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RESEARCH PAPER

Analysis of *pcC13-62* promoters predicts a link between *cis*-element variations and desiccation tolerance in Linderniaceae

Valentino Giarola, Niklas Udo Jung, Aishwarya Singh, Pooja Satpathy, and Dorothea Bartels*

Institute of Molecular Physiology and Biotechnology of Plants, University of Bonn, Kirschallee 1, 53115 Bonn, Germany

* Correspondence: dbartels@uni-bonn.de

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Abstract

Reproductive structures of plants (e.g. seeds) and vegetative tissues of resurrection plants can tolerate desiccation. Many genes encoding desiccation-related proteins (DRPs) have been identified in the resurrection plant *Craterostigma plantagineum*, but the function of these genes remains mainly hypothetical. Here, the importance of the DRP gene *pcC13-62* for desiccation tolerance is evaluated by analysing its expression in *C. plantagineum* and in the closely related desiccation-tolerant species *Lindernia brevidens* and the desiccation-sensitive species *Lindernia subracemosa*. Quantitative analysis revealed that *pcC13-62* transcripts accumulate at a much lower level in desiccation-sensitive species than in desiccation-tolerant species. The study of *pcC13-62* promoters from these species demonstrated a correlation between promoter activity and gene expression levels, suggesting transcriptional regulation of gene expression. Comparison of promoter sequences identified a dehydration-responsive element motif in the promoters of tolerant species that is required for dehydration-induced β-glucuronidase (GUS) accumulation. We hypothesize that variations in the regulatory sequences of the *pcC13-62* gene occurred to establish *pcC13-62* expression in vegetative tissues, which might be required for desiccation tolerance. The *pcC13-62* promoters could also be activated by salt stress in *Arabidopsis thaliana* plants stably transformed with promoter::*GUS* constructs.

Keywords: Desiccation tolerance, dehydration-responsive element, gene regulation, LEA-like protein, resurrection plants, stress protein.

Introduction

Tolerance to desiccation in plants is found in specialized tissues, such as spores, seeds, or pollen, in most angiosperms, and in vegetative tissues of a small group of resurrection plants. Resurrection plants comprise several distantly related plant lineages that tolerate the loss of most of their cellular water and suspend all metabolic activities, but restart normal physiological processes upon rewatering. The most widely accepted hypothesis locates the evolution of the desiccation tolerance trait at the beginning of land colonization (Cushman and Oliver, 2011). Later during their evolution, plants lost the ability to tolerate vegetative desiccation but retained genes relevant for desiccation tolerance in tissues such as spores, seeds, and pollen. More recently, these genes were apparently reprogrammed to be expressed in the vegetative tissues of angiosperm resurrection

Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; DRE, dehydration-responsive element; DRP, desiccation-related protein; GFP, green fluorescent protein; GUS, β-glucuronidase; LEA, late embryogenesis abundant; PIF, P instability factor.

plants, restoring their ability to tolerate desiccation. Studies of seeds and resurrection plants support this theory, because it has been shown that similar molecular programs are activated in vegetative tissues and seeds or pollen (Bartels and Salamini, 2001; Giarola *et al.*, 2017). Desiccation tolerance is acquired following a tightly controlled developmental or dehydration-induced process, which involves the accumulation of protective proteins such as the late embryogenesis abundant (LEA) proteins, reactive oxygen species (ROS) scavengers, ultraviolet protective compounds, nitrogen-rich metabolites (e.g. amino acids), polyols, and non-reducing sugars. In conditions of mild dehydration, sugars are required for osmoregulation, whereas during severe dehydration they may participate in hydrogen interactions with other polar macromolecules, leading to the suspension of metabolic activities and the formation of the so-called glassy state (Burke, 1986).

LEA genes are the most abundant group of protective genes activated in desiccation-tolerant tissues. The name LEA was assigned to these genes as they were initially discovered to be abundantly expressed during the final stage of cotton seed development (Dure *et al.*, 1981). However, these genes are also expressed during dehydration in vegetative tissues of both desiccation-tolerant and desiccation-sensitive plants. Conserved amino acid motifs and sequence similarities have been used to divide LEA proteins into different subclasses (Hundertmark and Hincha, 2008; Jaspard *et al.*, 2012). It has been hypothesized that high expression levels of LEAs are required for the acquisition of desiccation tolerance (Bartels and Salamini, 2001). Although LEA genes have similar expression patterns, they encode proteins with variable amino acid compositions and cellular localizations (Tunnacliffe *et al.*, 2010). This suggests that LEA genes may have multiple functions that are required for tissue protection under stress. Protection-related functions such as binding to membranes or macromolecule structures, or scavenging of reactive oxygen species and ions, have been demonstrated for some LEAs (Tunnacliffe *et al.*, 2010). LEAs are predicted to participate in the formation of the glassy state together with sugars (Hoekstra *et al.*, 2001).

The South African resurrection plant *Craterostigma plantagineum* has been studied to understand the molecular mechanisms underlying desiccation tolerance. Many different mRNAs encoding desiccation-related proteins (DRPs) have been identified (Bartels *et al.*, 1990; Ditzer *et al.*, 2001; Rodrigo *et al.*, 2004). All DRPs are abundantly expressed in desiccated plant leaves. Some DRPs could be assigned to the LEA proteins, as they contain conserved LEA motifs in their amino acid sequences, but others do not contain LEA motifs and thus are generally referred to as LEA-like DRPs. Some *C. plantagineum* DRPs have been studied in detail. For example, the LEA-like DRP CDeT11-24 was shown to protect enzymes from desiccation-related damage (Petersen *et al.*, 2012). Although the expression of other *C. plantagineum* DRPs predicts their involvement in desiccation-tolerance-related functions, these functions remain mainly unknown. The LEA-like DRP pcC13-62 was also associated with desiccation tolerance, but no function could be inferred and no sequence similarity was found with other proteins in public repositories (Bartels *et al.*, 1990; Piatkowski *et al.*, 1990).

Since desiccation tolerance genes appear to be ubiquitous in plants, the rewiring of regulatory networks is predicted to play a major role in the (re-)evolution/(re-)activation of vegetative desiccation tolerance. Therefore, the study of regulatory networks is essential to decipher what is required for desiccation tolerance. Several factors may be involved in gene regulation, including *cis*- and *trans*-acting elements and short and long non-coding RNAs. The same *trans*-acting elements appear to be activated upon dehydration in desiccation-tolerant and -sensitive species, and no desiccation-tolerance-specific *cis*-elements have been identified so far (Giarola *et al.*, 2017). In *C. plantagineum*, dehydration-specific non-coding RNAs, namely *CDT-1* and *28852*, have been identified and have been linked to desiccation tolerance, but their function remains mainly unknown (Hilbricht *et al.*, 2008; Giarola and Bartels, 2015). Abscisic acid (ABA) and ABA-related pathways seem to play an important role in regulating gene expression relevant for desiccation tolerance (Bartels and Salamini, 2001). In *C. plantagineum*, DRP transcripts accumulate and DRP promoters are activated following ABA treatments, suggesting transcriptional regulation of gene expression (Bartels *et al.*, 1990; Michel *et al.*, 1993; Michel *et al.*, 1994; Velasco *et al.*, 1998; Rodrigo *et al.*, 2004; Ditzer and Bartels, 2006). ABA-responsive elements (ABREs) are found in promoters of ABA-inducible genes, including *C. plantagineum* DRP genes. ABA-independent transcriptional regulation is also found in dehydration-responsive genes and it is mediated by factors binding to the dehydration-responsive element (DRE) promoter motifs (Todaka *et al.*, 2015).

The family of Linderniaceae represents a good source of plants for comparative studies as it groups desiccation-tolerant species such as *C. plantagineum* and *Lindernia brevidens* together with species that are mostly sensitive to desiccation, like *Lindernia subracemosa*. Thus, regulatory desiccation-related signatures can be inferred by comparing the promoter regions of protective genes in these species. Using such an approach, the high expression level observed for the *CDeT11-24* LEA-like protective gene in tolerant species was linked to promoter architecture and the presence of *cis*-elements (van den Dries *et al.*, 2011). This finding supports the role of certain *cis*-elements in the rewiring of regulatory networks of desiccation-tolerance genes. However, data are still limited to draw general conclusions.

In this study we compared the expression and promoter activity of the DRP gene *pcC13-62* in closely related desiccation-tolerant and desiccation-sensitive Linderniaceae. We found that the accumulation of 13-62 transcripts is controlled at the promoter level and involves a DRE motif that occurs only in tolerant species. Our data show the importance of DRE motifs for the regulation of desiccation-tolerant genes in resurrection plants and suggest the contribution of nucleotide variations in regulatory regions for the (re-)establishment of desiccation tolerance in vegetative tissues.

Materials and methods

Plant materials

Craterostigma plantagineum Hochst., *Lindernia brevidens* Skan, and *Lindernia subracemosa* De Wild plants were grown as described by Bartels *et al.*

(1990) and Dinakar and Bartels (2012). For the dehydration treatment, fully grown 6- to 8-week-old plants were gradually dehydrated in pots. Relative water content (RWC) measurements were made according to Bernacchia *et al.* (1996). *In vitro* cultures of *C. plantagineum* for transient expression experiments were grown on Murashige and Skoog (MS) medium at day/night temperatures of 22/18 °C with 80 µmol m^{-2} s^{-1} light for 16 h per day.

Arabidopsis thaliana (Col-0) wild-type and transgenic seeds were germinated and plants were grown on soil under short-day conditions (120–150 µmol m^{-2} s^{-1} light at 22 °C with a light/dark cycle of 8/16 h) for 4 weeks and then moved to long-day conditions (16 h light/8 h dark) to induce flowering.

Transgenic plants were selected on MS medium containing 50 µg ml^{-1} kanamycin under the same conditions as described for *in vitro* cultures and transferred to soil.

Molecular biology techniques and DNA sequence analysis

Molecular biology techniques were performed according to Green and Sambrook (2012). DNA sequencing was carried out by GATC Biotech (https://www.gatc-biotech.com/en/index.html) and primer synthesis by Eurofins MWG Operon (http://www.eurofinsgenomics.eu). Signal peptides were predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen, 2017). Sequence alignment figures were obtained with the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/index.html) using the alignment file generated with T-Coffee software (Notredame *et al.*, 2000). All primers used are listed in Supplementary Table S1 at *JXB* online.

Identification of Cp pcC13-62 *homologs and phylogenetic analysis*

Putative *Cp pcC13-62* homologs were identified from *L. brevidens* and *L. subracemosa* transcriptome data (data not published) or GenBank using the *C. plantagineum pcC13-62* predicted protein sequence (GenBank accession number AAA63616) as the query. The sequence alignment of pcC13-62 homologs was generated with T-Coffee (Notredame *et al.*, 2000) and used for phylogenetic analysis in MEGA6 (Tamura *et al.*, 2013). Phylogenetic analysis was performed using the maximum likelihood method based on the JTT matrix-based model (Jones *et al.*, 1992). The tree with the highest log likelihood is shown in Fig. 1B.

RNA isolation and cDNA synthesis

Total RNA was isolated as described by Valenzuela-Avendaño *et al.* (2005). The concentration and purity of the RNA was determined using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Japan). RNA integrity was verified by 2% (w/v) agarose gel electrophoresis. A 4 µg aliquot of total RNA was treated with DNase I (Thermo Fisher Scientific, St. Leon-Rot, Germany) to remove any DNA; 2 µg of the DNase I-treated RNA was reverse transcribed into cDNA using the RevertAid Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer's instructions. The remaining 2 µg of RNA served as a control to monitor genomic DNA contamination in cDNA preparations. The cDNA was diluted 15 times with diethylpyrocarbonate-treated water and 5 µl was used as a template for a 20 µl reverse transcription–quantitative PCR (RT–qPCR).

Primer design and RT–qPCR analysis

RT–qPCR analysis was performed as described by Giarola *et al.* (2015). The specificity of the primers was verified by melting curve analysis (Supplementary Fig. S1) and sequencing. To prepare template samples for standard curves, the 13-62 amplicons generated with RT–qPCR primers (Supplementary Table S1) were cloned into pJET1.2 vectors (Thermo Fisher Scientific). Plasmids containing the amplicons were linearized using the *Hind*III restriction enzyme. Linearized DNAs were purified after gel electrophoresis using the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL, Düren, Germany) and quantified with a BioSpec-nano spectrophotometer (Shimadzu Biotech). Standard curves were obtained by the amplification of 1/10 dilutions of linearized vectors, starting from 10^{10} copies. The amount of linearized plasmid required to obtain 10^{10} copies was calculated as described in Giarola *et al.* (2015). The transcript copy number for 5 µl of cDNA template was calculated from Cq values using standard curves.

Isolation of 13-62 *5′ gene sequences and mutagenesis*

Genomic DNA was extracted from leaves according to Rogers and Bendich (1985) and used to prepare genome walking libraries with the GenomeWalker™ universal kit (Clontech, Heidelberg, Germany). Genomic fragments corresponding to the 5′ *13-62* gene sequence were amplified from *C. plantagineum* and *L. subracemosa* genome walking libraries with gene-specific (Supplementary Table S1) and library-specific (AP1 and AP2; GenomeWalker™ universal kit manual) primers. The amplification from *L. brevidens* libraries was unsuccessful and thus primers designed to match conserved sequence regions of *C. plantagineum* and *L. subracemosa* genomic fragments (Lb13-62g_F and Lb13-62g2_R; Supplementary Table S1) were used to obtain the 5′ *13-62* sequence from *L. brevidens* genomic DNA. The sequence between the β-galactosyltransferase and the *13-62* translational start codons from *C. plantagineum* (962 bp for isoform1 and 1029 bp for isoform2) and *L. subracemosa* (723 bp) was used as the *13-62* promoter sequence in the promoter analyses. A transposon is inserted between the β-galactosyltransferase and the *13-62* genes in *L. brevidens*, and thus the sequence between the transposon terminal repeats and the *13-62* translational start codon was used as the promoter sequence for this species. The promoter sequences were screened for *cis*-acting regulatory elements using the PLACE (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?lang=en&pj=640&action=page&page=newplace) (Higo *et al.*, 1999) and TRANSFAC (http://genexplain.com/transfac/) (Wingender *et al.*, 1996) databases.

*Preparation of promoter::*GUS *constructs and mutagenesis*

The *13-62* promoter sequences from *C. plantagineum* (*Cp13-62iso1* and *Cp13-62iso2*), *L. brevidens* (*Lb13-62*), and *L. subracemosa* (*Ls13-62*) were amplified by PCR from genomic DNA with species-specific *GUS* primers (listed in Supplementary Table S1) to add restriction sites required for cloning the promoter fragment into pBT10GUS vectors (Sprenger-Haussels and Weisshaar, 2000). The *Cp13-62iso1*, *Cp13-62iso2*, and *Ls13-62* promoters were cloned using *Nco*I and *Xba*I restriction, sites whereas the *Lb13-62* promoter was cloned using *Nco*I and *Sal*I restriction sites. Mutagenesis of predicted *cis*-elements in the promoter sequences was achieved with primers (Supplementary Table S1) using the Quick-change II Site-Directed Mutagenesis kit (Stratagene, Heidelberg, Germany).

Analysis of promoter activity using transient transformation

Relative promoter activities were determined using the transient transformation method described by van den Dries *et al.* (2011). Briefly, the *Cp13-62*, *Lb13-62*, and *Ls13-62* promoter::*GUS* fusion constructs in pBT10GUS vectors were co-bombarded together with a vector carrying the CaMV35S::*GFP* construct. The activity of promoter fragments was assayed in both homologous and heterologous genetic backgrounds. The number of green fluorescent protein (GFP)-expressing cells per leaf was determined 16 h after particle bombardment with an inverted confocal laser-scanning microscope (Nikon Eclipse TE2000-U/D-Eclipse C1; Nikon, Düsseldorf, Germany). For dehydration treatments, the bombarded leaves were kept on filter paper for 4 h at room temperature. Leaves were histochemically stained with a solution containing 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc, Gold Biotechnology, St. Louis, USA). The number of β-glucuronidase (GUS) spots per leaf was determined using a stereoscopic microscope with a binocular eyepiece tube (Nikon SMZ 800). Relative promoter activities were calculated by dividing the number of GUS spots by the number of GFP spots. Mean values and standard deviations were calculated from at least four independent replicates.

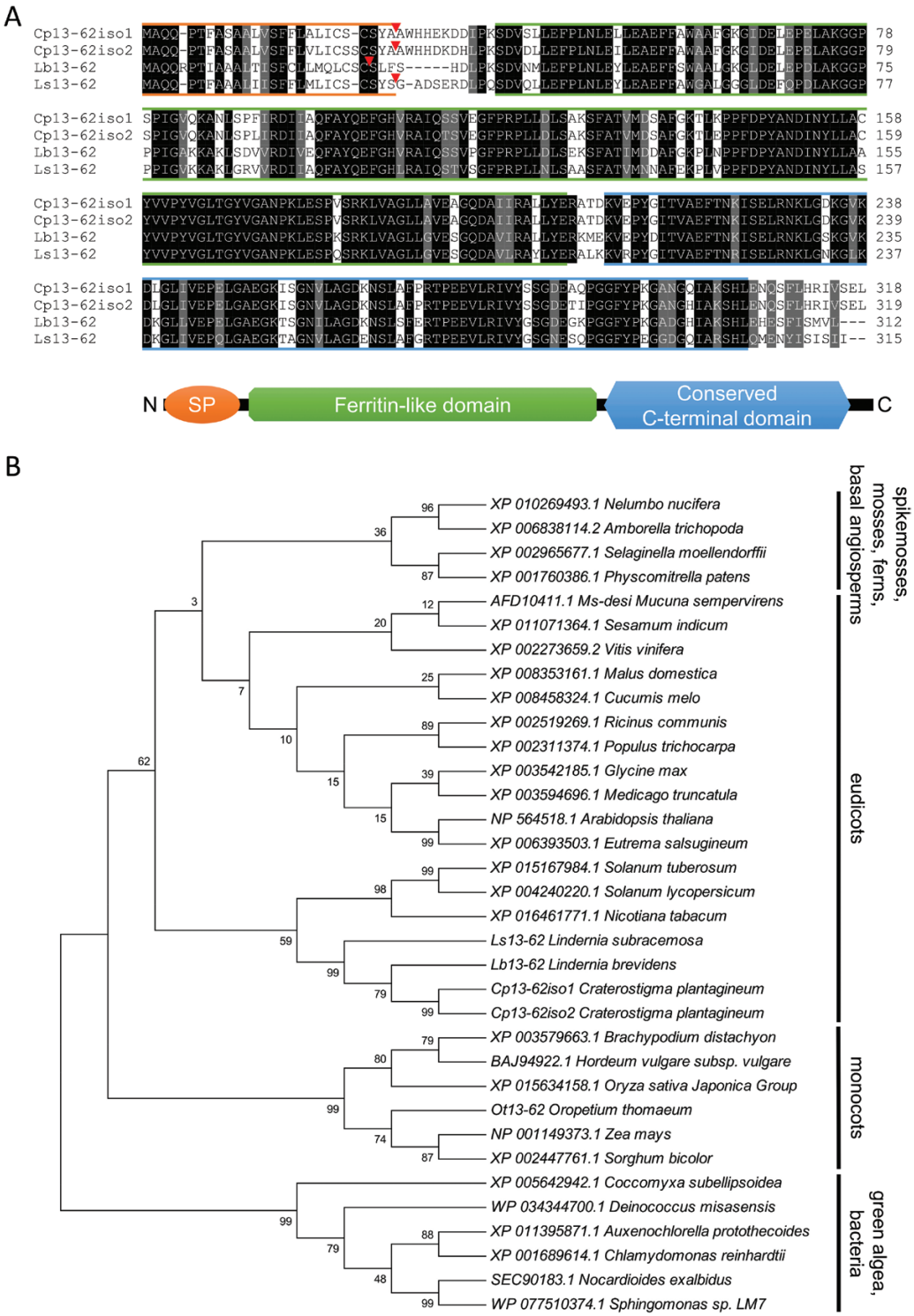

Fig. 1. Alignment of *Craterostigma plantagineum*, *Lindernia brevidens*, and *Lindernia subracemosa* 13-62 amino acid sequences and phylogenetic analysis of selected 13-62 protein homologs. (A) Alignment of the predicted 13-62 amino acid sequences. Identical (black) and conserved (grey) amino acids are indicated. Coloured lines are used to show the different protein domains. Orange: predicted signal peptide (SP); red triangles indicate the predicted SP cleavage site; green: ferritin-like domain (pfam13668); blue: conserved C-terminal domain. (B) phylogenetic analysis of 13-62 protein homologs. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Phylogenetic analysis was performed using the maximum likelihood method based on the JTT matrix-based model (Jones *et al.*, 1992). The tree with the highest log likelihood is shown. (This figure is available in colour at *JXB* online.)

Analysis of promoter activity in stably transformed A. thaliana *plants*

Stably transformed *A. thaliana* plants were generated by the floral dip method (Clough and Bent, 1998) using pBIN19 binary vectors (Bevan, 1984). Promoter::*GUS*-expressing cassettes were excised from pBT-10GUS vectors using the *Bgl*II restriction enzyme and inserted within the *Bam*HI site of pBIN19 vectors. The presence of the promoter::*GUS* cassette in transgenic plants was confirmed by PCR. As *13-62* transcript expression is also induced by salt stress (Supplementary Fig. S2),

we analysed the activity of promoter::*GUS* constructs in transgenic lines subjected to salt stress treatment. This allows testing of a stress response in a more uniform and reproducible way than by dehydration. Transgenic plants at the flowering stage were watered for 2 weeks with 150 mM NaCl or water for salt stress and control experiments, respectively. GUS expression was analysed in rosette leaves, cauline leaves, siliques, roots, open flowers, and floral clusters. Four-leaf-stage seedlings were removed from soil and incubated for 16 h in MS solution (control) or MS solution containing 150 mM NaCl. Plant material was vacuum-infiltrated for 3 min with a 0.5 mg ml^{-1} X-Gluc solution (Gold Biotechnology) and then incubated for 16 h at 37 °C. After destaining, tissues were observed using a Nikon SMZ 800 stereoscopic microscope (Nikon, Tokyo, Japan) and pictures were taken with a digital camera connected to the microscope (Nikon Digital Sight DS-2Mv).

Results

Genomic organization of 13-62 *genes and quantification of 13-62 transcripts*

The *C. plantagineum* DRP *pcC13-62* gene has been previously described (Bartels *et al.*, 1990; Piatkowski *et al.*, 1990; Schneider *et al.*, 1993). To gain more insight into the role of this gene in desiccation tolerance, we compared the *pcC13-62* gene structures and gene expression in the desiccation-tolerant species *C. plantagineum* and *L. brevidens* and in the desiccation-sensitive species *L. subracemosa*. Previous DNA blot analysis predicted the presence of two to three copies of *pcC13-62* in the genome of *C. plantagineum* (Bartels *et al.*, 1990; Piatkowski *et al.*, 1990). Here, we identified two *13-62* isoforms in the *C. plantagineum* genome, which were named *Cp13-62iso1* and *Cp13-62iso2* (GenBank accession numbers MH247237 and MH247238, respectively). The protein sequences predicted for *Cp13-62iso1* and *Cp13-62iso2* are very similar (98% similarity; Fig. 1A). *Cp13-62* homologs were identified in *L. brevidens* and *L. subracemosa* and were termed *Lb13-62* (GenBank accession number MH247239) and *Ls13-62* (GenBank accession number MH247240), respectively. The *13-62* predicted protein sequences from all three species showed more than 74% identity and 83% similarity between each other, with the N-terminal region being the most variable (Fig. 1A). All proteins contain N-terminal signal peptides and ferritin-like domains (pfam13668). The C-terminal regions of these proteins are very conserved. Homologs of *13-62*, sometimes referred to as DRPs, are found in the genomes of several groups of bacteria, a few Chlorophyta, and most Embryophyta (Carniel *et al.*, 2016). The overall conservation is quite high in Embryophyta, including the *C. plantagineum*, *L. brevidens*, and *L. subracemosa* genes (Supplementary Fig. S3): all genes contain the conserved domains and form a separate cluster, as shown by phylogenetic analysis (Fig. 1B).

The 13-62 protein coding sequence is conserved across desiccation-tolerant and non-tolerant species. However, abundant expression of the *13-62* transcript is found in desiccation-tolerant tissues. Fig. 2 shows that *pcC13-62* transcripts are expressed at a high level in response to dehydration in the desiccation-tolerant species *C. plantagineum* and *L. brevidens*, but only weakly in the desiccation-sensitive plant *L. subracemosa*. *pcC13-62* transcripts are also abundant in dehydrated leaves of the monocot resurrection plant *Oropetium thomaeum* (Supplementary Fig. S4). Correlation of *13-62* expression and desiccation tolerance is supported by transcriptomic studies of selected unrelated monocot and dicot plants, which show high accumulation of *pcC13-62* homologs in desiccation-tolerant structures such as seeds and pollen (Table 1). In *C. plantagineum*, *L. brevidens*, and *L. subracemosa*, *pcC13-62* transcripts are also expressed in response to mannitol and salt treatments (Supplementary Fig. S2). Some *13-62* homologs also accumulate in response to osmotic, salt, and/or cold stress in various species, but the expression in stressed tissues is much lower than in reproductive organs (data not shown).

The 13-62 promoter structure

The genomic regions of *Cp13-62iso1*, *Cp13-62iso2*, *Lb13-62*, and *Ls13-62* were isolated and analysed to identify regulatory motifs that cause high expression of *13-62* transcripts in vegetative tissues of desiccation-tolerant species (Fig. 3). The genomic regions of the 13-62 locus are conserved and similarly organized: the β-galactosyltransferase protein coding sequence is upstream of the *13-62* gene, and the *13-62* coding sequence has the same exon-intron structure (Fig. 3). The distance between the β-galactosyltransferase coding sequence and the *13-62* coding sequence varies among the different species. The distance is ~700 bp in *L. subracemosa* and nearly 1000 bp in *C. plantagineum*, whereas it is more than 4000 bp in *L. brevi*dens (Fig. 3). This large difference is due to the insertion of a transposable element ~800 bp upstream of the *Lb13-62* gene translational start codon. The transposable element contains features similar to the maize P instability factor (PIF) such as short terminal inverted repeat sequences and the coding sequence for a transposase (Supplementary Fig. S5) (Zhang *et al.*, 2001).

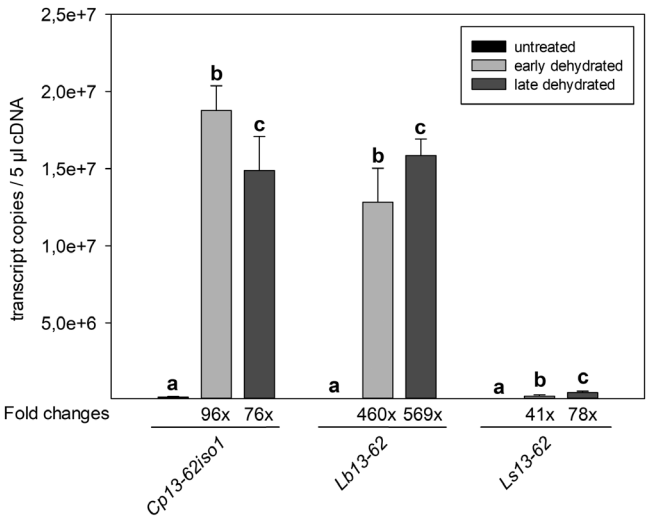

Fig. 2. Absolute quantification of *13-62* transcripts in *Craterostigma plantagineum*, *Lindernia brevidens*, and *Lindernia subracemosa* by RT–qPCR analysis. cDNA was prepared from total RNA isolated from leaves of untreated, partially dehydrated (relative water content 50–60%), and late dehydrated (relative water content 30–40%) plants and amplified using 13-62-specific primers. Transcript copy numbers were calculated from three different biological replicates (mean +SD). Different letters above bars denote statistically significant differences within each group of samples ($P<0.05$; one-way ANOVA).

Table 1. *Expression patterns of predicted 13-62 homologs in selected dicots and monocots*

Gene name	GenBank Ac.	Species	Expression	References
AT1G47980	NP_564518.1	*Arabidopsis thaliana*	Em, Fl, Po, Ro, Se, Sm; OS, SS leaves	Schmid *et al.* (2005); Brady *et al.* (2007) Kilian *et al.* (2007)
AT3G62730	NP_191832.1	*Arabidopsis thaliana*	Em, Ro, Se, Si, Sm, St; OS, SS roots	Schmid *et al.* (2005); Brady *et al.* (2007) Kilian *et al.* (2007)
Medtr2g033580	XP_003594696.1	*Medicago truncatula*	Se	Benedito *et al.* (2008)
Medtr2g033520	XP_003594692.1	*Medicago truncatula*	Se	Benedito *et al.* (2008)
Glyma15g14720	XP_003546306.2	*Glycine max*	Fl, GP, Se	Libault *et al.* (2010); Severin *et al.* (2010)
Glyma13g11550	XP_003542185.1	*Glycine max*	Po, Se	Libault *et al.* (2010); Severin *et al.* (2010)
LOC_Os03g22470	XP_015632037.1	*Oryza sativa*	Em, En, In, Ro, Se, Sg	Jain *et al.* (2007); Li *et al.* (2007)
LOC_Os04g33150	XP_015634158.1	*Oryza sativa*	Em, En, In, Ro, Se	Jain *et al.* (2007); Li *et al.* (2007)
GRMZM2g085260	NP_001149373.1	*Zea mays*	En, Ro, Se, Ta	Downs *et al.* (2013)
GRMZM2g327051	NP_001150304.1	*Zea mays*	Ta	Downs *et al.* (2013)

CS, Cold stress; Em, Embryo; En, Endosperm; Fl, Flowers; GP, Green pods; In, Inflorescence; OS, Osmotic stress; Pl, Pollen; Po, Pods; Ro, Roots; Se, Seeds; Sg, Stigma; Si, Siliques; Sm, Stamens; SS, Salt stress; St, Stem; Ta, Tassel.

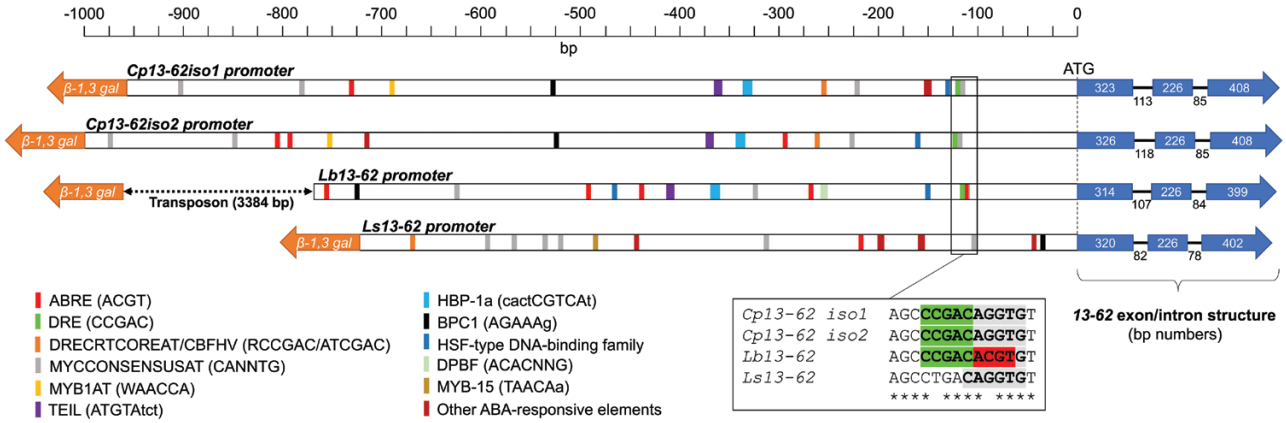

Fig. 3. Structure of the *13-62* gene locus in *Craterostigma plantagineum*, *Lindernia brevidens*, and *Lindernia subracemosa*. Putative *cis*-acting regulatory elements associated with abscisic acid (ABA)- and dehydration-responsive gene expression are indicated by coloured boxes. The box indicates the position of the conserved drought-responsive element (DRE) in the promoter of desiccation-tolerant species.

Elements that are related to ABA and drought promoter responsiveness were predicted upstream of the *Cp, Lb*, and *Ls13-62* gene translational start sites (Fig. 3). Only a few structural elements are conserved in the desiccation-tolerant species. The promoter of the desiccation-sensitive species *L. subracemosa* showed almost no conservation of *cis*-elements (Fig. 3). Most of the sequence conservation was observed within the first 170 bp upstream of the translational start site (Supplementary Fig. S6). In this region we identified a putative consensus sequence (CCGAC) for a DRE and binding sites for Myc factors (Fig. 3). The putative DRE motif is conserved in the desiccation-tolerant species *C. plantagineum* and *L. brevidens* but not in the desiccation-sensitive species *L. subracemosa*, in which a single-nucleotide variation was found (Fig. 3).

Functional analysis of Cp, Lb, *and* Ls13-62 *promoters in response to dehydration*

To test which promoter sequences are essential for transcriptional activation of the *13-62* genes, the above-described promoter regions were fused to *GUS* as a reporter gene and the promoter activities were determined. The response of the *Cp13-62iso1*, *Cp13-62iso2*, *Lb13-62*, and *Ls13-62* promoters to dehydration was evaluated by monitoring GUS accumulation in dehydrated leaf tissues transiently transformed with *Cp*, *Lb*, and *Ls13-62* promoter::*GUS* constructs. Only promoters from the desiccation-tolerant species (*C. plantagineum* and *L. brevidens*) showed activities in response to dehydration when tested in the corresponding species (Fig. 4A). We then analysed the activity of the *Ls13-62* promoter in *C. plantagineum* and the *Cp13-62* promoter in *L. subracemosa* to determine whether the lack of *Ls13-62* promoter activity upon dehydration was due to the promoter structure *per se* or the absence of transcription factors in the desiccation-sensitive species. The *Cp13-62* promoter was functional in the *L. subracemosa* background, which suggests that *trans*-acting factors are present in *L. subracemosa*. However, the *Ls13-62* promoter did not function in the *C. plantagineum* background, which indicates a lack of *cis*-elements essential for promoter activation in response to dehydration (Fig. 4B).

Next, we tested whether the conserved 170 bp region from the desiccation-tolerant promoters could drive *GUS* expression in response to dehydration. A promoter::*GUS* construct containing this region derived from the *Cp13-62iso1* promoter was sufficient to obtain dehydration-induced promoter activation in *C. plantagineum* (Fig. 4A). This fragment contains the DRE motif conserved in the tolerant species (Fig. 3). Thus, the functionality of this element was tested; promoter::*GUS*

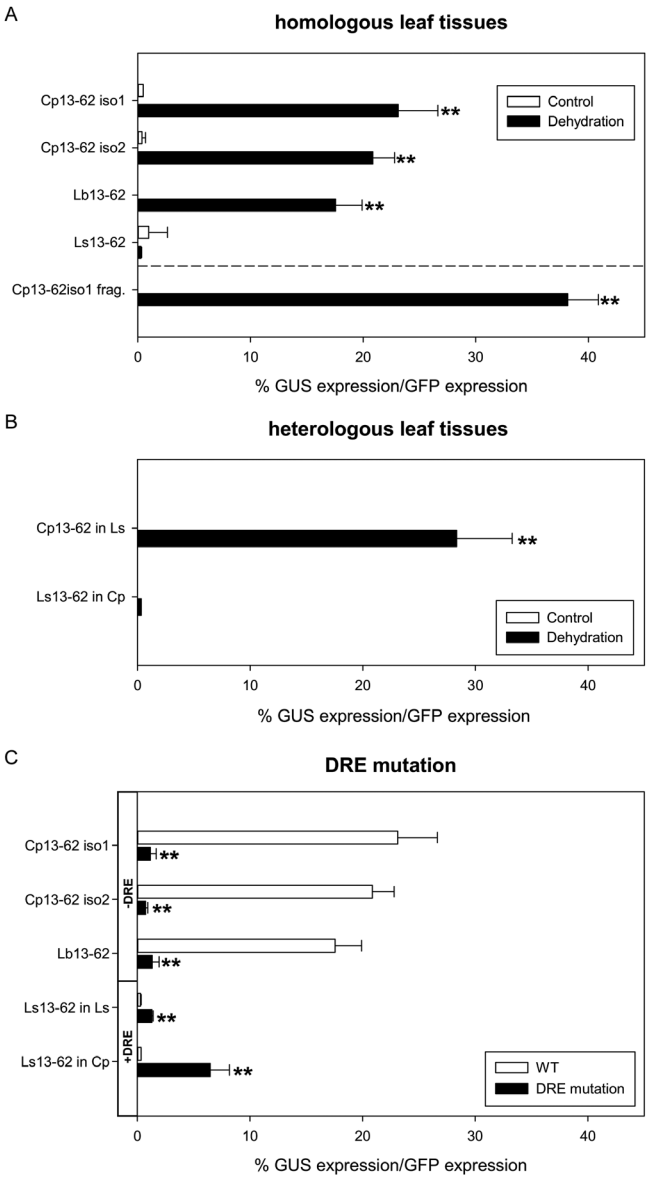

Fig. 4. Activity of the *13-62* promoter in response to dehydration. Wild-type and mutated *Craterostigma plantagineum* (Cp), *Lindernia brevidens* (Lb), and *Lindernia subracemosa* (Ls) promoter fragments were fused to the *GUS* reporter gene and tested for their activity upon dehydration in homologous and heterologous leaf tissues using a transient expression assay. (A) Response to dehydration of *13-62* wild-type promoters in homologous tissues. (B) Response to dehydration of *Cp13-62iso1* and *Ls13-62* promoters in heterologous tissues. (C) Effect of mutation of the DRE motif on activity of the *13-62* promoter in homologous and heterologous tissues. The DRE consensus sequence was either impaired (-DRE; promoters of desiccation-tolerant species) or restored (+DRE; promoter of desiccation-sensitive species) and the response to dehydration was compared using wild-type and mutated promoters. A construct expressing green fluorescence protein (GFP) under the control of the CaMV35S promoter was used to normalize all experiments. Bars indicate the relative promoter activities, expressed as a percentage of *13-62* promoter fragment activity relative to that of the CaMV35S promoter. The values are calculated from at least four independent experiments (mean +SD) for each treatment. Statistically significant differences from the control mean in (A) and (B) or wild-type expression in (C) of each promoter fragment are indicated above the error bars: **P*<0.05, ***P*<0.01 (Student's *t*-test).

constructs with mutated DRE motifs (C*C*GAC mutated to C*T*GAC in the tolerant species, or C*T*GAC into C*C*GAC in the sensitive species) were created and used in expression experiments. The mutated promoters from the desiccation-tolerant species were unable to drive dehydration-induced *GUS* expression, suggesting that this motif was essential for the dehydration response (DRE-; Fig. 4C). By contrast, when the DRE element of the desiccation-sensitive species *L. subracemosa* was restored to C*C*GAC, a small increase of promoter activity was measured in response to dehydration (DRE+; Fig. 4C). The activity increased when the *Ls13-62*(+DRE) promoter was tested in the *C. plantagineum* background, but it did not reach the same level as observed for the wild-type promoters of desiccation-tolerant species (Fig. 4C). Taken together, these results suggest that the DRE element is essential but not sufficient for dehydration-induced activation of the *13-62* promoter.

Cp, Lb, *and* Ls13-62 *promoter activities in* A. thaliana

We tested whether the *Cp*, *Lb*, and *Ls13-62* promoter::*GUS* constructs were also functional when stably integrated into the *A. thaliana* genome. Transgenic plants were assayed for GUS activity at different developmental stages and upon salt stress. Salt stress was chosen instead of dehydration because this allowed more robust testing than dehydration and because it was previously shown that *pcC13-62* accumulated to high levels following NaCl treatments (Smith-Espinoza *et al.*, 2003). The *GUS* reporter gene was expressed in transgenic seeds, suggesting promoter activation during seed development (Fig. 5). GUS activity was not detected in seedlings that were germinated under control conditions, but the *13-62* promoters from both desiccation-tolerant and desiccation-sensitive species (except for *Cp13-62iso1*) could be activated in seedlings subjected to salt stress (Fig. 5). This indicates that *trans*-acting elements from Arabidopsis that are active upon salt stress can trigger transcription of the *GUS* reporter gene from *13-62* promoters. Next, transgenic plants were grown to the flowering stage to allow assessment of whether the *13-62* promoters were activated in different plant organs under control and stress conditions. GUS staining was barely detected in plant organs from unstressed control plants (Fig. 6). When plants were subjected to salt stress, mainly leaves but also roots showed GUS accumulation. Most activity was observed in leaves under stress conditions when GUS expression was under the control of the *Lb13-62* promoter (Fig. 6). It cannot be concluded from these experiments whether other *cis*-elements as well as the DRE are involved in the response to salt stress.

Discussion

The DRP *pcC13-62* gene was originally classified among genes restricted to the resurrection plant *C. plantagineum* as no significant sequence similarities were identified (Bartels *et al.*, 1990). Advances in genome sequencing and transcriptome analyses revealed that genes similar to *pcC13-62* are present in bacteria, green algae, mosses, ferns, and angiosperms (Table 1; Fig. 1B) (Battista *et al.*, 2001; Zha *et al.*, 2013; Carniel *et al.*, 2016; Kitajima *et al.*, 2017). This observation corroborates the hypothesis that protective genes important for desiccation tolerance in resurrection plants are present in the genome of desiccation-sensitive species. All pcC13-62-related proteins

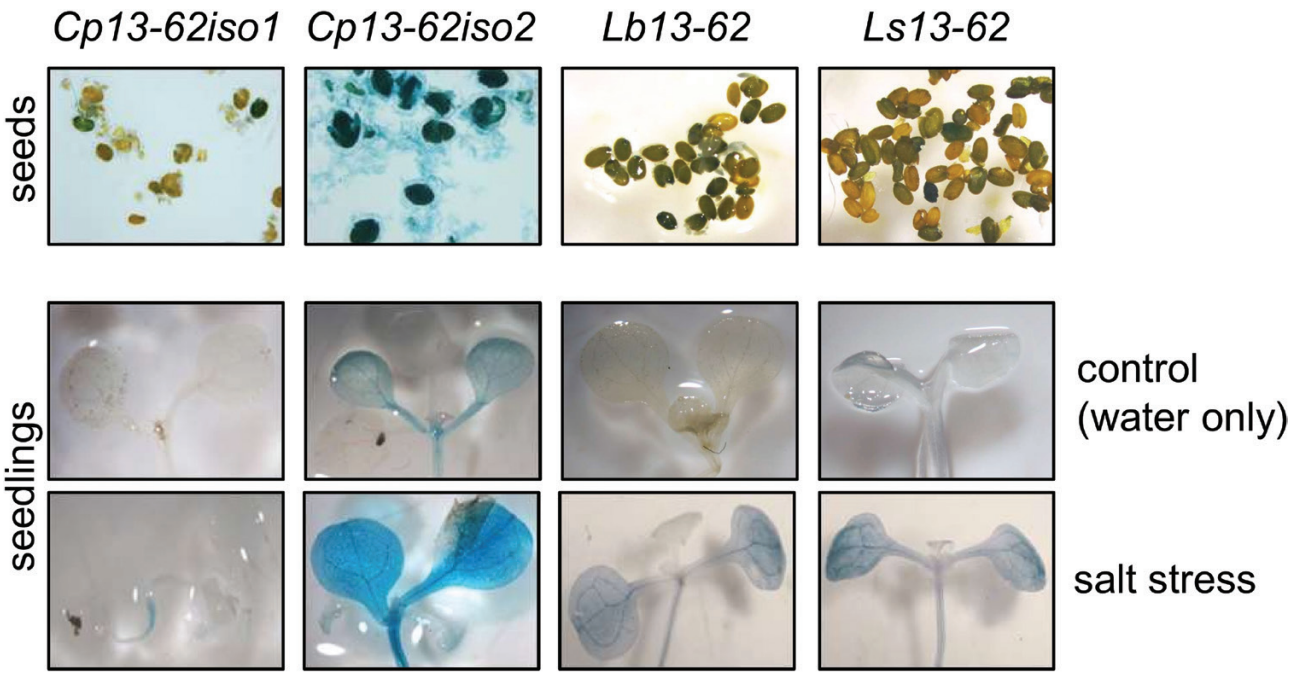

Fig. 5. Activity of the *13-62* promoter in *A. thaliana* seeds and seedlings. Seeds and seedlings from stably transformed Arabidopsis plants containing the *Cp*, *Lb*, *and Ls13-62* promoter::*GUS* constructs were analysed for *GUS* expression. Seedlings were germinated on soil and incubated with water (control) or 150 mM NaCl (salt stress) for 16 h before staining for GUS activity. Representative pictures from different lines are shown.

contain a ferritin-like domain and a conserved C-terminal domain (Carniel *et al.*, 2016). Enzymatic assays with recombinant pcC13-62-related proteins from sap of the lacquer tree *Toxicodendron vernicifluum* (TvFe2D) or floral nectar of the bean *Mucuna sempervirens* (MS-desi) support a biochemical function for this family of proteins (Zha *et al.*, 2013; Kitajima *et al.*, 2017). The C-terminal domain of TvFe2D suppresses laccase and peroxidase colorimetric reactions (Kitajima *et al.*, 2017), whereas the MS-desi protein was able to inhibit citrate synthase activity (Zha *et al.*, 2013).

The expression of *pcC13-62* and *pcC13-62*-related genes is linked to desiccation tolerance. Transcripts of *13-62* accumulated abundantly in desiccated tissues of the desiccation-tolerant plants *C. plantagineum* and *L. brevidens*, but only to a very low level in desiccated leaves of the desiccation-sensitive plant *L. subracemosa* (Fig. 2). This result could be explained by the lack of selective pressure for desiccation tolerance in the habitat of *L. subracemosa*. The correlation of *pcC13-62* and desiccation tolerance is supported by observations from several other species. Deletion of the *Deinococcus radiodurans* locus DRB0118, which encodes a *pcC13-62* homolog, was responsible for the loss of viability of desiccated cultures (Battista *et al.*, 2001). Expression of the *pcC13-62* gene increases in dehydrating leaves of the resurrection grass *O. thomaeum* (Supplementary Fig. S4). The *pcC13-62* family underwent large expansions and diversifications in the desiccation-tolerant lichen photobiont *Trebouxia gelatinosa*. Members of the *13-62* family were among the most up-regulated genes during desiccation of *T. gelatinosa* (Carniel *et al.*, 2016). One of the two *pcC13-62* homologs in *A. thaliana* (At3g62730) is specifically expressed in immature seeds and progressively accumulates during seed development (Becerra *et al.*, 2006). A *pcC13-62*–related expressed sequence tag was also found among genes expressed in spores of the aquatic fern *Ceratopteris richardii* (Salmi *et al.*, 2005). In several non-resurrection species, *pcC13-62* genes are mainly expressed in desiccation-tolerant reproductive tissues and organs, including seeds and pollen (Table 1). Taken together, these results suggest that *pcC13-62* homologs are highly expressed in bacteria, lichens, spores, seeds, pollen, and vegetative tissues of resurrection plants, and that the expression is linked to desiccation tolerance. The function of the encoded proteins might be relevant for enzymatic reactions, perhaps inhibiting enzymes that are involved in degradation processes.

The *Cp*, *Lb*, and *Ls*13-62 genes showed conserved structural features, supporting their common origin (Fig. 3). Intriguingly, we identified a transposable element similar to members of the PIF/Harbinger superfamily inserted between the *Lb13-62* and β-1,3 galactosyltransferase coding sequences. PIF/Harbinger elements are DNA-mediated elements that contain coding sequences for two proteins required for their mobilization, namely a transposase and a Myb-like domain-containing protein (Feschotte and Pritham, 2007). The element in *L. brevidens* carries mutations in the transposase coding sequence, suggesting a lack of autonomous transposition (data not shown). The *Lb13-62* promoter sequence used for the analysis in this study was restricted in its length to the region between the predicted transposon terminal inverted repeat sequence and the ATG start codon of *Lb13-62*. Our analysis showed that regulatory *cis*-elements important for dehydration-induced activation appear to be within this sequence (Fig. 4).

Cp, *Lb*, and *Ls13-62* promoter activities correlated with transcript expression levels, suggesting that expression is regulated mostly at the transcriptional level. *Cp* and *Lb13-62* promoters showed comparable dehydration-induced activities, whereas the *Ls13-62* promoter could not be activated upon dehydration (Fig. 4A). Promoter activity is the result

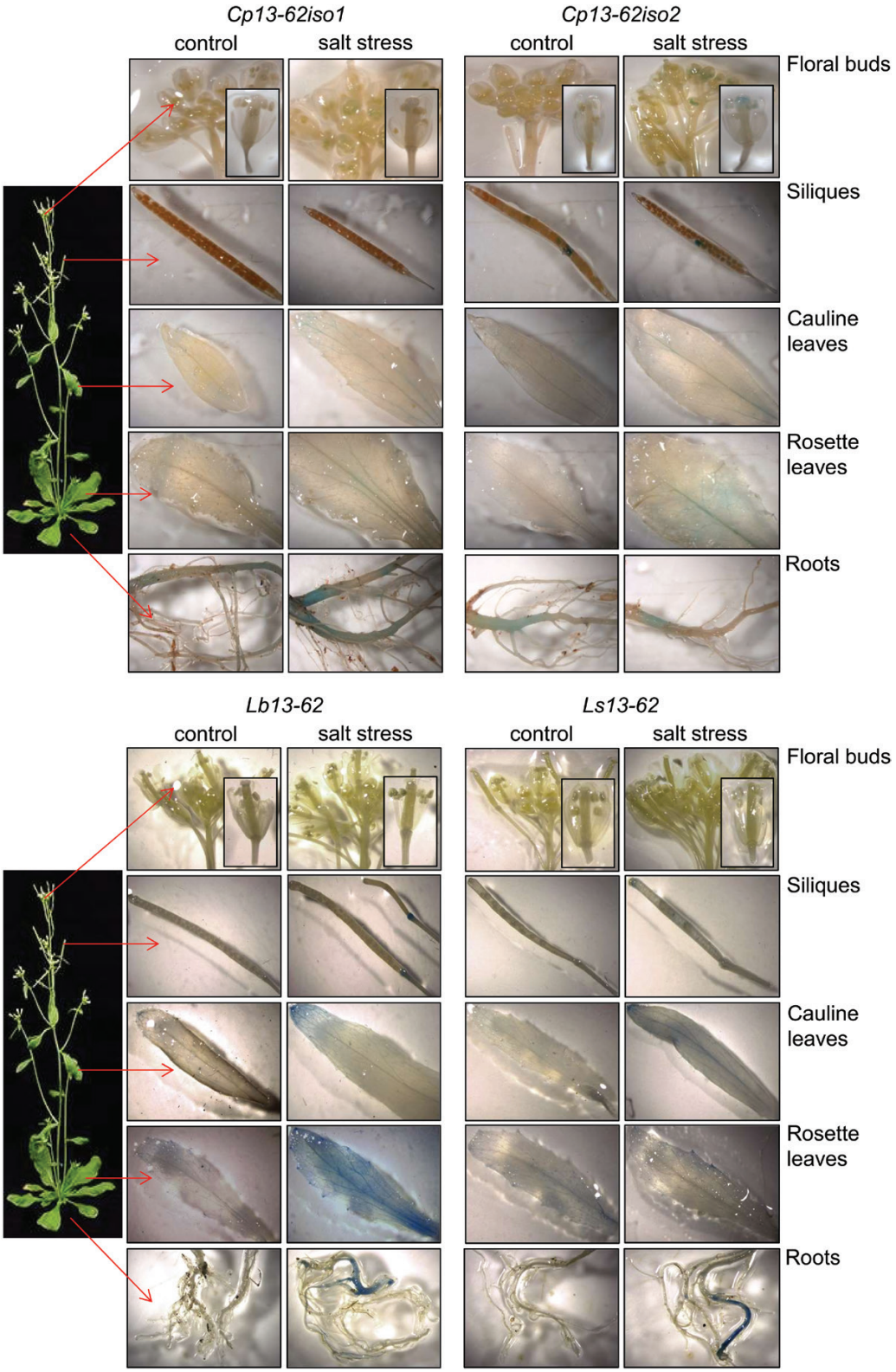

Fig. 6. Activity of the *13-62* promoter in untreated and stressed *A. thaliana* plants. Stably transformed Arabidopsis plants containing the *Cp*, *Lb*, *and Ls13-62* promoter::*GUS* constructs were watered for 2 weeks with water (control) or 150 mM NaCl (salt stress), and tissues were subsequently analysed for GUS expression. Different lines were analysed and representative pictures of plant organs stained for GUS activity are shown.

of the interaction of *trans*-acting binding elements with their corresponding *cis*-acting elements. Since the promoter from *C. plantagineum* was active in dehydrated *L. subracemosa* leaves but the *L. subracemosa* promoter was not functional in dehydrated *C. plantagineum* leaves, the lower activity in *L. subracemosa* can be explained by the lack of essential *cis*-acting elements (Fig. 4B). The availability of *trans*-acting elements required for promoter activation in response to dehydration

but a lack of proper *cis*-acting elements was also demonstrated for the *CDeT11-24* gene in *L. subracemosa* (van den Dries *et al.*, 2011). Transcriptomic studies suggest that most dehydration-induced protective genes of resurrection species are similar to genes whose transcripts accumulate abundantly during the acquisition of desiccation tolerance in seeds (Rodriguez *et al.*, 2010; Costa *et al.*, 2017). *pcC13-62* homologs from non-desiccation-tolerant plants are mainly expressed in seeds, and the *Cp*, *Lb*, and *Ls13-62* promoters showed activity in Arabidopsis seeds. We hypothesize that regulatory elements for seed-specific expression are conserved among species. Hence, *pcC13-62* must be linked to desiccation tolerance, and the (re-)activation of *pcC13-62* expression in vegetative tissues may have contributed to the (re-)establishment of desiccation tolerance in resurrection plants.

Promoters of dehydration-induced genes contain conserved dehydration-responsive *cis*-acting DNA elements such as DRE-element/C-repeat (DRE/CRT) and ABA-response elements (ABRE) (Nakashima and Yamaguchi-Shinozaki, 2010). *In silico* analysis revealed the presence of several ABA- and dehydration-related *cis*-elements in the *Cp*, *Lb*, and *Ls13-62* promoters. However, the spatial organizations of these *cis*-elements mainly differ, indicating limited conservation of the promoter architecture among the three species (Fig. 3). However, some elements, such as TEIL, HBP-1a, HSF, DRE, and MYC, were found in the promoters of the two desiccation-tolerant species (Fig. 3). The first 170 bp upstream of the ATG start codon shows the highest conservation across the three species and it is sufficient for dehydration-responsive gene activation. This sequence contains the only DRE element in the desiccation-tolerant species *C. plantagineum* and *L. brevidens*, and the deletion of the DRE element almost completely abolished dehydration-induced promoter activation (Fig. 4C). In *L. subracemosa*, a single-nucleotide mutation in the corresponding promoter region suppressed part of the dehydration responsiveness. When the DRE element was introduced into the *L. subracemosa* promoter, dehydration responsiveness was partially restored (Fig. 4C). Therefore, we assume that other *cis*-acting elements in the first 170 bp are possibly missing in the *L. subracemosa* promoter and may work as coupling elements for the DRE. The importance of the DRE element in the promoter of desiccation-tolerant species has previously been demonstrated for the CDeT11-24 promoter. The CDeT11-24 gene requires the DRE element for high promoter activity upon stress (van den Dries *et al.*, 2011). It has been hypothesized that desiccation tolerance in vegetative plant tissues (re-) evolved through mutations in the regulatory sequences of desiccation-related genes (van den Dries *et al.*, 2011). Here, we provide support for this hypothesis by showing that the desiccation-related *pcC13-62* gene from desiccation-tolerant Linderniaceae was reprogrammed to be expressed at high levels in vegetative tissues. Similar to the *11-24* gene, a DRE motif is involved in dehydration-induced gene expression in the *pcC13-62* promoter. This allows us to conclude that such elements, in combination with unknown coupling elements, are important for the regulation of desiccation-tolerance genes in resurrection species.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. List of primers used in this study.

Fig. S1. Specificity of the primer pairs used to amplify *13-62* in RT–qPCR amplifications.

Fig. S2. RT–PCR analysis of *Craterostigma plantagineum*, *Lindernia brevidens*, and *Lindernia subracemosa 13-62* expression in response to salt and osmotic stress.

Fig. S3. Alignment of amino acid sequences of *13-62* protein homologs in selected Embryophyta.

Fig. S4. RT–PCR analysis of *Oropetium thomaeum 13-62* (*Ot13-62*) expression during dehydration and rehydration.

Fig. S5. Sequence of the transposable element identified in the *Lb13-62* genomic locus.

Fig. S6. Putative *cis*-acting elements identified in the *13-62* promoter.

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