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High level transcription of a member of a repeated gene family confers dehydration tolerance to callus tissue of *Craterostigma plantagineum*

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An experimental system has been developed which allows the identification of intermediates in the abscisic acid (ABA) signal transduction pathway leading to desiccation tolerance in plants. Desiccation tolerance in callus of the resurrection plant *Craterostigma plantagineum* is mediated via the plant hormone ABA, which induces the expression of gene products related to desiccation tolerance. Based on T-DNA activation tagging, a gene (*CDT*-1) was isolated which encodes a signalling molecule in the ABA transduction pathway. Constitutive overexpression of *CDT*-1 leads to desiccation tolerance in the absence of ABA and to the constitutive expression of characteristic transcripts. *CDT*-1 represents a novel gene with unusual features in its primary sequence.The *CDT*-1 gene resembles in several features SINE retrotransposons. Mechanisms by which *CDT*-1 activates the pathway could be via a regulatory RNA or via a short polypeptide.

Keywords: abscisic acid/activation tagging/desiccation tolerance/regulatory RNA/resurrection plant

Introduction

Plants respond to dehydration stress by complex physiological and morphological changes, including irreversible damage of cell membranes and organelles followed by cessation of growth and general metabolism (Hsiao, 1973). Abscisic acid (ABA), a plant growth factor stimulating cellular responses to water stress (for review, see Zeevaart and Creelman, 1988; Bray, 1993), plays a central role in controlling the expression of drought-responsive genes (Skriver and Mundy, 1990; Giraudat *et al.*, 1994; Ingram and Bartels, 1996). Drought responses are also modulated by ABA-independent signalling pathways, and it has been shown that some drought-responsive genes are activated during dehydration in ABA-deficient mutants (Gosti *et al.*, 1995; Yamaguchi-Shinozaki *et al.*, 1995).

Desiccation-tolerant plants provide excellent models for genetic and biochemical studies (Bartels *et al.*, 1990; Ingram and Bartels, 1996). *Craterostigma plantagineum*, a species of *Scrophulariaceae*, can tolerate up to 96% loss of its relative water content and recovers within several hours from such extreme dehydration (Gaff, 1971; Bernacchia *et al.*, 1996). This 'resurrection' response is expressed in differentiated tissues. *In vitro* propagated callus is not normally desiccation tolerant and it has a strict requirement for exogenously applied ABA in order to survive a severe dehydration. Dehydration of *Craterostigma* plants, as well as ABA treatment of vegetative organs and callus tissues, result in the induction of similar sets of drought-responsive genes (Bartels *et al.*, 1990). Several of these genes code for late embryogenesis abundant (Lea) proteins (Piatkowski *et al.*, 1990; Ingram and Bartels, 1996). Lea proteins occur abundantly and ubiquitously in embryos during the final stages of seed maturation or in immature embryos treated with ABA (Dure *et al.*, 1989; Ingram and Bartels, 1996). Lea proteins are thought to play an important role in protecting cellular structures during water loss (Skriver and Mundy, 1990; Dure, 1993). The expression of Lea genes in *Craterostigma* organs or calli, therefore, serves as a suitable marker to identify mutants altered in ABA and/or drought signalling.

Unlike the genetic model plant *Arabidopsis*, the polyploid *C.plantagineum* is a poor target for mutational studies using chemical or insertional mutagens, such as transposons (Baker *et al.*, 1986; Balcells *et al.*, 1991) or the T-DNA of *Agrobacterium* (Koncz *et al.*, 1989, 1994; Feldmann, 1991). However, an efficient transformation system in *C.plantagineum* (Furini *et al.*, 1994) permits the isolation of dominant mutants obtained by T-DNA activation tagging (Hayashi *et al.*, 1992). This approach is based on the observation that enhancers carried by T-DNA transformation vectors can induce the transcription of silent plant genes located in the vicinity of T-DNA insertions in the genome (Walden *et al.*, 1994). The fact that wild-type callus tissues of *Craterostigma* do not survive desiccation without ABA treatment offers an ideal way to select for dominant mutations that activate the ABA and/or drought signalling pathways and thus confer desiccation tolerance to calli. Here we describe the T-DNA tagging of a *Craterostigma* gene that induces desiccation tolerance by activating ABA-inducible genes.

Results

Isolation of ABA-independent desiccation-tolerant callus mutants

T-DNA insertions into the plant genome have the potential to generate dominant mutants. After integration of correctly engineered T-DNA into the plant genome, flanking genes may become activated and thus lead to dominant mutations. This allows selection of tagged mutants at the level of primary transformants (Walden *et al.*, 1994). The selection of dominant *C.plantagineum* mutants made use of a particular behaviour of *in vitro* grown calli: callus tissue of this plant is only desiccation tolerant when it is treated with ABA prior to the dehydration treatment

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Fig. 1. Callus phenotypes: (**a**) wild-type callus; (**b**) T-DNA-tagged desiccation-tolerant callus; (**c**) ABA-treated wild-type callus; (**d**) callus transformed with the *pg5–CDT-1* promoter–gene construct.

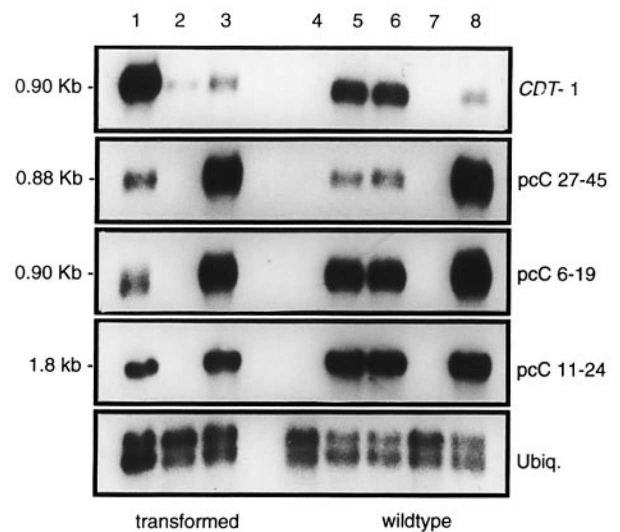

Fig. 2. Northern analyses showing the expression patterns for the *CDT-1* gene and the ABA- and desiccation-induced genes CDeT-27-45, CDeT-6-19 and CDeT-11-24. Poly$(A)^+$ RNA was isolated from the following tissues of the T-DNA-tagged plant: callus (lane 1), leaves (lane 2) and dried leaves (lane 3); and from the following tissues of wild-type plants: callus (lane 4), ABA-treated callus (lane 5), ABA-treated and dried callus (lane 6), untreated leaves (lane 7) and dried leaves (lane 8).

(Bartels *et al.*, 1990). The ABA treatment induces the expression of a specific set of of desiccation-related genes

For mutant isolation, leaf explants of *C.plantagineum* were transformed with an activator T-DNA tagging vector, pCVHPT (see Materials and methods). The T-DNA of this vector carried an enhancer domain from the gene 5 promoter of *A.tumefaciens* close to its left border. The gene 5 enhancer is inactive in non-dividing cells, but capable of activating transcription in proliferating tissues, such as calli grown in the presence of auxin (Körber *et al.*, 1991). Following transformation, hygromycin-resistant calli were isolated. The transformed calli were subjected to selection by dehydration to select for dominant mutations that confer desiccation tolerance in callus tissues. For this, calli were dehydrated rapidly without ABA treatment and viable calli were selected (see Materials and methods). For selection of true mutants, the selection cycles were repeated. From 25 000 transformed calli, one transformant passed the rigorous selection and proliferated. In contrast to the green wild-type callus (Figure 1a), the selected transgenic callus was more friable and displayed a reddish colour (Figure 1b). The untransformed callus turned to a similar colour only a few days after ABA treatment which confers desiccation tolerance (Figure 1c). Upon continuous proliferation of the transgenic line, embryogenic structures developed. Subculturing in a shoot differentiation medium allowed individual embryoids to germinate, which then gave rise to green shoots and eventually to fully developed plants. These were morphologically not different from wild-type plants. Callus was again generated from these plants and phenotypically it resembled the original transformed callus line. The physiological properties of the mutant callus and the callus resulting from the retransformation could not be distinguished from ABA-treated wild-type callus. All callus samples including untreated wild-type callus lost 90% of their initial fresh weight during the dehydration treatment. Only ABA-treated wild-type callus, mutant callus and callus resulting from the retransformation exhibited the same degree of viability measured after 2 and 4 weeks of regrowth; no viable callus resulted from untreated wild-type callus. Viability of callus was measured by the ability of the cells to reduce 2,3,5-triphenyltetrazolium chloride (Chandler *et al.*, 1997).

In order to assess whether the ABA level was affected through the transformation events, the endogenous ABA level was measured in wild-type and transformed callus by an enzyme-linked immunosorbent assay (ELISA) (Weiler, 1986). A similar ABA content was found in both callus lines, indicating that the T-DNA insertion did not affect the ABA biosynthesis (46.23 ± 13.50 pmol/g fresh weight and 47.12 ± 7.93 pmol/g fresh weight, respectively for the wild-type and the transgenic callus).

Since ABA induces drought-responsive genes in wild-type *Craterostigma* callus and leaf tissues (Schneider *et al.*, 1993), the steady-state mRNA levels of three different drought- and ABA-responsive genes CDeT-27-45, CDeT-6-19 and CDeT-11-24 (Bartels *et al.*, 1990) were compared in wild-type and mutant callus by Northern hybridization (Figure 2). None of the transcripts were detected in RNA samples of the wild-type callus in the absence of ABA (lane 4), and only treatment with exogenous ABA induced accumulation of the transcripts to levels specific for each class (lanes 5 and 6). The transcript expression pattern was changed in the mutant callus: all the transcripts were constitutively expressed and detected in the absence of ABA. The CDT-1 transcript was highly abundant in callus tissue (lane 1). In leaves of the mutant plants, the CDT-1 transcript was expressed at very low levels, but dehydration led to increased levels in mutant and wild-type but not to the same extent as for the other three transcripts. The mutant phenotype, i.e. constitutive expression in the absence of exogenous ABA,

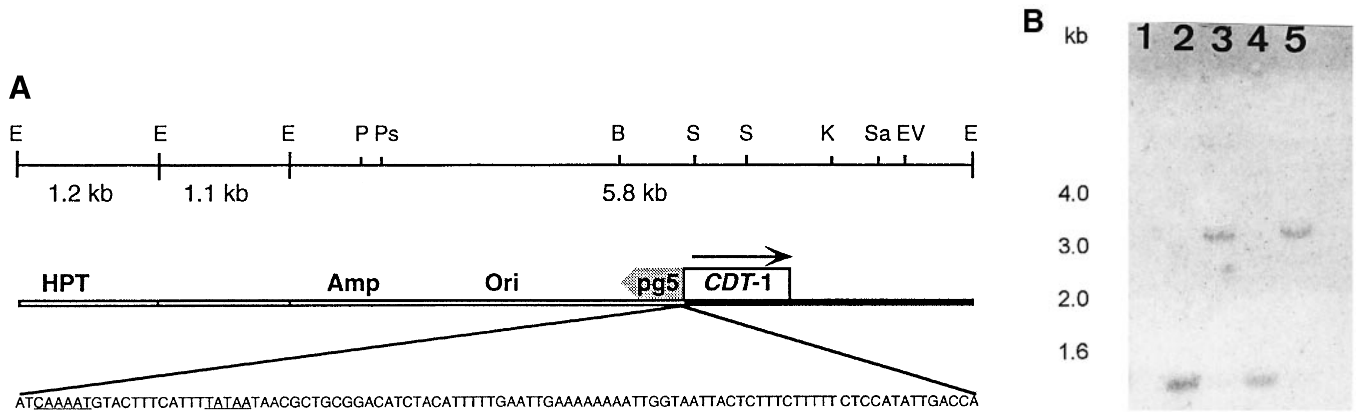

Fig. 3. (**A**) Map of the tagged genomic clone containing the T-DNA and the gene *CDT-1*. The open line represents sequences present in the T-DNA. The black line represents flanking genomic DNA sequences. The ampicillin resistance gene (Amp), the origin of replication in *E.coli* (Ori), the 645 bp of the gene 5 promoter (pg5) and the *CDT-1* gene are located on the 5.8 kb *Eco*RI fragment. Part of the hygromycin resistance gene (*HPT*) is located on the 1.2 kb *Eco*RI fragment, and the other hybridizing fragments are sequences present in the T-DNA. Partial *pg5* DNA sequence upstream of the *CDT-1* gene is shown and potential CAAT and TATA boxes are underlined. Vertical bars represent *Eco*RI sites, other indicated restriction sites are: P (*Pvu*II); Ps (*Pst*I); B (*Bgl*II); S (*Sac*I); K (*Kpn*I); Sa (*Sal*I); and EV (*Eco*RV). (**B**) Southern blot analysis. Lane 1, genomic DNA isolated from an untransformed plant and digested with *Eco*RI; lanes 2–5, genomic DNA isolated from two plants regenerated from the T-DNA tagged callus, DNA from plant 1 digested with *Eco*RI (lane 2) and digested with *Bam*HI (lane 3), DNA from plant 2 digested with *Eco*RI (lane 4) and *Bam*HI (lane 5). A ^{32}P-labelled fragment of the hygromycin gene (*HPT*, A) was used as hybridization probe.

was maintained when callus was again obtained from plants regenerated from the original mutant callus. These data support the assumption that the mutant phenotype is the result of gene activation mediated by the T-DNA-linked gene 5 enhancer, which stimulates transcription in calli but not in leaves. Furthermore, the constitutive expression of ABA-inducible genes in the mutant callus suggested that a T-DNA tag could have activated a pathway which is induced by ABA in the wild-type.

Segregation analysis and rescue of the T-DNA-tagged mutant locus

The mutation did not affect the process of organ differentiation, because the plants regenerated from the mutant callus had morphologically the same appearance as wild-type plants. It was also possible to induce callus formation from the regenerated plants under the same experimental conditions as from wild-type plants, thus the mutation did not interfere with somatic embryogenesis. The plants derived from the mutant callus, however, displayed reduced fertility: after selfing, only a small number of progeny were obtained and used for segregation analysis. To test the co-segregation of the T-DNA-encoded hygromycin resistance marker and the desiccation-tolerant mutant phenotype, calli were induced from leaves of an F2 family represented by 143 seeds. During repeated segregation analyses, we noted that although seeds were carrying the transgene, the number of viable seedlings was reduced. Out of 143 seeds, 42 surviving seedlings were obtained. These seedlings were resistant to hygromycin, and all calli derived from these plants were resistant to desiccation without exogenous ABA treatment. These data suggest a linkage between the mutant and the T-DNA insertion and exclude somaclonal variation as an explanation for the mutant phenotype.

Using a fragment of the pCVHPT vector as probe, Southern DNA hybridization analysis revealed that the mutant carried a single T-DNA insertion, as one internal *Eco*RI fragment was obtained and a larger *Bam*HI fragment (only one cleavage site) (Figure 3A and B). The T-DNA-tagged locus was isolated by screening a genomic library constructed from nuclear DNA of the regenerated mutant plant. Fifteen clones were isolated which hybridized to the T-DNA probe. The clones contained overlapping fragments of the same T-DNA insertion, including identical junction sequences between the left border of the T-DNA and the adjacent plant DNA (Figure 3A). To investigate whether the gene 5 enhancer was responsible for gene activation in the mutant line, DNAs from three representative genomic clones were restricted with *Eco*RI, and each isolated *Eco*RI fragment was hybridized to a Northern blot carrying mRNA from various plant tissues. A fragment of 5.8 kb containing the inserted T-DNA hybridized to a transcript of ~0.9 kb (Figure 2), indicating that an expressed gene (or part of it) was present in this 5.8 kb fragment. This *Craterostigma* desiccation-tolerant gene, now termed *CDT*-1, was transcribed in all transgenic tissues, although at a very high level only in callus. In wild-type *Craterostigma*, the *CDT*-1 gene expression was modulated in a similar way to other ABA- and desiccation-inducible genes: it was transcribed in dried leaves (Figure 2, lane 8) and in ABA-treated calli (lanes 5 and 6) but it was not transcribed in untreated callus (lane 4) and in hydrated leaves (lane 7).

The experiments described support the conclusion that the transcription of plant DNA sequences located downstream of the gene 5 enhancer was responsible for the gain-of-function phenotype observed in the desiccation-tolerant mutant. In addition, the accumulation of the 0.9 kb transcript coincided with the constitutive expression of ABA-inducible marker genes in the callus of the mutant. This was taken as evidence that the T-DNA insertion had activated an ABA- and desiccation-inducible gene necessary for the expression of other genes downstream of an ABA-mediated pathway.

The CDT-1 gene conferring desiccation tolerance to Craterostigma callus

To prove that a plant gene linked to the gene 5 enhancer was capable of activating desiccation tolerance as a

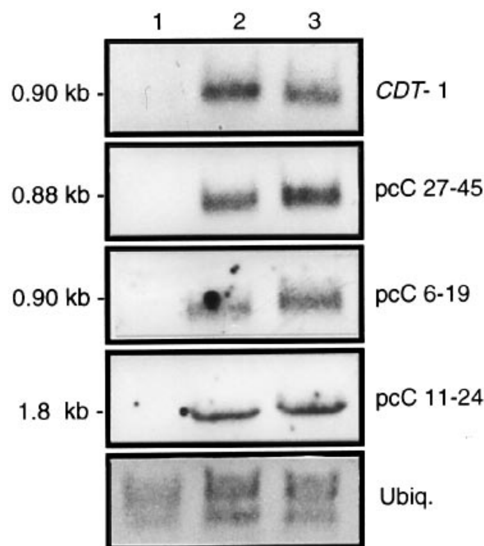

Fig. 4. RNA blot analysis: total RNA isolated from wild-type callus (1), ABA-treated callus (2) and callus transformed with the *pg5–CDT-1* chimeric gene (3) was separated in a denaturing gel and transferred to Nylon filters. Identical filters were hybridized with ^{32}P-labelled probes from *CDT-1* cDNA, pcC 27-45, pcC 6-19 and pcC 11-24: equal loading of RNAs was monitored by re-hybridizing a filter with a ubiquitin probe.

dominant trait, the 5.8 kb *Eco*RI fragment of the genomic clone which contained the T-DNA–plant DNA junction and the *CDT*-1 gene (Figure 3A) was inserted into the pBIN19 plant transformation vector (Bevan, 1984). In another construct, a smaller fragment extending from a *Bgl*II site upstream of the gene 5 enhancer to a *Sal*I site in the plant DNA (Figure 3A) was inserted in the pPCV811 transformation vector (Koncz *et al.*, 1994). Leaf explants of *C.plantagineum* were transformed with both constructs to yield two different transgenic lines. Transformation events were confirmed by PCR analysis and Southern hybridization (data not shown). When the calli from both lines were dehydrated, transformants from both lines were able to withstand desiccation in the absence of ABA. In contrast, calli transformed with control vectors only did not survive dehydration. The desiccation-tolerant transformants accumulated anthocyanins, and their phenotype was indistinguishable from the original T-DNA-tagged mutant line (Figure 1, compare b and d). RNA hybridization analysis confirmed that the desiccation-tolerant transformants contained high levels of the 0.9 kb transcript and constitutively expressed the ABA-responsive marker genes (Figure 4), as was observed in the T-DNA-tagged mutant.

CDT-1 codes for an unusual RNA

The 5.8 kb *Eco*RI fragment conferring desiccation tolerance to the callus was used as a probe to isolate 10 clones from a cDNA library constructed from poly(A)$^+$ RNA of ABA-treated wild-type callus tissues. All 10 clones were sequenced and the DNA sequences were aligned with the DNA sequence of the 5.8 kb genomic *Eco*RI fragment which contained the T-DNA junction and the adjacent plant DNA. One clone contained a 900 bp long insert which was identical in DNA sequence to the corresponding fragment in the T-DNA-tagged locus. The size of the cDNA corresponded to the transcript size detected in RNA hybridizations with the mutant as well as with the desiccation-tolerant transformants carrying the gene 5 promoter–*CDT*-1 gene in the pPCV811 or pBIN19 vector. The 5′ end of this cDNA matched the genomic sequence in the mutant, starting with the sixth base pair downstream of the T-DNA left border. All cDNA clones analysed were identical except that most of them were not full-length clones; therefore, all the further analysis was done with the *CDT*-1 cDNA clone. Screening of cDNA libraries and Northern analyses suggested that the 5.8 kb fragment contains the coding capacity for one transcript.

The nucleotide sequence of the *CDT*-1 cDNA with its putative open reading frames (ORFs) is shown in Figure 5A. As expected, the 3′ end of the cDNA contained a poly(A) track. Surprisingly, an oligo(A) track of 18–21 nucleotides (from nucleotides 193 to 213 in Figure 5A) was also found in the 5′ region. This was present in all cDNAs and genomic clones sequenced (see below). Large ORFs starting with an AUG codon were not found in the sequence. A putative AUG translation start codon is located at position 126, but is followed by a UAA stop codon after 22 triplets. The other two large ORFs are 179 and 169 amino acids in size, but do not contain an AUG start codon. No homology to sequences published in the sequence data library was detected, with the exception that some fragments following the short peptide shared some homology with extensin-like proteins but never in-frame with an AUG. To investigate the coding potential of the *CDT*-1 gene, RNA was synthesized by *in vitro* transcription and translated *in vitro* in a cell-free, nuclease-treated rabbit recituculyte lysate. The putative products were analysed by SDS–PAGE, and no translation products could be assigned to the *CDT*-1 transcript. A control RNA included in the same assay was translated correctly.

The oligo(A) segments present in the 5′ part of the *CDT*-1 transcripts varied between 18 and 21 nucleotides in the different *CDT*-1 homologous cDNA clones. This sequence variation suggested that several *CDT*-1 alleles and/or *CDT*-1 like genes are expressed in wild-type *C.plantagineum*.

Characterization of CDT-1 gene homologues in C.plantagineum

Southern hybridization of genomic DNA with the *CDT*-1 cDNA probe resulted in a smear, indicating that *CDT*-1 sequences are probably represented by a large gene family in the *Craterostigma* genome (Figure 6). Screening of a wild-type genomic library with a 5′ end probe of the T-DNA-tagged *CDT*-1 gene, resulted in numerous clones. It was estimated that 0.1–0.2% of the clones contained *CDT*-1-related sequences, indicating a high frequency of *CDT*-1 genomic sequences in the *C.plantagineum* genome. Several of these clones were characterized by comparison of genomic and cDNA sequences. Genes corresponding to four cDNAs showing variation in the length of the 5′ oligo(A) segment were identified, indicating that the variability detected in the cDNAs was not due to a cloning artefact but reflected a sequence heterogeneity between members of the *CDT*-1 gene family. The structure and putative coding domain of *CDT*-1 homologues were highly conserved. None of the genes contained an intron and each gene had an oligo(A) segment in the 5′ region that

A

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  1 TTGAAATTACGCTTGTCTCAAAAAACCCTTCTCTCTTATTTCCCTCCCATCTCACTAAAAACCCTACCTCCACCGCCGCCACACACAACCAAAACCT
a   L K L R L S Q K T L L S L I F P P I S L K T L P P P P P H T T K T F
b   * N Y A C L K K P F S L L F S L P S H * K P Y L H R R H T Q P K P
c   E I T L V S K N P S L S Y F P S H L T K N P T S T A A T H N Q N L

101 TCCCTCTCCTCCCCCTCCTCCATTAATGGAGGCAGGGGGAGCTCTCTCTTCTCTTGTCTTTTTTATTGCTTTCTGTTTTTATATATTCATATAAAAAAAA
a   P L L P L L H * W R Q G E L S L L L S F L L L S V F I Y S Y K K K
b   S L S S P S S I N G G R G S S L F S C L F Y C F L F L Y I H I K K K
c   P S P P P P P L M E A G G A L S S L V F F I A F C F Y I F I * K K

201 AAAAAAAAAAAAAATTGTGCCGCCACCACCACCGTCGCCCACCACCGTTGCCACCATCGCCAACCGCCTACTGCCGGTCGCCACCGACGCCACCACTCCCA
a   K K K K L C R H H H R R P P P L P P S P T A Y C R S P P T P P L P
b   K K K N C A A T T T V A H H R C H H R Q P P T A G R H R R H H S H
c   K K K K I V P P P P P S P T T V A T I A N R L L P V A T D A T T P T

301 CGGTCTTCATCCACAGTTCACATCCACCGCTACGACCACCGCCTTCCACCTGTCCCGTCCTCGCTGCCCCTCATCTGCTGCCCGCCGCCCCCTGTCGCCC
a   R S S S T V H I H R Y D H R L P P V P S S L P L I C C P P P P V A H
b   G L H P Q F T S T A T T T A F H L S R P R C P S S A A R R P L S P
c   V F I H S S H P P L R P P P S T C P V L A A P H L L P A A P C R P

401 ACGGCCAGCCGCGCCGCCAAGGGAAGCCGCCAGCTAAGCAGCTCGCGTTGTCCGCCTGTCGCCAGGACTCGCCGGCAGCCAGTCGCTTCGCCCGCTCGGT
a   G Q P R R Q G K P P A K Q L A L S A C R Q D S P A A S R F A R S V
b   T A S R A A K G S R Q L S S S R C P P V A R T R R Q P V A S P A R C
c   R P A A P P R E A A S * A A R V V R L S P G L A G S Q S L R P L G

501 GCCCCCCTCATATCGCTGTCTGGAGTCTCCCAGTTTGTGGGAGCTCGTCTCTCCCGATCCTCGGATCGTGAGCCTTTTCTCCCTTGTGGAGCCCTCAGTC
a   P P S Y R C L E S P S L W E L V S P D P R I V S L F S L V E P S V
b   P P H I A V W S L P V C G S S S L P I L G S * A F S P L W S P Q S
c   A P L I S L S G V S Q F V G A R L S R S S D R E P F L P C G A L S P

601 CAACGTAGACAAAACCTAGAAGTCGAAAACCAAGTTAAAATGAAGAGTATCGTAGATTTAGCTTAATAGCTTTTTCTGTTATAGGTTCTTTAGTTCTTG
a   Q R R Q N L E V E N Q V K M K S I V D L A * * L F L L * V L * F L V
b   N V D K T * K S K T K L K * R V S * I * L N S F F C Y R F F S S W
c   T * T K P R S R K P S * N E E Y R R F S L I A F S V I G S L V L G

701 TCGTAGATAATGGGTTTAGTTTTCTGTTGGTTTTTTGTCCCTTGTGGACCTTTTTTGTCAGTGCTTGTACCCCGTTGTGGGTTGTGTTGTGCTCCTTGTG
a   V D N G F S F L L V F C P L W T F F V S A C T P L W V V L C S L *
b   S * I M G L V F C W F F V P C G P F L S V L V P R C G L C C A P C E
c   R R * W V * F S V G F L S L V D L F C Q C L Y P V V G C V V L L V

801 AGCCTTTGTTCTCCCGGTTTTCGATGTACCGGTGAGACTCCGTGGGCTATTTAGCCCTGTATCTTTGTTATTAATAAAAGTATCCGTTTCCGGGAAAAAA
a   A F V L P V F D V P V R L R G L F S P V S L L L I K V S V S G K K
b   P L F S R F S M Y R * D S V G Y L A L Y L C Y * * K Y P F P G K K
c   S L C S P G F R C T G E T P W A I * P C I F V I N K S I R F R E K K

901  AAAAAAAAAAAA
a    K K K K
b     K K K
c      K K K
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B

Clone	5′ flanking sequence	Identical sequence	3′ flanking sequence
1	TTGCATCGTTGAACAATGGCTTCGAAAAAA	aaaaaacccttc......$A_{(25)}$	GAACAATGCTTTCGAAATT
2	AAGAGAGAGAATCTGATATACTCCACGCTTGTCTC	aaaaaacccttc......$A_{(17)}$	GAGAGAGAATCTGATGCCT
3	ACTCAACTTTTTTTCTCTACTCACGGTCTC	aaaaaacccttc......$A_{(15)}$	TTTTTTTCTCTATTCACA
4	AAATGACACGTTTGAAATTACGCTTGTCTC	aaaaaacccttc......$A_{(10)}$	GAAATTACGCTTGCCTCGT
Tagged	GACCAACACGTTTGAAATTACGCTTGTCTC	aaaaaacccttc......$A_{(23)}$	CCCTACCATTTCAGAGGGC

Fig. 5. (**A**) Nucleotide sequence and putative ORFs of the *CDT-1* cDNA. The ORF starting with an AUG codon is underlined and the poly(A) track in the 5′ region is highlighted. (**B**) Comparison of the 5′- and 3′-flanking sequences of four genomic clones and the tagged genomic clone containing the *CDT-1* retroposon-like element. The column on the left shows the 5′ sequences flanking the identical sequence parts. Nucleotides 1–19 of the *CDT*-1 cDNA sequence (A) are contained in the 5′-flanking sequence of the genomic clone (B). The middle column represents the start of the identical sequences (corrresponding to nucleotides 20–31 of the cDNA clone in A) and the length of the poly(A) track which flank the identical sequences at the 3′ end. (The CDT-1 cDNA clone contains only the first 18 As out of the 23 As present in the genomic clone.) The third column of (B) contains the sequences which flank the oligo(A) track. Shaded boxes represent direct repeats. The arrow indicates the border of the inserted T-DNA in the tagged genomic clone.

was also present in the corresponding cDNA clone. The genomic sequences of different *CDT*-1 homologues were completely divergent 5′ upstream and 3′ downstream of the conserved sequences corresponding to the cDNA clones. The 5′ and 3′ ends of the conserved *CDT*-1 coding domains were marked by short sequence duplications that varied in length between the different genes coding for *CDT*-1 homologues (Figure 5B). These sequence duplications showed a remarkable similarity to target site duplications of eukaryotic SINE elements that represent a family of repetitive sequences moving in the genome by RNA intermediates (see Discussion).

The nucleotide sequences at the 5′ termini of the *CDT*-1 genomic clones indicate that direct repeats—or parts of them—generated by insertion events have been transcribed, and that the new elements have been re-inserted

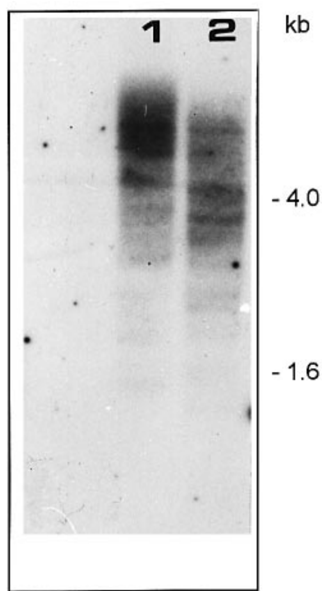

Fig. 6. Southern blot analysis. *Craterostigma* genomic DNA was isolated from wild-type plants, 10 μg were digested to completion with *Bgl*II (1) and *Hin*dIII (2), electrophoresed on an agarose gel and transferred onto a nitrocellulose membrane; the filter was hybridized with a ^{32}P-labelled internal *Sac*I fragment of the *CDT*-1 cDNA clone.

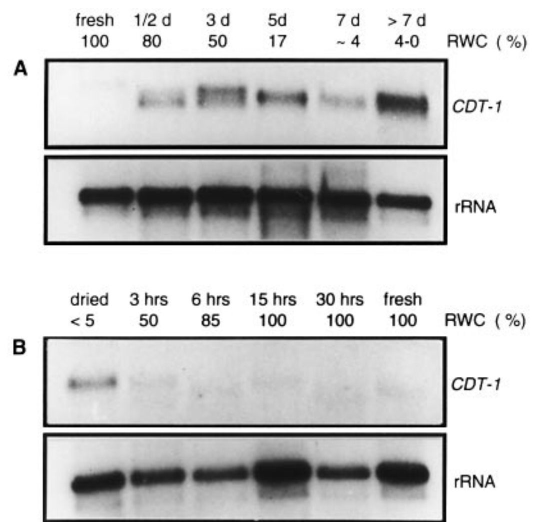

Fig. 7. Northern hybridization experiments showing the transcript accumulation of the *CDT-1* gene during desiccation (**A**) and rehydration (**B**) of wild-type leaves. For this, RNAs were isolated from leaf samples dried or rehydrated for the different times indicated; the corresponding RWC (relative water content) is given as a percentage of the untreated sample. To monitor loading of the RNAs, the filters were reprobed with an rRNA probe.

at other positions in the genome (compare in Figure 5B the sequence gaaattacgttgTCTC in genomic clone No. 4 with the sequence CACGTTTgaaattacgttgTCTC in the tagged clone below: the nucleotides GAAATTACGCTTG belong to the 5′ direct repeat of clone 4, but are part of the putative transposed element in the tagged clone).

The fact that cDNA clones corresponding to *CDT*-1 and to four sequenced homologous genomic clones were identified in wild-type *Craterostigma* indicates that the amplification of *CDT*-1 sequences in the genome (see Discussion) did not ultimately result in the evolution of a family of silent genes. Hybridization analysis with the *CDT*-1 cDNA probe demonstrated that the *CDT*-1 transcript(s) accumulated in the wild-type specifically during dehydration. The *CDT*-1 probe detected an accumulation of transcripts in wild-type leaves dried for 7 days, at a stage when the relative water content of tissues is already in equilibrium with the air present in the environment (Figure 7A). *CDT*-1 transcripts were expressed in wild-type *Craterostigma* throughout the desiccation process, but the expression was suppressed as soon as water was supplied to dried tissues during rehydration (Figure 7B).

Discussion

Genetic identification of the CDT-1 gene activating Craterostigma desiccation tolerance

Drought and ABA signals induce the expression of many proteins that have a protective role during dehydration in the desiccation-tolerant resurrection plant *C.plantagineum.* These gene products are expressed in all vegetative tissues (Schneider *et al.*, 1993). In desiccation-sensitive species such as *Arabidopsis* and crop plants, genes like those encoding Lea proteins are expressed mainly in the final stage of maturation of embryos (Dure *et al.*, 1989; Parcy *et al.*, 1994). Remarkably, the conversion of differentiated tissues to dedifferentiated calli suppresses the ability of *Craterostigma* cells to withstand a rapid dehydration treatment. *Craterostigma* calli are sensitive to desiccation, and only when the callus is pre-treated with ABA is the desiccation tolerant phenotype restored (Bartels *et al.*, 1990). The ABA treatment induces the same set of genes in the callus as does dehydration for the whole plant. Because the resurrection phenomenon depends on the expression of a large number of structural genes (Bartels *et al.*, 1990), it is necessary to isolate regulatory genes, which control the expression of a set of structural genes, if modulating desiccation tolerance is the ultimate goal.

Craterostigma has a chromosome number (n = 28) which indicates a polyploid state of the genome. This impairs the search for recessive mutants. An alternative approach is the screening of dominant mutants by activation tagging (Koncz *et al.*, 1994; Walden *et al.*, 1994). A tissue culture transformation system available in *Craterostigma* facilitates the induction of dominant insertional mutations using the T-DNA of *Agrobacterium* as the insertional element. T-DNA vectors carrying the enhancer domain of the cauliflower mosaic virus (CaMV) 35S promoter are used with success in tobacco and *Arabidopsis* to activate the expression of silent plant genes by T-DNA insertion (Walden *et al.*, 1991). Here we have used a modified T-DNA insertion element carrying the *A.tumefaciens* gene 5 enhancer to select desiccation-tolerant callus tissues of *Craterostigma.* In contrast to the CaMV 35S enhancer which is active in most vegetative plant organs, as well as in callus, the gene 5 enhancer preferentially promotes transcription in proliferating dedifferentiated cells in the presence of plant hormones (Körber *et al.*, 1991).

A selection for a gain-of-function phenotype in T-DNA-transformed *Craterostigma* cells identified a mutant line with one T-DNA insertion that displayed a desiccation-tolerant phenotype in dedifferentiated callus tissues. In

this mutant, the gene 5 enhancer was placed upstream of the *Craterostigma* gene *CDT*-1 that showed a high level of expression in the mutant callus but was silent in the wild-type callus. The *CDT*-1 gene was isolated in linkage with the gene 5 enhancer from the mutant and inserted into the wild-type *Craterostigma* via transformation. The callus resulting from this transformation experiment again exhibited desiccation tolerance and was characterized by the expression of desiccation-inducible transcripts in the absence of exogenous ABA. This demonstrates that the high level of expression of the *CDT*-1 gene is sufficient to induce desiccation tolerance in calli in the absence of ABA. As in the wild-type, the desiccation-tolerant phenotype was correlated with the expression of specific genes. The ABA content in wild-type and transformed callus indicated that the integrated T-DNA did not affect ABA accumulation. Thus, the gene tagged by T-DNA integration must act downstream of the initial ABA signal.

The CDT-1 gene is a member of a large family of potential retrotransposons

Hybridization analysis with the cDNA probe revealed that the *CDT*-1 gene is a member of a gene family. Sequence analysis of the tagged *CDT*-1 gene and the homologous genomic and cDNA clones indicates that the genes do not resemble canonical members of so far known plant signalling pathways, since they code for unusual primary transcripts with no large ORFs for proteins. The *CDT*-1 homologues differ slightly from each other in the length of an oligo(A) sequence located in the 5′ region of their transcribed domains. Since these sequence variations are also represented in the cDNAs, it is likely that in addition to *CDT*-1 some other members of the gene family are transcribed, but this is difficult to establish without gene-specific probes. The *CDT*-1 gene and its homologues do not contain introns. Furthermore, the nucleotide sequences of the cDNA of the *CDT*-1 homologues agree 100% with corresponding fragments of the *CDT*-1 genomic clones. However, genomic DNA sequences flanking the highly conserved transcribed regions represented by the cDNAs are completely divergent between the different *CDT*-1 homologues.

Transcribed domains of the *CDT*-1-like genes are flanked by short direct sequence repeats. A sequence alignment of these direct repeats (Figure 5B) shows that they are of different lengths, but carry common core sequences. This feature of repeats suggests a close analogy to sequence duplications flanking the integration target sites of eukaryotic retrotransposons. In different genomic clones, the lengths of the repeats seem to increase, suggesting that the genomic DNA sequences involved are copied during successive turns of retrotransposition. The direct repeats consist of 12–17 nucleotides. Direct repeats of similar lengths are typically seen at the site of insertion of plant non-long terminal repeat (LTR) retrotransposons [4–13 bp in the *del2* element of *Lilium speciosum* (Leeton and Smyth, 1993) and 3–16 bp in *Cin4* of maize (Schwarz-Sommer *et al.*, 1987)].

Although in the *CDT*-1-like genes no sequences were found encoding reverse transcriptase, their structures otherwise resemble a family of eukaryotic repetitive elements that transpose via RNA intermediates, such as the mammalian interspersed repeated DNA elements (SINEs) like Alu (Deininger, 1989). It has been proposed that the RNAs of these or similar elements are able to prime the reverse transcription reaction (Jagadeeswaran *et al.*, 1981), or that a priming takes place via intermolecular intermediates (Van Arsdell *et al.*, 1981). Their retro-transcription may be RNA mediated, as described for the *R2Bm* RNA element of *Bombyx mori* (Luan *et al.*, 1993). In such a case, the requirement will be that the RNA template has, at its 3′ end, the same sequence as an active non-LTR retrotransposon, so that the process can be mediated by the reverse transcriptase of that element. Several classes of non-LTR retrotransposons have been described for plants (Schwarz-Sommer *et al.*, 1987; Flavell *et al.*, 1992; Grandbastien, 1992; Voytas *et al.*, 1992). In this context, it should also be mentioned that Maestre *et al.* (1995) have demonstrated that human HeLa cells can 'retropose' the mRNA of a reporter gene via an endogenous reverse transcription activity not restricted to transcripts of transposable elements.

Regulation of desiccation tolerance by the CDT-1 function

Heat shock, UV radiation, steroids and cAMP are known to increase the transcription of retrotransposons as well as the frequency of retrotransposition (Brandshaw and McEntee, 1989; Strand and McDonald, 1989). Furthermore, induction of cell proliferation in plant protoplasts or calli, as well as microbial infections, have been shown to activate the transcription of *Tnt*, *Tto1* and *Tto2* retroelements in tobacco (Pouteau *et al.*, 1991, 1994; Hirochika, 1993; Moreau-Mhiri *et al.*, 1996). It is thus conceivable that transcription of the *CDT*-1 or of its homologues, induced by stress conditions, could also lead to a higher frequency of retroposition of *CDT*-1 sequences. The oligo(A) motif within the 5′ region of the gene may represent a potential *cis*-element targeted by stress signalling. It has been observed that many stress-responsive genes, such as alcohol dehydrogenase in *Arabidopsis*, the heat shock protein genes in soybean or chalcone synthase in parsley (Elliston and Messing, 1989), code for mRNAs with adenine-rich segments of 12–20 nucleotides in their untranslated 5′ regions. These oligo(A) motifs were suggested to control translation of these transcripts during periods of stress.

While retroposition of *CDT*-1 may ultimately reveal a mechanism by which acquisition of desiccation tolerance has been acquired progressively during evolution, the mechanism that links the expression of *CDT*-1 sequences and desiccation tolerance of the callus in the absence of ABA remains to be clarified. Although the *CDT*-1 mRNA does not carry a long ORF starting with AUG and no protein product is detected by *in vitro* translation, it cannot be excluded that the *CDT*-1 mRNA is translated into a short peptide of 22 amino acids starting from the AUG codon located at position 126. Moreover, other codons may initiate translation under particular conditions (Boeck and Kolakofsky, 1994). Alternatively, translation of *CDT*-1 mRNA may be controlled by UAA suppression (Zerfass and Beier, 1992). Reports on the *ENOD*40 gene in *Medicago* suggest that the organogenesis of *Rhizobium*-induced N_2-fixing nodules is controlled by the *ENOD*40 transcript (Crespi *et al.*, 1994); it was shown recently that the activity of this gene may be mediated via a 10 amino

acid long peptide encoded by *ENOD*40 (van de Sande *et al.*, 1996). As the *CDT-1* gene has the coding potential for a 22 amino acid long peptide, a mechanism analogous to that reported for *ENOD*40 should also be considered. Future experiments will test whether the putative peptide encoded by *CDT*-1 has any biological activity. Considering all the possibilities, it is also conceivable that the polyadenylated transcript of the *CDT*-1 gene may act as regulatory RNA (for review, see Nowak, 1994). Examples of regulatory RNAs are documented, such as the inactivation of X-linked gene expression in human (Penny *et al.*, 1996) or the translation control of *lin-14* in *Caenorhabditis elegans* (Lee *et al.*, 1993). Other examples of polyadenylated and non-translated mRNAs with potential regulatory functions have also been reported (Brannan *et al.*, 1990; Brockdorff *et al.*, 1992; Brown *et al.*, 1992; Rastinejad *et al.*, 1993).

Agrobacterium gene 5 DNA sequences control the CDT-1 transcription

In the transformed callus line, 645 bp (–684 to –39) of the *Agrobacterium* gene 5 promoter (*pg5*) precede the *CDT-1* gene. This suggests that these sequences are involved in the activation of the tagged gene. Gene 5 is one of the octopine Ti-plasmid T_L-DNA genes. The T_L-DNA encodes essential functions involved in the neoplastic transformation of plant cells (Gielen *et al.*, 1984). The expression of the tagged *CDT-1* gene, as studied by Northern analysis, reveals similarities with data concerning gene expression mediated by the gene 5 promoter (Koncz and Schell, 1986). This suggests that the tagged gene is under the control of gene 5 DNA sequences. Conclusive proof of the role of *pg5* DNA sequences in the activation of *CDT-1* was obtained by transformation of *C.plantagineum* leaf explants with the *pg5–CDT-1* construct. In those transgenic desiccation-tolerant calli, *CDT-1* is constitutively expressed. The highest expression was detected in dedifferentiated callus tissue, and the lowest level of expression was observed in fully differentiated leaves of transgenic plants. The finding that the expression of ABA- and desiccation-related genes was not observed in the tissues of regenerated transformed plants, but that it was again initiated when callus was obtained from such leaves, supports the assumption that the *CDT-1* gene is under control of *Agrobacterium* gene 5 DNA sequences.

Materials and methods

Plant material

Craterostigma plants and calli were propagated and maintained under controlled environmental conditions as described by Bartels *et al.* (1990) and Furini *et al.* (1994).

Agrobacterium strains, vectors, selection conditions and transformation

The *A.tumefaciens* strain GV3101pMP90RK (Koncz and Schell, 1986) was used as the host strain for the hygromycin vectors pCVHPT (Koncz and Schell, 1986) and pPCV811 (Koncz *et al.*, 1994). The strain LBA4404 (Ooms *et al.*, 1982) was used for the transformation with the pBin19 vector (Bevan, 1984) carrying the kanamycin gene. The T-DNA tagging vector pCVHPT is based on the pPCV002 vector (Koncz and Schell, 1986): it contains left and right T-DNA border fragments encompassing gene 5 (*pg5*) promotor sequences including the gene 5 enhancer, the hygromycin resistance gene (*HPT*) with the nopaline synthase promoter (*pnos*), the *Escherichia coli* origin of replication (*ori*), and the ampicillin (Amp) resistance gene.

A 2.3 kb *Bgl*II–*Sal*I fragment of the tagged genomic clone, containing the 645 bp of the *Agrobacterium* gene 5 promoter (Gielen *et al.*, 1984), together with the adjacent *CDT-1* gene were cloned in the *Bam*HI–*Sal*I sites of the pUC18 and then subcloned in the *Eco*RI–*Hin*dIII sites of the pPCV811 binary vector (Koncz *et al.*, 1994). In addition, the 5.8 kb *Eco*RI genomic DNA fragment was cloned in the pBin19 vector (Bevan 1984).

Agrobacterium transformation was achieved by high voltage electroporation (Wen-jun and Forde, 1989). *Craterostigma* transformation, media for callus induction, maintenance and plant regeneration were as previously described (Furini *et al.*, 1994).

Identification of desiccation-tolerant calli

About 25 000 calli were selected on hygromycin-containing medium and maintained by subculturing at 3 week intervals for 2–3 months. Calli were then transferred to sterile filter paper and dried in a constant air stream of a ventilating hood for 24 h at 20°C. During this time, the calli lost ~94–96% of fresh weight. Untreated calli (negative control) and ABA-treated calli (positive control) were also included in each drying experiment. Dried calli were then transferred on callus medium and screened for viability after 2 days using a dissecting microscope.

Measurement of ABA content

ABA was extracted from lyophilized samples and quantified using a competitive ABA ELISA as described in detail by Weiler (1986). Briefly, the assay was done on microtitre plates using a mouse monoclonal antibody to (+)ABA. ABA was measured via a phosphatase reaction using ABA standards ranging from 10 fmol to 10 nmol.

Recombinant DNA techniques

Standard procedures for recombinant DNA techniques were used essentially according to Sambrook *et al.* (1989).

Nucleic acid preparation, gel electrophoresis and hybridizations

Total RNA was isolated from *C.plantagineum* tissues according to Pawlowski *et al.* (1994). Poly(A)$^+$ mRNA was purified using oligo(dT) chromatography (Bartels *et al.*, 1990). The *C.plantagineum* genomic DNA was isolated as described by Doyle and Doyle (1990).

Northern and Southern blot analyses of RNA and DNA were performed as described in Bartels *et al.* (1990) For Northern analysis, 2 μg of poly(A)$^+$ mRNA or 30 μg of total RNA were fractionated on a formaldehyde-containing agarose gel. Probes for Northern hybridization were the 5.8 kb *Eco*RI fragment of the genomic clone containing the inserted T-DNA and the tagged gene, the *CDT-1* cDNA clone and the *C.plantagineum* cDNA clones pcC 6-19, pcC 27-45 and pcC 11-24 (Bartels *et al.*, 1990). To standardize the amounts of RNA loaded, the filters were reprobed with a barley ubiquitin cDNA clone (Gausing and Barkardottir, 1986).

For Southern blot analysis, 10 μg of plant DNA were digested with selected restriction enzymes, separated by electrophoresis on a 0.8% agarose gel and transferred onto Hybond-N membrane. The membranes were pre-hybridized for at least 3 h and then hybridized for 16 h at 65°C in 0.6 M NaCl, 10 mM PIPES pH 6.8, 0.1% (w/v) SDS, 0.2% Ficoll, 0.2% (w/v) polyvinylpyrrolidone and 10 μg/ml sonicated calf thymus DNA. The filters were washed three times for 10 min at 65°C with 2× SSC and 0.1% SDS and then exposed to X-ray films (Kodak X-Omat) with Trimax intensifying screens. ^{32}P-labelled probes were obtained by random priming (Feinberg and Vogelstein, 1984).

Isolation of genomic and cDNA clones

Genomic DNA from leaves of transformed plants regenerated from the mutant callus was partially digested with *Mbo*I. After separation, fragments of 14–20 kb were isolated on a 0.7% agarose gel and ligated into the *Bam*HI-cut EMBL4 λ vector, packaged *in vitro* and plated on K803 bacterial cells to yield up to 10^6 recombinants. The library was amplified and ~5×10^5 plaques were screened using part of the T-DNA as a probe to isolate the tagged gene, which was designated *CDT-1*. To isolate the wild-type genes, the genomic library was rescreened with the isolated *CDT-1* cDNA.

A cDNA library in λ Uni-Zap ™X R vector (Stratagene) was constructed previously (Eggestein, 1993) using cDNA derived from poly(A)$^+$ mRNA of ABA-treated *C.plantagineum* callus. A 5.8 kb *Eco*RI fragment of the genomic clone was used to screen for the corresponding cDNA clones.

DNA sequencing and computer analysis

The isolated genomic clones and cDNA clones were sequenced on both strands, using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) with an automatic DNA sequencer (Type 373A, Applied Biosystems). Computer alignments and sequence analyses were performed with the WISGENE software (version 7.1) of the University of Wisconsin Genetic Computer Group (Devereux *et al.*, 1984) and with the FASTA program (Pearson and Lipman, 1988).

In vitro transcription and translation assays and protein analysis

For *in vitro* transcription assays, the *CDT-1* cDNA was subcloned into pBluescript II SK +/–. The *in vitro* transcription was carried out in a final volume of 50 µl containing 5 µl of 10× transcription buffer (10× buffer: 400 mM Tris pH 7.5, 60 mM $MgCl_2$, 20 mM spermidine, 50 mM NaCl), 5 µl of dithiothreitol (DTT) (100 mM), 2.5 µl each of ATP, CTP, UTP (10 mM) and GTP (1 mM), 5 µl of m7G(5)pppG(5), 1.5 µl of RNase inhibitor (50 U/µl), 5 µg of DNA template and 1 µl of T3 RNA polymerase (10 U/µl). After incubation at 37°C for 30 min, a phenol/chloroform extraction was performed and RNA was precipitated with 1/10 volume of Na-acetate (3 M) and 2 volumes of ethanol. RNA was recovered and dissolved in water. For the *in vitro* translation, the *in vitro* transcribed RNA was heated for 10 min at 65°C and used in a cell-free, nuclease-treated rabbit reticulocyte lysate system (Amersham Buchler, Braunschweig) incorporating [^{35}S]methionine. Electrophoresis of the *in vitro* translation products and fluorography were performed as in Bartels *et al.* (1990).

PCR analysis

Transformation with (i) the *BglII*–*SalI* fragment containing the *pg5* promoter and the *CDT-1* gene and with (ii) the 5.8 kb *Eco*RI fragment inserted in the pCV811 and pBin19 vector, respectively, was verified by PCR analysis of DNAs from transformed calli. PCR primers were designed between the *CDT-1* gene and the *pg5* (5′-CTCAGCATCG-AAATATTCGCC-3′, and 5′-CTTTTCTCCATATTGAC-3′) to amplify a fragment of 560 bp. To exclude problems of plasmid contamination, a PCR reaction was performed with primers designed outside of the T-DNA (5′-AGCTTGCTTGGTCGTTCCG-3′, and 5′-AGCTCCACCGG-GTGCAAAG-3′) to amplify a fragment of 290 bp in the control plasmid. The PCR reaction (50 µl volume; 35 cycles) contained genomic DNA (30–50 ng) 1× *Taq* buffer (Gibco-BRL), 1.5 mM $MgCl_2$, 0.12 mM dNTP, 0.25 µM of each primer and 2.5 U of *Taq* DNA polymerase (Gibco-BRL).

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