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ORIGINAL ARTICLE

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The novel gene *CpEdi-9* from the resurrection plant *C. plantagineum* encodes a hydrophilic protein and is expressed in mature seeds as well as in response to dehydration in leaf phloem tissues

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Abstract The resurrection plant *Craterostigma plantagineum* Hochst. is used as an experimental system to investigate desiccation tolerance in higher plants. A search for genes activated during early stages of dehydration identified the gene *CpEdi-9*, which is expressed in mature seeds and in response to dehydration in the phloem cells of vascular tissues of leaves. Elements for the tissue-specific expression pattern reside in the isolated promoter of the *CpEdi-9* gene, as shown through the analysis of transgenic plants. The *CpEdi-9* promoter could be a suitable tool for expressing genes in the vascular system of dehydrated plants.*CpEdi-9* encodes a small (10 kDa) hydrophilic protein, which does not have significant sequence homologies to known genes. The predicted protein CpEDI-9 shares some physicochemical features with LEA proteins from plants and a nematode. Based on the unique expression pattern and on the nucleotide sequence we propose that *CpEdi-9* defines a new class of hydrophilic proteins that are supposed to contribute to cellular protection during dehydration. This group of proteins may have evolved because desiccation tolerance requires the abundant expression of protective proteins during early stages of dehydration in all tissues.

Keywords *Craterostigma* · Early dehydration-induced protein · Hydrophilic protein · Phloem promoter analysis · Resurrection plant

Abbreviations ABA: Abscisic acid · ABRE: ABA-responsive element · Edi: Early dehydration induced · GUS: Glucuronidase · LEA: Late embryogenesis abundant · MU: Methylumbelliferone

Introduction

Dehydration is a common environmental stress to which plants are exposed. Tolerance to dehydration rarely occurs in vegetative plant tissues while angiosperm seeds and pollen are able to survive extreme dehydration conditions (Leopold 1992; Bartels et al. 1996; Phillips et al. 2002). A unique exception among higher plants is the small group of resurrection plants, which have developed the ability to recover from severe water loss in vegetative tissues (Gaff 1971). A representative of the resurrection plants is *Craterostigma plantagineum*, a member of the Scrophulariaceae, which can lose most of its cellular water and recover from such severe dehydration within 24 h of contact with water (Bartels et al. 1990). Hence *C. plantagineum* provides an excellent system to study the molecular basis of desiccation tolerance in higher plants. During dehydration, many new transcripts rapidly accumulate in vegetative tissues of *C. plantagineum* and it has been supposed that the corresponding gene products contribute to the protection of cellular structures and metabolites during severe water stress (Bockel et al. 1998; Bartels and Salamini 2001).

This article is dedicated to Prof. Dr. Francesco Salamini on the occasion of his 65th birthday and his departure from the Max Planck Institute in Köln

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It has previously been shown that a diverse group of hydrophilic proteins, called LEA (= Late Embryogenesis Abundant) proteins are one of the most abundant groups of proteins that accumulate in the vegetative tissues of *C. plantagineum* in response to water stress (Piatkowski et al. 1990; Schneider et al. 1993; Velasco et al. 1998; Ditzer et al. 2001). LEA proteins were first characterised in cotton embryos where they accumulate to high levels during the last stage of seed formation (Galau et al. 1986). Besides dehydration, the expression of LEA proteins can be induced in vegetative tissues in response to freezing or salt stress, or by treatment with the phytohormone abscisic acid (ABA; Piatkowski et al. 1990; Schneider et al. 1993). The endogenous ABA concentration increases in vegetative tissues upon dehydration and at late stages of seed formation. It is known to play an important role in several aspects of seed development, and it is crucial for the plant responses to environmental stress (Leung and Giraudat 1998). LEA proteins have been classified into six groups on the basis of sequence similarities (Ingram and Bartels 1996; Colmenero-Flores et al. 1997; Cuming 1999). Often no significant homology on the sequence level has been detected between LEA proteins of different families, although most of them share common features such as high hydrophilicity, biased amino acid composition, stability after boiling and the prediction to exist as random coil structures. Some conserved motifs within LEA proteins are predicted to form amphipathic α-helices, which may be important for their function (Dure 1993; Ingram and Bartels 1996; Close 1997). Recent findings suggest that α-helices preferentially occur under dehydration conditions (Goyal et al. 2003). The accumulation pattern of LEA proteins and structural characteristics suggest that they function in the protection of plant cells during dehydration or other stresses with a water-deficiency component. Additional evidence supports this hypothesis: firstly, functional in vitro protection assays have shown that several LEA proteins are able to protect diverse enzyme activities against different stresses (e.g. Rinne et al. 1999; Hara et al. 2001; Bravo et al. 2003) and secondly, transgenic and genetic approaches have also shown the relationship between the accumulation of some LEA proteins and enhanced stress protection (Imai et al. 1996; Xu et al. 1996; Ismail et al. 1999; Zhang et al. 2000; Hara et al. 2003).

LEA protein members of groups 2, 3, 4 and 5 from *C. plantagineum* have been characterised. They accumulate during dehydration and have been found in all cell types, accumulating abundantly in the cytoplasm or plastids (Michel et al. 1993, 1994; Schneider et al. 1993; Furini et al. 1996; Velasco et al. 1998; Ditzer et al. 2001). Promoters of four *C. plantagineum* LEA genes have been analysed in tobacco and *Arabidopsis*. All of them were highly active in seeds and pollen, two naturally dehydration-tolerant tissues (Michel et al. 1993, 1994; Furini et al. 1996; Velasco et al. 1998). Several *cis*-elements involved in ABA-induced gene expression have been identified in promoters of *Lea*genes from *C. plantagineum* (Bartels and Salamini 2001). One important group are the ABREs (ABA-responsive elements) which contain the G-box ACTG core motif, but they do not seem to be the sole determinants for ABA and dehydration response (Michel et al. 1993, 1994).

Here we report the characterisation of a novel early dehydration-inducible gene *CpEdi-9*from *C. plantagineum*. *CpEdi-9* is expressed in response to plant dehydration, salt stress and ABA treatment in vegetative tissues and constitutively in mature seeds. Sequence analysis of *CpEdi-9* did not reveal significant homology with known genes, although it shares some predicted structural features with LEA proteins. In contrast to the *C. plantagineum Lea* genes studied, expression in leaves in response to dehydration is earlier and restricted to the phloem cells of the vascular bundles. Like other promoters of *Lea* genes and ABA-responsive genes, the *CpEdi-9* promoter is active in stomatal guard cells, pollen and mature seeds, when analysed in transgenic plants. The *CpEdi-9* promoter directs reporter gene expression in response to dehydration and ABA treatment and its activity is mainly localised in phloem cells of the vascular tissue. *CpEdi-9* encodes a hydrophilic boiling-stable protein, which may extend the known classes of Lea genes. It could be involved in the protection of vascular tissues of *C. plantagineum* during early phases of dehydration.

Materials and methods

Plant material

Plants of *Craterostigma plantagineum* Hochst., originally collected in South Africa, were grown under controlled environmental conditions as described by Bartels et al. (1990). In dehydration experiments, mature plants were removed from their pots and water was withheld. The water content of untreated plants was set to 100% of relative water content and the water loss was monitored during the dehydration process (Smith-Espinoza et al. 2003). Plants were treated for salt stress in hydroponic cultures in Hoagland solution supplemented with the indicated NaCl concentrations. For ABA treatments, excised leaves were placed on water containing 100 μM ABA (+/− *cis trans* isomer; Sigma–Aldrich, München, Germany).

Plants of *Nicotiana tabacum* L. cv Samsun and *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia) were propagated and grown as described by Velasco et al. (1998). Seeds were obtained from the collection at the Max Planck Institute, Köln, Germany.

Six-week-old *Arabidopsis*plants were treated by submerging the aerial parts in an aqueous solution with or without 100 μM ABA for 10 s twice a day for 2 days. In dehydration experiments water was withheld for 5 days from soil-grown *Arabidopsis* plants.

The ABA treatment in transgenic tobacco was applied to 8-day-old in vitro-grown seedlings by transferring them to medium without or with 100 μM ABA for 24 h.

Nucleic acid analysis

Extraction of nucleic acids, RNA and DNA blot analyses were carried out as described in Bartels et al. (1990). The CpEdi-9 cDNA insert was labelled with ^{32}P by random prime labelling (Feinberg and Vogelstein 1984).

General molecular biology methods were performed according to standard procedures (Sambrook et al. 1989).

Isolation of the CpEdi-9 cDNA and genomic clone

The CpEdi-9 cDNA clone was isolated by differentially screening a cDNA library constructed from mRNA isolated from 1-h-dehydrated *C. plantagineum* leaves (Bockel et al. 1998). The genomic library was constructed by cloning partially digested DNA fragments into the phage lambda vector EMBL4 (Michel et al. 1994). The library was screened using the CpEdi-9 cDNA clone as a probe.

Production of recombinant EDI-9 protein

The recombinant EDI-9 protein was produced and purified as polypeptide containing an N-terminal His-tag carried in the pQE30 vector following the instructions of the manufacturer (QIAexpress; Qiagen, Germany). Recombinant EDI-9 protein was affinity-purified on Ni-NTA Agarose resin (QIAexpress; Qiagen) under native conditions. Purified EDI-9 protein was precipitated with 2.5 vol. of acetone and resuspended in 150 mM potassium phosphate buffer (pH 7.5) to obtain a protein concentration of 1 μg μl^{-1} and stored at −20°C until used.

DNA sequencing and computer analysis

DNA was sequenced using a dideoxynucleotide sequencing kit with the T7 polymerase (Pharmacia LKB, Freiburg, Germany) and the Ready reaction Dideoxy Terminator Cycle Sequencing Kit Prism (Applied Biosystems, Darmstadt, Germany) with a model 373A DNA sequencer (Applied Biosystems). DNA and protein sequences were analysed using programs from the University of Wisconsin Genetics Computer Group (Devereux et al. 1984).

Primer extension analysis

A 17-mer oligonucleotide complementary to the strand position +73 to +89 was end-labelled with ^{33}P and hybridised to 10 μg poly(A)$^+$RNA isolated from 2-h-dehydrated *C. plantagineum* leaves. The hybridisation was carried out for 3 h at 42°C in 250 mM KCl and 10 mM Tris–HCl (pH 7.9). First-strand cDNA synthesis was performed in 50 mM NaCl with 20 units of AMV reverse transcriptase (Life Sciences, St. Petersburg, USA) for 1 h at 42°C. The extended product was analysed in a 6% polyacrylamide–urea gel using the 17-mer oligonucleotide for sequencing the genomic *CpEdi-9* clone as reference.

Construction of the *CpEdi-9* promoter β-glucuronidase(*GUS*) gene, and plant transformation

A promoter fragment (1,293 bp in length) of the *CpEdi-9* gene was amplified by PCR and subcloned into the binary vector pBI101.2 containing the *GUS* gene coding sequence and the nopaline synthase (NOS) polyadenylation signal (Jefferson et al. 1987) creating the plasmid pBIpEdi9. The pBIpEdi9 plasmid was transferred to *Agrobacterium tumefaciens* (strain LBA4404) for plant transformation. Leaf discs of *Nicotiana tabacum* (cv. Samsun) were transformed as described by Horsch et al. (1985). After inoculation with *Agrobacterium* harbouring pBIpEdi-9, transformed cells were selected in a shoot-inducing medium supplemented with 50 mg l^{-1} kanamycin (Sigma) and 500 mg l^{-1} cefotaxime (Hoechst). Young shoots were transferred to a root-inducing medium containing 100 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. Rooted plantlets were transferred to soil and grown in the greenhouse.

Plants of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia) were transformed using the vacuum method as described by Bechtold et al. (1993).

The insertion of the pBIpEdi-9 plasmid in transgenic plants was confirmed by PCR using an internal primer of the *GUS* gene and a primer located in the *CpEdi-9* promoter.

In-situ hybridisation of RNA

Leaves from *C. plantagineum* plants dehydrated for 4 h were fixed in 4% formaldehyde, 0.1% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 16–24 h at 4°C. The plant material was dehydrated in a graded ethanol–xylene series and embedded in Paraplast. Sections (10 μm thick) were prepared using a microtome (Leica RM 2065). The Edi-9 cDNA clone was subcloned in the vector pBluescript SK (Stratagene, Heidelberg, Germany) and used as template for synthesis of sense and antisense digoxigenin-labelled ribo probes following the manufacturer's instructions (Boehringer, Mannheim, Germany). Probes were hydrolysed to an average size of 150 bp before hybridisation. Mounted slides were hybridised overnight at 50°C. After hybridisation the sections were incubated with 40 μg ml^{-1} RNase A and then washed several times with 2 × SSPE (1 × SSPE = 0.18 M NaCl, 10 mM NaH_2PO_4, 2 mM EDTA) at 45°C. Digoxigenin-labelled RNA probes were detected using anti-digoxigenin alkaline-phosphatase conjugate (Boehringer). Slides

were incubated with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT), rinsed with water and counterstained with Safranin O.

Detection of GUS activity

Transformed tobacco and *Arabidopsis* plants were examined at different developmental stages and upon dehydration or ABA treatment for GUS activity. For quantitative GUS assays, protein extracts from transgenic plants were mixed with the substrate solution [10 mM 4-methylumbelliferyl-D-glucuronide (MUG), 50 mM NaH_2PO_4, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, pH 7.0] and assayed as described by Jefferson et al. (1987). The GUS activity was quantified with a fluorometer (LS30; Perkin Elmer, Rodgau, Germany). Total protein concentrations were determined using the BioRad (Munich, Germany) protein assay kit. GUS activity is expressed as pmol 4-methylumbelliferone (MU) mg^{-1} min^{-1}.

For histochemical GUS staining the samples were immersed in GUS staining solution [1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) in 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide and 0.01% Triton X-100], vacuum-infiltrated and incubated overnight at 37°C. Plant pigments were extracted with three changes of 95% (v/v) ethanol. Tissue for microscopic sections (12 μm thick) was dehydrated in a graded ethanol–xylene series, embedded in Paraplast and sectioned using a microtome (Leica RM 2065). Paraplast was removed from sections by treatments with xylene. Hand-cut sections were prepared from fixed, stained tissue using a razor blade. Tobacco and *Arabidopsis* epidermal peels were removed from the abaxial surface of the leaves.

Results

Isolation and sequence analysis of the *CpEdi-9* gene from *Craterostigma plantagineum*

In a search for genes expressed early in response to dehydration, the *CpEdi-9* (= C*raterostigma*p*lantagineum* early dehydration-induced) gene was identified (Bockel et al. 1998). The CpEdi-9 cDNA clone isolated from the library was 375 bp long containing the 3′poly(A)-tail but truncated at the 5′-end. Screening of a genomic library (Michel et al. 1994) resulted in the isolation of a genomic clone with a 2.9-kb *EcoR*I fragment containing the *CpEdi-9* gene. The genomic clone was identical to the cDNA and contained an intron of 96 bp with the conserved GT/AT splicing site (Fig. 1a). The 5′-end of the CpEdi-9 transcript was determined to be 41 nucleotides upstream of the ATG (Fig. 1b). The deduced amino acid sequence indicates the presence of a termination codon 410 nucleotides downstream of the transcription start-site. The deduced CpEDI-9 protein is 91 amino acids long with a predicted size of 10.1 kDa. The CpEDI-9 protein is very hydrophilic, soluble after boiling (Fig. 2), and the carboxy terminus of the protein is predicted to form an amphiphilic α-helix (Fig. 1a). The composition of the CpEDI-9 protein is biased towards Lys (12.1%) and Glu (16.5%), and lacks Cys, Phe and Trp. Genomic DNA blot analysis suggests that *CpEdi-9* is present as a single-copy gene in the *C. plantagineum* genome (data not shown). When the CpEDI-9 sequence was compared with nucleotide and protein sequences available in public databases, no significant homology was found. However, when database searches were carried out without filter settings, some hits were obtained. Among proteins with the closest similarities are LEA proteins, and alignments are shown in Fig. 3. CpEDI-9 displays some features of LEA proteins from plants, like the maize group-3 LEA protein or the dehydrin-like BDN1 protein from the resurrection plant *Boea crassifolia*, and a group-3 LEA-like protein AavLEA1 from the nematode *Aphelenchus avenae*. The conservation of Lys and Asp/Glu residues is the most remarkable feature (Fig. 3), and sequence identity between EDI-9 and the aligned LEA-like proteins ranged between 32 and 22% identity. No continuous stretches of sequence homologies were identified and therefore CpEDI-9 defines a new class of small hydrophilic proteins.

The CpEdi-9 transcript is expressed in response to dehydration, salt stress or ABA treatment and accumulates in mature seeds

We analysed the effect of dehydration and salt stress on the expression of the *CpEdi-9* gene in *C. plantagineum* leaves and roots (Fig. 4). No expression was detected in vegetative tissues of untreated plants, but the transcript accumulated rapidly in dehydrated leaves and roots. Highest expression levels were reached during the first hours of dehydration, and then the expression in leaves declined to relatively low levels, whereas in roots the transcript level was maintained during further dehydration. The CpEdi-9 transcript accumulated faster in response to dehydration than previously isolated *Lea* genes from *C. plantagineum*, e.g. the dehydrin CDeT6-19 transcript (Fig. 4; Schneider et al. 1993). The *CpEdi-9* gene was also induced by application of exogenous ABA or salt treatment (Fig. 4). The *CpEdi-9* gene is constitutively expressed in mature seeds, a naturally dehydrated tissue. The transcript level was lower in seeds than in 4-h-dehydrated leaves but similar to that found in seeds for the Lea-type dehydrin CDeT6-19 (Fig. 4).

The spatial distribution of the CpEdi-9 transcript was investigated by in situ hybridisation using the RNA transcript of CpEdi-9 as a probe on sections of 4-h-dehydrated leaves of *C. plantagineum*. This analysis showed that the CpEdi-9 transcript was mainly localised

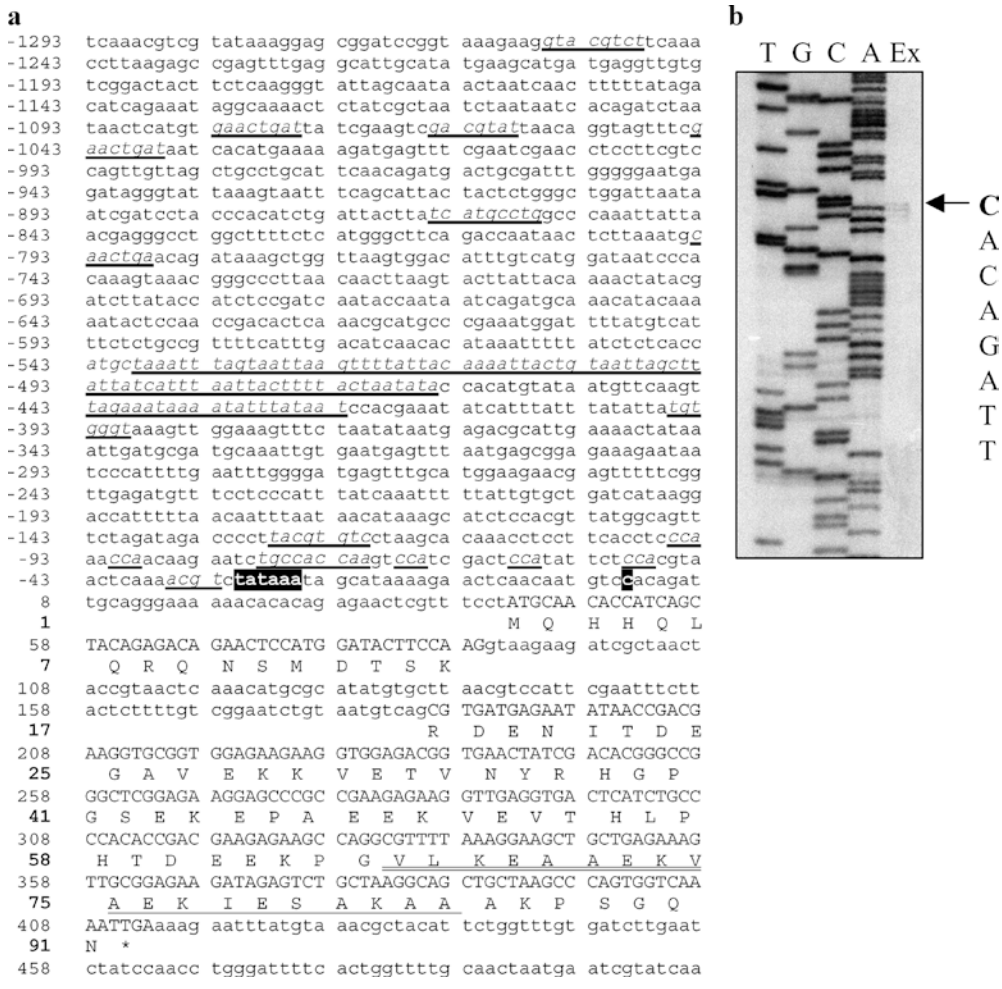

Fig. 1 **a** Nucleotide sequence of the *Craterostigma plantagineum CpEdi-9* gene and the deduced protein sequence. Exons are printed in *upper-case letters* and introns or untranslated sequences are shown in *lower-case letters*. Amino acid residues corresponding to a putative α-helix are marked with a *double line*. The nucleotides and amino acids are numbered on the *left-hand side*. The transcription start and the putative TATA box are indicated in *reverse print*. Putative regulatory *cis*-elements are in *italics and underlined*. The sequence has been submitted to GenBank database and has been given the accession number AY382595. **b** Determination of the transcription start site of the *CpEdi-9* gene by primer-extension analysis. A 17-mer oligonucleotide was hybridised to poly(A^+) RNA isolated from 2-h-dehydrated *C. plantagineum* leaves. The oligonucleotide was used for the reference sequence. *A*,*C*,*G*, and *T* Dideoxynucleotides; *Ex*, extension product

in the phloem cells of the vascular bundles (Fig. 5). In summary, the *CpEdi-9* gene represents a novel gene, rapidly induced during dehydration and mainly restricted to the phloem and mature seeds of *C. plantagineum*.

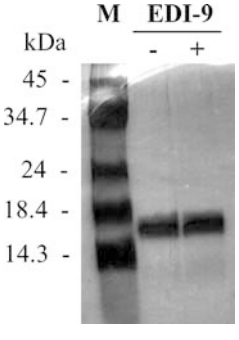

Fig. 2 The recombinant *C. plantagineum* EDI-9 protein is soluble after boiling. An aliquot of the EDI-9 protein (1 μg μl^{-1} in 150 mM potassium phosphate buffer pH 7.5), was heated at 95°C for 10 min, then cooled to room temperature and centrifuged at 12,000 *g* for 15 min at 4°C. Four μl of the heated supernatant (+ *lane*) and of the untreated purified protein (− *lane*) were separated in a 16% SDS–PAGE gel and stained with Coomassie brilliant blue R250 (Merck)

Search for potential regulatory sequences in the *CpEdi-9* promoter

The PlantCARE database (Lescot et al. 2002) was searched to identify several potential regulatory

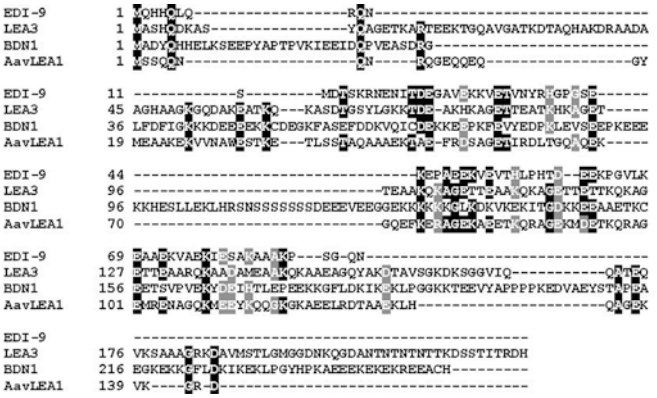

Fig. 3 Alignment of the amino acid sequences of *C. plantagineum* EDI-9 (CpEDI-9; AY382595), the maize protein LEA3 (Q42376), the dehydrin-like BDN1 protein from the resurrection plant *B. crassifolia*(AAF01465) and the group-3 LEA-like protein AavLEA1 from the nematode *A. avenae* (Q95V77). Amino acids that are identical or conservative substitutions in at least three of the sequences are *boxed* in *black* or *grey*, respectively

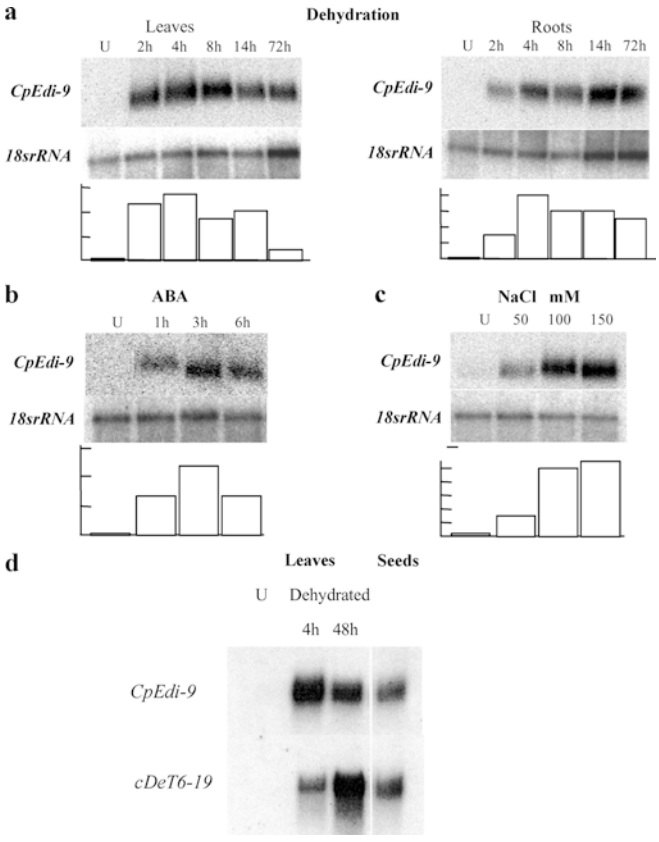

Fig. 4 a–c RNA blot analysis showing the expression of the *C. plantagineum* CpEdi-9 transcript in response to **a** dehydration, **b** ABA (100 μM) and **c**salt treatment (NaCl for 6 h). Membranes carrying 2 μg of poly(A)$^+$RNA were probed with the CpEdi-9 cDNA insert. All filters were hybridised with a ribosomal probe to monitor loading of RNA. The relative intensity of the hybridising bands was calculated using the 18srRNA signal as a reference. The graph below each autograph gives the relative signal intensity. In the dehydration kinetics the relative water content (RWC) was set to 100% in untreated plants (*lane U*) and the corresponding RWC values for dehydrated plants were 62%, 39%, 25%, 18% and 15% for 2, 4, 8, 14 and 72 h of dehydration. **d** The CpEdi-9 transcript accumulates in mature seeds. Total RNA (30 μg in each lane) from untreated (*U*) and dehydrated (4 h and 48 h) leaves and mature seeds of *C. plantagineum* was hybridised with the CpEdi-9 cDNA insert to compare relative expression levels. The blot was also hybridised with the dehydrin CDeT6-19 cDNA insert to compare expression levels (Bartels et al. 1990)

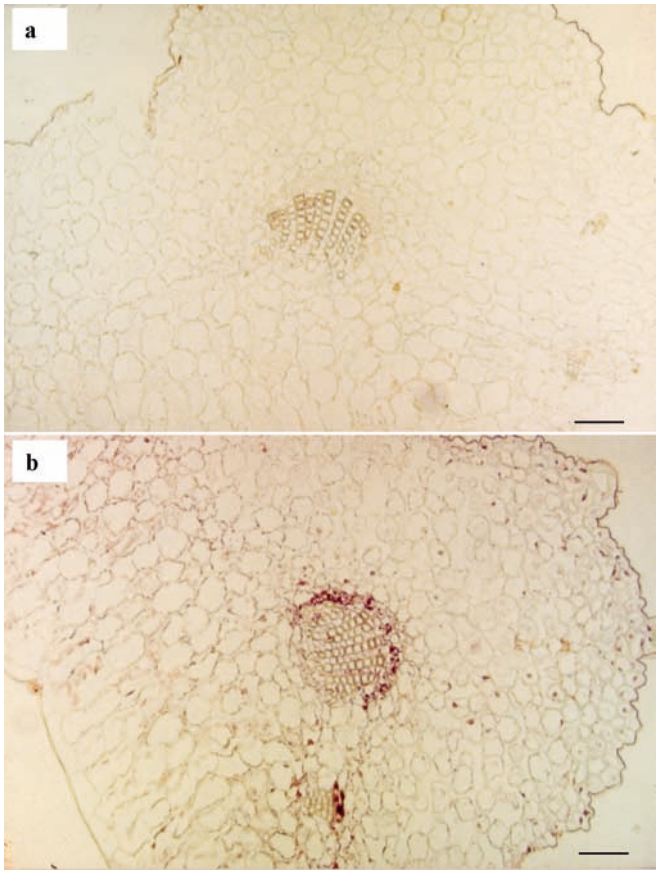

Fig. 5a,b In situ hybridisation of CpEdi-9 to sections of 4-h-dehydrated *C. plantagineum* leaves. Bright-field micrographs are shown of tissue sections hybridised with a digoxigenin-labelled CpEdi-9 riboprobe. **a** Leaf section hybridised with a sense probe. **b** Leaf section hybridised with an antisense probe. Bars = 200 μm

sequences in the *CpEdi-9* promoter. Motifs known to play a role in transcriptional control were found: a TATA box at −31 and several ABA- and stress-responsive elements (Table 1). At least six motifs with the core sequence ACGT, known as the G-box of the ABREs, were found, and a sequence with a high degree of homology to a 'coupling element' (CE1), which is active in combination with ABREs but not alone, was also identified. Further, one Myb and two Myb-like recognition sites, and one sequence which closely resembles an ABA-inducible Sph-containing element or Sph box (ASCE), which is conserved in many seed-specific promoters (Hattori et al. 1992), were identified (Table 1). Two regions rich in A/T are located between −539 and −424 bp, and they may act as general enhancers of gene expression (Bustos et al. 1989). We also identified the sequence of the PB core motif, which is present in promoters of pollen-specific genes of tomato (Twell et al. 1991). A sequence comparison of the *CpEdi-9* 5′ regulatory sequence with other phloem-specific promoters revealed the presence of motifs related to phloem-specific expression. First, CCA sequence repeats were identified, which have been suggested to contribute to phloem specificity (Hehn and Rhode 1998). The second element related to phloem expression in the *CpEdi-9* promoter is a 13-bp motif, which is located close to the TATA box, as in other phloem-specific promoters (Yoshida et al. 2002). The third element is a GATA motif found in the rice tungro bacilliform virus and known to be important for phloem-specific gene expression (Yin et al. 1997). Sequences matching the consensus sequence of the GATA motif, $A(N)_3GATA$, were found at −1044, −947 and −789 bp in the *CpEdi-9*promoter (Table 1). The same motif was found also in the promoters of plant genes expressed in the phloem: glutamine synthase from pea (Brears et al. 1991), a plasma membrane H^+-ATPase from *Arabidopsis* (DeWitt et al. 1991) and a potato invertase (Hedley et al. 2000).

Table 1 Putative regulatory elements in the promoter of the *Craterostigma plantagineum CpEdi-9*gene

Motif	Position	Sequence in *CpEdi-9*
ABRE core ACGT[a]	−1254	GTACGTCTT
	−1064	CGACGTATT
	−157	CCACGTTA
	−127	TACGTGTC
	−48	CCACGTAA
	−26	AAACGTCT
CE1-like element[b]	−79	TGCCACCAA
Myb recognition site PyAACTG	−794	CAACTGAA
Myb-like recognition site	−1083 and −1044	GAACTGAT
ASCE-like[c]	−864	CATGCCTG
A/T rich regions	−539 to −465	86% A/T
	−443 to −424	95% A/T
PB-core like[d]	−396	TGTGGGT
CCA repeated sequence	−96 to −50	7 times CCA
Phloem-specific element (13 bp)	−199	ATAAGGACCATTT
$A(N)_3GATA$ motif	−1044	AACTGATA
	−947	ATGAGATA
	−789	AACAGATA

[a]The ACGT motif is the core sequence of ABRE G-box elements (Busk and Pages 1998)
[b]CE1: Coupling Element 1 acts in combination with a G-box-type ABRE. The sequence of the CE1 element is TGCCACCGG (Busk and Pages 1998)
[c]The ASCE (ABA-inducible Sph-Containing Element) motif is CATGCATG (Hattori et al. 1992)
[d]The sequence of the PB core motif identified in the promoters of pollen-specific genes of tomato is TGTGGTT (Twell et al. 1991)

Analysis of the *CpEdi-9* promoter in transgenic plants

In order to confirm regulatory elements which determine the specific expression pattern of *CpEdi-9*, 1,293 bp of the promoter region including 41 bp of the leader sequence of the *CpEdi-9* gene were fused to the *GUS* gene (for details, see Materials and methods). GUS expression was analysed by in situ tissue staining and in quantitative fluorometric assays. The results shown here were obtained from two representative tobacco lines (4.4 and 4.8) and three *Arabidopsis* lines (4.2, 4.4 and 4.5). In tobacco, T_0 and T_1plants were analysed, and in *Arabidopsis*, T_1 and T_2.

Mature tobacco seeds showed high GUS activity, which was reduced by a factor of 3–6 after 1 day of seed imbibition in water (Fig. 6). During plantlet development, GUS activity progressively decreased in transgenic tobacco seedlings. In 8-day-old seedlings, GUS activity was 100- to 50-times lower than in mature seeds. Histochemical localisation showed staining mainly in embryos of mature seeds and low GUS expression in the surrounding endosperm tissue (Fig. 7a). After 1 day of germination, GUS activity was located at the emerging radicle. In 3-day-old seedlings, GUS expression was restricted to the main vein of the cotyledons and the transition zone between stem and root (Fig. 7b). In mature tobacco plants, low GUS activity was found in leaves, ranging between 20 and 50 pmol MU min^{-1} mg^{-1} protein in the different transgenic lines. Histochemical analysis of GUS expression in leaves showed a strong signal in stomatal guard cells (Fig. 7c) and trichomes (Fig. 7d), and a weak staining in some of the leaf veins (data not shown). In the leaf pedicel and stem the GUS activity was restricted to the phloem, mainly to the internal phloem, and to some guard cells and trichomes (Fig. 7e–g). High GUS expression also occurred in desiccated pollen grains (data not shown). ABA treatment increased GUS activity in 8-day-old tobacco seedlings (Fig. 8) and histochemical analysis showed that expression was localised to the vascular system of the cotyledons and the root (Fig. 7h,i). Induction of GUS activity was also detected by dehydration stress in detached leaves from mature plants (Fig. 8).

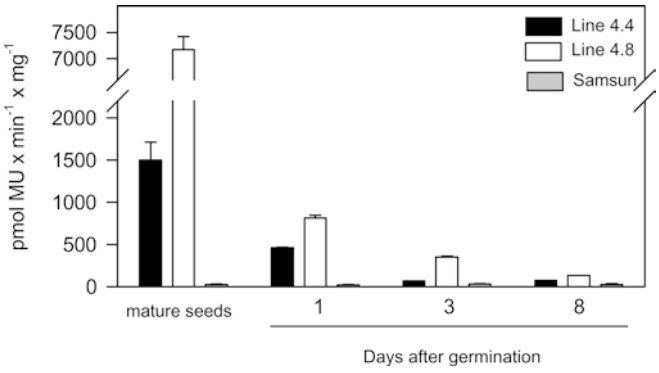

Fig. 6 Time-course of promoter GUS activity during seed germination of tobacco (*Nicotiana tabacum*) transformed with the *CpEdi-9* promoter *GUS* construct. Seeds from two independent transformed lines (lines 4.4 and 4.8) and untransformed wild-type plants (Samsun) were analysed. Data are the mean of three independent measurements. The SE is indicated

Similar results to those with tobacco were obtained from transgenic *Arabidopsis* plants. The highest GUS activity in 6-week-old *Arabidopsis*plants was found in mature pollen grains (Fig. 7j), followed by the roots and stems (Fig. 7k); a very weak activity was detected in leaves (Fig. 7m). Histochemical staining of roots, stems and leaves revealed that GUS expression was restricted to the vascular system, and only to the main vein in leaves. Detailed analysis of GUS expression in *Arabidopsis* stems showed that the activity was localised to the phloem of vascular bundles (Fig. 7l). When 6-week-old *Arabidopsis* plants were treated with ABA an increase in GUS activity was observed mainly associated with the vascular system, but when plants were subjected to dehydration stress the GUS signal was more intense and was also present in the minor-vein system, more specifically in the free vein endings in the centre of the areoles (Fig. 7n,o).

Discussion

Many genes are differentially expressed during dehydration of the desiccation-tolerant plant *C. plantagineum*

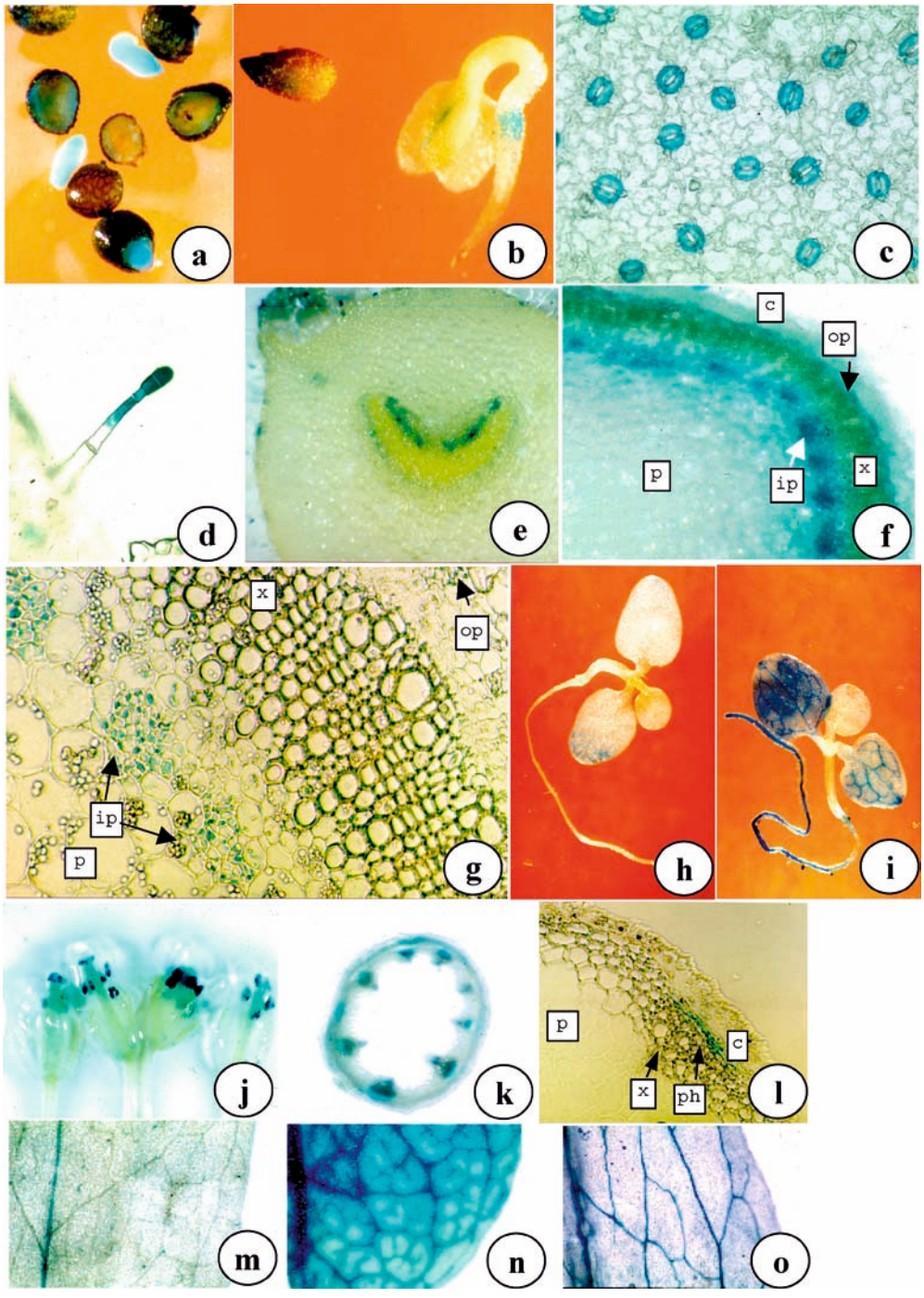

Fig. 7a–o Histochemical localisation of GUS expression directed by the *CpEdi-9* promoter in tobacco (**a–i**) and *Arabidopsis* (**j–o**) plants transformed with the *CpEdi-9*promoter *GUS* construct. **a** Mature tobacco seeds (×10). **b** One-day-old (*right*) and 3-day-old (*left*) germinating tobacco seedlings (×8). **c** GUS activity in guard cells in an epidermal peel of a tobacco leaf (×150). **d** Trichomes of a tobacco leaf (×150). **e** Tobacco petiole section (×12). **f,g** Tobacco stem section (**f**; ×5) and magnification of the vascular tissue (**g**; ×400). **h,i** Eight-day-old tobacco seedling untreated (**h**) and treated (**i**) with ABA (100 µM) for 24 h (×2). **j** Mature *Arabidopsis* flowers (×4). **k,l** Stem section of a 6-week-old *Arabidopsis* plant that had not been watered for 5 days (**k**; ×10) and magnification of the a vascular bundle (**l**; ×400). **m–o** *Arabidopsis* rosette leaves from a 6-week-old untreated plant (**m**), a plant not watered for 5 days (**n**) and a plant treated with ABA (100 µM) for 2 days (**o**; ×3). *c* Cortex, *ip* internal phloem, *p* pith parenchyma, *ph* phloem, *op* outer phloem, *x* xylem

(Bartels et al. 1990; Bockel et al. 1998) and they appear to be important in preventing the deleterious effects of extreme water loss on cellular structures and metabolism (reviewed in Bartels and Salamini 2001). A relevant role in cellular protection has been attributed to LEA proteins (Schneider et al. 1993; Velasco et al. 1998; Ditzer et al. 2001), to detoxifying enzymes, e.g. aldehyde dehydrogenase (Kirch et al. 2001; Sunkar et al. 2003), and to some heat-shock proteins (Alamillo et al. 1995). Besides proteins, the accumulation of sucrose also seems to be of importance. In work presented here we describe *CpEdi-9*, a novel, early dehydration-responsive gene. The expression behaviour of *CpEdi-9* is different from previously described dehydration-induced genes. It was surprising that no significant sequence similarities were detected, either on the nucleotide or on the protein level, to genes present in public databases. However, when criteria such as structural and physicochemical characteristics of the predicted protein are analysed, resemblance is found to LEA proteins. The *CpEdi-9* gene encodes a protein which does not coagulate after boiling and which has a high content of hydrophilic amino acids, lacking aromatic amino acids and Cys. These characteristics are the main features of group-2 LEA

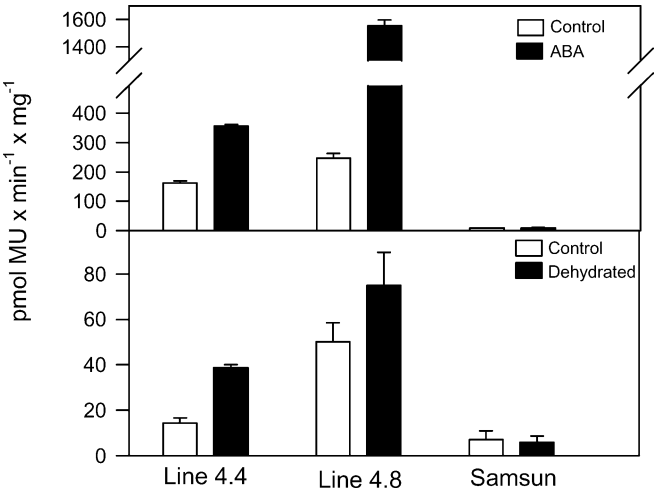

Fig. 8 The *CpEdi-9* promoter is responsive to dehydration and ABA in transgenic tobacco. GUS activity in 8-day-old seedlings treated for 24 h with 100 μM ABA (*upper graph*) or in detached leaves dehydrated for 4 h (*lower graph*). Dehydration treatment was given to the youngest fully developed leaf of each plant above the 5th internode. The Samsun line represents untransformed tobacco plants and seedlings. Data are shown as the mean of at least three independent measurements and error bars indicate the SE

proteins (dehydrins; Close 1997; Cuming 1999). Moreover, amino acid sequence analysis predicts that the protein exists mainly as random-coil structure except for the presence of an amphipathic α-helix at the C-terminus, also a structural characteristic of dehydrins and group-3 LEAs (Dure et al. 1989; Ingram and Bartels 1996; Close 1997; Cuming 1999). The predicted amphipathic α-helix in dehydrins, termed the K segment, is highly conserved and present in one to several copies. This structure may be of functional importance and has been suggested to allow interactions with other hydrophobic or hydrophilic cellular surfaces, stabilizing them during dehydration (Close 1997; Cuming 1999). Recent structural analysis of the Aav LEA1 nematode protein showed that it exists in an unfolded structure, but an increased formation of α-helices and possibly coiled-coil formation are observed under dehydration conditions (Goyal et al. 2003). Similar observations were reported for the dehydrin-like CDeT 6-19 protein from *C. plantagineum* (Lisse et al. 1996); EDI-9 may likewise exist as an unfolded molecule in hydrated conditions. The fact that CpEDI-9 shares features with group-2 and group-3 LEA proteins but does not fall into either group (Fig. 3) supports the suggestion that *CpEdi-9* represents a novel Lea-like gene which so far is unique to *C. plantagineum*. This gene may have been evolved under the pressure of extreme dehydration tolerance, which requires abundant expression of protective hydrophilic proteins.

The expression pattern of *CpEdi-9* is similar to that of other known dehydration-induced genes and very consistent with a protective function for CpEDI-9. The CpEdi-9 transcript accumulates in mature seeds of *C. plantagineum* (Fig. 4d), and transgenic plants show that the high promoter activity in the desiccated embryo decreases rapidly during germination (Fig. 6), which again points to a role during dehydration. Expression of the *CpEdi-9* gene is induced by dehydration, salt treatment and exogenous application of ABA (Fig. 4). In contrast to all previously described dehydration-induced genes, including *Lea*genes from *C. plantagineum* (Piatkowski et al. 1990; Schneider et al. 1993; Ditzer et al. 2001),*CpEdi-9* is expressed in leaves early during dehydration and it is spatially restricted to the phloem (Fig. 5). To investigate whether the phloem-specific expression is retained in the promoter of the *CpEdi-9* gene, promoter GUS activities were analysed in transgenic plants. This analysis was performed in heterologous systems like *Arabidopsis* and tobacco in order to test whether the *CpEdi-9* promoter has biotechnological potential and is suitable to drive the expression of desired target genes in a stress- and tissue-specific manner. The analysis of transgenic plants supported the in situ hybridisation results and suggests that the function of CpEDI-9 might be restricted to vascular tissues. Accumulation of CpEDI-9 could provide the vascular tissue of the resurrection plant with an early defence against desiccation.

The fact that the expression of *CpEdi-9* is restricted to phloem cells of the vascular bundles during dehydration of *C. plantagineum*(Fig. 5) makes it an excellent candidate to analyse promoter elements responsible for this specific expression pattern. This work has identified in the 5′ regulatory region of the *CpEdi-9* gene a number of *cis*-acting elements required for ABA-inducibility in many stress-responsive genes (Table 1; Busk and Pages 1998; Leung and Giraudat 1998; Rock 2000). However, the functionality of these motifs in the *CpEdi-9* promoter remains to be demonstrated since modified core ABREs present in other *Lea* gene promoters from *C. plantagineum* are not always a major determinant of ABA or drought responsiveness (Michel et al. 1993, 1994). The *CpEdi-9* promoter drives expression of the reporter gene to phloem cells (Fig. 7e–g,k,l). Interestingly, in leaves from dehydrated transgenic *Arabidopsis* plants, in contrast to ABA-treated plants, GUS expression was not just restricted to the main vein system but was also found in minor veins (Fig. 7n), which are sites of intensive phloem loading. Several promoters from plants (Brears et al. 1991; DeWitt et al. 1991; Hedley et al. 2000; Yoshida et al. 2002),*Agrobacterium* (Guevara-Garcia et al. 1993) and viruses (Bhattacharyya-Pakrasi et al. 1993; Hehn and Rohde 1998) have been reported to drive expression of the reporter gene in the phloem of transgenic plants. The *CpEdi-9* promoter contains three types of element related to phloem cell-specific expression (Table 1). The contribution of these motifs to phloem-specific expression in other genes has been demonstrated by loss-of-function analysis (Yin et al. 1997; Hehn and Rohde 1998). The phloem-specific 13-bp element and the GATA motifs are conserved between virus and plant promoters, implying an important role for these motifs for gene expression in the phloem. *Lea* gene promoters from *C. plantagineum* and other higher plants have been reported to be highly active in

the naturally desiccation-tolerant tissues pollen and seeds (specifically embryos; Cuming 1999; Bartels 1999), which supports a role for these proteins in desiccation. Previous studies of seed-specific genes have shown that A/T-rich sequences in the promoter can act as general enhancers of seed specific expression (Bustos et al. 1989). Such sequences are present in the *CpEdi-9* promoter and could promote expression in seeds. Concerning the *CpEdi-9*promoter activity in pollen, a sequence was identified resembling the PB core motif that is present in the pollen-specific LAT genes of tomato (Twell et al. 1991) and in the *Lea AtEm1* gene from *Arabidopsis* (Hull et al. 1996); it may function as transcriptional enhancer in pollen.

In conclusion, we propose that *CpEdi-9* defines a new class of genes. The predicted protein shares some physiological and biochemical properties with *LEA* proteins. The localisation in the phloem tissue is consistent with the hypothesis that *CpEdi-9* might contribute to a specialised protecting function of the vascular bundles during the desiccation process.

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