Retrotransposons and siRNA have a role in the evolution of desiccation tolerance leading to resurrection of the plant *Craterostigma plantagineum*

Tobias Hilbricht¹, Serena Varotto², Vittorio Sgaramella³, Dorothea Bartels⁴, Francesco Salamini³ and Antonella Furini⁵

¹Max-Planck-Institut für Züchtungsforschung, Carl-von-Linne Weg 10, D-50829 Köln, Germany; ²Dipartimento di Agronomia Ambientale e Produzione Vegetale, University of Padova, Agripolis-Viale dell’Università 16, 35020 Legnaro, Padova, Italy; ³Fondazione Parco Tecnologico Padano, CERSA, via Einstein – Località Cascina Codazza, 26900 Lodi, Italy; ⁴Molekulare Physiologie und Biotecnologie der Pflanzen, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany; ⁵Dipartimento Scientico e Tecnologico, University of Verona, Strada Le Grazie 15, 37134 Verona, Italy

**Summary**

- *Craterostigma plantagineum* can lose up to 96% of its water content but fully recover within hours after rehydration. The callus tissue of the plant becomes desiccation tolerant upon pre-incubation with abscisic acid (ABA). In callus and vegetative organs, ABA addition and water depletion induce a set of dehydration-responsive genes.

- Previously, activation tagging led to the isolation of *Craterostigma* desiccation tolerant (*CDT-1*), a dehydration-related ABA-inducible gene which renders callus desiccation tolerant without ABA pre-treatment. This gene belongs to a family of retroelements, members of which are inducible by dehydration.

- *Craterostigma plantagineum* transformation with mutated versions of *CDT-1* indicated that protein is not required for the induction of callus desiccation tolerance. Northern analysis and protoplast transfection indicated that *CDT-1* directs the synthesis of a double-stranded 21-bp short interfering RNA (siRNA), which opens the metabolic pathway for desiccation tolerance.

- Via transposition, these retroelements have progressively increased the capacity of the species to synthesize siRNA and thus recover after dehydration. This may be a case of evolution towards the acquisition of a new trait, stimulated by the environment acting directly on intra-genomic DNA replication.

**Key words:** abscisic acid (ABA), *Craterostigma plantagineum*, desiccation tolerance, retrotransposon, siRNA.


**Introduction**

The resurrection plant *Craterostigma plantagineum* can tolerate up to 96% loss of its relative water content and recover from such extreme dehydration within several hours (Bartels & Salamini, 2001 and Supplementary Material Fig. S1). By contrast, callus tissue of this plant has a strict requirement for exogenous abscisic acid (ABA) in order to survive severe water loss (Bartels et al., 1990 and Supplementary Material Fig. S1), and water depletion and ABA treatment result in the induction of the same set of dehydration- and ABA-responsive genes (Bartels et al., 1990). T-DNA activation tagging has allowed the isolation of callus lines tolerant to desiccation even in the absence of ABA (Furini et al., 1997; Smith-Espinoza et al., 2005). The first gene isolated from these lines, *Craterostigma* desiccation tolerant (*CDT-1*; Furini et al., 1997), when transcribed at a high level in the callus as a result of the nearby insertion of the *Agrobacterium tumefaciens* gene 5.

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promoter (pg5), induces ABA- and dehydration-responsive genes represented in this plant by the genes encoding the cDNA p6c-19, pc11-24 and pc27-45 (Bartels et al., 1990).

CDT-1 is an unusual gene: (1) the longest open reading frame (ORF) starting with ATG is followed by 20 triplets and ends with TAA; (2) no translation products have been observed; (3) no sequences comparable to that of CDT-1 have been found in databases; (4) a poly(A) tract of 17–22 nucleotides has been reported in the 5′ region of all cDNA and genomic clones, and a 3′ poly(A) tail is present in genomic clones; (5) the structure of CDT-1 is reminiscent of that of retrotransposons; it is intronless, flanked by direct repeats and present in multiple copies in the genome; (6) most importantly, CDT-1 transcript has never been detected in fresh wild-type leaves; in the callus it is up-regulated by ABA, and in wild-type vegetative organs it is induced by dehydration and repressed by rehydration (Furini et al., 1997).

Retrotransposons are genetic elements that transpose through reverse transcription of RNA intermediates; in plants they play a significant role in genome structure, organization and evolution (Kumar & Bennetzen, 1999; Brosius, 2003). The process of retrotransposition is highly invasive: retrotransposons do not excise from the genome and their copy number increases exponentially with transposition (Beguiristain et al., 2001). Plant genomes have mechanisms to control retrotransposition which limit the dangerous effects of new insertions, as well as excessive growth of the retrotransposon population (Liu & Wendel, 2000). Plant retrotransposons quiescent during normal plant development may respond to environmental stress, tissue culture conditions and pathogen attack (Kalander et al., 2000; Beguiristain et al., 2001). Active retrotransposons can affect transcription of adjacent genes: for wheat insertion sequence Wu 2-1A, a wheat Long-Terminal-Repeats (LTRs)-retroelement, a direct link exists between retrotransposon transcription, readout activity from the LTRs and silencing or activation of the adjacent genes by means of antisense or sense RNA, respectively (Kashkush et al., 2003).

Transcriptional interference is also a mechanism by which retrotransposons might affect gene expression (Whitelaw & Martin, 2001). In plants, the notion that short interfering RNAs (siRNAs) can be essential molecules for retrotransposon regulatory mechanisms is not new. The presence of siRNAs was investigated in three different plant retrotransposons (Hamilton et al., 2002): the tobacco (Nicotiana tabacum) LTR-retrotransposon transposon Nicotiana tabacum (Tnt1) and two short interspersed elements (SINEs), TS SINE of tobacco and ArSn1 of Arabidopsis. Small RNAs of both sense and antisense polarities were detected in equal abundance. Based on the size of these siRNAs, the authors suggest that retroelement siRNAs are produced by mechanisms similar, but not identical, to those involved in transgene RNA silencing. Because retrotransposons are often transcriptionally silent, it has been suggested that siRNAs play a role in transcription inactivation (Hamilton et al., 2002). Akin to DNA transcription into active siRNA (Mallory & Vaucheret, 2006), SINE-like DNA sequences could be first transcribed into single-stranded RNA (ssRNA) and then converted into functional double-stranded siRNA. In plants, this would lead to the formation of transacting (ta) siRNA (Dunoyer et al., 2005; Yoshikawa et al., 2005).

In this study we investigated the role of CDT-1 in the pathway for desiccation tolerance. First we provide evidence that CDT-1 translation is not required, and then we show that an siRNA is responsible for opening the ABA and desiccation tolerance pathways. We lastly suggest that the ability to survive extreme conditions may have been stimulated by the environment acting on intra-genomic duplication of DNA elements.

Materials and Methods

Plant material, constructs and transformation

Craterostigia plantagineum Hochst. plants were maintained and propagated in vitro as previously described (Bartels et al., 1990). The transformation procedure and the media for callus induction and maintenance were as reported by Furini et al. (1994), and identification of desiccation-tolerant calli, including untreated calli (negative control) and ABA-treated calli (positive control), was performed as detailed in Furini et al. (1997). ABA treatments were performed by adding 10 µM ABA for 4 d to in vitro grown calli and plants.

The Agrobacterium tumefaciens strain GV3101 pMP90RK (Koncz & Schell, 1986) was used as the host strain for the pPCV811 (Koncz et al., 1994). An EcoRI-HindIII fragment containing the A. tumefaciens gene 5 promoter (pg5) and the CDT-1 cDNA (accession number Y11822) was cloned adjacent to the hygromycin-resistance (hpt) gene into the pPCV811 vector (control construct a) (Furini et al., 1997). In addition, three mutated forms of CDT-1 (constructs b, c and d) were cloned under the control of pg5 into pPCV811 and used for C. plantagineum transformation (Fig. 1a). In construct b, the triplet ATG was changed to GGG, in construct c the 3′ end was deleted up to nucleotide 424 and in construct d 30 bp were deleted between positions 131 and 160. In constructs a, b and d the native CDT-1 poly(A) tail is recognized as a termination signal. In construct c transcription termination relies on the nopaline synthase polyadenylation site present in the pPCV811 vector downstream of CDT-1 insertion (Koncz et al., 1994). All mutated forms of CDT-1 were generated using the mutagenesis kit from Amersham Biosciences (Buckinghamshire, UK), with the CDT-1 cDNA as template. Agrobacterium tumefaciens transformation was achieved by high-voltage electroporation (Wen-jun & Forde, 1989). Transformation with the different constructs was confirmed by PCR analysis of DNA isolated from transformed calli. PCR primers were designed between the CDT-1 gene and pg5. To exclude problems of plasmid contamination, a PCR reaction was performed with primers designed outside of the T-DNA. Primers and conditions were as described previously (Furini
et al., 1997). Calli resistant to hygromycin and tested for effective transformation were 13, 13, 10 and 9 for constructs a, b, c and d, respectively; fresh callus material was sampled from several independent callus lines.

Nucleic acid extraction and analysis

Poly(A)⁺ RNA extraction and RNA gel blot analysis were as described by Bartels et al. (1990). Total RNA was extracted from calli using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Genomic DNA was isolated using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Probes for RNA blot were CDT-1 cDNA and the C. plantagineum cDNA clones from genes pc6-19, pc11-24 and pc27-45 (Bartels et al., 1990). To test for the presence of sense and antisense CDT-1 transcripts, 30 µg of total RNA extracted from leaves and callus were subjected to RNA blot hybridization analysis. Probes were CDT-1 cDNA labeled by specific priming incorporation of [α-32P]dCTP. For strand-specific probes, 100 ng of CDT-1 cDNA was combined with 1 µg of RT-CDT-1-A primer (5′-AAGATACAGGGCTAAATAGCC-3′) or RT-CDT-1-T primer (5′-TTGTGCCGCCACCCACCACCTG-3′) for sense or antisense DNA labeling, respectively, in a total volume of 14 µl and DNA was denatured at 95°C for 5 min; 2.5 µl of 0.5 mM dNTP mix (minus dCTP), 2.5 µl of Klenow buffer, 1 µl (5 U) of Klenow DNA polymerase and 5 µl of [α-32P]dCTP (50 µCi) were then added to the mixture to a final volume of 20 µl. Incubations were for 1 h at 37°C. Probes were purified with MicroSpin™ G50 columns from Amersham Bioscience.

In situ hybridization

In situ hybridization experiments were carried out as described in Varotto et al. (2003). In situ hybridization was performed on wild-type ABA-treated calli, calli transformed with construct a, and roots and leaves of ABA-treated plants. The tissues were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 16 h at 4°C and embedded in Paraplast Plus (Sigma-Aldrich, St Louis, MO, USA). Sections (7–10 µm) were cut using the microtome RM 2135 (Leica, Nussloch, Germany) and collected on xylane-coated slides. Slides were deparaffinized, treated with 5 µg ml⁻¹ proteinase K and hybridized with sense and antisense riboprobes in 50% formamide at 50°C overnight. CDT-1 cDNA was cloned into the pGem-T vector (Promega, Madison, WI, USA) and transcribed in vitro to obtain DIG-UTP (Roche Applied Science, Mannheim, Germany) labeled RNA probes using T7 and Sp6 RNA polymerases. As control, DIG-labeled sense and antisense riboprobes were transcribed using a 450-bp cDNA fragment of the pc27-45 gene cloned into the pGem-T vector (Promega). After hybridization the slides were extensively washed in 2x saline sodium citrate (SSC) at 50°C and treated with 20 µg ml⁻¹ RNaseA (Roche Applied Science). DIG detection and signal visualization...
were carried out using NitroBlue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) staining following the manufacturer’s instructions (Roche Applied Science). Images were acquired using the DC 300F camera (Leica).

Small RNA analysis

Small RNA analysis was carried out as described by Hamilton & Baulcombe (1999), with slight modifications. Total RNA was extracted from wild-type ABA-treated and -untreated calli, and calli transformed with construct a, using Trizol reagent (Invitrogen). The RNA pellet was resuspended in 0.5 ml of sterile diethyl pyrocarbonate (DEPC)-treated water, heated at 65°C for 5 min and placed on ice. Polyethylene glycol 8000 (5%)-0.5 M NaCl was added, and the RNA preparation was kept on ice for 30 min and then pelleted by centrifugation at 10,000 g for 10 min. The supernatant enriched with low-molecular-weight RNA was washed twice in 3 M sodium acetate, pH 5.2. Prehybridization and hybridization were performed as described by Hamilton et al. (2000). The solution was neutralized with 20 μl of 1 M Tris base and 5 M NaCl, pH 5.5, after incubation overnight at 25°C in the dark, with gentle shaking (30 rpm). The average protoplast yield was 3 × 10⁵ protoplasts per gram of callus. Protoplasts were sequentially filtered through two sieves (300 and 85 μm), washed in solution I (0.4 M mannitol and 3.6 mM 2-(N-morpholino)ethanesulfonic acid (Mes), pH 5.5) and resuspended in solution II (solution I plus 0.1 mM CaCl₂) to a density of 2 × 10⁶ protoplasts ml⁻¹ and kept on ice for 1 h before electroporation. Protoplasts were then divided into four aliquots: (1) untreated control, (2) protoplasts for transfection with siRNA designed based on 595–615 bp of CDT-1 cDNA, (3) protoplasts for transfection with siRNA designed based on 634–654 bp of CDT-1 cDNA, and (4) protoplasts treated with ABA (10 μM) 36 h before harvesting. All protoplast samples underwent electroporation and were collected 36 h later. siRNAs corresponding to the regions 595–615 bp and 634–654 bp of the CDT-1 cDNA were designed with 5′ phosphate, 3′ hydroxyl, and two-base overhangs at the 3′ end of each strand as indicated previously (Krichevsky & Kosik, 2002) and synthesized by Qiagen. The siRNA primer sequences corresponding to the region 634–654 bp were: sense strand 5′-UAAAGGAAGAGAAUGAd(TT)-3′ and antisense strand 5′-UAGGUAACUCUCAUUUAd(AC)-3′. The siRNA primer sequences corresponding to the region 595–615 bp were similarly prepared. Annealing for duplex siRNA formation was performed by incubation at 90°C for 1 min and at 37°C for 1 h. Protoplasts (aliquots of 10⁶) were mixed with 3 μg of siRNA in a cuvette with a 0.4-cm gap and electroporated at 0.3 kV and 125 μF as described previously (Vanitharani et al., 2003). After electroporation, protoplasts were transferred to a new tube containing 1 ml of solution II, kept on ice for 30 min, collected by centrifugation at 70 g for 5 min and then cultured in 1 ml of culture solution as described by Qi et al. (2004). The experiment was repeated three times.

Quantification of gene-specific transcripts by real-time PCR

Total RNA was isolated from protoplasts and collected 36 h post-electroporation using the RNeasy Plant Mini Kit.
library was amplified and ~5 × 10^5 copies were obtained. Forward and reverse primers were designed based on cDNA sequences for genes 5′-TGAGCTTGATAAAGGACATCG-3′ and reverse 5′-TGAGACATGGAATCTGGA-3′; 5′-GGGATGAAGGACAAGTGA-3′ and reverse 5′-GGCTTGACCCATGTTTCTA-3′. All data were normalized to a reference cDNA fragment of rct3, a transketolase gene constitutively expressed in C. plantagineum (Bernacchia et al., 1995), amplified in the same conditions using primers TKT3-forward (5′-GGCCACACTGATATTGCTTT-3′ and TKT3-reverse (5′-TTAATAGCAGCGCGAATCTT-3′). The data were organized according to the 2^−ΔΔCT method for relative gene expression analysis (Livak & Schmittgen, 2001).

**Isolation of genomic clones**

A genomic library was constructed from leaves of C. plantagineum as previously described (Furini et al., 1997). The library was amplified and -5 × 10^5 plaques were screened using the CDT-1 cDNA as a probe.

**Results**

**CDT-1 translation is not required for desiccation tolerance**

Although CDT-1 translation products were not detected in previous work (Furini et al., 1997), the translation of the relevant ORF into a 21-amino acid peptide still remained a possibility. To test this hypothesis, C. plantagineum leaf discs were transformed with constructs carrying mutated versions of the wild-type CDT-1 cDNA fused to pg5 (Fig. 1a). Construct a was used as a control, as the overexpression of the wild-type CDT-1 cDNA in callus tissues led to the acquisition of desiccation tolerance and the expression of dehydration- and ABA-induced genes (Furini et al., 1997). Calli transformed with construct b were desiccation tolerant, acquired the typical color of ABA-treated calli (Fig. 1b, upper panels; compare with Supplementary Material Fig. S1) and expressed dehydration- and ABA-inducible genes (Fig. 1c). A similar phenotype was observed for calli transformed with construct d. In construct b, the hypothetical ATG starting codon was mutated into GGG. This change did not alter the desiccation tolerance of the transgenic callus. Construct d contains a 30-bp deletion which drastically reduces the length of the hypothetical ORF and alters the sequence of the corresponding peptide, if any: still, it induced the transcription of dehydration-related genes and supported desiccation tolerance (Fig. 1c). Conversely, a version of CDT-1 truncated at position 424 bp, and thus missing 488 bp of the 912 bp of the CDT-1 cDNA, but still containing the ORF (construct c), neither elicited desiccation tolerance nor induced genes pg6-19, pg11-24 and pg27-45 (Fig. 1c). Desiccation-sensitive calli showed a yellowish-green color (Fig. 1b, lower panels), like the wild-type ABA-untrated callus (phenotypes of calli transformed with constructs a, b, c and d are presented in Supplementary Material Fig. S4).

**Detection of CDT-1 sense and antisense RNA and in situ hybridization**

Transformation with pg5-CDT-1 constructs allowed the detection of high levels of CDT-1 transcript in callus tissues, as pg5 is active only in tissue with a high level of internal auxin (Koerber et al., 1991). Furthermore, RNA blot analyses indicated that the transcribed region of CDT-1 was c. 900 bp (Furini et al., 1997 and Fig. 1c) and that signals of comparable sizes were detected for both strands (Fig. 2a). In differentiated leaves, CDT-1 transcript was detected throughout the dehydration process and suppressed as soon as water was supplied to dried leaves (Furini et al., 1997). In situ hybridization with CDT-1 RNA probes was carried out on callus and on differentiated plant tissues (Fig. 2b). In CDT-1-overexpressing calli and in wild-type ABA-treated calli, the CDT-1 signal, with antisense probe, was localized specifically in cells with substantial cell wall thickening (Fig. 2b, panels 1, 2 and 3). CDT-1 signal on the same type of cells was also detected with sense probe in CDT-1-overexpressing calli (Supplementary Material Fig. S2, panel 3) and in wild-type ABA-treated calli (not shown). An attempt was made to localize CDT-1 transcript in a dehydrated plant. As desiccation is a process affecting both the nucleous and the cytoplasm,
the hybridization signal could not be detected in dried tissues; however, ABA-treated root apexes gave a strong signal restricted to a thin layer of parenchyma cells adjacent to the epidermis (Fig. 2b, panels 4 and 5). Experiments with wild-type ABA-untreated root apexes showed only minimal background signals (Supplementary Material Fig. S2, panels 4 and 5). To exclude the possibility that the detection of CDT-1 transcripts was a result of artefacts, ABA-treated wild-type callus was hybridized with a cDNA fragment of the pC27-45 gene (Bartels et al., 1990). In this case only the antisense probe yielded positive signals (Supplementary Material Fig. S2, panels 1 and 2).

Small RNA analysis

The detection of sense and antisense CDT-1 RNA prompted us to test whether the desiccation-tolerant phenotype was mediated by small RNA. No specific size of low-molecular-weight RNA was detected with sense and antisense riboprobes obtained from in vitro transcription followed by hydrolysis of the CDT-1 template. Instead, a smear was observed (Fig. 2c), indicating the accumulation of RNA of variable length. After testing several oligonucleotides (see Materials and Methods) designed based on the 488-bp segment of CDT-1 deleted in construct c, positive results were obtained when low-molecular-weight RNA was hybridized with sense and antisense 21mers (GTTAAAATGAAGATCGTA) corresponding to the region from 634 to 654 bp in the CDT-1 cDNA (Supplementary Material Fig. S3). This oligonucleotide had some similarity to microRNA 159 (Achard et al., 2004), which is highly conserved in evolution. Both sense and antisense probes gave a hybridization signal with low-molecular-weight RNA from desiccation-tolerant calli but no accumulation of small transcripts was evident when desiccation-sensitive wild-type calli were examined (Fig. 2d). We propose that this desiccation-dependent small RNA plays
a regulatory role in the process leading to desiccation tolerance and should be considered an siRNA.

If this double-stranded RNA has an siRNA function, it should re-activate the genes induced by dehydration or ABA, when fed directly to wild-type callus-derived protoplasts. *Craterostigma plantagineum* wild-type callus-derived protoplasts were therefore transfected with the double-stranded CDT-1 siRNA corresponding to the GTTAAATGAAGAG-TATCGTA oligonucleotide sequence, from 634 to 654 bp in the CDT-1 cDNA, which gave a strong signal in RNA blot analysis. The transient expression of dehydration-responsive genes, quantified by real-time PCR 36 h post-transfection, showed that protoplasts treated with siRNA (634–654 bp) expressed desiccation- and ABA-inducible genes, as did the ABA-treated protoplasts in the absence of siRNA. Conversely, desiccation- and ABA-responsive genes were not induced in control protoplasts nor in protoplasts transfected with siRNA derived from the region from 595–615 bp in the CDT-1 cDNA sequence (Fig. 2e). The induction of these genes in protoplasts transfected with a specific CDT-1 siRNA indicated that a mechanism of RNA interference may be responsible for the induction of the dehydration tolerance pathway in *C. plantagineum*.

**CDT-1 retrotransposition**

The demonstration that CDT-1 siRNA (634–654 bp) is expressed in desiccation-tolerant calli and is responsible for the induction of ABA- and dehydration-responsive genes prompted us to screen the genomic library to isolate CDT-1 homologs and to analyze the features of several genomic and cDNA clones. The structure of genomic clones (schematized in Fig. 3a), indicates that the CDT-1 elements (Fig. 3a, regions b and c) are flanked by different DNA sequences characterized by direct repeats of 5 to 22 bp (Fig. 3a,b, regions a and d). Furthermore, the length of the 3′ poly(A) tail (from 10 to > 60 bp; Fig. 3b, region c) and the presence of direct repeat core sequences (colored in Fig. 3b,c) appearing in more than one clone made it possible to reconstruct, at least in part, the temporal series of transpositions (Fig. 3c).

Sequencing of CDT-1 cDNA clones revealed that several loci in *C. plantagineum* are transcribed into essentially identical CDT-1 mRNA. The derived cDNAs are almost invariant, except for the length of the 5′ oligo(A) sequence (17, 18, 20 or 21 bp), which was of 19, 21 and 22 bp in three different sequenced genomic clones.

**Discussion**

Retrotransposons are the most abundant class of transposable elements in plants (Feschotte *et al*., 2002). They are often considered as inert components of the genome, but their presence in databases of expressed sequence tags (Vicien *et al*., 2001; Nigumann *et al*., 2002) indicates that many have retained the capacity to initiate transcription. Moreover, the abundance of retrotransposons in most eukaryotic genomes (Lander *et al*., 2001; Feschotte *et al*., 2002), together with data indicating that retrotransposons are induced by various signals (Mhiri *et al*., 1997; Takeda *et al*., 1999), supports the idea of retrotransposons as controlling elements (Kashkush *et al*., 2003). It has also been reported that pseudogenes which do not produce functional full-length proteins are actively transcribed and regulate the expression of other related genes by mechanisms based on the functional significance of noncoding RNAs (Korneev *et al*., 1999; Hirotsume *et al*., 2003). The results of this study indicate that translation of the retroelement CDT-1 is not required for desiccation tolerance of the callus. However, the transcript of the 3′-end sequence of CDT-1 deleted in construct c (Fig. 1c) contains information necessary for desiccation tolerance. Another retroelement, CDT-2, isolated from a new T-DNA-generated desiccation-tolerant mutant, differs in sequence from CDT-1, but the highest sequence similarity between them is found in the region deleted in construct c. Furthermore, the CDT-2 sequence does not include the 5′ region of CDT-1 that may potentially code for a short peptide (Smith-Espinoza *et al*., 2005). In addition to being desiccation tolerant, transgenic calli overexpressing CDT-1 have a reddish color similar to wild-type ABA-treated calli, reinforcing the observation that a common response is elicited by CDT-1 overexpression and ABA. The obvious link between the two responses is that CDT-1 itself is ABA inducible (Furini *et al*., 1997). In fact, unlike vegetative organs of *C. plantagineum*, wild-type calli are not desiccation tolerant (Bartels *et al*., 1990). However, when they are treated with ABA, or transformed with CDT-1, they transcribe dehydration-related genes and become desiccation tolerant.

The analysis of the Arabidopsis genome indicates antisense RNA expression of c. 20% of the pseudogenes annotated as not-expressed sequences (Yamada *et al*., 2003). Moreover, transposable elements have the potential to generate double-stranded RNA as a result of promoters in their terminal repeats and/or because of their random integration in the genome (Raizada *et al*., 2001). In our work, in situ hybridization revealed sense and antisense RNA signals, and RNA blot analysis confirmed that both strands of CDT-1 are transcribed. Low-molecular-weight RNA analysis and the activation of the desiccation and ABA pathway in protoplasts transfected with a double-stranded siRNA identified in the 488-bp region deleted in construct c indicate that the siRNA resulting from CDT-1 transcription activates a desiccation-related response. We can speculate that the siRNA is processed from a longer double-stranded RNA precursor and represses gene expression through sequence-specific interactions with sequences of the *C. plantagineum* genome. The abundance of CDT-1 transcripts induced by ABA treatment, by dehydration in differentiated tissues and by the CDT-1 transgene in the callus may be recognized by the cell as a signal of stress and,
through the formation of double-stranded RNA, the transcripts may be converted to small RNAs which in turn might control the expression of genes responsible for desiccation tolerance to avoid deleterious stress effects on the cell. Because in *C. plantagineum* only a limited number of genes implicated in the revival of dried tissues have been identified, with respect to the complexity of the desiccation tolerance phenomenon, the gene(s) potentially recognized by *CDT-1* siRNA is as yet unknown. The fact that *CDT-1* siRNA was detected in transgenic callus as well as in ABA-treated wild-type callus suggests an identical role for *CDT-1* in both types of tissue.

Many of the newly identified tiny RNAs are not expressed at distinct stages of development but only in particular cell types (Bartels, 2004). In the callus and *in planta*, *CDT-1* expression is restricted to special cells or cell layers (in the case of the root); however, all cells from both the callus and the root are capable of resurrecting, making it likely that the effect of the *CDT-1* gene or gene products can be transferred between cells or between organs. In plants, it is known that systemic signals with RNA components (Voinnet & Baulcombe, 1997; Voinnet et al., 1998) convey RNA interference between cells. One component of this process is vascular dependent and transmits silencing signals between distinct organs. A
second type of movement involves cell-to-cell contact via plasmodesmata and is based on reiterated short-distance signaling events involving 21-nt siRNA (Himber et al., 2003; Dunoyet et al., 2005). In specific cases, plant micro RNAs (miRNAs) or siRNAs are active when added exogenously to cultured plant cells (Vanitharani et al., 2003).

What is special in the case of CDT-1 siRNA is the functional link provided by the association of C. plantagineum retroelements with the increase in the level of their siRNA transcription. In establishing a mechanism capable of mediating between environment and DNA, the evolutionary relevance of the transposition of siRNA-carrying retroelements can hardly be assessed directly, but can be conjectured. The starting point is the consideration that CDT-1 mRNA accumulates in wild-type plants only during dehydration (Furini et al., 1997). This implies that the environment controls both the level of transcription of retroelements with potential siRNA activity and their genomic reinsertion. Plants do not have a sequestered germline: in meristematic cells new insertions of the element should contribute to increased desiccation tolerance in the progeny of the plant, provided that they can be transcribed under stress.

CDT-1 cDNAs vary only in the length of the 5′ oligo(A) sequence. Based on available data, a minimum of five CDT-1 elements are transcribed. This evidence was difficult to obtain because of problems with PCR amplification of genomic or transcribed CDT-1 elements across the region containing the 5′ A tract. Nevertheless, the four possible lengths of the 5′ oligo(A) sequence in CDT-1 cDNA (17, 18, 20 or 21 bp), together with the longest CDT-1 cDNA sequence already reported (Furini et al., 1997 and Supplementary Material Fig. S3), indicate that at least five CDT-1 genes are transcribed. However, a larger number is expected, based on the high frequency of CDT-1 elements in the genome, and the quasi invariance of their sequence as evident from the data on genomic clones. In sequenced cDNA clones the length of the 5′ poly(A) tract was 17, 18, 20, or 21 bp; the same tract had a length of 19, 21 and 22 bp in three different genomic clones. The multiplicity of CDT-1 transcripts is also supported by the mRNA analyses of dehydrated and rehydrated plants: a limited but clear size variability of CDT-1 transcripts has been observed (Furini et al., 1997). The sequence of genomic clones indicates that the CDT-1 genes are flanked by different DNA sequences. This, together with the high level of reiteration of the element in the genome, supports the conclusion that CDT-1 elements, after transcription under stress, are variably transcribed into the genome. As a result, there is an increasing probability that the CDT-1 element will land in a DNA sequence capable of directing transcription under stress. When repeated, such a process should result, even without selection, in a progressively higher level of synthesis of the CDT-1 siRNA, which eventually triggers the onset of desiccation tolerance. In this respect, it is worth recalling the two components of Lamarck’s model of organic change (Burkhardt, 1995): the ‘power of life’, or the natural progress of organic development, can be seen as the ‘cause that tends to make organization increasingly complex’; and the modification of this progress by constraining circumstances suggests that ‘they arise from the influence of the climate, variations in temperature of the atmosphere, ... from diversities of places, ... from habits of movements’ (Lamarck, 1801, 1802).

Acknowledgements

The authors wish to dedicate this paper to their colleague and friend Dr Lorena Borgato in recognition of her invaluable contribution to the research prior to her untimely death earlier last year.

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Craterostigma plantagineum plant and calli.

Fig. S2 In situ hybridization with sense and antisense CDet-27-45 and sense CDT-I riboprobes.

Fig. S3 Nucleotide sequence of the CDT-I element.

Fig. S4 Phenotypes of calli transformed with constructs a, b, c and d.

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