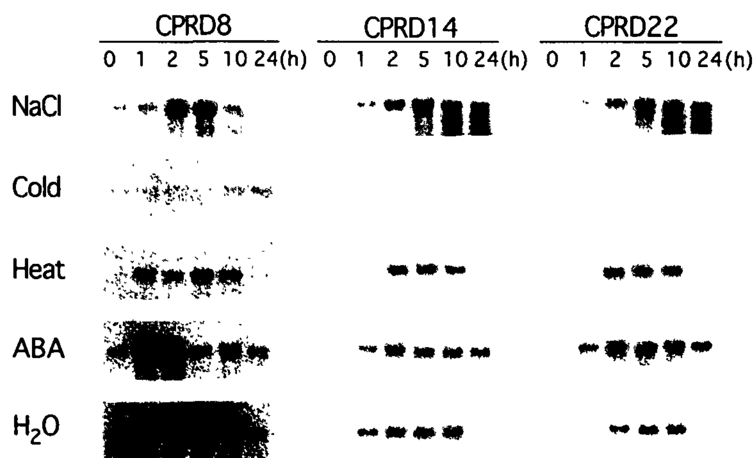

Fig. 6 Northern blot analysis of the induction of the CPRD8, CPRD14, and CPRD22 genes by high salinity (NaCl), high temperature (Heat), low temperature (Cold), and the application of abscisic acid (ABA). One-month-old cowpea leaves were cut from plants at the petiole and placed in tubes that contained 250 mM NaCl, 100 μM ABA, or water. Tubes, that contained cowpea leaves in water were also incubated at 40°C (Heat) or 4°C (Cold). Total RNA was isolated from the leaves after treatment. Each lane was loaded with 10 μg of total RNA. The number above each lane indicates the duration (hour) of treatment. For other details, see legend to Figure 2.

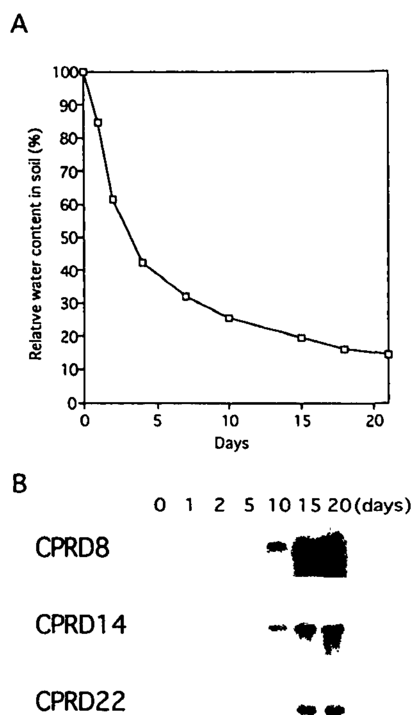

Fig. 7 Changes in the relative water content of the soil (A) and Northern analysis of the induction of the CPRD8, CPRD14, and CPRD22 genes by dehydration (B). Cowpea plants that had been grown in soil under normal conditions for 1 month were exposed to progressive dehydration by withholding. Total RNA was prepared from cowpea leaves that had been dehydrated in soil for 0, 1, 2, 5, 10, 15, or 20 days. Each lane was loaded with 10 μg of total RNA. Northern hybridization was carried out as described in the legend to Figure 2.

sion of the corresponding genes. The various cultivars of cowpea exhibit different levels of drought tolerance and cowpea is, therefore, a very suitable plant for the molecular analysis of drought tolerance. For our experiments, we chose the cowpea cultivar IT84S-2246-4, which is very tolerant to drought stress and produces large numbers of seeds even under water-deficit conditions (Singh 1993). We cloned ten independent cDNAs for genes that respond to dehydration by differential screening (Table 1). To analyze the drought-inducible expression of these genes, we performed Northern blot hybridization. All the genes corresponding to CPRD cDNAs, with the exception of CPRD29, were induced by dehydration exclusively, and they were not induced to any significant extent by water treatment. These results suggest that nine of the CPRD genes were drought-inducible genes while the CPRD29 gene was not. Under rehydration conditions, levels of all the CPRD mRNAs, apart from the CPRD29 and CPRD46 mRNAs, decreased significantly. The CPRD46 mRNA decreased and then it increased transiently under rehydration conditions. The level of the CPRD46 gene might be responsive to osmotic changes during dehydration and rehydration.

In dehydrated plants, levels of endogenous ABA increase significantly, and the accumulated ABA induce the expression of many genes that might be involved in dehydration tolerance. We analyzed the accumulation of ABA in cowpea leaves under dehydration conditions. When cowpea leaves were subjected to dehydration, they gradually lost 35% of their net weight over the course of 24 h (Fig. 1). Accumulation of ABA became apparent 2 h after the start of dehydration treatment and reached a maximum level at 10 h. The 10-h-dehydrated cowpea plants contained

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endogenous ABA at about 10 nmol $(gFW)^{-1}$, and this level was 160 times higher than that found in unstressed plants. The maximum level of accumulated ABA in dehydrated *Arabidopsis* rosette plants was, by contrast, only four times higher than that found in unstressed plants (Kiyosue et al. 1994). The level of ABA in dehydrated *Arabidopsis* plants was about 160 pmol $(gFW)^{-1}$, which was about 2% of that in dehydrated cowpea. Thus, the level of accumulated ABA under dehydration conditions seems to be extremely high in cowpea, as well as in other legumes, when compared to that in *Arabidopsis* (Walton et al. 1979). These observations suggest that ABA might be important in drought tolerance in the cowpea.

To determine whether ABA is involved in the induction of CPRD genes, we performed Northern hybridization experiments to quantitate the transcripts of three CPRD genes. The CPRD8 and CPRD22 genes responded significantly to exogenous ABA, but CPRD14 did not (Fig. 6). These findings suggest the existence of ABA-independent and ABA-dependent signal-transduction cascades between the initial signal of drought and the expression of specific genes in cowpea, as well as in *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki 1996). These three CPRD genes were induced by high salinity but not by low temperature. The transcripts of these three genes accumulated slightly and transiently upon heat treatment. However, the levels of induction of these three genes by heat treatment were the same as those by water treatment, a correlation that suggests that the accumulation of the transcript might have been due to some other stress that was related to manipulation of the leaves. The CPRD8, CPRD14 and CPRD22 genes were gradually induced in cowpea plants in soil from 5 to 10 days after water was withhold (Fig. 7). Thus, it appears that these genes respond naturally to a water deficit, and that our experimental conditions for the isolation of cDNAs for drought-inducible genes were appropriate.

The deduced amino acid sequence of the CPRD22 protein revealed structural features homologous to those of group 2 LEA proteins. At least 30 different putative proteins have been identified as group 2 LEA proteins in a variety of plants (Bray 1993). A conserved repeat sequence, EKKGIMDKIKEKLPG, and a cluster of serine residues are usually found in group 2 LEA proteins. However, one of the repeat sequences and the cluster of serine residues were completely absent in the CPRD22 protein (Fig. 5B). We found similar structural features in soybean MAT1 (Chyan et al. 1992) and pea DHN1 (Roberton and Chandler 1992), which are both group 2 LEA proteins. Group 2 LEA proteins are thought to function in the maintenance of protein structure (Bray 1993). The CPRD22 protein might also function in protecting cowpea cells from dehydration. There were five direct repeats of the sequence GTTGGFTGDTGRQ in the amino-terminal half of CPRD22, a feature that has not been reported in other group 2 LEA proteins. The expression of the CPRD22 gene was up-regulated by drought, high salinity, and treatment with exogenous ABA, as is the expression of other LEA genes. Typical drought-inducible genes (e.g., LEA genes) were also isolated from cowpea in our library, providing another indication that the dehydration conditions in our experiments were appropriate.

We identified two novel genes, CPRD14 and CPRD8, that were induced by dehydration stress in cowpea. The amino acid sequence of the putative CPRD14 protein was similar to those of dihydroflavonol-4-reductase (DFR) and vestitone reductase (VR; Fig. 4). DFR is involved in the biosynthesis of anthocyanin. VR is involved in the biosynthesis of medicarpin. Medicarpin is the major phytoalexin in alfalfa. Both anthocyanin and medicarpin are synthesized via the phenylpropanoid pathway (Dixon and Palva 1995, Guo et al. 1994). Secondary metabolites produced in this pathway include a number of compounds derived from phenylpropanoid and flavonoid compounds. These products are involved in protection of cells from UV light, defense against attack by pathogens, legume nodulation and pollen viability (Li et al. 1993, Hahlbrock and Scheele 1989, Lamb et al. 1989, Long 1989). The product of the CPRD14 gene might be involved in the synthesis of such compounds, which protect plant cells from stress conditions.

The sequence of the putative CPRD8 protein was similar to those of old yellow enzyme from *saccharomyces*, morphinone reductase from *Pseudomonas*, estrogen-binding protein from *Candida*, and bile acid-inducible proteins of *Eubacterium* (Fig. 3B). These proteins belong to a family of flavoprotein α/β-barrel oxidoreductases (French and Bruce 1995). The CPRD8 protein might have similar enzymatic activity to that of flavoprotein α/β-barrel oxidoreductases because both the active domain and an FMN-binding site were found in the CPRD8 protein (Fig. 3B). Although the physiological functions of old yellow enzyme, morphinone reductase and estrogen-binding protein remain unclear, these proteins are all known to have a high affinity for steroids (French and Bruce 1995). Furthermore, bile acid-inducible proteins C and H, two bacterial α/β-barrel flavoenzymes, are thought to be involved in steroid metabolism (Mallonee et al. 1990). In plants, brassinosteroids have structures typical of steroid derivatives and function as growth regulators. Brassinosteroids also increase resistance to stress in plants under high-temperature, high-salt, and low-temperature conditions (Katsumi 1991, Kulaeva et al. 1991). Under stress conditions, brassinosteroids induce a set of proteins that might function in the protection of cells against heat and salt stress (Kulaeva et al. 1991). The drought-inducible CPRD8 protein might interact with brassinosteroids to protect plant cells from dehydration and salt stress.

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