Comparative and functional promoter analysis of desiccation-related genes from three closely related plant species differing in desiccation tolerance

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To my parents

Je kijkt naar me
zoals mijn vader vroeger keek

Hij zei niets
maar het was net
of hij iets wilde zeggen

Je blik is me
vreemd vertrouwd
nee, je hoeft niets uit te leggen

Ik weet
dat je nog van me houdt

Ton van den Dries

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Abbreviations

3-AT	3-amino-1,2,4-triazole	kDa	KiloDalton
A (1)	Adenine	l	Liter
A (2)	Absorbance	*Lb*	*Lindernia brevidens*
ABA	Abscisic acid	LB	Luria Bertani media
ABF	ABRE-binding factor	LEA	Late embryogenesis abundant
ABRE	ABA-responsive elements	Leu	Leucine
AD	Activation domain	*Ls*	*Lindernia subracemosa*
At	*Arabidopsis thaliana*	m	Meter
bp	Nucleotide base pair	M	Molar concentration
BSA	Bovine serum albumin	miRNA	MicroRNA
bZIP	Basic-domain leucine zipper	MOPS	3-(N-morpholino) propanesulfonic acid
C	Cytosine	MPa	MegaPascal
c	Concentration	mRNA	Messenger ribonucleic acid
CaMV	Cauliflower mosaic virus	MS	Murashige and Skoog
cDNA	Complementary DNA	nt	Nucleotide
CE	Coupling element	oilgo(dT)	Oligodeoxythymidylic acid
ChIP	Chromatin immunoprecipitation	PCR	Polymerase chain reaction
Cp	*Craterostigma plantagineum*	PEG	Polyethylene glycol
CRT	C-repeat	pGAL	*GAL* promoter
CTAB	Cetyl trimethyl ammonium bromide	PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
dATP	Deoxyadenosine triphosphate	PVP	Polyvinylpyrrolidone
dCTP	Deoxycytidine Triphosphate	RNA	Ribonucleic acid
DEPC	Diethyl pyrocarbonate	Rnase	Ribonuclease
dGTP	Deoxyguanosine triphosphate	rpm	Revolutions per minute
DMF	N,N-dimethylformamide	RT	Reverse transcriptase
DMSO	Dimethyl sulfoxide	SC	Synthetic complete
DNA	Deoxyribonucleic acid	SD	Standard deviation
Dnase	Deoxyribonuclease	SDS	Sodium dodecyl sulfate
dNTPs	Deoxynucleotide triphosphate	SSC	Saline sodium citrate
DPBF	Dc3 promoter-binding factor	T	Thymine
DRE	Dehydration-responsive element	Ta	Annealing temperature
DREB	DRE-binding protein	TAE	Tris-acetate-EDTA
DSP	Desiccation stress protein	TE	Tris-EDTA
dTTP	Deoxythymidine triphosphate	TKT3	Transketolase 3
dw	Dry weight	Tm	Melting temperature
E/m2/s	Einsteins per square meter per second	Tris	Tris(hydroxymethyl)aminomethane
EDTA	Ethylenediaminetetraacetic Acid	Triton X-100	Polyethylene glycol p-iso-octylphenyl ether

ELIP	Early light-inducible protein	Trp	Tryptophan
EMSA	Electrophoretic mobility shift assay	U	Unit
EST	Expressed sequence tag	UV	Ultraviolet
EtBr	Ethidium bromide	V	Volts
fw	Fresh weight	v/v	Volume/volume
g	Gram	w/v	Weight/volume
G	Guanine	WC	Water content
GFP	Green fluorescent protein	X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
GUS	β–glucuronidase	X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
His	Histidine	Y1HS	Yeast one hybrid system
IPTG	Isopropylthio-b-D-galactoside	YEPD	Yeast extract peptone dextrose
kb	Kilobase		

1. Introduction

1.1. Water is essential for plants

Water plays a crucial role in plant growth and development. It is involved in many important biological processes, like photosynthesis and nutrient transport. Non-woody plants are primarily composed of water. In higher plants, water is mainly taken up from the soil through the roots and released for the most part by transpiration through stomata in the leaf surface.
In order to extract water from the soil, the water potential in the roots must be more negative than the water potential in the soil. Water molecules always have the tendency to move across a cell wall or cell membrane from an area of higher water potential to an area of lower water potential. Water potential is dependent on pressure potential and osmotic potential.
After absorption by the roots, water is transported through the xylem to the leaves, where the process of photosynthesis takes place. Water is lost in the leaves by transpiration or during photosynthesis when water is split into oxygen. However, some of the water that remains in the leaves is used to transport sugars, which are produced during photosynthesis, through the phloem to other parts of the plant.

1.2. Water deficit affects plant growth

In periods of drought, the soil dries out and the soil water potential becomes more negative. During a drought, the plant must therefore be able to reduce the water potential in order to absorb water from the dry soil. A molecular response that allows plants to lower their water potential is osmotic adjustment (Bray, 1993). The synthesis of osmolytes, like compatible solutes generates a more negative osmotic potential and thereby decreasing the water potential (Hanson *et al*., 1982). Furthermore, compatible solutes are also associated with protective functions, such as protein stabilization (Hoekstra *et al*., 2001).
Plants have developed several other adaptive strategies to avoid water stress. One of them is the closing of stomata. Stomatal closure decreases transpiration and thereby minimizes the loss of water. However, closed stomata prevent the plant to take up more carbon dioxide from the atmosphere and this affects the rate of photosynthesis. Thus, water availability is a limiting factor for photosynthesis and affects the energy production. When a plant is no longer able to extract water from the soil the wilting

point is reached. This means that the plant is not able to maintain the turgor pressure of its cells. The cell turgor pressure is essential for cell expansion and growth.
Hence, water deficit has a negative effect on plant growth and biomass production. Even though, most plants are able to withstand mild water deficit, a prolonged period of severe drought is lethal to most plants.

1.3. Water scarcity is posing a threat for agriculture and food security

Drought has a huge impact on agriculture. Since water stress is a major factor affecting crop yield. To date, water shortage is already a worldwide problem and this problem is only getting larger. Water is becoming scarce in many parts of the world, especially in developing countries as can be seen in Figure 1 (Comprehensive Assessment of Water Management in Agriculture, 2007).
One-fifth of the world's population (more than 1.2 billion people) is living in countries where there is physical water scarcity, which means that there is an imbalance between water availability and demand (Comprehensive Assessment of Water Management in Agriculture, 2007). In addition, about 1.6 billion people are living in areas that are approaching a physical water scarcity in the near future. Limited water availability for agricultural purposes will have a significant impact on food production. It has been estimated by the Food and Agriculture Organization (FAO) of the United Nations that approximately 850 million people worldwide are undernourished (FAO, 2006). This number will certainly rise when water availability is anticipated to increase as water availability decreases in more global areas.
Another contributory to water scarcity and food shortage is the expansion of the biofuel market (FAO, 2008). To meet increasing energy demands, it is predicted that increased areas will be used to grow biofuel crops in the future. The growing production of biofuels will further increase the demand for water. Currently, already many biofuel crops are grown in areas of the world where water is already scarce. Eventually, it is very likely that biofuel and food crops will have to compete for the available water. Additionally, future effects of climate change will probably have a negative impact on water resources and could make the situation of water shortage even worse. So, in the future, water availability will pose a real threat on food security and could lead to a severe food crisis in several regions of the world. Hence, it is of extreme importance to increase water productivity in agriculture.

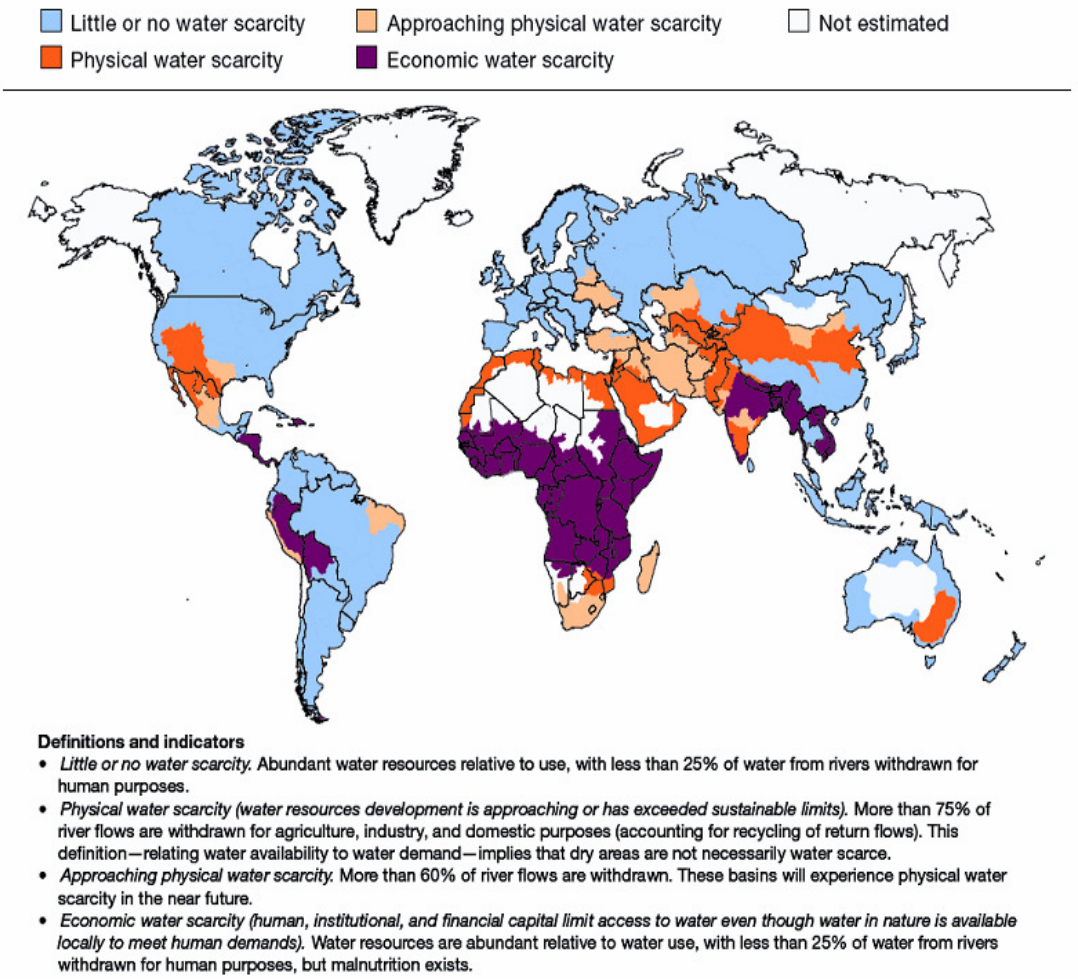


Figure 1. Water scarcity in different regions of the world (Source of the picture: Comprehensive Assessment of Water Management in Agriculture, 2007).

Optimization of irrigation management practices will contribute to decreased water use in agriculture. A complementary approach is the development of more dehydration tolerant crops varieties. Improved dehydration tolerance of agriculturally relevant plants would reduce the demand for water and potentially increase crop yield in dry areas. The enhancement of dehydration tolerance in major crop plants could be achieved by conventional breeding methods or with the help of biotechnology tools. However, knowledge about plant responses to water stress is essential to be able to successfully enhance dehydration tolerance of crops. Understanding of mechanisms of desiccation tolerance can be of great value for the development of dehydration tolerant crops.

1.4. Resurrection plants as model systems to study desiccation tolerance

Resurrection plants are useful models to study desiccation tolerance mechanisms, because these plants have the remarkable ability to survive desiccation of their vegetative tissues (Gaff, 1971). Desiccation is described as less than 0.1 g of water per 1 g of dry weight (Wood and Jenks, 2007). Vegetative desiccation tolerance is only found in some members of angiosperm families (Oliver *et al*., 2000). There are several different types of resurrection plants, like mosses and ferns. Most resurrection plants grow in areas with seasonal water availability and many desiccation tolerant plants are found in the semi-arid regions of southern Africa (Moore *et al*., 2009). In dry conditions, resurrection plants equilibrate the water content in their leaves to the relative humidity of the air. Some resurrection plants are even able to withstand complete desiccation and survive in dry air with a relative humidity of 0% (Gaff, 1987). During desiccation, resurrection plants curl their leaves and keep them in an air-dried state until water becomes available. After rehydration, desiccated resurrection plants are able to recover their tissue and resume full physiological activity.

Resurrection plants can be differentiated into two types: homoiochlorophyllous and poikilochlorophyllous, respectively (Tuba *et al*., 1998). The homoiochlorophyllous desiccation tolerant plants retain their chlorophyll and keep their photosynthetic structures intact during desiccation. This gives them the ability to recover rapidly when water becomes available, but light absorption during water stress might result in the formation of reactive oxygen species. Therefore, homoiochlorophyllous resurrection plants must have protective mechanisms against these reactive oxygen species. Poikilochlorophyllous resurrection plants lose their chlorophyll during dehydration. This means that upon rehydration, poikilochlorophyllous desiccation tolerant plants need to resynthesize their photosynthetic system.

Most resurrection plants are sensitive to the rate at which cellular water is lost (Oliver and Bewley, 1997). Rapid desiccation of tissue is lethal to the majority of resurrection plants. Gradual dehydration is crucial for most resurrection plants in order to protect themselves against damage caused by desiccation. This means that these resurrection plants need time to switch on a protective gene expression program. Resurrection plant species employ different mechanisms to survive desiccation of their vegetative tissues (Moore *et al*., 2009).

1.5. The resurrection plant *Craterostigma plantagineum*

The homoiochlorophyllous resurrection plant *Craterostigma plantagineum* Hochst. is one of the most extensively studied resurrection plants (Bartels, 2005). The species *C. plantagineum* is a member of the Linderniaceae family in the order Lamiales (Rahmanzadeh *et al*., 2005). This plant is mainly distributed in eastern and southern Africa, where it grows in areas with highly variable water availability, such as seasonally filled pools and rocky outcrops (Fischer, 2004; Fischer, 1992).
The molecular basis of desiccation tolerance in *C. plantagineum* has been examined in various studies. For example, the expression of genes linked with desiccation tolerance has been studied (Rodrigo *et al*., 2004; Bockel *et al*, 1998). The promoters sequences of a number of desiccation-related genes from *C. plantagineum* have been analyzed (Ditzer and Bartels, 2006; Hilbricht *et al*., 2002; Michel *et al*., 1994). Furthermore, proteomic studied of post-translational modifications, such as dehydration-induced protein phosphorylation, have been conducted in *C. plantagineum* (Röhrig *et al*., 2008; Röhrig *et al*., 2006). Results from these studies demonstrated that the resurrection plant *C. plantagineum* is an excellent model system for obtaining more insight into the mechanisms of desiccation tolerance. In addition, *C. plantagineum* is such a good model system, because desiccation tolerance can be studied in both callus tissue and the whole plant.

1.6. Desiccation of seeds

The ability to withstand severe dehydration can, however, also be found in plant embryos from many types of desiccation sensitive plants. Seeds that have the ability to survive drying are also called “orthodox seeds”. When orthodox seeds undergo maturation, normally after completion of morphogenesis, most water is lost from the seed tissue due to the accumulation of dry matter. During the final stages of seed development, the plant embryos acquire desiccation tolerance (Golovina *et al*., 2001). Mature orthodox seeds contain 5% to 10% of water and many of these seed can even reach water levels of only 1% to 5% (Manfre *et al*., 2009).
Various protection mechanisms are important for the acquisition of desiccation tolerance and most mechanisms are involved in the stabilization of protein and cell structures during water loss (Hoekstra *et al*., 2001). Orthodox seeds and desiccation tolerant plants use very similar cellular mechanisms for desiccation tolerance. During dehydration, cells undergo structural and biochemical changes to protect themselves against damaging effects of desiccation, such as the accumulation of polysaccharides

and synthesis of hydrophilic proteins with protective functions (Golovina *et al*., 2001). During final stages of seed development, orthodox seeds eventually enter into a metabolically quiescent state and are able to maintain this dormant state for years. The desiccated seeds, however, remain viable and under suitable conditions dry seeds take up water and subsequently germinate (Bewley, 1997). Even though, seed desiccation tolerance is observed in many types of plants, the ability to tolerate desiccation of vegetative tissues is only found in the so-called resurrection plants.

1.7. Evolution of vegetative desiccation tolerance.

It is very likely that mechanisms of desiccation tolerance have evolved in primitive plants, bryophytes. Bryophytes are non-vascular land plants and can be classified in three separate groups: (1) liverworts, (2) hornworts and (3) mosses. Bryophytes were the first plants that moved from water and colonized the land (Mishler and Churchill, 1985). It is believed that higher land plants have evolved from bryophytes. The colonization of land meant that bryophytes needed to adapt to life in this relatively dry environment and this resulted in evolutionary changes (Charron and Quatrano, 2009; Floyd and Bowman, 2007). The initial evolution of vegetative desiccation tolerance was probably essential for bryophytes to colonize the land. The earliest mechanisms of desiccation tolerance were very similar to those found in current desiccation tolerant bryophytes. It has been hypothesized that mechanisms of desiccation tolerance that are observed in seeds have evolved from these primitive mechanisms of vegetative desiccation tolerance (Oliver *et al*., 2000).
Eventually, higher plants lost the ability to tolerate desiccation in their vegetative tissue. Desiccation tolerance was probably lost, because it was no longer required or not efficient to maintain. There appears to be a trade-off between desiccation tolerance and growth (Alpert, 2006). Protection against desiccation is energetically very costly. Metabolic rates in desiccation tolerant plants are much lower as compared to those in desiccation sensitive plants. However, it is thought that desiccation tolerance re-evolved independently in some plants species, the modern-day resurrection plants (Proctor *et al*., 2007). This hypothesis means that genes involved in the acquisition of desiccation tolerance are also present in most desiccation sensitive plant species.

1.8. Protection mechanisms against desiccation damage

During desiccation, resurrection plants have to cope with different types of stress, which have been divided into three categories: (1) mechanical stress causes by the loss

of turgor pressure, (2) disturbance of cell membrane integrity through the disruption of phospholipids in cell membranes and (3) interruption of metabolism caused by oxidative stress (Vicre *et al*., 2004). The resurrection plant *C. plantagineum* has developed several protection mechanisms against the different categories of damage.

1.8.1. Late embryogenesis-abundant proteins

Desiccation tolerance in *C. plantagineum* has been associated with the accumulation of late embryogenesis-abundant (LEA) proteins (Bartels and Salamini, 2001). LEA proteins were discovered more than two decades ago in the embryos of cotton (Galau *et al*., 1986). In the meantime, many LEA proteins have been identified in various plant species. In many types of higher plants, LEA proteins are related with the development of desiccation tolerance in seeds. LEA proteins accumulate at high levels in seeds at the later stages of embryo development. This increase in LEA proteins occurs just before the seed begins to dry out.

In *C. plantagineum*, LEA proteins accumulate abundantly in different vegetative tissues during dehydration (Bartels, 2005). The exact functions of LEA proteins in the process of desiccation tolerance still remain to be elucidated, the accumulation of LEA proteins is linked with the establishment of desiccation tolerance (Close, 1996). It is generally accepted that LEA proteins have protective roles against desiccation damage (Hoekstra *et al*., 2001). LEA proteins share several common features, such as high hydrophilicity, thermal stability and unordered structures in solution (Battaglia *et al*., 2008). Based on expression patterns, amino acid and structure similarities, LEA proteins have been divided into different groups (Dure, 1993; Wise and Tunnacliffe, 2004). To date, at least six groups of LEA proteins have been described (Battaglia *et al*., 2008). The main characteristics of each group of LEA proteins are described below and listed in Table 1

Group 1 LEA proteins are extremely hydrophilic and even remain soluble after boiling. Therefore it is speculated that these proteins have a disordered structure in solution. LEA proteins from group 1 harbor a conserved motif of twenty amino acids (TRKEQ[L/M]G[T/E]EGY[Q/K]EMGRKGG[L/E]) that is very hydrophilic. The LEA proteins from group 1 have also been found in different soil bacterial species. In plants, LEA group 1 proteins accumulate specially during seed development.

Group 2 LEA proteins are mainly found in plants and are also called “dehydrins”. This group of LEA proteins is well characterized in plants. Groups 2 LEA proteins are highly hydrophilic and contain three conserved amino acid sequence motifs: (1) The Y-segment ([V/T]D[E/Q]YGNP), (2) the S-segment (a serine-rich region) and (3) a lysine-rich motif of a fifteen amino acids (EKKGIMDKIKEKLGP), named the K-

segment. The K-segment is principally located near the carboxyl-terminus and is predicted to form amphipathic α–helices.

Group 3 LEA proteins are characterized by an 11-amino acid motif, which is repeated within the protein sequence (Dure, 2001). It is assumed that this 11-mer motif can form an amphipathic α–helix. However, variability in the 11-mer motif has been observed and therefore LEA group 3 proteins are further divided into subgroups.

Group 4 LEA proteins can be found in many types of plants and are characterized by a conserved amino acid motif at their amino-terminus (AQEKAEKMTA[R/H]DPXK EMAHERK[E/K][A/E][K/R]), which is predicted to form amphipathic α–helical structures. At their carboxyl-terminus, LEA group 4 proteins are unstructured. Other motifs can be found in sequences of LEA proteins from group 4 and therefore these LEA proteins are also subdivided into subgroups.

Group 5 LEA proteins are thermally unstable and are not resistant to high temperature treatment and compared to LEA proteins from other groups, group 5 LEA proteins contain more hydrophobic amino acids. Consequently, all LEA proteins that have a high proportion of hydrophobic residues are classified in this group.

Group 6 LEA proteins are distinguished by their relatively small size (approximately 7 to 14 kDA). Furthermore, LEA proteins from group 6 contain four distinct motifs, from which two are highly conserved.

Some other hydrophilic proteins are considered as members of the LEA protein family and are classified as group 7 LEA proteins (Battaglia *et al*., 2008). However, the group 7 LEA proteins contains only a few proteins and therefore the characteristics of proteins belonging to this group are not further described here.

Table 1. Main characteristics of LEA protein groups.

Name of group	Characteristics
Group 1 LEA proteins	Conserved 20-mer amino acid sequence motif: (TRKEQ[L/M]G[T/E]EGY[Q/K]EMGRKGG[L/E])
Group 2 LEA proteins	Three conserved amino acid sequence motifs: (1) Y-segment: ([V/T]D[E/Q]YGNP) (2) S-segment: (a serine-rich region) (3) Lysine rich 15-mer sequence motif: (EKKGIMDKIKEKLGP)
Group 3 LEA proteins	A repeating 11-mer amino acid sequence motif
Group 4 LEA proteins	Conserved amino acid motif at their amino-terminus: (AQEKAEKMTA[R/H]DPXKEMAHERK[E/K][A/E][K/R])
Group 5 LEA proteins	High content of hydrophobic amino acid residues Thermally unstable
Group 6 LEA proteins	Small in size (approximately 7 to 14 kDa) Four distinct amino acid motifs, from which two are highly conserved

1.8.1.1. Expression patterns of genes encoding LEA proteins

Members of LEA protein families show similar properties and also most genes encoding LEA proteins have similar expression patterns. It is known that the expression of genes for different types of LEA or LEA-like proteins is induced in *C. plantagineum* leaves by dehydration in vegetative tissues (Bartels, 2005). For example, the transcript encoding the LEA-like 11-24 protein was found to accumulate in *C. plantagineum* leaves during desiccation (Bartels *et al*., 1990). The activity of the *C. plantagineum LEA-like 11-24* promoter was studied experimentally in transgenic *Arabidopsis* and tobacco plants and this promoter showed responsiveness to the plant stress hormone abscisic acid (ABA) and dehydration (Velasco *et al*., 1998). Velasco *et al*. (1998) showed that the amino acid sequence of the LEA-like 11-24 protein of *C. plantagineum* displayed high sequence similarity to two desiccation-responsive proteins from *Arabidopsis*: RD29A (responsive to dehydration 29 A) and RD29B, respectively. The highest degree of protein sequence identity (37%) was found between the LEA-like 11-24 protein and the RD29B protein. Both *RD29A* and *RD29B* genes encode for hydrophilic proteins (Yamaguchi-Shinozaki and Shinozaki, 1993).

The accumulation of hydrophilic proteins RD29A and RD29B in response to water stress was observed in *Arabidopsis thaliana*. Nakashima *et al*. (2006) showed that

transcripts encoding for RD29B were highly abundant in dry *A. thaliana* seeds. Furthermore, it was demonstrated that the expression of *RD29A* and *RD29B* in 2-day-old seedlings and 2-week-old plants was induced by exposing the plants to dehydration. In addition, *RD29A* and *RD29B* expression could be induced by ABA and salt treatment (Nakashima *et al*., 2006).
Dehydration also induced the expression of LEA proteins in non-plant species, like the anhydrobiotic nematode *Aphelenchus avenae* and some microorganisms (Browne *et al*., 2002; Dure, 2001). The observation that LEA proteins also accumulate under water deficit stress conditions in these non-plant species indicates that plants, invertebrates and microorganisms use common protective mechanisms against desiccation and that LEA proteins are playing important roles in these mechanisms. The findings that the expression of genes encoding LEA proteins is up-regulated in different organisms by dehydration provide support that these proteins are involved in the adaptation to water stress.

1.8.1.2. LEA proteins and their roles in desiccation tolerance

It is generally accepted that LEA are essential for the acquisition desiccation tolerance. Even though, the precise functions of LEA proteins remain so far unknown, potential roles of LEA proteins in desiccation tolerance have been hypothesized.
It has been proposed that LEA proteins play fundamental roles in the protection of cell structures, like cell membranes, against dehydration damage (Hoekstra *et al*., 2001). It is assumed that LEA proteins can bind water and function as water replacement molecules under drought conditions (Dure, 1993). Furthermore, it appears that LEA proteins support the stabilization of other proteins, like enzymes, during water stress (Tunnacliffe and Wise, 2007). LEA proteins are probably important for the protection of enzyme functionality during stress. It was reported that some dehydrins can provide protection to other proteins against freezing (Bravo *et al*., 2003; Sánchez-Ballesta *et al*., 2004). Reyes *et al*. (2005) showed in an *in vitro* enzymatic assay that LEA proteins were able to protect the enzymatic activity of the enzymes malate dehydrogenase and lactate dehydrogenase during water stress. There is evidence that LEA proteins function as chaperones and are capable to prevent protein aggregation due to desiccation (Goyal *et al*., 2005).
Although, most LEA proteins do not have a secondary structure in solution, it has been predicted that these LEA proteins change confirmation and become more structured on dehydration (Wise and Tunnacliffe, 2004). This change in confirmation enables the LEA proteins to perform their protective functions. The protective roles of LEA

proteins against desiccation stress have been supported by transgenic expression studies. For example, improved tolerance to dehydration was observed in transgenic rice, expressing a LEA protein from barley (Xu *et al*., 1996). Hence, the production of LEA proteins seems to be crucial for the acquisition of desiccation tolerance

1.8.2. Carbohydrate metabolism

It has been hypothesized that changes in carbohydrate metabolism are involved in the acquisition of desiccation tolerance in *C. plantagineum* (Bartels and Sunkar, 2005). During desiccation, the C8 sugar 2-octulose, which is present in large amounts in the leaves of *C. plantagineum*, is converted into sucrose (Bianchi *et al*., 1991). After desiccation, sucrose makes up around 40% of the dry weight of the dehydrated plant. Upon rehydration of the desiccated plant, the process is reversed and sucrose is converted back into 2-octulose (Figure 2).

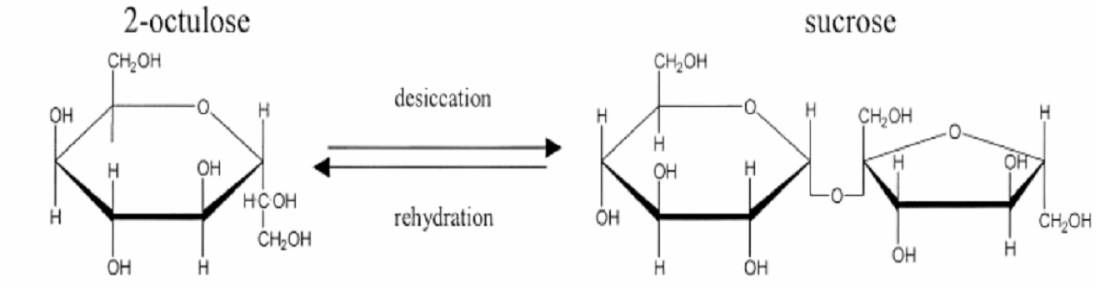


Figure 2. Carbohydrate conversion in *C. plantagineum* leaves upon desiccation and rehydration. In untreated leaves the sugar 2-octulose is present in high concentrations. Upon desiccation this sugar is converted into sucrose. In dehydrated leaves sucrose is the most abundant carbohydrate. When the plant is rehydrated, sucrose is converted back into 2-octulose (Figure modified from Bartels and Salimini, 2001).

The exact metabolic pathways for conversion of 2-octulose into sucrose and visa-versa conversion still have to be resolved. It has been described that the expression of two classes of transketolases was increased in *C. plantagineum* during rehydration (Bernacchia *et al*., 1995). Based on these expression pattern and enzymatic assays, it has been assumed that these transketolases are involved in the conversion of sucrose into 2-octulose (Willige *et al*., 2008).

In other species of resurrection plants, the accumulation of the sugar trehalose has been reported (Drennan *et al*., 1993). The resurrection plant *Myrothamnus flabellifolius* accumulated a small amount of trehalose in its leaves during desiccation. Consequently, the accumulation of carbohydrates in the absence of water has been correlated with the protection against desiccation stress. It is believed that sugars support the formation of

an intracellular glass (Koster, 1991). The glass forming properties of carbohydrates may, for example, protect phospholipid bilayers against the damaging effects of water stress (Crowe *et al*., 1992). Furthermore, it has been suggested that an interaction between LEA proteins and sugars, which results in the formation of a tight hydrogen bonding network, stabilizes cellular structures (Wolkers *et al*., 2001).
However, the exact relationship between carbohydrates and desiccation tolerance is still not clear, as it has also been shown that seeds are able to acquire desiccation tolerance without the presence of sugars (Black *et al*., 1999). Despite the fact that the precise role of sugars in desiccation tolerance is not yet fully understood, it is generally accepted that carbohydrates have an important function in the process of desiccation tolerance.

1.8.3. Effects of ABA on desiccation tolerance

The plant hormone ABA has a role in various developmental and physiological processes in plants. This phytohormone controls, for instance, seed development and maintenance of seed dormancy (Bewley, 1997). Four decades ago, it was discovered that ABA induces stomatal closure and thereby preventing water loss (Mittelheuser and Steveninck, 1969). Years later, it was reported that the production of ABA in roots was induced in response to dehydration (Zhang and Davies, 1987). Nowadays, ABA has a well established role in responses to abiotic-stress, such as drought.
The hormone ABA act as endogenous messenger and regulates plant responses to drought stress (Christmann *et al*., 2006). During soil water deficit, the concentration of ABA in the roots system dramatically increases. Signaling pathways that result in the accumulation of ABA still remain unknown. It is speculated that changes in cell turgor pressure can lead to the accumulation of ABA (Verslues and Zhu, 2005). The accumulation of ABA under drought stress is probably controlled by induction of ABA synthesizes and the inhibition of ABA degradation.
In higher plants, it is assumed that two pathways can lead to the synthesis of ABA (Seo and Koshiba, 2002). Either, ABA is synthesized directly from the C_{15} compound farnesyl pyrophosphate or it is produced indirectly by the cleavage of the C_{40} carotenoid precursor, which is the main way of ABA production (Zeevaart and Creelman, 1988; Zeevaart, 1999). The biosynthesis of the carotenoid precursor occurs in plastids, but ABA can be produced in various parts of the plant. The expression of several genes involved in ABA biosynthesis is enhanced by osmotic stress.
Besides stomatal closure, ABA plays a role in the regulation of expression of many genes. The up-regulation or down-regulation of expression of various genes in response to ABA has been investigated in different expression studies (Hoth *et al*., 2002; Seki *et*

al., 2002). However, the exact molecular mechanisms that lead to this change in expression are not yet clarified. Calcium ions are believed to be important components of ABA signal transduction pathways that regulate stomata closure and ABA-responsive gene expression (Webb *et al*., 2001). The presence of ABA can trigger cytosolic calcium elevations. It is believed that calcium functions as second messenger in ABA signaling pathways. Furthermore, various protein phosphorylation cascades are presumed to be involved in the ABA signaling network (Zhu, 2002). In addition, there is evidence that sugar sensing and reactive oxygen species are playing a role in ABA responses (Verslues and Zhu, 2005).

The expression of genes encoding LEA proteins in seed and vegetative tissue is also known to be induced by ABA (Ingram and Bartels, 1996). Therefore, ABA is also associated with desiccation tolerance. This is supported by the fact that undifferentiated callus tissue of *C. plantagineum* needs to be pre-treated with ABA in order to obtain tolerance to desiccation (Bartels, 2005). Therefore, it is clear that ABA must play a crucial role in responses to desiccation stress in *C. plantagineum*.

1.9. Regulation of dehydration-responsive gene expression

During drought, the expression of numerous genes is changed (Bray, 1993). The products of these genes can be divided into two groups according to their function: (1) genes, which products are predicted to play a role in signal transduction pathways and gene regulation and (2) gene products that could be involved in cellular adoption mechanisms to dehydration stress (Bray, 2004).

The earlier discussed LEA proteins belong to the latter group of gene products and it has been shown that their expression is affected by dehydration stress. The expression levels of transcripts encoding for different types of LEA proteins, like 6-19 (LEA group 2), 3-06 (LEA group 3), 27-45 (homologous to the *Arabidopsis* LEA 14 protein) and LEA-like protein 11-24 were increased in *C. plantagineum* leaves by dehydration (Piatkowski *et al*., 1990; Bartels *et al*., 1990). Not just LEA genes, but also other genes, which products are assumed to have protective functions, have been found to be regulated by dehydration. An example of such a gene, is the gene encoding the desiccation stress protein 22 (DSP22) in *C. plantagineum*. The DSP22 protein has similarity to early light-inducible proteins (ELIP) and was reported to accumulate in the chloroplasts of desiccated leaves (Bartels *et al*., 1992). It has been suggested that DSP22 protects photosynthetic structures against damage caused by desiccation (Alamillo and Bartels, 2001).

It is thought that at least two different signaling pathways are responsible for the activation of gene expression in response to dehydration. Gene expression in response to drought, but also other abiotic stress conditions is regulated by an ABA-dependent and ABA-independent signaling pathway (Zhu, 2002). The molecular mechanisms by which the ABA-dependent and ABA-independent signal transduction pathways control gene expression are still not completely known. However, several *cis*-acting regulatory elements and transcription factors that participate in these signaling pathways have been identified. Probably the ABA-independent signaling pathway is involved in a rapid response to dehydration. The ABA-dependent signal transduction pathway plays a role in the late response to dehydration, because this pathway requires an increase in endogenous ABA levels.

1.9.1. Regulation of gene expression through *cis*-acting regulatory elements and *trans*-acting factors

Gene expression is mainly controlled by a segment of DNA upstream of the transcription start site of a gene, called the promoter region. This promoter region contains the core-promoter and various specific protein binding sites, termed *cis*-acting regulatory elements. Transcription factors (*trans*-acting elements) can specifically bind to these *cis*-acting regulatory elements and change the transcription rate of a particular gene. Transcription factors have the capacity to activate or repress the transcription of genes (Singh, 1998). Transcription factors bind often to a number of *cis*-elements and are able to interact with each other. Therefore, the distribution and spacing between the different *cis*-acting regulatory elements is important for the functionality of transcription factors (Ludwig, 2002). Thus, *cis*-acting factors and *trans*-acting factors both contribute to regulation of gene expression. Especially *cis*-acting regulatory elements are believed to play a major role in the regulation of gene expression (Ludwig, 2002).
Wittkopp *et al*. (2004) investigated the distribution of *cis*- and *trans*-regulatory changes essential for expression differences between closely *Drosophila* species, *D. melanogaster* and *D. stimulans*. In the study of Wittkopp *et al*. (2004), the relative abundance of species-specific transcripts in F_1 hybrids was compared. Differential expression of species-specific alleles in the same hybrid genetic background would imply *cis*-regulatory differences. Variation in the ratio of species-specific transcripts between F_1 hybrids and the parental species would point to *trans*-regulatory differences. This study revealed that interspecific expression difference between *D. melanogaster* and *D. stimulans* were almost always caused by *cis*-regulatory changes.

1.9.2. Evolution of *cis*-acting regulatory elements

Evolution is dependent on changes in genetic material. A large portion of the genome is made up of non-coding DNA. Nucleotides can sometimes change dramatically within non-coding DNA sequences (Ludwig, 2002). A large part of non-coding DNA is involved in the regulation of transcription. Therefore, nucleotide changes in non-coding DNA sequences can have major effects on gene expression patterns. Point mutations within promoter regions can evolve quickly and are able to generate new *cis*-acting regulatory elements (Stone and Wray, 2001). However, these mutations may also disturb the functionality of existing *cis*-acting regulatory elements. Furthermore, gene duplication events can change the distribution and spacing of *cis*-acting regulatory elements and may affect transcription regulation. Therefore, sequence alternations within promoter regions are playing an important role in the evolution of *cis*-acting regulatory elements.

The short-term evolutionary dynamics of *cis*-regulatory regions in *A. thaliana* have been studied (de Meaux *et al*., 2005). Within-species nucleotide variation in a putative promoter region was examined. Nucleotide and functional variation of the *cis*-regulatory region of the enzyme chalcone synthase (CHS) was determined. CHS is a key enzyme in the flavonoid pathway, which produces secondary metabolites that are associated with stress adaptation. The 5'- upstream region of *CHS* from 28 different *A. thaliana* accessions was analyzed and various nucleotide polymorphisms were found in the *CHS* promoter region. Distinct parental promoter alleles were paired within F_1 heterozygotes and functional *cis*-regulatory variation was analyzed. In heterozygous F_1 individuals, parental alleles are analyzed in a common *trans*-regulatory environment. The relative expression levels of parental-specific alleles were determined and the influence of different stimuli (dark, light and insect-feeding) on CHS transcription was analyzed. Functional variations were found, which seem to be independent of the *trans*-regulatory background. De Meaux *et al*. (2005) showed that a few point mutations could already cause functional changes in promoters. The study indicated that changes in *cis*-acting regulatory elements can have major impacts on gene regulation.

Since, *cis*-acting regulatory elements play a major role in controlling gene expression, it is assumed that functional important *cis*-acting regulatory elements are preserved between species (Ludwig, 2002). However, it is difficult to detect conserved *cis*-acting regulatory elements, because the lengths of *cis*-acting regulatory elements are quite short. For this reason, it is also hard to study the distribution and spacing of conserved *cis*-acting regulatory elements. Comparative analysis of non-coding DNA sequence

from closely related species is an approach that is often used for the detection of conserved *cis*-acting regulatory elements.

1.9.3. *Cis*-acting regulatory elements and *trans*-acting factors involved in ABA-dependent gene expression

It is established that specific c*is*-acting regulatory elements, called ABA-responsive elements (ABREs) are involved in ABA-induced gene expression (Guiltinan *et al*., 1990). Different types of ABREs have been identified within ABA-inducible promoters. A typical ABRE consist of 8-10 bp long sequence motif and contains an ACGT-core sequence (Busk and Pages, 1998). However, also ABREs without an ACGT-box have been found (Hattori *et al*., 2002). Furthermore, not every ACGT-containing sequence within an ABA-inducible promoter functions as an ABRE. The sequence flanking this ACGT-core motif is very important for the functionality of the element. Many functional ACGT-containing ABREs have a conserved CACGTG sequence, also called the "G-box" (Hattori *et al*., 2002). In addition, in order to confer an ABA response, an ABRE needs to work together with other *cis*-acting regulatory elements (Shen *et al*., 1996). MYC and MYB recognition sites in promoters also have a function in ABA-responsive gene expression (Iwasaki *et al*., 1995)

Transcription factors are other essential components involved in ABA signal transduction. The gene *abscisic acid-insensitive3* (*ABI3*) from *Arabidopsis* and the maize *viviparous1* (*VP1*) gene encode for homologues transcription factors, which are essential for ABA signaling in seeds. Mutation of these genes reduced ABA-responsiveness in seeds. Seeds of *ABI3* mutant plants showed decreased seed dormancy and were not able to acquire desiccation tolerance. The mutation of *VP1* resulted in premature germination of the embryo (Nambara, 1995; Ooms *et al*., 1993; Robertson, 1955). However, also in vegetative tissue transcription factors are required to induce an ABA response. For example, MYC and MYB type of transcription factors are involved in ABA-induced gene expression. Overexpression of a basic helix-loop-helix protein, named AtMYC2 and a MYB-related protein, called *At*MYB2 in transgenic *Arabidopsis* plants increased ABA-induced expression of the dehydration-responsive gene, *RD22* (Abe *et al*., 1997). In addition, it was later reported that overexpression of both *At*MYC2 and *At*MYB2 enhanced the expression of several other ABA-inducible genes (Abe *et al*., 2003).

It is generally accepted that basic-domain leucine zipper (bZIP) transcription factors are playing a key role in ABA signal transduction, although their exact function is not clear. Most types of ACGT-containing ABRE motifs are recognized by bZIP proteins

(Yamaguchi-Shinozaki and Shinozaki, 2005). For example, ABRE binding factors (ABFs) from *Arabidopsis*, encoding a distinct subfamily of bZIP proteins and are assumed to play a role in ABA-responsive gene expression (Choi *et al*., 2000). Members of ABFs are able to interact with ABREs and their expression is induced by ABA. Furthermore, the *Arabidopsis* ABI5 protein, a member of the ABF family is also associated with ABA signal transduction. The *Arabidopsis ABI5* mutant plants displayed a reduced ABA sensitivity and this mutation led to an altered expression pattern of some ABA-regulated genes (Finkelstein and Lynch, 2000). In *in vitro* experiments, it was demonstrated that different types of bZIP proteins are able to bind ABREs (Uno *et al*., 2000; Guiltinan *et al*., 1990). In addition, the transcription of several genes encoding bZIP proteins is up-regulated in response to ABA (Leung and Giraudat, 1998). For these reasons, it is well believed that bZIPs are important transcription factors in ABA signaling pathways.

1.9.4. *Cis*-acting regulatory elements and *trans*-acting factors involved in ABA-independent gene expression

The dehydration-responsive element (DRE)/C-repeat (CRT) has been identified as c*is*-acting regulatory element that is involved in ABA-independent gene expression in response to dehydration (Shinozaki *et al*., 2003). It was demonstrated that the presence of the DRE motif, with the 9 bp long conserved sequence TACCGACAT, in the promoter of the gene *RD29A* was sufficient to induce expression in response to dehydration or salt stress (Yamaguchi-Shinozaki and Shinozaki, 1994). Furthermore, the expression of the *RD29A* gene was reported to be induced by low-temperature stress. This indicated that DRE also has a role in cold-induced gene expression. There are indications the DRE motif might also be involved in ABA-induced gene expression. It was reported that there exist interaction between ABRE and DRE elements (Narusaka, *et al*., 2003). Deletion and base-substitution analysis of the promoter region of the *RD29A* gene suggest that the DRE sequence may function as coupling element of ABRE and could be involved in ABA-induced expression.
Transcription factors that are able to bind to the DRE sequence have been identified. Two members of the DRE-binding protein (DREB) family, respectively, DREB1A and DREB2A were shown to interact specifically with the DRE motif (Liu *et al*., 1998). In the same study, it was found that the expression of *DREB1A* gene was induced by cold and that dehydration or high-salt stress induced the expression of the *DREB2A* gene. In another study, it was reported that the promoter regions of *DREB2A* and a homologue of the *DREB2A* gene, called *DREB2B,* were able to stimulate the expression of a

reporter gene in response to dehydration and high salinity-stress (Nakashima, *et al*., 2000). Therefore, it is assumed that DREB1 transcription factors participate in cold stress signaling pathways, whereas DREB2 proteins function in dehydration signal transduction. However, overexpression of DREB2A in transgenic *Arabidopsis* did not enhance dehydration tolerance of the transgenic plants (Liu *et al*., 1998). It was suggested that DREB2 proteins require post-translational modification, such as phosphorylation, to become functional.

1.10. Regulation of *LEA* gene expression in *C. plantagineum*

The transcription of genes encoding LEA or LEA-like proteins is also at least regulated trough an ABA-dependent and ABA-independent signal transduction pathway. It was demonstrated that the accumulation of *LEA* transcripts in barley embryos was regulated by ABA and by mannitol-induced osmotic stress (Espelund *et al*., 1995). The transcriptional regulation of some *LEA*-type genes in response to dehydration in *C. plantagineum* have been described (Ingram and Bartels, 1996). It is assumed that all the *cis*-acting regulatory elements and *trans*-acting factors, which have been mentioned earlier, are involved in dehydration-induced *LEA* gene expression in *C. plantagineum*. The regulatory mechanisms of dehydration-induced *LEA* gene expression are shown in a simplified model (Figure 3). However, one should keep in mind that other signaling pathways that control the expression of *LEA* genes may also exist.

Dehydration stress causes a stimulus that is detected by a sensor. However, it is still not clear how plants sense dehydration stress. It is assumed that water-deficit causes the loss of cell turgor pressure, which triggers a molecular response (Shinozaki and Yamaguchi-Shinozaki, 1997). This response is able to activate the ABA-dependent and ABA-independent signal transduction pathway. In the ABA-response pathway, the endogenous ABA level is increased. The activation of this signaling pathway results in the induction or activation of transcription factors. The transcription factors ABF, MYB and MYC are major components that participate in ABA-induced gene expression. These ABF, MYC and MYB transcription factors bind to ABRE, MYC and MYB recognition sites, respectively. In the ABA-independent pathway, the DREB2 class of transcription factors is related with dehydration-induced *LEA* gene expression. The DREB2 proteins specifically interact with DRE/CRT motifs. Upon binding of the transcription factors to the specific *cis*-acting regulatory elements within the promoter sequence, the transcription of the *LEA* gene is stimulated. There are indications that these two signaling pathways are not completely separate from each other. It has been

suggested that the DRE sequence can function as coupling element for ABRE and thus is involved in ABA-induced gene expression (Narusaka, *et al*., 2003).

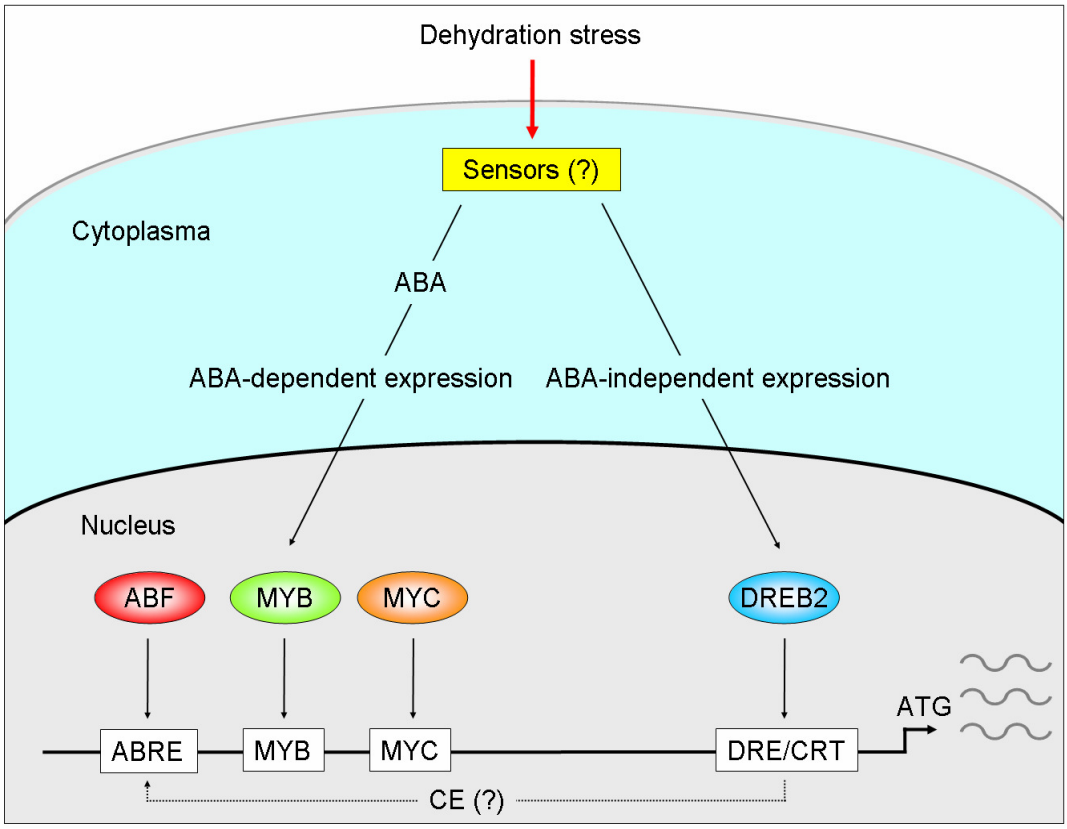


Figure 3. Regulation of dehydration-responsive expression of *LEA* genes. Dehydration-responsive *LEA* gene expression is mediated by an ABA-dependent and ABA-independent signal transduction pathway. Initially, dehydration stress is sensed by the plant. The sensors that detect dehydration stress are unknown. After perception of dehydration stress, the ABA-dependent and ABA-independent signaling pathways are triggered and this lead to the induction or activation of transcription factors. The transcription factors ABF, MYB and MYC are the major components in the ABA-dependent signaling pathway. These transcription factors interact, respectively, with the ABRE, MYB and MYC sequences within the promoter of the relevant *LEA* gene. The DREB2 transcription factors are involved in ABA-independent *LEA* gene expression signaling pathway. Upon binding of the transcription factors to the *cis*-acting elements, the *LEA* transcription is induced. It is believed that these two pathways can interact and that the DRE motif may have a role in ABA-dependent gene expression as it could function as coupling element (CE) for ABRE in ABA-dependent gene expression. This model of regulation of *LEA* gene expression is based on a model described in Shinozaki *et al*. (2003).

In reality, the situation is, however, much more complex than as it is described in the model, in Figure 3. Second messengers, like calcium ions and various phosphorylation cascades are also involved in controlling gene expression. Furthermore, presumably cross-talk between the two described signaling pathways occurs. In addition, other *cis*-acting elements and transcription factors participate in the regulation of *LEA* gene expression. For example, in the promoter of the *LEA 27-45* gene in *C. plantagineum*, a

novel 29 bp *cis*-regulatory region that responded to ABA was detected (Nelson *et al*., 1994). Later on, a new transcription factor that interacted with this 29 bp sequence domain was identified (Hilbricht *et al*., 2002).

1.11. Desiccation tolerance within the Linderniaceae family

Several members within the family of Linderniaceae are resistant to desiccation (Fischer *et al*., 1992). It is known that all *Craterostgma* species are desiccation tolerant, but also a few members of the *Lindernia* genus have the ability to withstand desiccation.

Recently, it has been reported that *Lindernia brevidens* Skan, a close relative of *C. plantagineum* has the ability to recover after extreme desiccation (Phillips *et al*., 2008). The plant *L. brevidens* is endemic to tropical rainforests in eastern Africa (Fischer *et al*., 1992). Therefore, it is surprising that *L. brevidens* exhibits desiccation tolerance, because in these areas the plant is never exposed to prolonged periods of drought. It was proposed that *L. brevidens* retained desiccation tolerance through genome stability (Phillips *et al*., 2008). In the same study by Phillips *et al*. (2008), it was described that *L. brevidens* uses similar mechanisms as *C. plantagineum* to protect itself against desiccation, such as the accumulation of LEA proteins and alterations in carbohydrate metabolism.

Although, some members of the *Lindernia* genus are desiccation-tolerant, the greater part of the *Lindernia* species are sensitive to desiccation. One of these desiccation-sensitive *Lindernia* species is *Lindernia subracemosa*. The phylogenetic relationship between *C. plantagineum*, *L. brevidens*, *L. subracemosa* and other Linderniaceae family members has been studied (Rahmanzadeh *et al*., 2005). This phylogenetic analysis showed that the species *L. brevidens* and *L. subracemosa* are closely related to *C. plantagineum*. In a phylogenetic tree inferred from sequences of the chloroplast maturase gene (*MATK*), *L. brevidens* and *L. subracemosa* are localized, together with the species *Torenia vagans*, in the same branch as members of the *Craterostigma* genus (Phillips *et al*., 2008) (Figure 4).

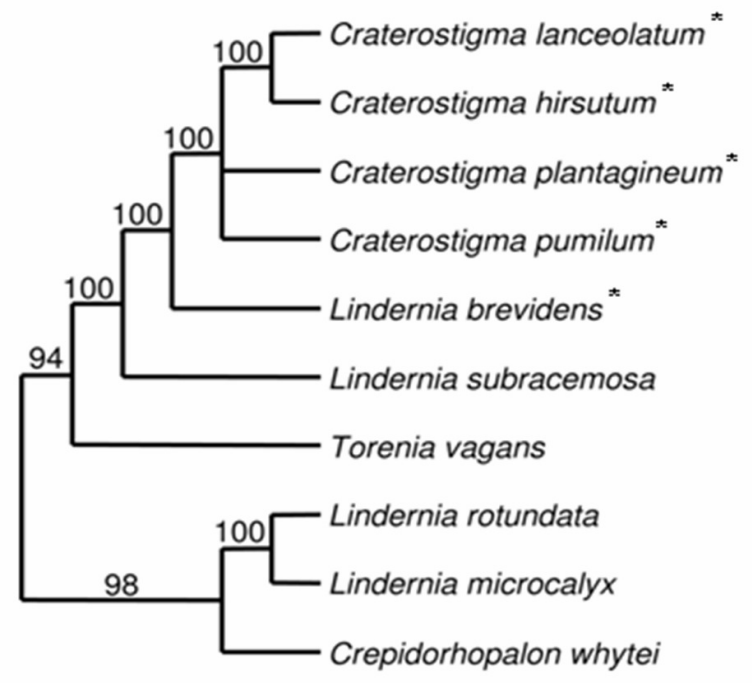


Figure 4. Phylogenetic relationship between selected members of the Linderniaceae family. The phylogenetic tree was based on sequences of the *MATK* gene. Bootstrap percentages are indicated above the branches. The desiccation-tolerant species are marked with an asterisk. The phylogenetic tree in this form was published by Phillips *et al*. (2008), but the data derived from the study of Rahmanzadeh *et al*. (2005).

The species *C. plantagineum*, *L. brevidens* and *L. subracemosa* share very similar flower morphology (Figure 5). These three members of the Linderniaceae family produce flowers with similar structures and purple white colored petals. On the other hand, although closely related, the morphological features of the two *Lindernia* species, *L. brevidens* and *L. subracemosa* differ from those of *C. plantagineum*. Especially, the stem and leaf morphology between *C. plantagineum* and the two *Lindernia* species are quite different. Compared to *C. plantagineum*, the two *Lindernia* species have relatively long stems and internodes. The leaves of the *L. brevidens* and *L. subracemosa* are hairless, thinner and smaller in size than *C. plantagineum* leaves. The resurrection plant *C. plantagineum* has leaves, which are rather thick and contain spreading hairs beneath.

Figure 5. Flower morphology of *C. plantagineum*, *L. brevidens* and *L. subracemosa*. (A) Flower of *C. plantagineum*, (B) flower of *L. brevidens* and (C) flower of *L. subracemosa*

1.12. The plant species *L. brevidens* and *L. subracemosa* as experimental systems

Only a few studies on mechanisms involved in desiccation tolerance acquisition have been done so far in the plant *L. brevidens* (Phillips et al., 2008; Smith-Espinoza *et al*., 2007). However, much more information and understanding about desiccation tolerance could be gained from this resurrection plant.

The desiccation-sensitive *L. subracemosa* is an interesting plant to study desiccation tolerance, since it is so closely related to the desiccation-tolerant species *C. plantagineum* and *L. brevidens*. The species *L. subracemosa* is a very useful model system for comparative studies on mechanisms of desiccation tolerance between desiccation tolerant and desiccation sensitive plants.

For the resurrection plants *C. plantagineum* and *L. brevidens*, *Agrobacterium*-mediated transformation procedures have been developed (Furini *et al*., 1994; Smith-Espinoza *et al*., 2007). The transformation protocol that was developed for *L. brevidens,* probably also functions for other *Lindernia* species, such as *L. subracemosa*. However, a major drawback of the available transformation methods is that these procedures require long periods of time.

1.13. Objectives of the study

Desiccation tolerant and desiccation sensitive plants differ in their ability to survive extreme desiccation of their vegetative tissues. Vegetative desiccation tolerance is only found in resurrection plants. However, various types of desiccation sensitive plants are able to acquire desiccation tolerance in orthodox seeds during seed development. Therefore, it is thought that genes involved in the acquisition of desiccation tolerance are present in desiccation tolerant and desiccation sensitive plant species. Differential expression of genes involved in desiccation tolerance is probably the reason for the phenotypic difference between desiccation tolerant and desiccation sensitive plant species. To test this hypothesis, 5'-upstream regions from several desiccation-responsive genes will be analyzed. This analysis will provide more insight in the mechanisms of transcriptional regulation during desiccation. Here, it is further hypothesized that promoter architecture and functionality is conserved among the desiccation tolerant species. To examine this last hypothesis, variation in promoters of homologous genes in evolutionarily-related plant species that vary in desiccation tolerance will be explored. In addition, functionality of promoters will be investigated by functional *cis*-acting regulatory element mapping.
The desiccation tolerant plants *C. plantagineum, L. brevidens* and the desiccation sensitive plant *L. subracemosa* will be used as experimental model systems. These plants provide the ability to investigate the relationship between promoter conservation and desiccation tolerance. In this study the putative promoter regions of the following genes will be analyzed:

- *LEA 6-19*
- *LEA-like 11-24*
- *LEA 3-06*
- *DSP22*
- *LEA 27-45*

However, promoter regions are difficult to study, because these upstream regulatory sequences often lack sequence homology. Therefore, promoter architecture and distribution of putative *cis*-acting regulatory elements will be investigated. Conservation of *cis*-acting regulatory elements among promoters from different species may indicate that these elements have an important *cis*-regulatory function. Mutational approaches will be applied to identify essential *cis*-acting regulatory elements. The functions of *cis*-acting regulatory elements within the *LEA-like 11-24* promoter

sequences in desiccation-responsive gene expression will be analyzed in a transient expression assay. It will, therefore, be necessary to develop an efficient transient expression assay in *C. plantagineum*, *L. brevidens* and *L. subracemosa* for measuring relative promoter activities. The activities of *LEA-like 11-24* promoters from *C. plantagineum*, *L. brevidens* and *L. subracemosa* under exogenous ABA and osmotic treatment will be analyzed. Furthermore, *trans*-acting components that are able to interact with the *C. plantagineum LEA-like 11-24* promoter sequence will be examined.

2. Materials and methods

2.1. Chemicals, enzymes and kits

Chemicals, enzymes and kits used in this study were purchased from the following manufacturers: 5 Prime (Hamburg, Germany), Amersham (Braunschweig**,** Germany), Biomol (Hamburg, Germany), Bio-Rad (Munich, Germany), Boehringer (Mannheim, Germany), Clontech (Heidelberg, Germany), Difco (Augsburg, Germany)**,** Fermentas (St. Leon-Rot, Germany), Invitrogen (Karlsruhe, Germany), Macherey-Nagel (Düren, Germany), Merck (Darmstadt, Germany), Qiagen (Hilden, Germany), Roche (Mannheim, Germany), Roth (Karlsruhe, Germany), Sigma (Deisenhofen, Germany) and Stratagene (Heidelberg, Germany).

2.2. Plasmids

The following plasmid DNAs were used in this study:

- pBluescript® SK(-) (Stratagene, Heidelberg, Germany)
- pBT10 GUS (Kleines, 1997)
- pCK-GFP-S65C (Reichel *et al*., 1996)
- pCR2**.**1®-TOPO® vector (Invitrogen, Karlsruhe, Germany)
- pGJ280 (G. Jach, Max-Planck-Institute, Cologne, Germany)
- pJET1.2 (Fermentas, St. Leon-Rot, Germany)
- pPC86 (Chevray and Nathans, 1992)
- pSK1 (Kim *et al*., 1997)
- Uni-ZAP® XR (Stratagene, Heidelberg, Germany)

Detailed maps of these plasmids can be found in the supplemental data.

2.3. Primers

All primers that were employed in this study are listed in Table 2. The characters A, C, G and T stand for the nucleotides: adenine, cytosine, guanine and thymine, respectively.

Most primers were designed with primer design software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3.cgi). In order to design optimal primers the following guidelines were used:

- The length of the primers should be between 15 and 30 nucleotides.
- Primers should have a GC content of approximately 50%, which is distributed equally along the primer.
- The primers are not allowed to be self-complementary or complementary to other primers in the reaction mixture.
- The 3' end of the primers should contain at least one G or C to ensure optimal binding of the 3' end.
- The melting temperature (T_m) of the primers should be approximately 60°C and melting temperatures of both primers in the reaction must be similar.

For most primers the approximate T_m can be calculated with the following formula:

$$T_m = 4(G+T) + 2(A+T)$$

Where G, C, A and T are the number of respective nucleotides in the primer.

Site-directed mutagenic primers were designed with either the QuikChange® Primer design program (http://www.stratagene.com/sdmdesigner/) or with the PrimerX design program (http://www.bioinformatics.org/primerx/). Primers were synthesized by Sigma-Aldrich (Steinheim, Germany).

Table 2. Primers used in this study

GenomeWalker primers	Sequence (5' to 3')	Restriction site
GW Cp3-06 f1	GAGAATTTGGGACGAAGGGTAAGGTAAG	-
GW Cp3-06 f2	AAGGACTTGGAGATGCTGAGAACTGTG	-
GW CpDSP22 f1	TGCTCCTCCTTCTCATTGTCCTCTCT	-
GW CpDSP22 f2	GTAATAGTGCTTTGTCCTCGGCAAGAC	-
GW Lb11-24 f1	CTCCGGTTCCCCTAACCCTAGAAATC	-
GW Lb11-24 f2	GTCAGGGGTTTCTTCTGCTGAGTAGC	-
GW Lb27-45 f1	ACGCAGATCTTGGCGAGGTAAGTGAT	-
GW Lb27-45 f2	AGCATCGTCTTATCGTTTCCCTTCAAC	-
GW LbDSP22 f1	CCTCTTCAGTGGCAAGAGGTTGTTAGG	-
GW LbDSP22 f2	GCGTAGCAAGTAGCAGAACTCATCACG	-
GW Ls11-24 f1	ACCTTCTTCAGCATGGACTTCTTCTCTC	-
GW Ls11-24 f2	CTCCACCATTTCATTGTCTGAAGCAAC	-
GW Ls3-06 f1	GGTCGAAGGTTAAGGTAAGTGGGATTG	-
GW Ls3-06 f2	GAAGGAGGACTTGGAGAGGTTAAGAACTG	-
GW Ls6-19 f1	CACCACCAGTCATACCGTGTTGACCA	-
GW Ls6-19 f2	CAGTCTGTCTCCCACCGTATTTCTCAC	-
GW LsDSP22 f1	TTAGGGCCAACCCTTGTGAGAGTAGTT	-
GW LsDSP22 f2	TCCACAAGACACTGAAAGCATAGCATTAC	-
GW2 Lb11-24 f1	GTTCATGTCCTTGAGGCCTTGTGTAAGA	-
GW2 Lb11-24 f1	GTTCATGTCCTTGAGGCCTTGTGTAAGA	-
GW2 Lb11-24 f2	CCAACATCTGCTTCTTTAACACCTTTGC	-
GW2 Lb11-24 f2	CCAACATCTGCTTCTTTAACACCTTTGC	-
GW2 Lb3-06 f1	GTCACTTGTGTGGTACCAATCTTTCT	-
GW2 Lb3-06 f2	ATACATATGAGTGTAGAGCCGAGCTAA	-
GW2 LbDPS22 f1	CTTAGACTCGTGAAATACTTAGCACCT	-
GW2 LbDSP22 f2	GTCTCTGAATCTCTTTCTGCTTCATAC	-
GW2 Ls11-24 f1	ATGTTTCTTGATGGTCCCCTTGATCTTC	-
GW2 LsDSP22 f1	CTCCTCCTCCTCGGACATACTTCTAACC	-
GW2 LsDSP22 f1	CTCCTCCTCCTCGGACATACTTCTAACC	-
GW3 Lb11-24 f1	TTTCCTCGAATGACGATGTAGAGAAGGA	-
GW3 Lb 11-24 f2	GCGGAGGAGAGAGGATATGAGTGACAAG	-
GW3 Lb11-24 f1	TTTCCTCGAATGACGATGTAGAGAAGGA	-
GW3 Lb11-24 f2	GCGGAGGAGAGAGGATATGAGTGACAAG	-
GW3 Ls11-24 f1	GCTGAGGGTGTTCTTGCTCACTAGG	-
GW3 Ls11-24 f2	TGCGATTCCATCTTCTATTCTCTCACTTC	-
***Lb 27-45* insertion primers**	**Sequence (5' to 3')**	**Restriction site**
Lb27-45 insertion left	GGTGAAAATATGCACTTCACGA	-
Lb27-45 insertion right	GCGTATTTAAAAAGTATGCACGTAA	-
GUS fusion primers	**Sequence (5' to 3')**	**Restriction site**
Cp11-24-GUS left	TTT**GAATTC**TGGGCCGAAACA	*Eco*RI
Cp11-24-GUS right	TTT**CCATGG**TACAATATTCAGATTCTCT	*Nco*I

Lb11-24-GUS leftA	CCCTAATCCCAATGTTCTACCA	-
Lb11-24-GUS leftB	TTT**GAATTC**TTGGGCCTAAACTATCTACT	*Eco*RI
Lb11-24-GUS rightA	TTT**CCATGG**TCTTGTTCTATTTTT	*Nco*I
Lb11-24-GUS rightB	CGGAGGAGAGAGGATATGAGTG	-
Ls11-24-GUS left	TTT**GAATTC**TTGGGCCTAAACTG	*Eco*RI
Ls11-24-GUS right	TTT**CCATGG**TCTATTCTCTCACTTCTTT	-
Ls11-24-GUSlong left	TTT**GAATTC**CTATAGGGCCGTCA	*Eco*RI
Ls11-24-GUSlong right	TTT**CTCGAG**TCTATTCTCTCACTTCTTT	*Xho*I
Mutagenic primers	**Sequence (5' to 3')**	**Restriction site**
Cp11-24 M1 for	GGGACCCACGATCGTGCCATATCCCTCGACT	-
Cp11-24 M1 rev	AGTCGAGGGATATGGCACGATCGTGGGTCCC	-
Cp11-24 M2 for	TGTCGGTAGGCAAGTACCTCAGACATATGTGC TAACGAAT	-
Cp11-24 M2 rev	ATTCGTTAGCACATATGTCTGAGGTACTTGCC TACCGACA	-
Cp11-24 M3 for	ACACGTGTGCTAACGAATAGGCTGATATGTCC CTAATCTC	-
Cp11-24 M3 rev	GAGATTAGGGACATATCAGCCTATTCGTTAGC ACACGTGT	-
Cp11-24 MDRE for	CTTACAGGACAGGTGATGGTAGGCAAGTACC	-
Cp11-24 MDRE rev	GGTACTTGCCTACCATCACCTGTCCTGTAAG	-
Lb11-24 M1 for	TGTATAAGCCATATCCACGACTTA	-
Lb11-24 M1 rev	TAAGTCGTGGATATGGCTTATACA	-
Lb11-24 M2 for	AACCTCGGACATATGTGCTAATTA	-
Lb11-24 M2 rev	TAATTAGCACATATGTCCGAGGTT	-
Y1HS primers	**Sequence (5' to 3')**	**Restriction site**
YIHS frag1 for	AAA**GAGCTC**CACGTCCCTCGACTC	*Sac*I
YIHS frag1 rev	TTT**TCTAGA**GCACACGTGTCTGAGG	*Xba*I
YIHS frag2 for	TTT**TCTAGA**GCCACGTCCCTCGACT	*Xba*I
YIHS frag2 rev	AAA**ACTAGT**GCACACGTGTCTGAGG	*Spe*I
YIHS frag3 for	TTT**ACTAGT**GCCACGTCCCTCGACT	*Spe*I
YIHS frag3 rev	AAA**GGATCC**CACACGTGTCTGAGGTA	*Bam*HI
Sequencing primers	**Sequence (5' to 3')**	**Restriction site**
M13 for	GTAAAACGACGGCCAGT	-
M13 rev	CAGGAAACAGCTATGAC	-
pBT10 left	TTAATGCAGCGGATCA	-
pBT10 right	TTTTGATTTCACGGGT	-
pJETfor	CGACTCACTATAGGGAGAGCGGC	-
pJETrev	AAGAACATCGATTTTCCATGGCAG	-
pPC86 left	GGATGTTTAATACCAC	-
pPC86 right	TTGATTGGAGACTTGACC	-
T3	AATTAACCCTCACTAAAGGG	-
T7 promoter	TAATACGACTCACTATAGGG	-
T7 terminator	GCTAGTTATTGCTCAGCGG	-

Reverse transcriptase PCR primers	**Sequence (5' to 3')**	**Restriction site**
RT LEA 11-24 for	CGTTAGCAGGCCAAGAACAT	-
RT LEA 11-24 rev	CTGACGAGGGGTGAGATTGT	-
RT Cp TKT3 for	ACTACCCCTTGGATGGGAAA	-
RT Cp TKT3 rev	GTTGAGGTTTTGCTGGGAGA	-
RT Cp bZIP1 for	CAATTGTACCTCAGCGTTGC	-
RT Cp bZIP1 rev	CTCACATCAGAAGCAACTCGAA	-
cDNA synthesis primer	**Sequence (5' to 3')**	**Restriction site**
Hybrid oligo(dT) linker primer (Stratagene, Heidelberg, Germany)	GAGAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**TTTTTTTTTTTTTTTTT	*Xho*I

2.4. Biological material

2.4.1 Bacterial strains

Escherichia coli strain DH10B (Lorrow and Jessee, 1990). Genotype: F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ⁻.

E.coli strain SOLR™ (Stratagene, Heidelberg, Germany). Genotype: *e14*⁻ (*mcr A*) Δ(*mcr CB-hsd SMR-mrr*) 171 *sbc C rec B rec J uvr C umu C*::Tn 5 (Kan^{R}) *lac gyr* A96 *rel* A1 *thi*-1 *end* A1 λ^{R} {F' *pro AB lacI*^{Q}Z*ΔM15 Tn 10* (Tet^{R})} Su⁻.

E. coli strain TOP10 (Invitrogen, Karlsruhe, Germany). Genotype: F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G.

E. coli strain XL1-Blue (Stratagene, Heidelberg, Germany). Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*(F *proAB lacI*^{Q}*Z ΔM15* Tn*10* (Tet^{R})).

E.coli strain XL1-Blue MRF' (Stratagene, Heidelberg, Germany). Genotype: Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*) *173*, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac*(F' *pro*AB, *lac*I^{Q} ZΔM15 Tn10 (Tet^{R})).

2.4.2. Yeast strains

Saccharomyces cervisiae strain YM954 (Wilson *et al*., 1991). Genotype: *MAT*a *ura3-52 his3-200 ade2-101 lys2-801 leu2-3,112 trp1-901 gal4-542 gal80-538.*

2.4.3. Plant material

Craterostigma plantagineum Hochst was originally collected in eastern Africa, obtained from Prof. O. H. Volk (University of Würzburg, Germany) and subsequently maintained in the department.

Lindernia brevidens and *Lindernia subracemosa* were originally collected from different countries in Africa: Kenya and Rwanda, respectively. These plant species were kindly provided by Prof. E. Fischer (University of Koblenz, Germany). Both species were found in the tropical rainforests.

2.5. Sterilization of solutions, buffers and media

Prepared solutions, buffers and media used in this study were sterilized by either autoclaving at 120°C for 20 minutes or by filter sterilization.

2.6. Growth conditions

2.6.1. Bacterial cultivation conditions

E.coli strains DH10B, TOP10 and XL1-Blue were grown at 37°C in Luria-Bertani (LB) medium (1% (w/v) bactotryptone (Difco, Augsburg, Germany), 0.5% (w/v) yeast extract (Difco, Augsburg, Germany), 1% (w/v) NaCl, pH 7.0 and optionally 1.5 % (w/v) Select agar (Invitrogen, Karlsruhe, Germany). When necessary the LB medium was supplemented with appropriate antibiotics, e.g. ampicillin.
E.coli strains XL1-Blue MRF' and SOLR™ cells were cultured at 37°C or at 30°C in either LB medium supplemented with 10 mM $MgSO_4$ und 0.2% (w/v) maltose or in NZY medium (0.5% (w/v) NaCl, 0.2% (w/v) $MgSO_4$, 0.5% (w/v) yeast extract, NZ amine (Sigma, Diesenhofen, Germany), pH 7.5 and optionally 1.5% (w/v) Select agar).

2.6.2. Yeast cultivation conditions

Saccharomyces cerevisiae was grown at 30°C either in Yeast Extract Peptone Dextrose (YEPD) medium (0.5% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose and optionally 2% (w/v) Bacto agar (Difco, Augsburg, Germany)) or synthetic complete (SC) medium (0.67% (w/v) yeast nitrogen base without amino acids (Difco, Augsburg, Germany), 2% (w/v) glucose and optionally 2% (w/v) Bacto agar). The SC medium was further supplemented with 1x amino acid drop-out solution (Components of complete 10x amino acid drop-out solution are listed in Table 3). In order to select auxotrophic yeast transformants, the drop-out solution was lacking either, histdine (his), leucine (leu), tryptophan (trp) or a combination of these amino acids.

Table 3. Composition of complete 10x amino acid drop-out solution

Amino acid	Concentration
L-Tryptophan	0.2 mg/ml
L-Histidine HCl Monohydrate	0.2 mg/ml
L-Arginine HCl	0.2 mg/ml
L-Valine	1.5 mg/ml
L-Adenine Hemisulfate Salt	0.2 mg/ml
L-Tyrosine	0.3 mg/ml
L-Aspartate	1 mg/ml
L-Threonine	2 mg/ml
L-Lysine HCl	0.3 mg/ml
L-Methionine	0.2 mg/ml
L-Leucine	1 mg/ml
L-Phenylalanine	0.05 mg/ml
L-Isoleucine	0.3 mg/ml
L-Uracil	0.2 mg/ml
L-Glutamate	1 mg/ml

2.6.3. Plant cultivation conditions

2.6.3.1. Seed sterilization and germination

When plants were grown under sterile conditions, seeds were first sterilized before germination. Seeds were collected in an Eppendorf tube and washed with 70% (v/v)

ethanol for 2 minutes. After washing, the seeds were allowed to sink to the bottom of the tube and the ethanol was removed. Then, 1 ml of sterilization solution was added to the seeds and the mixture was incubated for 20 minutes. The sterilization solution was removed and seeds were washed 3 times with sterile water. Next, seeds were separated on a piece of sterile Whatman filter paper and air-dried in a sterile hood.
To start germination, seeds were spread on half-strength MS medium agar plates. Plates with seeds were then wrapped with parafilm (American National Can, Chicago, USA) and incubated in a cold room at 4°C for 3 days. Subsequently, plates were transferred to a growth chamber at day/night temperature of 22°C/18°C, in a 16 hours day/8 hours night regime, with a light intensity of 80 microEinsteins per square meter per second ($\mu E/m^2/s$) and 50% relative humidity. Seedlings were kept under these conditions until plants reached a length of 1 cm. Then, seedlings were transferred to pots.

2.6.3.2. Plant growth conditions

C. plantagineum was grown either under either *in vitro* under sterile conditions on Murashige and Skoog (MS) medium (4.3 g/l MS salts, 20 g/l sucrose, 1 ml/l vitamin solution (2 mg/l glycine, 0.5 mg/l Nicotine acid, 0.5 mg/l peridoxine-HCl, 0.1 mg/l thiamine-HCl), pH 5.8, 0.8% (w/v) Select agar (Invitrogen, Karlsruhe, Germany)) (Murashige and Skoog, 1962) or in pots filled with clay granulate (Seramis; Masterfoods, Verden, Germany). Plants were maintained in a growth chamber at day/night temperature of 22°C/18°C, in a 16 hours day/8 hours night regime, with a light intensity of 80 $\mu E/m^2/s$ and 50% relative humidity.
L. brevidens and *L. subracemosa* were grown either *in vitro* under sterile conditions on half-strength MS medium or in pots filled with soil that was treated with 20% (w/v) Confidor® (Bayer Cropscience, Monheim, Germany). Plants were maintained in a growth chamber at day/night temperature of 22°C/18°C, in a 16 hours day/8 hours night regime, with a light intensity of 80 $\mu E/m^2/s$ and 50% relative humidity.

2.7. Plant stress treatments

For general dehydration and rehydration treatments, plants were dried in pots under normal growth conditions for a period of 12 days. Dried plants were rehydrated by submerging the complete plant in water for 24 hours.
To analyze the effect of dehydration and rehydration on the *LEA-like 11-24* expression in leaves, whole plants were removed from their pots and dried on filter paper in a growth camber. Plants were dehydrated for 48 hours and rehydrated plants were

submerged in water for 24 hours. Leaf samples were collected after 24 hours of dehydration, 48 hours of dehydration and 24 hours of rehydration.
To determine the effect of ABA and mannitol treatments on *LEA-like 11-24* promoter activity, excised leaves were incubated for 48 hours in solutions containing 100 µM ABA or 0.8 M mannitol. Leaves were incubated under normal growth conditions in a growth chamber.

2.8. Determination of water content of plants

To analyze the water conditions during dehydration and rehydration, plants were dehydrated for 12 days. Plants were collected after 4, 6, 8, 10 and 12 days of dehydration, respectively. Rehydrated plants were collected after 12 days of dehydration and 24 hours of rehydration. Subsequently, the fresh weight and dry weight (dw), after 48 hours of incubation at 70°C, of the plants were measured and the water content (WC) was determined. The WC was expressed as gram of water per gram of dry weight ($g \cdot g^{-1}$ dw) and was calculated with the following formula:

$$WC = (\text{fresh weight} - \text{dry weight}) / \text{dry weight}$$

2.9. Quantification of nucleic acids by spectrophotometry

The concentration of DNA and RNA were quantified by spectrophotometry. Nucleic acids were diluted in dH_2O. Then, the absorbance of the solution was measured at a wavelength of 260 nm with a photospectrometer (Ultrospec 2000, Amersham Pharmacia Biotech, Freiburg, Germany). Nucleic acids have an absorption peak at this wavelength. The concentration of nucleic acids was calculated with Lambert Beer's law:

$$A = \varepsilon \cdot c \cdot d$$

Where A is the absorbance, ε is the molar extension coefficient of the analyzed compound, c is the concentration of the analyzed compound and d is the optical length of the cuvette. Multiply the concentration of nucleic acids by the dilution factor. An absorbance of 1 at 260 nm corresponds to 50 ng/µl of double-stranded DNA, whereas an absorbance of 1 at 260 nm corresponds to 40 ng/µl of single stranded RNA.
The purity of the nucleic acids sample was estimated by determining the absorbance ratio at 260 nm and at 280 nm. Since, proteins in solution have an absorption maximum

at 280 nm. Pure DNA should give an A260/A280 ratio of around 1.8 and pure RNA should have an A260/A280 ratio of around 2.

2.10. DNA sequence analysis

DNA sequencing reactions were performed by Macrogen DNA sequencing services (Seoul, Korea). Single extension sequencing reactions and nucleotide sequence analysis were conducted by Macrogen using the Applied Biosystems 3730xl and 9 ABI 3700 DNA analyzers (Applied Biosystems**,** Darmstadt, Germany).
The Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) was used to identify regions of sequence similarity between a query sequence and sequences in the database. Sequence alignments were performed with the multiple alignment program ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). DNA sequence were translated to protein sequences with the ExPASy translate tool (http://www.expasy.ch/tools/dna.html). Restriction sites in DNA sequences were detected with the program Restriction Summary (http://www.bioinformatics.org/ sms/rest_sum.html).

2.11. Standard cloning techniques

2.11.1. DNA digestion with restriction enzymes

For a typical DNA restriction enzyme digestion, the following components were added to an Eppendorf tube: 1 µl of 10 x digestion buffer, 2 µl of DNA (up to 1 µg), 1 - 10 Units (U) of restriction enzyme and distilled water was added up to a volume of 10 µl. The digestion mixture was incubated in a water bath at the recommended temperature (e.g. 37°C) for at least 1 hour. When a double digestion was performed a digestion buffer was chosen that was most compatible with both restriction enzymes. For some DNA restriction enzyme digestions, a longer incubation time or more restriction enzyme was required. However, the enzyme volume was never allowed to exceed 1/10 of the digest volume to avoid star activity.

2.11.2. DNA ligation

Ligation reactions were always performed with DNA molecules containing compatible sticky ends. For all reactions, the molar ratio of vector to insert was 1:4. The following components were added into a thin-wall PCR tube in this order: 2 µl of 5 x ligase

buffer, *x* µl of digested vector, *y* µl of digested insert DNA, 0.5 µl of T4 DNA ligase (Invitrogen, Karlsruhe, Germany) and distilled water was added to a final volume of 10 µl. The reagents were mixed by gently pipetting up and down. The ligation mixture was incubated overnight at 16°C.

2.11.3. Agarose gel electrophoresis of DNA fragments

DNA fragments were separated by agarose gel electrophoresis. A 1.5% (w/v) agarose gel solution was prepared by dissolving the agarose in 1x Tris-acetate-EDTA (TAE) buffer (50x TAE: 2 M Tris base, 1 M acetic acid, 100 mM EDTA pH 7.6) and melting the agarose in a microwave. The agarose solution was poured into a gel tray with comb and the gel was allowed to solidify. The solidified gel was then placed into an electrophoresis apparatus, which was filled with 1x TAE buffer. The comb was carefully removed from the gel. DNA samples supplemented with one tenth of volume 10x DNA loading buffer were loaded onto the agarose gel. Electrophoresis was carried out at 80V to 120V for approximately 1 hour. After electrophoresis, the agarose gel was removed from electrophoresis apparatus and stained with (0.5 mg/ml) ethidium bromide (EtBr) solution. Subsequently, the gel was rinsed with distilled water. DNA on the gel was visualized by exposure to ultraviolet (UV) light and a photograph was taken.

2.11.4. Extraction of DNA from agarose gels

After agarose gel electrophoresis, the appropriate DNA fragment was excised from the agarose gel with a clean scalpel and transferred to a clean Eppendorf tube. The excised DNA fragment was then extracted from agarose gel using the Nucleospin Extraction II kit (Macherey-Nagel, Düren, Germany). The kit is provided with silica membrane columns and specific buffers. The procedure was performed in accordance with the manufacturer's instructions. The gel slice containing the DNA fragment was dissolved in a buffer that contained chaotropic salt at 50°C. Subsequently, the solution was loaded on the column. In the presence of chaotropic salt, DNA binds to a silica membrane. Possible contaminants were removed by washing. The column was then dried and DNA was eluted from the column with a slightly alkaline elution buffer.

2.11.5. Standard PCR amplification

Polymerase chain reaction (PCR) is a technique to amplify specific DNA fragments. A standard PCR amplification procedure is described below. A typical PCR reaction mixture with a total volume of 50 µl was prepared with the following components: 5 µl PCR buffer (10x); 3 µl $MgCl_2$ (25 mM); 1 µl dNTP's (25 mM each dATP, dCTP, dGTP, dTTP); 1 µl forward primer (10 µM);1 µl reverse primer (10 µM); 1 µl template DNA (10 pg to 1 µg); 0.1 µl *Taq* polymerase (2.5 U/µl) and 38 µl nuclease-free water. As template DNA either plasmid DNA, genomic DNA, PCR products or a sample of an individual bacterial colony were taken.

A standard PCR amplification reaction was performed as followed:

One cycle of:	95°C for 3 minutes (initial denaturation)
Thirty cycles of:	95°C for 30 seconds (denaturation)
	T_a for 30 seconds (primer annealing)
	72°C for 1 minute (extension)
One cycle of:	72°C for 5 minutes (final extension)
Hold at:	4°C (storage)

Generally, the primer annealing temperature (T_a) was 5°C lower than the lowest T_m of the primers in the reaction. PCR amplification reactions were performed with a MJ Research PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, USA). In order to obtain the best results, each PCR reaction was optimized and modifications to this standard PCR procedure were made.

2.12. Bacterial transformation with plasmid DNA

2.12.1. Preparation of bacterial chemically competent cells

Cells from the *E.coli* strain DH10B were made chemically competent with a method that was adapted from Inoue *et al*. (1990). A single colony was incubated overnight in 5 ml LB media at 37°C with shaking at 220 rounds per minute (rpm). From the overnight culture, 2.5 ml was transferred to 250 ml SOB medium (2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) and grown at 18°C with shaking (250 rpm) to an optical density of 0.6 at 600 nm. The bacterial cell culture was chilled on ice for 10 minutes and then centrifuged at 4000

rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 80 ml TB (10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 15 mM $CaCl_2$, 250 mM KCl, pH 6.7, 55 mM $MgCl_2$). The suspension was placed on ice for 10 minutes and centrifuged again at 4000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 20 ml of ice-cold 7% Dimethyl sulfoxide (DMSO) (v/v) in TB buffer. Cells were put on ice for 10 minutes. Subsequently, 50 µl of cells were aliquoted into pre-cooled Eppendorf tubes. Cell aliquots were frozen in liquid nitrogen and stored at -80°C

2.12.2. DNA transformation in bacteria by heat-shock

For one transformation, 50 µl of chemically competent cells were thawed on ice. The DNA (1 to 10 ng) was added to the cells and the mixture was incubated on ice for 30 minutes. Subsequently, cells were heat-shocked in a water bath at 42°C without shaking for 30 seconds. Immediately after the transformation, cells were placed on ice for 5 minutes. Then, 250 µl of SOC medium (2% (w/v) bactotryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added and the cell mixture was incubated in a shaker (200 rpm) at 37°C for 1 hour. Multiple dilutions of cell suspensions were plated on LB agar plates containing selective antibiotics and incubated overnight at 37°C.

2.13. Screening of bacterial clones

2.13.1. Colony PCR

The colony to be analyzed was picked from LB medium selection plate with a sterile toothpick. A sample of the colony was transferred to a PCR master mix and a PCR amplification was performed to determine whether the colony contained the correct plasmid.

2.13.2. Blue-white screening of bacterial colonies

Blue-white screening is a method that allows the detection of positive transformants. Plasmids designed for blue-white screening contain the *LACZ* reporter gene. The *LACZ* gene codes for the enzyme β-galactosidase. This enzyme metabolizes the substrate 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) to a blue product. Isopropylthio-b-D-galactoside (IPTG) is used as an inducer of the *LAC* operon. In the

presence of X-gal and IPTG the colony turns blue. During cloning, a DNA fragment is inserted within the *LACZ* gene sequence and the synthesis of β-galactosidase is disrupted. The colony remains white in the presence of X-gal and IPTG. White colonies indicate that the ligation of the DNA fragment into the plasmid was successful.
Prior to transformation experiments, 40 µl of X-gal (40 mg/ml in N,N-dimethylformamide (DMF)) and 40 µl of 100 mM IPTG were spread onto LB medium plates. However, when TOP10 cells were used as host strain, it was not necessary to spread IPTG on the medium plates.

2.14. Preparation of bacterial glycerol stocks

A bacterial colony of interest was picked from a selection plate and grown overnight in 5 ml LB medium with the appropriate antibiotic at 37°C with shaking (220 rpm). The next day, 0.5 ml of the bacterial culture was transferred to an Eppendorf tube and 0.5 ml of 100% (v/v) glycerol solution was added. Subsequently, the sample was mixed by vortexing vigorously for 1 minute. The glycerol sample was frozen in liquid nitrogen and stored at -80°C.

2.15. DNA plasmid purification from bacteria

2.15.1. Small-scale DNA plasmid purification from bacteria

Plasmid DNA was isolated from bacteria using a method developed by B. Weishaar (Max-Planck-Institute, Cologne, Germany).
E. coli cells were cultured in 5 ml LB medium containing the appropriate antibiotic, grown overnight at 37°C with shaking (220 rpm). The next day, bacterial cells were collected by centrifuging for 2 minutes at 15000 rpm. The supernatant was discarded and the cell pellet was resuspended in 200 µl of TELT buffer (50 mM Tris-HCl pH 7.5, 62.5 mM EDTA, 2.5 M LiCl, 0.4% (v/v) polyethylene glycol p-iso-octylphenyl ether (Triton X-100)) and 20 µl of freshly prepared Lysozyme solution (10 mg/ml Lysozyme (Biomol**,** Hamburg**,** Germany) in Tris EDTA (TE) buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). The cell mixture was heated at 95°C for 3 minutes and then cooled on ice for 5 minutes. Subsequently, the cell mixture was centrifuged at 13000 rpm for 15 minutes at 4°C. After centrifugation, the slimy pellet was removed with a sterile tooth pick. Then, 100 µl of isopropanol was added to the supernatant and the mixture was shortly vortexed. The mixture was centrifuged again at 15000 rpm for 15 minutes at 4°C. Supernatant was removed and the pellet was washed with 200 µl of 70% (v/v)

ethanol and centrifuged briefly. The DNA pellet was air-dried at room temperature and resuspended in 50 µl of TE/R solution (10 µg/ml RNase from a RNA stock solution (10mg/ml RNase A (Sigma, Deisenhofen, Germany) in TE buffer and boiled for 15 minutes) in 50 mM Tris-HCl pH 7.5). After 30 minutes of incubation, 50 µl of water was added to the DNA solution. DNA was precipitated by the addition of 100 µl of 4 M NH_4Ac and 600 µl of absolute ethanol. The sample was mixed thoroughly by vortexing and DNA was collected by centrifuging at 15000 rpm for 15 minutes at 4°C. Pellet was washed with 200 µl of 70% (v/v) ethanol and dried at room temperature. After drying, the pellet was dissolved in 25 µl of 10mM Tris/HCl pH 8.0.

2.15.2. Large-scale DNA plasmid purification from bacteria

The NucleoBond® AX 500 plasmid DNA purification kit (Macherey-Nagel, Düren, Germany) was used in order to obtain high concentration of plasmid DNA. This plasmid purification kit utilizes a silica-based anion-exchange system. Plasmid DNA is bound to the anion-exchange resin. The NucleoBond® AX 500 plasmid DNA purification kit is supplied with NucleoBond® AX 500 columns, NucleoBond® filters, RNase A and specific buffers. Buffer compositions can be found in the user's manual. The procedure was performed according to the manufacturer's instructions and is briefly described below.

E. coli cells were cultured in a suitable volume of LB medium, containing the appropriate antibiotic, grown overnight at 37°C with shaking (220 rpm). On the following day, bacterial cells were harvested by centrifugation (15000 rpm) for 15 minutes at 4°C and bacterial cells were dissolved in resuspension buffer. Then, the cells were lysed with an alkaline/sodium dodecyl sulfate (SDS) lysis procedure. The RNA present in the cell suspension was cleaved by RNase A (100 µg/ml). Next, chromosomal DNA and other cell components were precipitated by adding 2.8 M potassium acetate, pH 5.1. The precipitate was separated from the lysate with a help of a NucleoBond® filter. After equilibration of the NucleoBond® column, the clarified lysate was loaded on the column. When the NucleoBond® column was empty, possible contaminants were removed from the column by washing steps. Subsequently, plasmid DNA was eluted from the column and collected. Then, 15 ml of isopropanol was added to the plasmid DNA and the mixture was centrifuged at 10000 rpm for 15 minutes. The supernatant was discarded and the DNA pellet was washed once with 70% (v/v) ethanol. After washing, the pellet was dried at room temperature and resolved in an appropriate volume TE buffer.

2.16. DNA and RNA extraction from plant material

2.16.1. Genomic DNA extraction from leaf tissue

Genomic DNA was extracted from leaf tissue with the CTAB method of Murray and Thompson (1980) with some modifications.
The genomic DNA was extracted from 2 g of fresh young leaf tissue. Leaves were frozen with liquid nitrogen and ground to a fine powder using a mortar and pestle with the addition of 0.5 g of sterile quartz sand. The plant powder was transferred to an organic solvent-resistant tube and 0.5 g of polyvinylpyrrolidone was added to eliminate polysaccharides contaminates. Then, 8 ml of warm 2-ME/CTAB solution (2% (w/v) Cetyl Trimethyl Ammonium Bromide (CTAB), 2% (v/v) β-mercaptoethanol, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl) was added to the sample, mixed by vortexing and incubated at 65°C for 1 hour. After incubation, 8 ml of 24:1 (v/v) chloroform-isoamylalcohol mixture was added and the sample was mixed by inversion. This was followed by centrifugation at 8000 rpm at 4°C for 5 minutes. After centrifugation, the upper aqueous phase was transferred to a clean tube and the volume of the aqueous phase was estimated. Then, one-tenth volume of warm CTAB/NaCl solution (10% (w/v) CTAB, 7 M NaCl) as added to the aqueous phase and mixed well by shaking the tube. This was immediately followed by the addition of one volume of 24:1 (v/v) chloroform-octanol mixture. The sample was mixed and centrifuged at 8000 rpm at 4°C for 5 minutes. Again the aqueous phase was recovered in a clean tube. Next, one volume of CTAB precipitation buffer (1% (w/v) CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) was added to the aqueous phase and mixed well by inversion. When a precipitate was visible in the solution, the sample was centrifuged at 2000 rpm at 4°C for 5 minutes. If no precipitate was visible, the sample was incubated at 65°C for 30 minutes before centrifugation. Supernatant was discarded and pellet was dissolved in 4 ml of high-salt TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0). DNA was precipitated by adding 2.4 ml of isopropanol. The sample was mixed by vortexing and centrifuged at 8000 rpm at 4°C for 15 minutes. The resulting DNA pellet was washed with 70% (v/v) ethanol, dried at room temperature and resuspended in 500 µl of 10 mM Tris/HCl pH 8.0.

2.16.2. Total RNA extraction from leaf tissue

Total RNA from leaf tissue was extracted with the method of Valenzuela-Avendaño *et al*. (2005) with minor modifications.
The RNA was extracted from 50 mg of leaf tissue. Dried leaves were frozen with liquid nitrogen and ground to a fine powder with an RNase-free mortar and pestle. The leaf powder was dissolved in 1.5 ml of extraction buffer (38% (v/v) buffer-saturated phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate pH 5.0, 5% (v/v) glycerol) and the mixture was homogenized. After homogenization, the mixture was incubated at room temperature for 10 minutes and then centrifuged at 10.000g at room temperature for 10 minutes. The supernatant was transferred to a clean Eppendorf tube and 300 µl of 24:1 (v/v) chloroform-isoamylalcohol mixture was added. The sample was vortexed vigorously for 10 seconds, followed by centrifugation at 10.000 rpm at 4°C for 10 minutes. After centrifugation, the clear upper aqueous phase was transferred into a clean Eppendorf tube. Then, 375 µl of isopropanol and 375 µl of 0.8 M sodium citrate/1 M sodium chloride mixture were added to the aqueous phase. The sample was mixed well by inversion and incubated at room temperature for 10 minutes. The sample was centrifuged at 12.000 rpm at 4°C for 10 minutes. Supernatant was discarded carefully and the pellet was washed with 1 ml of 70% (v/v) ethanol at -20°C. The sample was centrifuged again at 12.000 rpm at 4°C for 10 minutes. After elimination of the supernatant, the obtained pellet was dried at room temperature. The dried pellet was dissolved in 100 µl of DEPC-treated water (0.2% (v/v) diethyl pyrocarbonate (DEPC) in distilled water, stirred vigorously for 1 hour and then autoclaved). Subsequently, 167 µl of 4 M LiCl was added and the solution was kept on ice for 2 hours. The sample was centrifuged at 14.000 rpm at 4°C for 10 minutes and supernatant was removed. The pellet was washed twice with 1 ml of 70% (v/v) ethanol at -20°C, dried for 10 minutes at room temperature and resuspended in an appropriate volume of DEPC-treated water.

2.17. Detection of DNA and RNA sequences

2.17.1. Southern blot analysis

Southern blot analysis (Southern, 1975) was performed according to Sambrook *et al*. (1989) with minor modifications. Genomic DNA (20 µg) was digested overnight at 37°C with the desired restriction enzymes. The digested genomic DNA samples were loaded onto a 0.8% agarose gel in TAE and electrophoresis was done overnight at 20V.

After electrophoresis, the gel was stained with EtBr, shortly rinsed with distilled water and photographed. Depurination of the DNA was carried out by soaking the gel in 0.25 M HCl and incubation under agitation for 30 minutes. The HCl was removed and the gel was washed with distilled water. DNA was denatured by soaking the gel in 500 ml of denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 20 minutes under agitation for 20 minutes. This step was repeated once. After the second treatment, the denaturation solution was poured off and 500 ml of neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.0) was added and again incubated under agitation for 20 minutes. Then, the neutralization solution was refreshed and the gel was again incubated for 20 minutes.
The DNA was transferred from the gel to a Hybond™ N+ nylon membrane (Amersham, Braunschweig, Germany) by upward capillary transfer. To transfer DNA to the nylon membrane, a blotting tower was assembled (Figure 6).

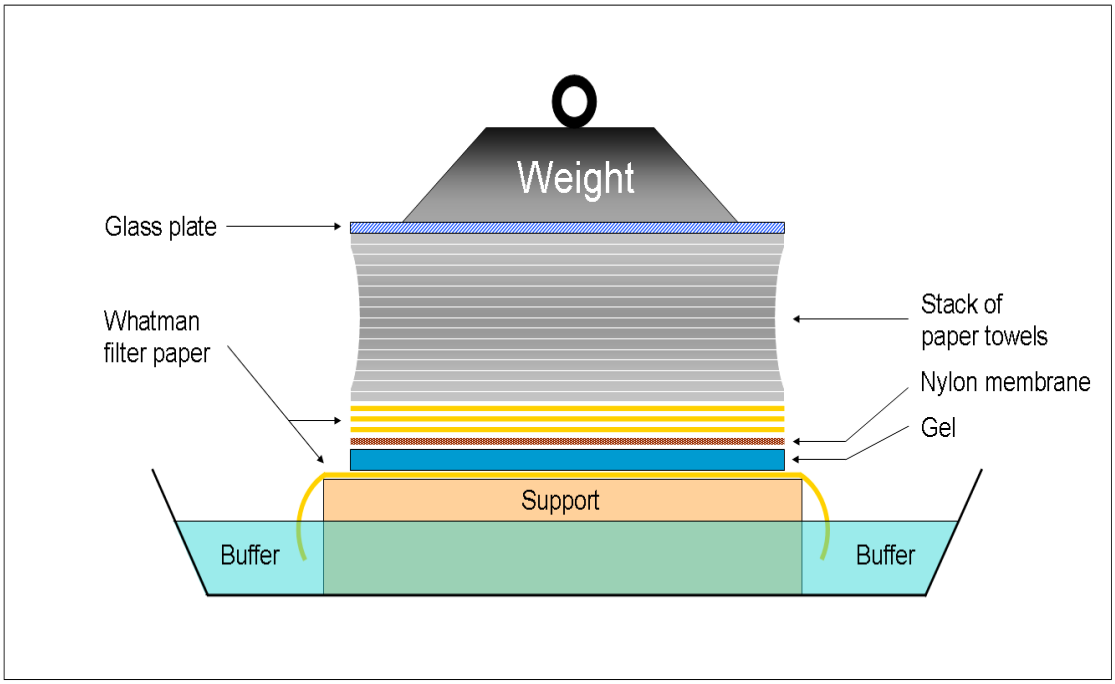


Figure 6. Schematic representation of a blotting tower used for nucleic acid blot analysis.

An inverted gel tray was used as a solid support for the agarose gel and placed in a container. A sheet of Whatman filter paper was placed onto the inverted gel tray with both sides of the Whatman paper reaching the bottom of the container. The container was filled with 20x saline sodium citrate (SSC) buffer (3 M NaCl, 300 mM Sodium citrate pH 7.0) to the surface of the inverted gel tray. Then, the gel was inverted and placed on an inverted gel tray. The edges of the gel were covered with four pieces of

Parafilm (American National Can, Chicago, USA). The nylon membrane, three sheets of Whatman filter paper and a stack of paper towels were cut in the same size as the gel. The corner of the nitrocellulose membrane was cut to mark the orientation. Then, the nitrocellulose membrane was wetted in distilled water and subsequently soaked in 20x SSC for 5 minutes. Carefully, the wetted nitrocellulose membrane was placed on the surface of the gel. Next, the three sheets of Whatman filter paper were wetted with 20x SSC and put on top of the nitrocellulose membrane. Possible air bubbles were removed by rolling a glass pipette over the surface. A stack of paper towels of about 10 cm in height was placed upon the three sheets of Whatman paper. Finally, a glass plate together with a weight of about 500 g was put on top of the blotting tower. The DNA transfer to the nylon membrane was done overnight. After transferring the DNA, the blotting tower was dismantled and DNA was fixed onto the nylon membrane by exposure to UV light for 3 minutes, followed by baking at 80°C for 2 hours.

2.17.2. RNA gel blot analysis

RNA gel blot analysis was adapted from Sambrook *et al*. (1989) with minor changes. RNA fragments were separated by gel electrophoresis that was carried out under denaturing conditions. First, a 1.2% (w/v) agrarose gel was prepared. For 150 ml of agarose gel, 1.8 g agarose was dissolved in 93 ml of DEPC-treated water, heated and then cooled down to 60°C in a water bath. Under a fume hood, 30 ml of 5x MOPS (200 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 50 mM sodium actetate pH 7.0, 0.5 mM EDTA) and 27 ml 37% (v/v) formaldehyde were added. The gel was poured into a gel tray and allowed to solidify at room temperature. After solidification, the gel was placed in a tank, which was filled with an adequate volume of RNA electrophoresis solution (1x MOPS, 2.96% (v/v) formaldehyde).
Then, the RNA blot sample buffer was prepared by pipetting 100 µl of 5x MOPS, 175 µl of 37% (v/v) formaldehyde and 500 µl of formamide together. Subsequently, the RNA (20 µg) was mixed with the RNA blot sample buffer at a volume ratio of 1:1. Next, one-tenth volume of 10x RNA loading dye (10 mM EDTA pH 8.0, 0.25% (w/v) bromophenol blue. 0.25% (w/v) xylene cyanol FF) was added and RNA samples were heated for 15 minutes at 65°C to denature the RNA. After heating, samples were briefly cooled on ice and then loaded onto the gel. Gel electrophoresis was performed at 65V until the RNA samples had migrated at least 4 cm into the gel.
After electrophoresis, RNA was transferred from the gel to a Hybond™ N+ nylon membrane (Amersham, Braunschweig, Germany) by upward capillary transfer (Figure

6). The RNA transfer and fixation to the membrane were done as described for the transfer of DNA.

2.17.3. Radiolabeling of DNA oligonucleotides with α-^{32}P-dCTP

Oligonucleotide DNA probes were radiolabeled with α-^{32}P-dCTP using the DecaLabel™ DNA labeling kit (Fermentas, St. Leon-Rot, Germany). This labeling procedure with random primers is based on the method of Feinberg and Vogelstein (1983).
One hundred ng of DNA template (10 ng/µl) was used for the labeling reaction. Ten µl of decanucleotides were added to the DNA probe and the volume was brought to 40 µl with nuclease-free water. The probe was denatured by heating at 95°C for 5 minutes, briefly centrifuged and cooled on ice. Next, 3 µl of Mix C (dNTPs without dCTP) was added to the probe mixture. Subsequently, 2 µl of α-^{32}P-dCTP (Hartmann Analytic, Braunschweig, Germany) and 1 µl of Klenow-mix were added. The mixture was gently mixed by pipetting up and down and incubated at 37°C for 10 minutes. Then, 4 µl of dNTP-mix was added and the mixture was incubated at 37°C for a further 5 minutes. The labeling reaction was stopped by adding 100 µl of 1x TE.
Non-incorporated nucleotides were separated from the labeled probe using a Sephadex G-50 column, which was equilibrated with 1x TE. The labeled probe was loaded on the column and eluted with 1x TE. In total, ten fractions of 100 µl each were collected. Fractions, which contained the radioactively labeled DNA fragments, were combined, heated at 95°C for 5 minutes, briefly centrifuged and used for hybridization reactions.

2.17.4. Southern and RNA blot hybridization with α-^{32}P-dCTP-labeled DNA probe

Southern and RNA blot hybridizations were performed as described by Sambrook *et al.*, (1989) with minor modifications.
Membranes were incubated in pre-hybridization solution to prevent non-specific binding of the probe during the hybridization reaction. Southern blots were incubated in DNA pre-hybridization solution (600 mM NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 0.1% (w/v) SDS, 10x Dernhardt's (100x Denhardt's: (2% (w/v) bovine serum albumin (BSA) Fraction V, 2% (w/v) Ficoll 400 and 2% (w/v) polyvinylpyrrolidone (PVP) 360.000), 100 µg/ml heat-denatured salmon sperm) at 65°C for at least 4h under continuous shaking. RNA blots were incubated in RNA pre-hybridization solution (50% (v/v) deionized formamide, 5x SSC, 10 mM PIPES pH 6.8, 1x Denhardt's, 0.1%

(w/v) SDS, 100 µg/ml heat-denatured salmon sperm) at 42°C for at least 4 hours under continuously shaking.
Pre-hybridization solution was replaced by hybridization solution. Southern blots were incubated in DNA hybridization solution (600 mM NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 0.1% (w/v) SDS, 10x Dernhardt's solution) and RNA blots in RNA hybridization solution (50% (v/v) deionized formamide, 5x SSC, 10 mM PIPES pH 6.8, 1x Denhardt's, 0.1% (w/v) SDS). Subsequently, the radiolabeled DNA probe was added to the hybridization solution and hybridization was performed overnight at 65°C for Southern blots or at 42°C for RNA blots.
After hybridization with the probe, the membrane was incubated with washing solution (0.1% (w/v) SDS and 2x SSC) for 30 minutes at 65°C for Southern blots or at 42°C for RNA blots. Washing steps were repeated until all unbound probe was removed. Then, the membrane was wrapped in plastic wrap and exposed to a phosphor screen (Amersham, Braunschweig, Germany) overnight.
The radioactive signal was detected with a phosphorimager Typhoon™ 9200 and Typhoon Scanner Control version 3.0 software (Amersham, Braunschweig, Germany).

2.18. Reverse transcriptase PCR analysis

Reverse transcriptase (RT) reactions were performed with 2 µg of total RNA that was extracted from *C. plantagineum* leaves treated with either 0.1 µM ABA, 0.8 M mannitol or water for 48 hours. Possible DNA contaminations in the RNA samples were removed by DNase treatment. The following reagents were added to the RNA sample: 1 µl of 10x DNase buffer (400 mM Tris-HCl pH 8.0, 60 mM $MgCl_2$), 1 µl DNase (10U/µl) (Boehringer, Mannheim, Germany) and 8 µl of distilled water. The reaction mixture was incubated at 37°C for 30 minutes. After incubation, 1 µl of 25 mM EDTA was added and the mixture was heated at 65°C for 10 minutes to inactivate the DNase activity. First-strand complementary DNA (cDNA) was synthesized using an oligo(dT) primer. First, 10 µl of DNase-treated RNA was transferred into an Eppendorf tube, 2 µl of oligo(dT) primer (25 pmol/µl) and 1 µl of NTPs (10 mM each) were added. The mixture was incubated at 65°C for 5 minutes, cooled down and briefly centrifuged. Then, 4 µl of 5x First-strand buffer (Invitrogen, Karlsruhe, Germany) and 2 µl of 0.1 M DTT were added to the sample. The mixture was incubated at 42°C for 2 minutes and 1µl Superscript II Reverse Transcriptase (200 U/µl) (Invitrogen, Karlsruhe, Germany) was added. Subsequently, the reaction mixture was further incubated at 42°C for 50 minutes. After incubation, the enzyme was inactivated by heating the sample at 70°C for 5 minutes. The synthesized cDNA samples were 5 times

diluted with distilled water and 1 µl of the diluted cDNA solution was used as template for PCR amplifications using transcript specific primers (Table 2). The PCR reaction mixtures were composed of 1 µl of cDNA template dilution, 1 µl of Forward primer (5 µM/µl), 1 µl of Reverse primer (5 µM/µl), 9 µl of RealMasterMix™ (0.05 U/µl HotMaster™ *Taq* Buffer, 10 mM magnesium acetate, 1 mM dNTPs) (5 PRIME, Hamburg, Germany) and 8 µl of distilled water.
All RT-PCR reactions were performed under the following conditions: one cycle of 95°C for 2 minutes, 33 cycles of 95°C for 15 seconds; 55°C for 20 seconds; 68°C for 30 seconds, hold at 4°C. Amplified PCR products were visualized on an EtBr-stained agarose gel.

2.19. Comparative EST analysis

In order to perform a comparative expressed sequence tag (EST) analysis, two cDNA collections were generated from desiccated *L. brevidens* and *L. subracemosa* leaves.

2.19.1. Construction of EST collections from dried leaf tissue

Messenger RNA (mRNA) extracted from dried leaf tissues was reverse transcribed into cDNA. The cDNA libraries were constructed with ZAP-cDNA® synthesis kit and ZAP-cDNA® Gigapack® III gold cloning kit (Stratagene, Heidelberg, Germany). All procedures were performed according to the manufacturer's manual. The different steps in the procedure are described briefly below.
First-strand cDNA was synthesized from the mRNA templates by reverse transcriptase with the StrataScript™ (Stratagene, Heidelberg, Germany) and the hybrid oligo(dT) linker primer (Table 2) that contained an *Xho*I restriction site. The dNTP mixture that was used for the reverse transcriptase PCR contained 5'-methyl dCTP instead of normal dCTP to protect generated cDNA from restriction enzymes. After reverse transcriptase PCR, mRNA was cleaved by treatment with RNase. The second-strand cDNA was synthesized using cleaved mRNA fragments as primers and DNA polymerase I (Stratagene, Heidelberg, Germany). When the second cDNA strand was synthesized, the double stranded cDNA fragments were blunt-ended with blunting dNTP mix and *Pfu* DNA polymerase (Stratagene, Heidelberg, Germany). This was followed by the ligation of adaptors containing an *Eco*RI overhangs to the blunt-ends of the cDNA fragments. To obtain cDNA fragments of the desired length, the cDNA fragments were separated with a size fractionation column. Subsequently, cDNA

fragments were digested with *Xho*I to realese adaptors and digested cDNA fragments were cloned into the UNI-ZAP® XR phage vector at *Eco*RI and *Xho*I sites.
The constructed UNI-ZAP® XR library was amplified in XL1-Blue MRF' cells (Stratagene, Heidelberg, Germany). Infected XL1-Blue MRF' cells were grown on NZY medium plates and the amplified λ-phage library was purified after plaque formation.
The UNI-ZAP® XR phage vector contained an excisable pBluescript phagemid, holding the cDNA fragments. A mass excision was performed to extract the pBluescript phagemids. The XL1-Blue MRF' strain was co-infected with the amplified UNI-ZAP® XR phage vector, containing the cDNA fragments and the ExAssist® helper phage. The ExAssist® helper phage produces components that are required for the *in vivo* excision of the pBluescript phagemid from the UNI-ZAP® XR vector. The excised single stranded pBluescript phagemids were packaged into λ-phage particles.
The XL1-Blue MRF' cells were infected with λ-phage library at a 1:10 λ-phage-to-cells ratio. Then, the ExAssist® helper phage was added at a 10:1 helper phage-to-cells ratio. After co-infection and growth of cells, cells were lysed by heating at 70°C and λ-phage particles were released. The excised pBluescript phagemids were separated from the cell debris by centrifugation.
Recovered phagemids were then introduced into SOLR™ cells (Stratagene, Heidelberg, Germany) to produce double stranded cDNA fragments. The ExAssist® helper phage contains a mutation and was therefore unable to grow in SOLR™ cells. Hence, only the excised pBluescript phagemid were allowed to replicate in SOLR™ cells. After infection, variable concentrations of infected SOLR™ cells were spread onto LB medium plates supplemented with (50 µg/ml) ampicillin. The pBluescript phagemids were extracted from obtained colonies and cDNA inserts sequenced.

2.19.2. Annotation of EST sequences and categorization

Sequences from the EST collections were annotated using the Gene Ontology (http://www.geneontology.org/) (The Gene Ontology Consortium, 2000) or the UniProt Knowledgebase (http://www.uniprot.org/) and categorized into different functional groups described in Bevan *et al*. (1998). The functional group: "Desiccation-related" was added to these groups.

2.20. Comparative promoter analysis

To conduct a comparative promoter analysis it was necessary to isolate 5'-upstream sequences from genes of interest.

2.20.1. Promoter isolation

The 5'-upstream putative promoter sequences were isolated using the GenomeWalker™ Universal kit (Clontech, Heidelberg, Germany). GenomeWalker™ reactions were performed as described in the user's manual. A schematic view of the GenomeWalker™ principle is shown in Figure 7.

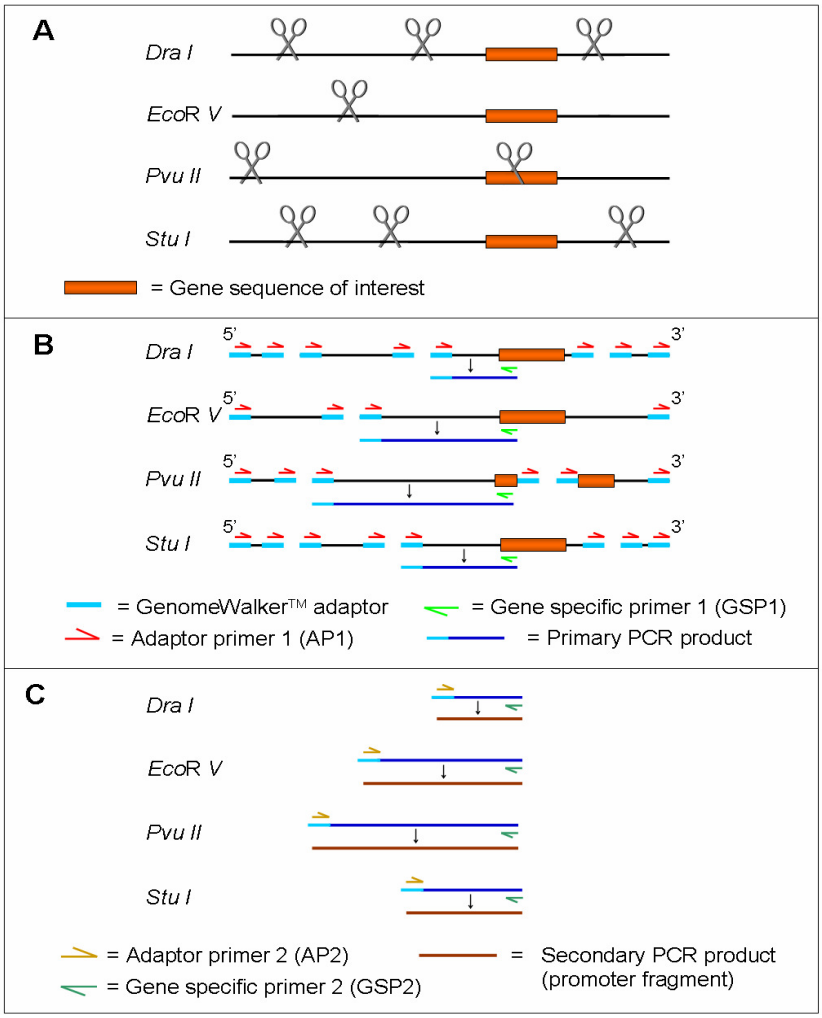


Figure 7. Principle of the GenomeWalker™ technique. (A) Contruction of libraries *Dra*I, *Eco*RV *Pvu*II and *Stu*I. (B) Ligation of GenomeWalker™ adaptor fragments and primary PCR amplification with primers AP1 and GSP1. (C) Secondary PCR amplification with primers AP2 and GSP2.

First, four genomic DNA libraries were generated by digesting genomic DNA with the restriction enzymes *Dra*I, *Eco*RV, *Pvu*II and *Stu*I (Figure 7A). After digestion, each of the libraries contained DNA fragments of varying length. Next, GenomeWalker™ adaptors were ligated to the 5'- and 3'-end of the digested DNA fragments. Then, a primary PCR with the primer set, Adaptor primer 1 (AP1) and Gene specific primer 1 (GSP1) was conducted (Figure 7B). Subsequently, a secondary PCR was performed, using the primary PCR products as templates and the primer pair, Adaptor primer 2 (AP2) and Gene specific primer 2 (GSP2). After the secondary PCR, promoter fragments of variable length were obtained (Figure 7C). All GenomeWalker™ PCR amplification was performed with the high-fidelity Platinum® *Taq* DNA Polymerase (Invitrogen, Karlsruhe, Germany).

2.20.2. Cloning of promoter fragments.

Isolated promoter fragments were cloned into either pPCR2.1® TOPO® vector using the TOPO TA Cloning® kit (Invitrogen, Karlsruhe, Germany) or pJET1.2 vector using the CloneJET™ PCR Cloning kit (Fermentas, St. Leon-Rot, Germany). Both these methods provide a cloning technique for direct insertion of PCR products into a plasmid vector. Both methods were performed according to the manufacturer's protocols.

The TOPO TA Cloning® method utilizes the enzyme DNA topoisomerase I. Topoisomerase I is an enzyme derived from the *Vaccinia* virus, where it functions both as restriction enzyme and ligase. The TOPO TA Cloning® kit is provided with a linearized pPCR2.1® TOPO® vector with topoisomerase I covalently bound to each 3´-phosphate. During a typical DNA amplification, *Taq* DNA polymerase adds a single deoxyadenosine to the 3'-ends of the PCR product. Therefore, the linearized pPCR2.1® TOPO® vector contains single 3'-thymidine overhangs. In the TOPO TA Cloning® reaction, the PCR product is ligated with the compatible ends of the linearized pPCR2.1® TOPO® vector.

The CloneJET™ PCR Cloning kit permits the direct cloning of either sticky- or blunt-end DNA fragments into the pJET1.2 vector. The kit is supplied with a DNA Blunting enzyme in order to clone PCR products with 3'-adenine overhangs or DNA fragments with sticky-ends. Furthermore, this kit is provided with a linearized blunt-end pJET1.2 vector. The DNA fragments are ligated into this blunt-end vector with the help of T4 DNA ligase. The pJET1.2 vector harbors a lethal restriction enzyme site, which is disturbed by the ligation of a DNA fragments into the vector and this means that only positive clones can grow.

2.20.3. Detection of *cis*-acting regulatory elements

Putative *cis*-acting regulatory elements were identified in promoter sequences using the plant *cis*-acting regulatory DNA elements (PLACE) database (http://www.dna.affrc.go.jp/ PLACE/index.html) (Higo *et al*., 1999).

2.21. Preparation of promoter::*GUS* reporter gene constructs

To analyze the activity of the *LEA-like 11-24* promoters, DNA fragments were fused to the *β–glucuronidase* (*GUS*) reporter gene. The *LEA-like 11-24* promoter regions were amplified with GUS fusion primers containing restriction sites at their 5'-ends (Table 2). The left primer held an *Eco*RI site and the right primer contained the either a *Nco*I or *Xho*I restriction site Amplified promoter fragments were digested with relevant restriction enzymes and inserted into compatible restriction sites of the pBT10GUS plasmid.

2.22. Biolistic transformation of leaf tissue

Leaf tissue was biolistically transformed with the different *LEA-like 11-24* promoter::*GUS* fusion constructs. The fusion construct were introduced in leaf tissue via a particle bombardment. Particle bombardments were carried out as described by Sanford *et al*., (1993) with minor modifications. Leaf tissue from *C. plantagineum*, *L. brevidens* and *L. subracemosa* were used for bombardments.

2.22.1. Preparations of gold microcarrier particles

Prior to the particle bombardment, leaves were detached from 3-month old *in vitro* grown plants and arranged in the center of a Petri dish containing half-strength MS medium. Finally, the leaves covered a space of approximately 16 cm^2.
Next, 30 mg microcarrier gold particles (1.6 µM in diameter (Bio-Rad, Munich, Germany)) were transferred into an Eppendorf tube. The gold particles were sterilized by adding 1 ml of 70% (v/v) ethanol. The mixture was vortexed vigorously for 5 minutes and the gold particles were incubated in 70% (v/v) ethanol for 15 minutes.
After sterilization, the supernatant was discarded. Gold particles were washed by adding 1 ml of sterile water and vortexing for 1 minute. Particles were allowed to settle for 1 minute and then the supernatant was carefully removed with a pipette. These washing steps were repeated three times.

After the third wash, all liquid was removed and gold particles were resuspended in 500 µl of sterile 50% (v/v) glycerol solution to bring the gold microcarriers to a concentration of 60 mg/ml.
Because of the high variability of particle bombardments, the *LEA-like 11-24* promoter::*GUS* constructs were co-bombarded with the pGJ280 plasmid (kindly provided by Dr. G. Jach, Max-Planck-Institute, Cologne, Germany). The plasmid pGJ280 harbours a cauliflower mosaic virus (CaMV) 35S promoter fused to the *green fluorescent protein* (*GFP*) gene. The GFP signal was used as an internal standard to measure the efficiency of each particle bombardment.
Before gold particles were coated with DNA, the gold particle solution was vortexed for 5 minutes to ensure an even suspension. For two bombardments, 50 µl (3 mg) of gold particles were pipetted into a sterile Eppendorf tube. Under continuous vortexing, 30 µl of plasmids DNA (15 µg of 35S CaMV::*GFP* and 15 µg of *LEA-like 11-24* promoter::*GUS* construct at a ratio of 1:1), 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine (Sigma, Deisenhofen, Germany) were added in this order to the gold particle solution. The mixture was vortexed for 5 minutes and left to settle for 1 minute before the liquid was removed. Gold particles were washed with 140 µl of 70% (v/v) ethanol, vortexed and liquid was removed carefully. This washing step was repeated once, but with 140 µl of absolute ethanol. Finally, DNA-coated gold particles were resuspended in 30 µl of absolute ethanol.

2.22.2. Particle bombardment of leaves

Leaf tissues were bombarded using a helium-driven microprojectile gene gun (Biolistic® Particle Delivery System-1000/He Device, Bio-Rad, Munich, Germany). All equipment used for the particle bombardment was pre-sterilized with 70% (v/v) ethanol. The principle of the gold particle bombardment is shown in Figure 8.

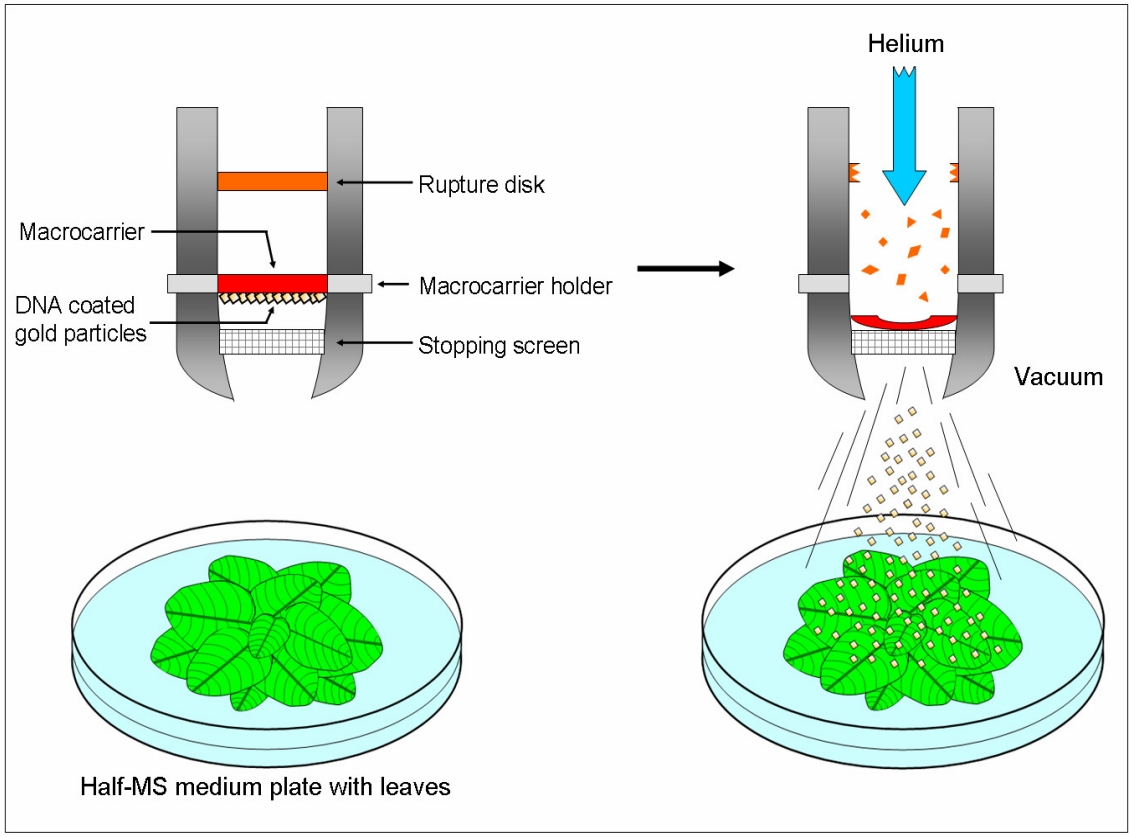


Figure 8. Principle of particle bombardment. All equipment was assembled into the launch assembly unit. A vacuum was applied to the bombardment chamber and helium pressure was increased until the rupture disk burst. The macrocarrier with DNA-coated gold particles was accelerated towards the leaf tissue. The macocarrier was then stopped by the stopping screen and DNA-coated gold particles were shot into the leaves.

The plastic macrocarrier disk was placed into the macrocarrier holder and 15 µl of DNA-coated gold particle suspension was transferred onto the center of the macrocarrier. The macrocarrier and stopping screen were placed into the launch assembly unit. The half-strength MS medium plate carrying the leaves was positioned 6 cm below the stopping screen. Vacuum was then applied to the bombardment chamber and helium pressure was increased until the burst of the rupture disk. This caused the macrrocarries, holding the DNA-coated gold particles to accelerate towards the leaves. The macrocarrier was held by the stopping screen and DNA-coated gold particles moved into the leaf tissue. The microcarrier gold particles were accelerated with a helium pressure of 1350 pounds per square inch (9.3 Mega Pascal (MPa)) under a vacuum of 27 mm of mercury (3.6 MPa).

Bombarded leaves were transferred to solutions with either 100 µM ABA (100 mM ABA (Sigma, Deisenhofen, Germany) stock solution in absolute ethanol), 0.8 M mannitol or water.

2.23. Detection of promoter activity

2.23.1. Determination of GFP activity in bombarded leaves

GFP activity in the leaves was analyzed 24 hours after the particle bombardment. A bombarded leaves was taken and sandwiched between two Micro Cover glass slides with a thickness of 0.13 to 0.17 mm (VWR International, Darmstadt, Germany). Subsequently, the leaf was analyzed for GFP activity. GFP fluorescence in bombarded leaves was visualized with an inverted confocal laser-scanning microscope (Nikon Eclipse TE2000-U/D-Eclipse C1, Nikon, Düsseldorf, Germany). Excitation of GFP was performed at a wavelength of 488 nm and detection was conducted at 515 nm. Chloroplast autofluorescence was excited at 543 nm and detected at 570 nm. Images were processed with EZ-C1 version 3.20 software (Nikon, Düsseldorf, Germany). All bombarded leaves were thoroughly screened for GFP signals and the number of GFP expressing cells per leaf was determined. After GFP analysis, leaves were placed back into the treatment solutions and treatment was continued for another 24 hours before GUS activity was determined.

2.23.2. Determination of GUS activity in bombarded leaves

The activity of the *GUS* reporter gene in bombarded leaves was screened 48 hours after the particle bombardment. First, leaves were histochemically stained with 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) (Jefferson *et al*., 1987). The GUS enzyme catalyzes the cleavage of the substrate X-Gluc, which results in the formation of an insoluble blue precipitate.
Bombarded leaves were submerged in freshly prepared X-Gluc solution (2 mM X-Gluc (in DMF), 0.1% (v/v) Triton X-100 and 0.1 M $NaPO_4$ pH 7.0) and incubated overnight at 37°C in the dark. After histochemical staining, the X-Gluc solution was removed and leaves were destained with 70% (v/v) ethanol at 80°C for 2 hours.
Leaves stained for GUS activity were analyzed with a stereoscopic zoom microscope with binocular eyepiece tube and digital camera system (Nikon SMZ 800, Nikon Digital Sight DS-2Mv, Nikon, Düsseldorf, Germany). Images were captured with NIS

Elements version D 2.2 software (Nikon, Düsseldorf, Germany). The number of blue GUS spots per leaf was determined.

2.23.3. Determination of *LEA-like 11-24* promoter activity

The relative activity of the different *LEA-like 11-24* promoter fragments from *C. plantagineum*, *L. brevidens* and *L. subracemosa* was determined using the method of Schenk *et al*., (1998). The number of GFP expressing cells per bombarded leaf was compared with the number of GUS producing spots on the bombarded leaves. A standardized value for each transient expression assay was calculated by dividing the number of GUS spots by the number of GFP expressing cells. From this standardized value, the percentage level of *LEA-like 11-24* promoter activity compared to the CaMV 35S promoter activity was calculated. The average of either four or seven independent experiments was determined and the standard deviation was calculated

2.24. Site-directed mutagenesis of promoter fragments

Introduction of site-directed mutations into the *LEA-like 11-24* promoter sequences was accomplished with three different methods

2.24.1. Introduction of mutations using an overlapping PCR extension technique

Site-directed mutations were generated into the promoter fragments using an overlapping extension PCR technique. The principle of this overlapping PCR method is shown in Figure 9. Two mutagenic primers carrying the desired mutation were designed: Mutagenic primer left and Mutagenic primer right. These mutagenic primers contained a small sequence overlap. In additions, two primers which were complementary to the ends of the promoter fragment were created: End primer left and End primer right. Then, two separate PCR amplifications were performed. One PCR reaction was done with a mutagenic primer left and a GUS fusion primer right (Table 2). The other PCR reaction was conducted with a Mutagenic primer right and a GUS fusion primer right. Each PCR reaction was amplifying a different part of the promoter sequence. PCR products were separated on an agarose gel and purified. Then, these two PCR products were fused together by conducting a second overlap PCR, which resulted in the amplification of the complete promoter fragment containing the desired mutation. The mutated promoter fragment was subsequently cloned into the pBT10GUS plasmid.

For all PCR amplifications, *Pfu Turbo*® DNA polymerase (Stratagene, Heidelberg, Germany) was used. This PCR technique demands an enzyme, like *Pfu Turbo*® DNA polymerase, because this enzyme has the ability to generate PCR products with blunt-ends. This is different from *Taq* DNA polymerase that adds a single deoxyadenosine to the 3'-ends of a PCR product. Furthermore, high-fidelity DNA synthesis was required and therefore the proofreading *Pfu Turbo*® DNA polymerases was chosen.

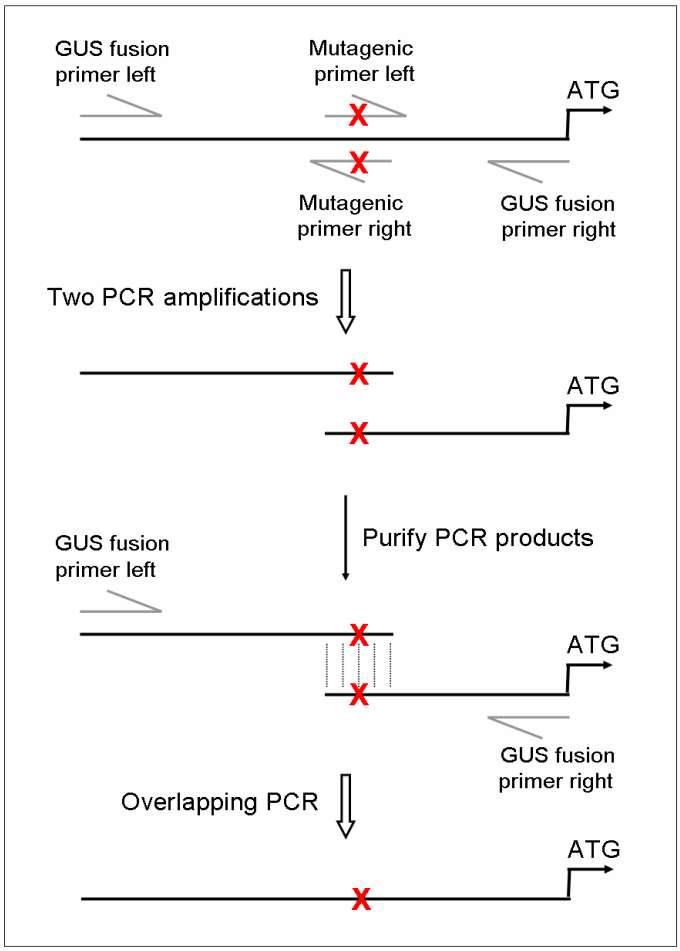


Figure 9. Principle of introducing site-directed mutations into promoter fragments using the overlapping PCR technique. First two separate PCR amplifications were performed either with primer pairs: GUS fusion primer left and mutagenic primer right or GUS fusion primer right and mutagenic primer left. The two promoter fragments were connected by an overlapping PCR with GUS fusion primer left and GUS fusion primer right.

2.24.2. Introduction of mutations using QuikChange® Site-Directed Mutagenesis

Alternatively, site-directed mutations were generated with the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). All procedures were done following the manufacturer's instruction. Specific mutations were introduced by performing a PCR reaction with mutagenic primers (Table 2). This PCR amplification generated a plasmid with the desired mutations. The parental non-mutated template was

eliminated by digestion with *Dpn*I. After digestion, the mutated DNA was transformed into XL1-Blue cells.

2.24.3. Introduction of mutations using a two stage PCR and QuikChange® Site-Directed Mutagenesis

The third procedure that was applied to introduce site-directed mutations into the promoter sequences was a method developed by Wang and Malcolm (1999). This procedure is based on the basic QuikChange® II Site-Directed Mutagenesis protocol (Stratagene, Heidelberg, Germany). However, with the method from Wang and Malcolm (1999), PCR amplifications were conducted in two steps. First, two pre-PCR reactions were performed in parallel. One pre-PCR was performed with a Mutagenic primer left for the coding strand of the promoter sequence and another pre-PCR was conducted with a Mutagenic primer right for the non-coding strand (Table 2). These two single-primer extension reactions amplified, respectively, the coding and non-coding strand of the promoter sequence. Subsequently, the two pre-PCR reactions were mixed together and a new PCR reaction was performed. This PCR generated a double-stranded promoter sequence. All PCR steps in this procedure used *Pfu Turbo*® DNA polymerase (Stratagene, Heidelberg, Germany). After the two pre-PCR amplifications, the standard QuikChange® II Site-Directed Mutagenesis procedure was followed.

2.25. Yeast one hybrid system

2.25.1. Preparations of the pSK1 (11-24) bait construct

To perform the yeast one hybrid system (Y1HS), a region of the *Cp LEA-like 11-24* promoter sequence was used as a DNA bait. This promoter region was amplified with different combinations of primers, which contained restriction sites at their 5'-ends (Table 2). The cloning strategy that was conducted for the construction of the pSK1 (11-24) bait vector is briefly described below and illustrated in Figure 10.

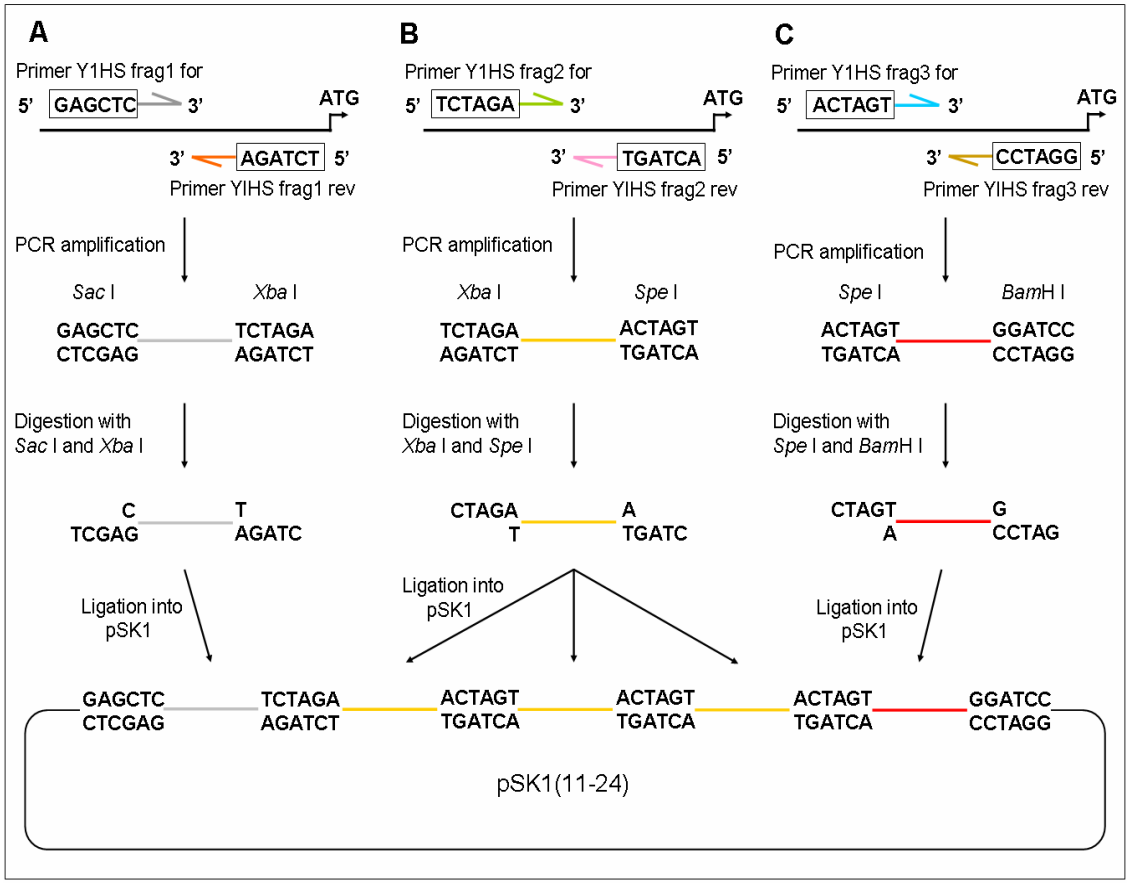


Figure 10. Cloning strategy for construction of the pSK1 (11-24) bait vector. In total five copies of a fragment of the *Cp LEA-like 11-24* promoter were cloned into the pSK1 vector. (A) First, the fragment of the *Cp LEA-like 11-24* promoter was amplified with Primer Y1HS frag1 for and Primer Y1HS frag1 rev. These primers introduced a *Sac*I and *Xba*I restriction sites at the ends of the promoter fragment (indicated in grey). The amplified promoter fragment was then digested with the restriction enzymes *Sac*I and *Xba*I and subsequently cloned into vector pSK1 at *Sac*I and *Xba*I sites. (B) Next, a PCR was conducted with primers Y1HS frag2 for and Y1HS frag2 rev, which added *Xba*I and *Spe*I restriction sites to the promoter fragment (indicated in yellow). After digestion with the relevant enzymes, the promoter fragment was ligated between the *Xba*I and *Spe*I sites of pSK1. However, because the *Xba*I and *Spe*I restriction sites at the end of this promoter fragment were compatible with each other, three copies of this promorer fragment were cloned into pSK1. (C) Finally, amplification with primers Y1HS frag3 for and Y1HS frag3 rev introduced *Spe*I and *Bam*HI restriction sites at the ends of the promoter fragment (indicated in red). After restriction enzyme digestion, this promoter fragment was cloned into the *Spe*I and *Bam*HI sites of pSK1.

The first PCR amplification with primers Y1HS frag1 for and Y1HS frag1 rev introduced a *Sac*I and *Xba*I restriction site at the ends of the promoter fragment, whereas PCR with primers Y1HS frag2 for and Y1HS frag2 rev added a *Xba*I and *Spe*I sites to the ends of the amplified promoter fragment. The ends of the promoter fragment that was amplified with primers Y1HS frag3 for and Y1HS frag3 rev was harboring *Spe*I and *Bam*HI restriction sites. All the amplified promoter fragments were

digested with the relevant restriction enzymes. Subsequently, the generated promoter fragments were inserted into the pSK1 plasmid.
The promoter fragment holding the *Sac*I and *Xba* I sites was first cloned into *Sac*I and *Xba*I sites of pSK1. This was followed by the cloning of the product harboring the *Xba*I and *Spe*I sites into compatible sites of the pSK1 vector. However, because, the *Xba*I and *Spe*I overhangs were compatible with each other, two additional copies of this promoter fragments were inserted into pSK1. Finally, the promoter fragment that exhibited the *Spe*I and *Bam*HI sites was cloned into pSK1 at *Spe*I and *Bam*HI sites. The final pSK1 (11-24) vector harbored five copies of a fragment of the *Cp LEA-like 11-24* promoter.

2.25.2. Preparations of the pPC86 prey construct

A cDNA library from dried *C. plantaginuem* leaves in the pPC86 vector was screened to isolate prey plasmids encoding putative DNA binding proteins (Ditzer and Bartels, 2006).

2.25.3. Yeast transformation

2.25.3.1. Preparation of competent yeast cells

A yeast pre-culture was prepared by inoculating a fresh yeast colony into 5 ml of YEPD media and incubating overnight at 30°C with shaking 200 rpm. The next day, the pre-culture was diluted into 250 ml YEPD media to an optical density of approximately 0.15 at a wavelength of 600 nm. The cells were then grown at 30°C with 250 rpm shaking to an optical density of 0.5 at a wavelength of 600 nm. Cells were pelleted by centrifugation at 2400 rpm at room temperature for 5 minutes. The pelleted cells were washed with 50 ml of sterile water and again centrifuged at 2400 rpm at room temperature for 5 minutes. After the second centrifugation step, cells were resuspended in 1 ml of 1 x LiCl and further diluted with 1 x LiCl to a density of 2 x 10^9 cells/ml (1 x 10^6 cells/ml corresponds approximately to an optical density of 1 at a wavelength of 600 nm). The yeast cell suspension could now be used for transformation.

2.25.3.2. Small-scale yeast transformation

The method that was used for small-scale yeast transformation was adapted from the method described by Gietz and Woods (2002) with minor modifications.
Fifty µl of yeast competent suspension was transferred to a clean Eppendorf tube. Cells were pelleted by centrifuging at 14000 rpm for 30 seconds. The supernatant was discarded and reagents were added to the cell pellet in the following order: 240 µl of 50% (w/v) polyethylene glycol (PEG) 4000, 36 µl of 10x LiCl, 50 µl of heat-denatured salmon sperm DNA (2 µg/µl) (Sigma, Deisenhofen, Germany), 33 µl of distilled water and 1 µl of plasmid DNA (1 µg/µl). The cell mixture was vortexed vigorously until the cell pellet was completely dissolved and then incubated at 30°C for 30 minutes. Next, the cells were heat-shocked in a water bath at 42°C for 30 minutes. Cells were cooled briefly on ice and collected by centrifugation (14000 rpm) at room temperature for 30 seconds. The supernatant was removed and the cell pellet was dissolved in 400 µl distilled water. Multiple dilutions of yeast cell suspensions were plated on SC medium selection plates to obtain single colonies.

2.25.3.3. Large-scale yeast transformation

The method for large-scale yeast transformation was adapted from the Matchmaker™ One-Hybrid System protocol (Clontech, Heidelberg, Germany) with minor changes.
The following components were added into an sterile 50 ml Erlenmeyer flask in the following order: 20 µg of cDNA library prepared from dried *C. plantagineum* leaves, 200 µl of heat-denatured salmon sperm DNA (2 µg/µl) (Sigma, Deisenhofen, Germany), 1 ml of yeast cell suspension and 6 ml PEG/LiCl. The yeast cells and transformation reagents were mixed well by vortexing. Cells were then heat-shocked at 42°C for 30 minutes. The transformation mixture was subsequently transferred to a 50 ml Falcon™ tube (Falcon-Becton Dickinson, Heidelberg, Germany) and the mixture was cooled on ice for 2 minutes. Next, the mixture was centrifuged at 2200 rpm at room temperature for 5 minutes. The supernatant was discarded and the pellet was resuspended in 50 ml of SC medium without histidine. The tube was placed in a 30°C incubator with shaking (220 rpm) for 1 hour. After incubation, the mixture was again centrifuged at 2200 rpm at room temperature for 5 minutes and supernatant discarded. The cell pellet was resuspended in 3.5 ml of TE buffer and aliquots of 500 µl were plated onto solid SC selection medium without histidine, leucine and tryptophan, but containing the appropriate concentration of 3-amino-1,2,4-triazole (3-AT) (3-AT stock solution: 2.5 M 3-AT) Plates were incubated at 30°C until colonies appeared.

2.25.4. Preparation of a yeast glycerol stock

A yeast colony of interest was isolated from a selection plate and grown overnight in 3 ml of the appropriate SC selection medium at 30°C with shaking (220 rpm). The next day, 0.5 ml of the yeast culture was transferred to an Eppendorf tube and 0.5 ml of 100% (v/v) glycerol solution was added. Then, the sample was mixed by vortexing vigorously for 1 minute. The glycerol sample was frozen in liquid nitrogen and stored at -80°C.

2.25.5. DNA purification from yeast

The procedure to purify plasmids from yeast was adapted from a protocol developed by Linda Hoskins (Hahn Laboratory**,** Seattle, USA) with minor modifications.
A yeast colony was picked and grown overnight in 5 ml of YEPD medium. The cell culture was centrifuged at 14000 rpm for 2 minutes. The cell pellet was then washed once with distilled water. After washing, cells were resuspended in 500 µl lysis buffer (0.1 M Tris pH 8.0, 50 mM EDTA, 1% (w/v) SDS). Acid-washed glass beads (425-600 microns) (Sigma, Deisenhofen, Germany) were added to yeast cell suspension. The mixture was vortexed vigorously for 30 seconds. Then, 25 µl of 5 M NaCl was added and the mixture was centrifuged for 2 minutes (14000 rpm). The lysed cells were transferred to a clean Eppendorf tube and 400 µl of TE saturated phenol was added and the mixture was centrifuged at 14000 rpm for 4 minutes. The upper phase was pipetted into an Eppendorf tube. Next, 400 µl of 1:4 (v/v) phenol:chloroform mixture was added and vortexed briefly. This was again followed by centrifugation at 14000 rpm for 4 minutes. Once more the upper phase was transferred to an Eppendorf tube and subsequently 1 ml of 96% (v/v) ethanol was added, followed by centrifugation for 6 minutes (14000 rpm). After centrifugation, the pellet was washed with 70% (v/v) ethanol. The pellet was dried at room temperature and dissolved in 50 µl TE buffer.

2.26. Determination of the subcellular location of the bZIP1 protein from *C. plantagineum*

The subcellular localization of the bZIP1 protein was investigated using the CaMV 35S::*bZIP1*::*GFP* fusion construct from Ditzer and Bartels (2006). The bZIP was fused to amino-terminus of the GFP in the plasmid pCK-GFP-S65C (Reichel *et al*., 1996). The expression of this fusion was driven by the CaMV 35S promoter. As control plasmid pGJ280 was used. The *CpbZIP1*::*GFP* fusion construct and plasmid pGJ280

were introduced into *C. plantagineum* leaf tissue by a particle bombardment. Bombarded leaves were incubated in water under normal growth conditions and the localization of the GFP fluorescence was examined 24 hours after the bombardment by confocal microscopy.

3. Results

3.1. Analysis of desiccation tolerance of *C. plantagineum*, *L. brevidens* and *L. subracemosa*

To examine the ability of *C. plantagineum*, *L. brevidens* and *L. subracemosa* to recover after severe drought stress, plants were subjected to a dehydration-rehydration cycle. Plants were dehydrated for a period of 12 days and subsequently rehydrated for one day. Representative photographs of *C. plantagineum*, *L. brevidens* and *L. subracemosa* before and after the treatments are shown in Figure 11.

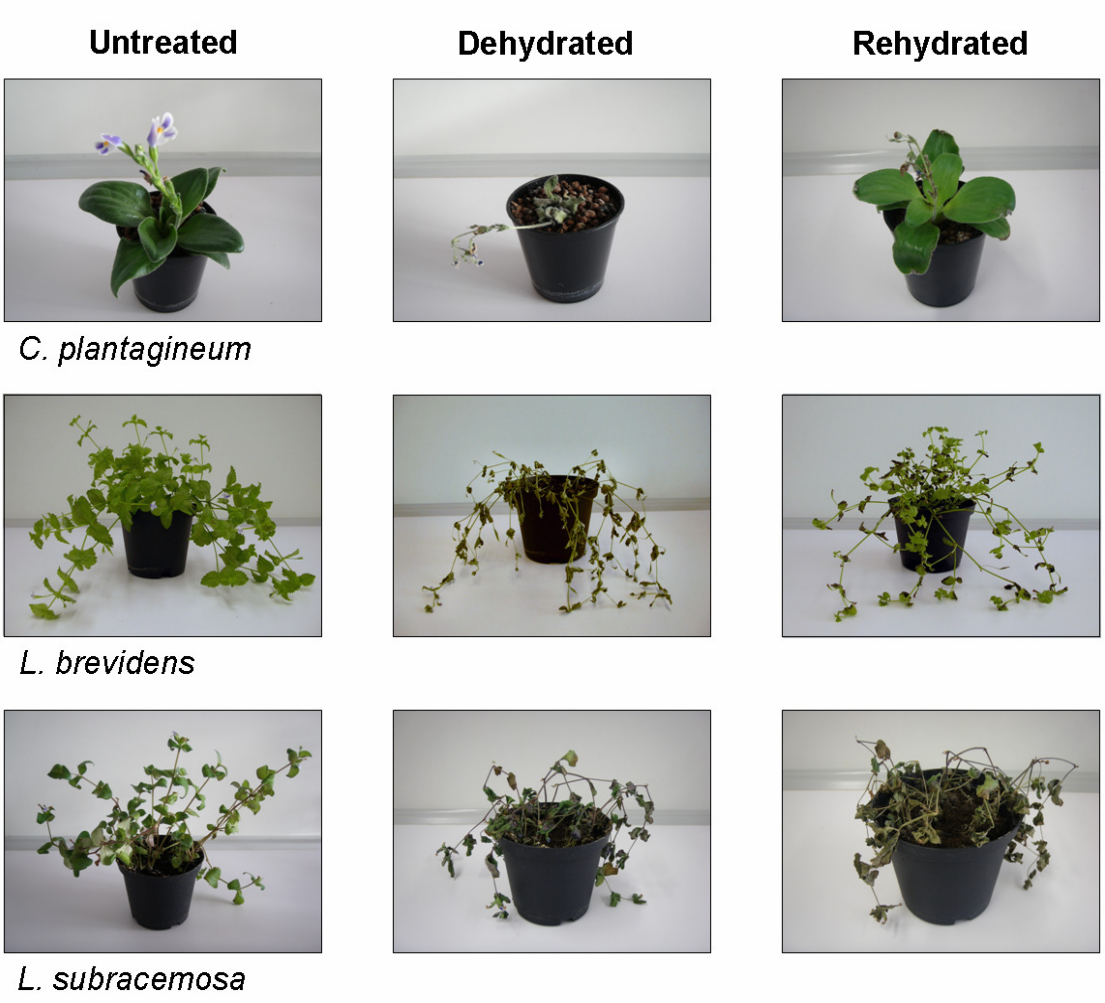


Figure 11. Pictures of untreated, dehydrated, rehydrated *C. plantagineum*, *L. brevidens* and *L. subracemosa* plants.

The relative water content of the plants was determined at different time points (untreated, 4 days of dehydration, 6 days of dehydration, 8 days of dehydration, 10 days of dehydration, 12 days of dehydration and one day of rehydration). A dehydration-rehydration profile for each species is shown in Figure 12.

The resurrection plants *C. plantagineum* and *L. brevidens* were able to withstand the drying process without loss of viability. Upon rehydration, these plant species fully recovered from desiccation. However, it was found that the recovery process in *L. brevidens* occurred at a slower rate than in *C. plantagineum* (Figure 12). During rehydration, *C. plantagineum* was able to take up water more rapidly than *L. brevidens*. *L. subracemosa* did not recover after rehydration and was not able to survive extreme dehydration. These results confirm that *C. plantagineum* and *L. brevidens* are indeed desiccation tolerant, whereas *L. subracemosa* is desiccation sensitive.

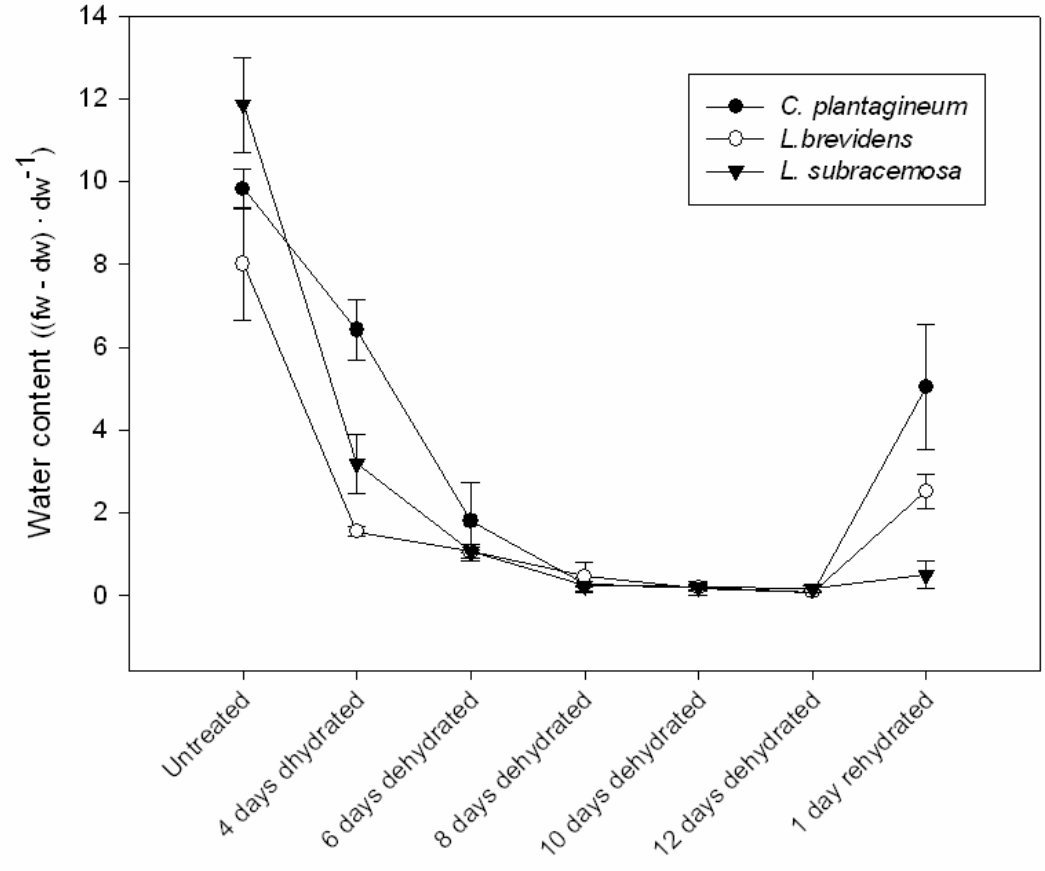


Figure 12. Dehydration and rehydration curves of *C. plantagineum*, *L. brevidens* and *L. subracemosa*. The relative water content ((fw-de) · dw^{-1}) was determined in untreated plants, 4 days dehydrated plants, 6 days dehydrated plants, 8 days dehydrated plants, 10 days dehydrated plants, 12 days dehydrated plants and in plants that were first dehydrated for 12 days and then rehydrated for 1 day. Values represent the mean relative water content with standard deviations (SD) and were derived from three independent experiments (n = 3).

3.2. Comparative analysis of dehydration-responsive transcriptomes of *L. brevidens* and *L. subracemosa*

In order to identify homologous sequences to the desiccation-related *C. plantagineum* genes, cDNA libraries from *L. brevidens* and *L. subracemosa* were constructed. The EST collections were generated from transcripts isolated from dried leaf tissue of either *L. brevidens* or *L. subracemosa*. These dehydration-responsive transcriptomes of *L. brevidens* and *L. subracemosa* were used to investigate potential differences in gene expression between closely related desiccation tolerant and desiccation sensitive plant species.

Comparative transcriptome analysis between *L. brevidens* and *L. subracemosa* was conducted. The EST sequences were first annotated using the Gene Ontology (http://www.geneontology.org/) and the UniProt Knowledgebase (http://www.uniprot.org/). Expressed transcripts were then categorized into different functional groups that have been described in the study of Bevan *et al*. (1998). After annotation of the EST sequences, putative cellular functions were predicted and ESTs were classified into the different groups. The focus of this study was on ESTs involved in desiccation responses, therefore a category "Desiccation-related" was added. Eventually, ESTs were distributed in the following functional groups:

- Metabolism
- Energy
- Cell growth and division
- Transcription
- Protein synthesis
- Protein destination and storage
- Transporters
- Intracellular traffic
- Cell structure
- Signal transduction
- Disease and defense
- Desiccation-related

In total 360 ESTs from *L. brevidens* were analyzed and putative functions were assigned to 234 of them (65%) (Table S1, Supplemental data). From *L. subracemosa*, 245 ESTs were analyzed and putative functions were assigned to 177 of these ESTs (72%) (Table S2, Supplemental data). The ESTs, which showed homology to genes of unknown function, were marked as "Unclear classification" and transcripts that did not

match to any sequences in the searched databases were sorted as "Unclassified". Comparisons showed that the distribution of ESTs from *L. brevidens* and *L. subracemosa* among the different groups was similar (Figure 13).

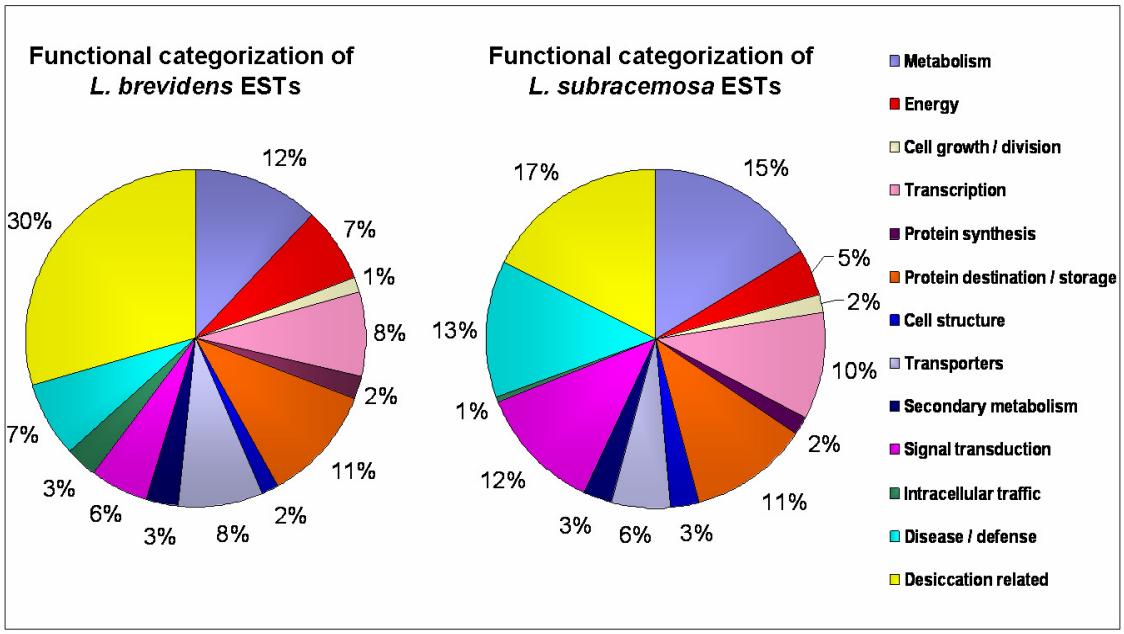


Figure 13. Pie charts showing the percentage of ESTs from *L. brevidens* and *L. subracemosa* classified in each of the functional groups.

In both EST collections, the functional category "Desiccation-related" contained the largest percentage of ESTs (for *L. brevidens* 30% and for *L. subracemosa* 17%, respectively). Especially, transcripts encoding LEA and LEA-like proteins were highly abundant within this category (Tables S1 and S2, Supplemental data).

In the *L. brevidens* EST collection, 24% of the total number of annotated ESTs were coding for LEA or LEA-like proteins. Transcripts encoding the LEA 6-19 protein were most abundant among the LEA proteins, but also transcripts for LEA 3-06 and LEA-like 11-24 proteins were found several times in this EST collection (Table S1, Supplemental data). The *L. brevidens* EST collection harbored one transcript that matched to the LEA 27-45 protein sequence.

In the *L. subracemosa* EST collection, 16% of total annotated ESTs showed identity to LEA or LEA-like proteins. Also here transcripts encoding LEA 6-19 proteins were most redundant among the LEA proteins that were found in this collection (Table S2, Supplemental data). Furthermore, this collection also contained several transcripts that encoded LEA 3-06 and LEA-like 11-24 proteins.

Other ESTs that were abundantly found in the EST collection of *L. brevidens*, were transcripts encoding DSP22 (16% of total annotated ESTs). In contrast, transcripts for

DSP22 comprised only a small part of the *L. subracemosa* EST collection (1%). A relatively large number of ESTs from the *L. subracemosa* collection were classified in the groups "Signal transduction" (12%) and "Disease and defense" (13%) as compared to the *L. brevidens* collection, where the groups made up, respectively, 6% and 7% of the total annotated ESTs.

The difference in the number of ESTs, which had functions related to desiccation tolerance, between *L. brevidens* and *L. subracemosa* indicate that expression of desiccation-related genes in response to dehydration is differentially regulated between these two closely related plant species.

Finally, the two EST collections were used to isolate desiccation-related genes in *L. brevidens* and *L. subracemosa*. These selected genes were: *LEA 6-19*, *LEA-like 11-24, LEA 3-06*, *DSP22* and *LEA 27-45*. Homologous transcript sequences to many of these genes of interest could be extracted from these collections. However, a *Cp LEA-like 27-45* gene sequence was absent from the EST collection of *L. subracemosa*.

3.2.1. Comparison of transcriptomes from resurrection plants *L. brevidens* and *Selaginella lepidophyla*

To examine similarities and differences between the transcriptome of *L. brevidens* and that of other resurrection plants, the *L. brevidens* transcriptome was compared to the transcriptome of the desiccation tolerant fern *Selanginella lepidophylla*. In a study performed by Iturriaga *et al*. (2006), a cDNA library from dehydrated *S. lepidophylla* microphyll fronds was constructed and EST sequences were classified into functional categories. Most transcripts from *S. lepidophylla* had photosynthetic-related functions (17%). The second most abundant category (14%) was comprised of ESTs related to primary metabolism. Within this category various ESTs coded for enzymes involved in sugar metabolism. The third largest category of ESTs was "Disease and defense-related". It must be mentioned that in the study of Iturriaga *et al*. (2006), EST sequences with putative desiccation-related functions were classified within this "Disease- and defense-related" category. Therefore, the majority of ESTs within this category were encoding proteins with putative protective functions, such as LEA proteins.

The high abundance of ESTs encoding LEA proteins was a common feature between the EST collections of *L. brevidens* and *S. lepidophylla*. Another similarity between the two EST collections was the high percentage of ESTs that showed similarities to proteins of unknown function or did not match to proteins in the databases, respectively 35% for *L. brevidens* and 38% for *S. lepidophylla*.

Differences between the transcriptomes of *L. brevidens* and *S. lepidophylla* were observed in the number of ESTs involved in photosynthesis. Compared to *L. brevidens*, a large portion of ESTs from *S. lepidophylla* were encoding proteins associated with photosynthesis. In *S. lepidophylla*, 17% of the ESTs were related to photosynthesis. The EST collection of *L. brevidens* did not contain a distinct functional group for ESTs associated with photosynthesis and transcripts that were putatively involved in photosynthesis were classified into the functional group "Energy". The functional category "Energy" comprised only 7% of total annotated *L. brevidens* EST sequences.

3.3. Comparative analysis of sequences from different desiccation-induced proteins

A comparative protein sequence analysis between *C. plantagineum*, *L. brevidens* and *L. subracemosa* was performed. Deduced amino acid sequences of the *LEA 6-19*, *LEA-like 11-24, LEA 3-06*, *DSP22* and *LEA 27-45* genes were compared. Putative protein sequence conservation was detected by performing a protein sequence alignment. Data from the protein alignments are shown in Figures S1 to S5 (Supplemental data). The percentages of sequence identity between proteins of the three species are given in Table 4.

The *Cp* LEA 6-19 protein shared 68% identical amino acids with the *Lb* LEA 6-19 protein and 49% with the *Ls* LEA 6-19 protein. The sequence identity between the *Lb* LEA 6-19 and *Ls* LEA 6-19 proteins was 48%. The *Cp* LEA-like 11-24 proteins exhibited 46% sequence identity with the *Lb* LEA-like 11-24 protein and 51% with the *Ls* LEA-like 11-24 protein. The *Lb* LEA-like and *Ls* LEA-like 11-24 proteins displayed 50% identity. The *Cp* LEA 3-06 protein exhibited 67% sequence identity with the *Lb* LEA 3-06 protein and 49% with protein *Ls* LEA 3-06. The *Lb* LEA 3-06 and *Ls* LEA 3-06 proteins had 56% identical amino acids. The *Cp* DSP22 protein possessed 71% identity with the *Lb* DSP22 protein and 56% with the DSP22 protein from *L. subracemosa*. Sequence identity between the *Lb* DSP22 and *Ls* DSP22 proteins was 53%. The *Cp* LEA 27-45 protein showed a high percentage of amino acid identity with the *Lb* LEA 27-45 protein (93%). Proteins from the desiccation tolerant species *C. plantagineum* and *L. brevidens*, with the exception of the LEA-like 11-24 protein, exhibited the highest sequence identity. Overall, there was a sequence identity of 69% between protein sequences of *C. plantagineum* and *L. brevidens*. This comparative amino acid analysis reveals that protein coding regions of the studied genes are well conserved between all three species.

Table 4. Amino acid similarity between desiccation related proteins

Protein:	Percentage of amino acid identity	
	Lb LEA 6-19	*Ls* LEA 6-19
Cp LEA 6-19	68	49
Lb LEA 6-19	-	48
Protein:	Percentage of amino acid identity	
	Lb LEA-like 11-24	*Ls* LEA-like 11-24
Cp LEA-like 11-24	46	51
Lb LEA-like 11-24	-	50
Protein:	Percentage amino acid identity	
	Lb LEA 3-06	*Ls* LEA 3-06
Cp LEA 3-06	67	49
Lb LEA 3-06	-	56
Protein:	Percentage of amino acid identity	
	Lb DSP22	*Ls* DSP22
Cp DSP22	71	56
Lb DSP22	-	53
Protein:	Percentage of amino acid identity	
	Lb LEA 27-45	
Cp LEA 27-45	93	

3.4. Comparative analysis of promoter sequences from five desiccation-related genes

Promoter sequences of *Cp LEA-like 11-24*, *Cp LEA 27-45*, *Cp LEA 6-19* and *Lb LEA 6-19* genes were already available in our laboratory. These promoters were analyzed in detail for function (Velasco *et al*., 1998; Hilbricht *et al*., 2002; Michel *et al*., 1994; Phillips *et al*., 2008). Therefore, these promoters were used as reference for the comparative promoter analysis. All other 5'-upstream putative promoter sequences were isolated using the GenomeWalking™ method. Promoter sequences of at least 500 bp in length were obtained (Table 5). These promoter regions were sequenced and subsequently computationally analyzed.

Table 5. Length of promoter fragments

Promoter	Length (bp)	Reference
Cp LEA 6-19	971	Michel *et al.*, 1994
Lb LEA 6-19	1029	Phillips *et al.*, 2008
Ls LEA 6-19	931	-
Cp LEA-like 11-24	1613	Velasco *et al.*, 1998
Lb LEA-like 11-24	1415	-
Ls LEA-like 11-24	500	-
Cp LEA 3-06	1310	-
Lb LEA 3-06	893	-
Ls LEA 3-06	589	-
Cp DSP22	672	-
Lb DSP22	671	-
Ls DSP22	980	-
Cp LEA 27-45	629	Hilbricht *et al.*, 2002
Lb LEA 27-45	1046	-

3.4.1. Identification of putative *cis*-acting regulatory elements in homologous promoter sequences

The isolated promoter regions from *C. plantagineum*, *L. brevidens* and *L. subracemosa* were screened for the presence of *cis*-acting regulatory elements that are potentially involved in dehydration- and ABA-responsive gene expression. The occurrence of these *cis*-acting regulatory elements was examined, using the Plant *cis*-acting regulatory DNA elements (PLACE) database (http://www.dna.affrc.go.jp/PLACE/).

Multiple putative *cis*-acting regulatory elements, which may be involved in ABA- and dehydration-mediated gene expression, were detected within the promoter homologs (Table 6). The exact number of *cis*-acting regulatory elements and their position within the different promoters are listed in Tables S3 to S7 (Supplemental data). The *cis*-acting regulatory elements that were detected in the different promoter regions are described in more detail below.

Multiple ACGT-containing ABA-responsive elements (ABRE) were found (Table 6). Studies have shown that these ABREs were able to stimulate gene expression in response to ABA and dehydration (Foster *et al.*, 1994 and Simpson *et al.*, 2003). Different types of ACGT elements were identified; ACGTCBOX, ABRELATERD1 and ACGTATERD1, respectively. The ABRELATERD1 and ACGTATERD1 motifs

were first discovered in the *ERD1* gene (early responsive to dehydration stress) in *Arabidopsis*. ABRE motifs are recognized by so-called ABF proteins, like bZIP transcription factors. Most ACGT-containing ABRE motifs are bound by bZIP transcription factors (Yamaguchi-Shinozaki and Shinozaki, 2005). Sequences flanking the ACGT-core motifs are important for the binding specificity of the bZIP proteins.

Two ABREDISTBBNNAPA motifs were detected, one in the *Lb LEA-like 11-24* promoter sequences and the other in *LEA-like 11-24* promoter of *L. subracemosa*. The ABREDISTBBNNAPA dist B (distal portion of B-box) element was first identified in the *NAPA* gene of *Brassica napus* and is required for seed specific expression and ABA-responsiveness (Ezcurra *et al*., 1999).

Several MYB recognition sites were recognized; MYBCORE and MYBATRD22, respectively. In *Arabidopsis*, the MYBCORE motif is a binding site for MYB1 and MYB2 proteins, where MYB2 is involved in dehydration-induced gene expression (Luscher and Eiseman, 2000). The MYBATRD22 motif was originally found in the promoters of the dehydration-responsive gene *RD22* in *Arabidopsis* (Abe *et al*., 1997).

Various MYC recognition sites were identified; MYCATRD22 and MYCCONSENSUSAT, respectively. The MYCATRD22 motif is associated with ABA-responsive gene expression and is a binding site for MYC transcription factors. The MYCCONSENSUSAT motif is a binding site of the *Arabidopsis* ATMYC2 protein (Abe *et al*., 2003). The MYCATRD22 and MYCCONSENSUSAT motifs and were discovered in the promoter of the dehydration-responsive gene *RD22* and (Abe *et al*., 1997; Abe *et al*., 2003).

Two types of dehydration-responsive elements were found; DRE1COREZMRAB17 and DRE2COREZMRAB17, respectively. These DRE motifs were detected for the first time in the promoter of the *RAB17* gene of *Zea mays,* which is both ABA- and dehydration-responsive (Busk *et al*., 1997). DREB proteins are able to interact with these DRE motifs. Furthermore, a number of core motifs of DRE/CRT (dehydration-responsive element/C-repeat) were found in the examined promoters. This element was initially identified in the promoters of many genes from *Arabidopsis* and rice. The DRE/CRT motif is, among other things, involved in dehydration-responsive expression (Dubouzet *et al*., 2003). The DRE/CRT motif is recognized by DREB/C**-**repeat**-**binding factor (CBF) proteins.

Many DPBFCOREDCDC3 core sequences were identified. The DPBFCOREDCDC3 motif is an ABA-responsive element that was first discovered in the *Dc3* gene promoter in *Daucus carota*. This element can bind the bZIP transcription factors, Dc3 promoter-binding factor (DPBF) 1 and 2 (Kim *et al*., 1997).

Table 6. Putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression found among the studied promoter orthologs from *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

Cis-element	Sequence	Description	Reference
ACGTCBOX	GACGTC	ABRE-like motif containing the "C-box" sequence Identified in the *ERD1* gene in *Arabidopsis* Binding site for ABF proteins	Foster *et al.*, 1994
ABRELATERD1	ACGTG	ACGT-containing ABRE-like motif Identified in the *ERD1* gene in *Arabidopsis* Binding site for ABF proteins	Simpson *et al.*, 2003
ACGTATERD1	ACGT	ACGT-core sequence Identified in *ERD1* gene in *Arabidopsis* Binding site for ABF proteins	Simpson *et al.*, 2003
ABREDISTBBNNAPA	GCCACTTGTC	Distal portion of B-box found in *NAPA* gene in *Brassica napus* and shows similarity to ABRE-like motifs	Ezcurra *et al.*, 1999
MYBCORE	CNGTTR	MYB-core motif Binding site for *Arabidopsis* MYB1 and MYB2 proteins	Luscher and Eiseman, 2000
MYBATRD22	CTAACCA	MYB recognition site Identified in the *RD22* gene in *Arabidopsis* Binding site for MYB transcription factors	Abe *et al.*, 1997
MYCCONSENSUSAT	CANNTG	MYC recognition site Identified in the RD*22* gene in *Arabidopsis* Binding site for MYC transcription factors	Abe *et al.*, 2003
MYCATRD22	CACATG	MYC-like motif Identified in the RD*22* gene in *Arabidopsis* Binding site for MYC transcription factors	Abe *et al.*, 1997
DRE1COREZMRAB17 DRE2COREZMRAB17	ACCGAGA ACCGAC	DRE-like motifs Identified in the *RAB17* gene in *Zea mays* Binding sites for DREB proteins	Busk *et al.*, 1997
DRE/CRT	RCCGAC	DRE/CRT motif Identified in many genes in *Arabidopsis* and rice Binding site of DREB/CBF proteins	Dubouzet *et al.*, 2003
DPBFCOREDCDC3	ACACNNG	DPBF binding core sequence Identified in the *Dc3* gene in *Daucus carota* Binding site for *Daucus carota* DPBF1 and DPBF2 proteins	Kim *et al.*, 1997

The table shows sequence motif (where N is any nucleotide and R is adenine or guanine), description of *cis*-acting element and reference. These c*is*-acting regulatory elements were identified using the PLACE database.

3.4.2. Sequence and structural similarities between homologous promoter regions

Comparative sequence alignment analysis between the promoter sequences was performed to detect similarities between the promoter regions. However, it is not straightforward to compare promoter sequences from different species. The rate of nucleotide changes due to mutations can be high in non-coding gene regulatory sequences (Ludwig, 2002). Mutations can generate novel *cis*-acting regulatory elements or disturb the function of existing regulatory motifs. In addition, not every *cis*-acting element that is found in a promoter sequence is functional. Therefore, special attention was paid to conservation of *cis*-acting regulatory elements among the different promoter regions. Furthermore, promoter architecture, distribution and spacing of *cis*-acting regulatory elements were analyzed.

3.4.2.1. Sequence and structural analysis of *LEA 6-19* promoter sequences

The first set of promoters chosen for the promoter comparison, were the dehydrin promoters. Fragments selected for the sequence alignment of *LEA 6-19* promoters had a length of 529 bp (Figure 14). Michel *et al*. (1994) demonstrated that a region of 448 bp of the *C. plantagineum LEA 6-19* promoter was sufficient to confer ABA-responsiveness.

Comparison between the *Cp LEA 6-19* and *Lb LEA 6-19* promoter regions gave a sequence identity of 51%. The sequence identity between the *Cp LEA 6-19* and *Ls LEA 6-19* promoter regions was 10%. There was also 10% of identity between the *Lb LEA 6-19* and *Ls LEA 6-19* promoter sequences. Several stretches of identical nucleotides were detected within the promoters, indicating that these regions were conserved between the promoters. Some of these conserved promoter regions held putative *cis*-acting regulatory elements. In order to obtain a better view of the distribution, spacing and conservation of the different putative *cis*-acting regulatory elements present within the compared *LEA 6-19* promoter sequences, promoters and *cis*-acting elements are schematically illustrated in Figure 15.

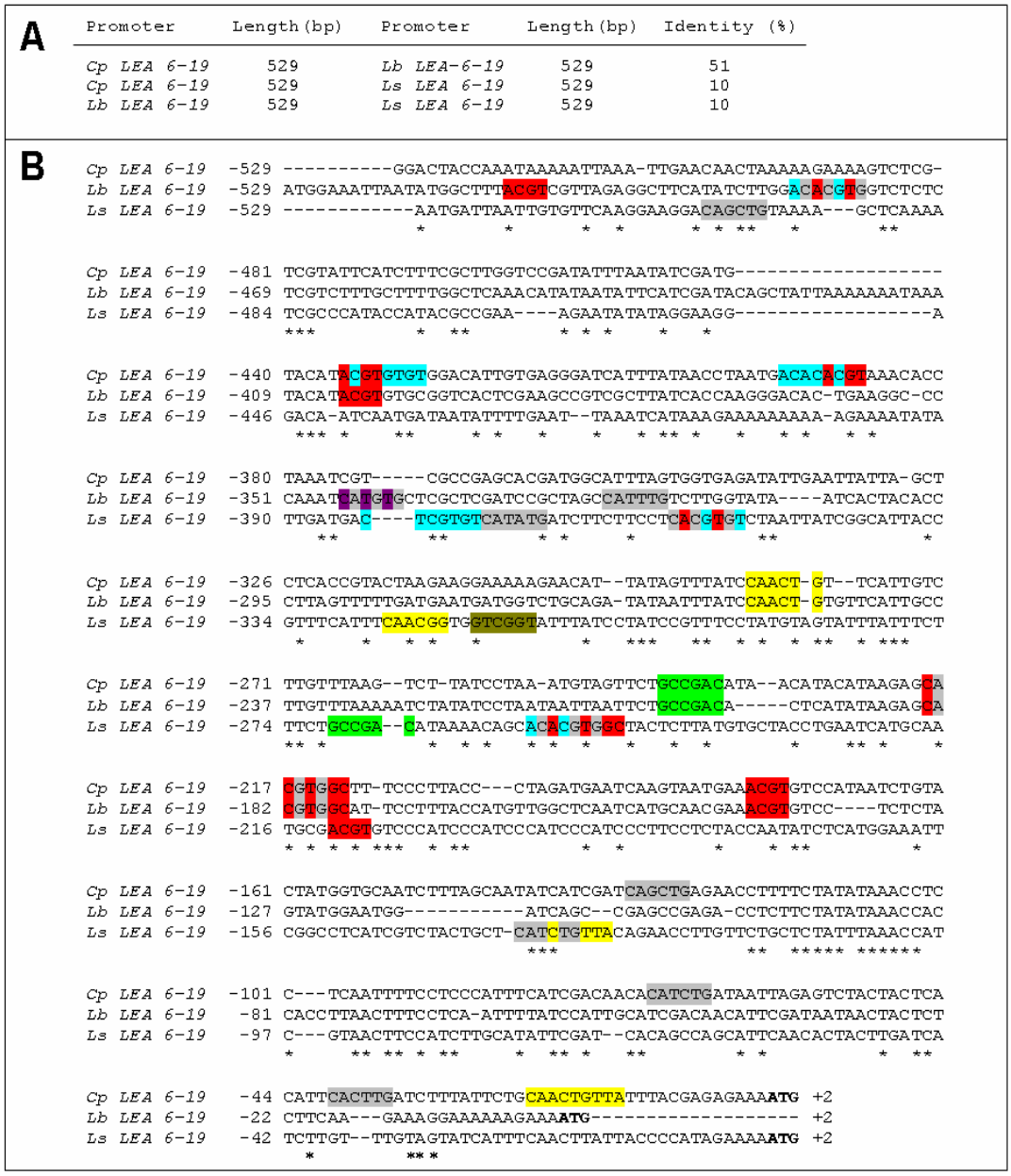


Figure 14. Sequence alignments between 529 bp *LEA 6-19* promoter regions from *C. plantagineum*, *L. brevidens* and *L. subracemos*a. (A) Nucleotide sequence identity between promoter regions. (B) Alignment data showing identities and putative *cis*-acting regulatory elements involved in ABA- and dehydration-responsive gene expression. Asterisks indicate nucleotides which are identical between all three promoter regions. Putative *cis*-acting regulatory elements are displayed in colored boxes: ACGT-containing ABRE elements are highlighted in red boxes, MYCATRD22 motif (CACATG) is displayed in purple, MYCCONSENSUSAT sequences (CANNTG) are shown in grey boxes, MYBCORE sequence (CNGTTR) is displayed in yellow, DPBF-core sequences (ACACNNG) are shown in blue boxes, DRE2COREZMRAB17 element (ACCGAC) is indicated in dark yellow and DRE/CRT motifs (RCCGAC) are displayed in green, respectively. Overlapping *cis*-regulatory elements are indicated with mixed colored boxes. The ATG start codons are shown in bold characters.

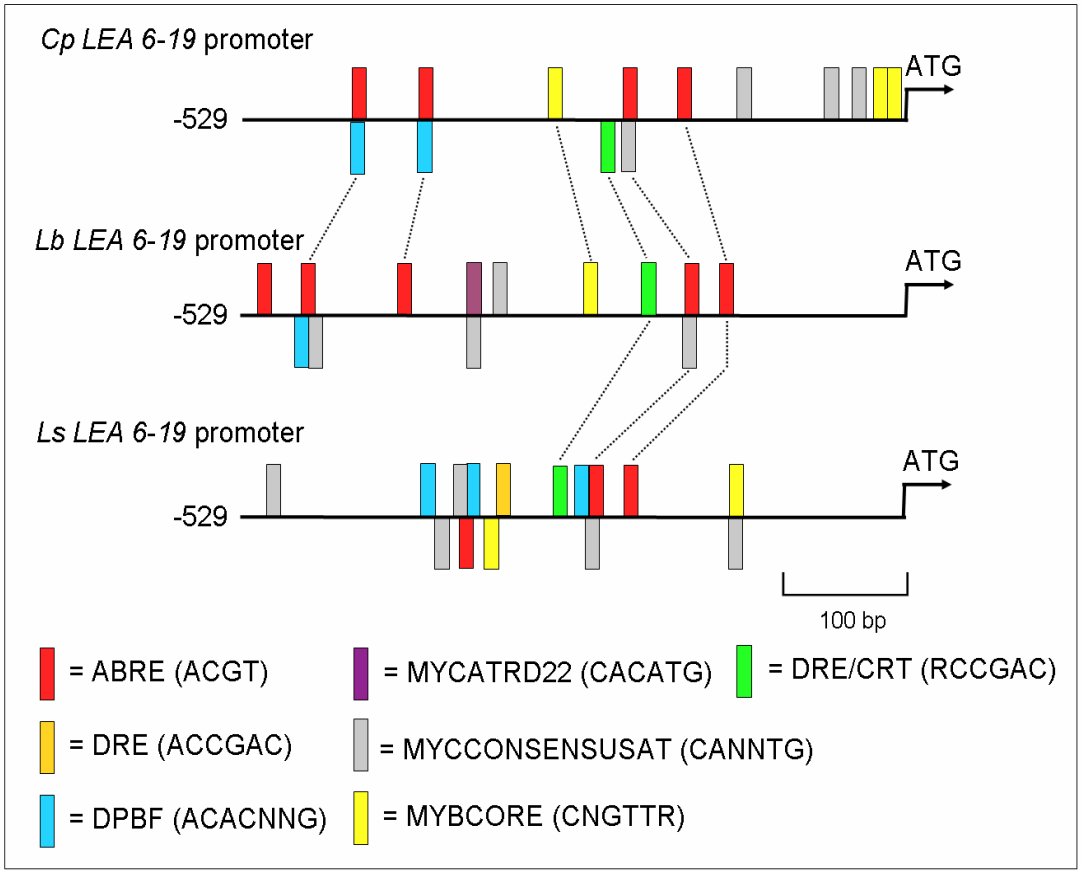


Figure 15. Distribution and conservation of putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression among 529 bp *LEA 6-19* promoter sequences from *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Conserved *cis*-acting regulatory elements found in the promoter sequences are linked together with dotted lines.

3.4.2.2. Sequence and structural analysis of *LEA-like 11-24* promoter sequences

Comparison of *LEA-like 11-24* promoter regions was done with upstream sequences of 500 bp in length (Figure 16). Velasco *et al.* (1998) showed that a 667 bp fragment of the *LEA-like 11-24* promoter from *C. plantagineum* was sufficient for dehydration and ABA-responsiveness.

The *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter regions had a nucleotide identity of 51%. The *Cp LEA-like 11-24* and *Ls LEA-like 11-24* promoters displayed 53% identity. Between the *Lb LEA-like 11-24* and *Ls LEA-like 11-24* promoter regions also 53% of nucleotide identity was observed.

The *LEA-like 11-24* promoter regions contained multiple *cis*-acting regulatory elements that were conserved between promoters (Figure 17). Also, the order of several of the *cis*-acting regulatory elements within the aligned *LEA-like 11-24* promoter regions was very similar (Figure 16). For example, the first three ABRE motifs in the *Cp LEA-like*

11-24 and *Lb LEA-like 11-24* promoters were arranged in a similar way. Furthermore, the arrangement of MYC consensus sequences in the promoters exhibited a certain similarity.

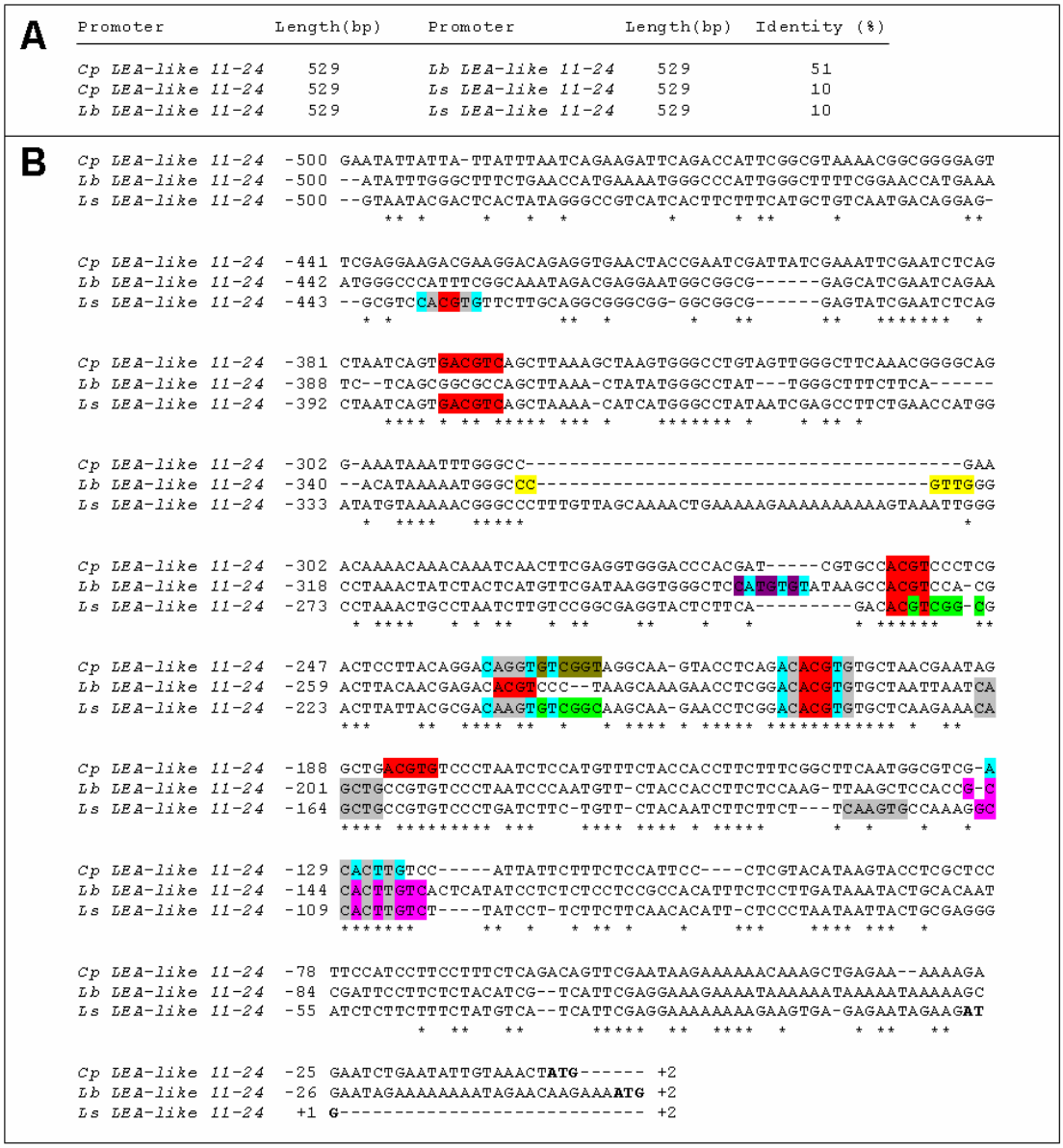


Figure 16. Sequence alignments between 500 bp *LEA-like 11-24* promoter regions from *C. plantagineum*, *L. brevidens* and *L. subracemos*a. (A) Nucleotide sequence identity between promoter regions.
(B) Alignment of *LEA-like 11-24* promoter sequences. Asterisks indicate nucleotides which are identical between all three promoter regions. Putative *cis*-acting regulatory elements involved in dehydration- and ABA-responsive gene expression are highlighted in colored boxes: ACGT-containing ABRE elements are displayed in red boxes, MYCATRD22 motif (CACATG) is highlighted in purple, MYCCONSENSUSAT sequences (CANNTG) are shown in grey boxes, MYBCORE sequence (CNGTTR) is displayed in yellow, DPBF-core sequences (ACACNNG) are shown in blue boxes, DRE2COREZMRAB17 element (ACCGAC) is indicated in dark yellow, ABREDISTBBNNAPA motifs (GCCACTTGTC) are shown in pink and DRE/CRT motifs (RCCGAC) are displayed in green, respectively. Overlapping *cis*-regulatory elements are indicated with mixed colored boxes. The ATG start codons are shown in bold characters.

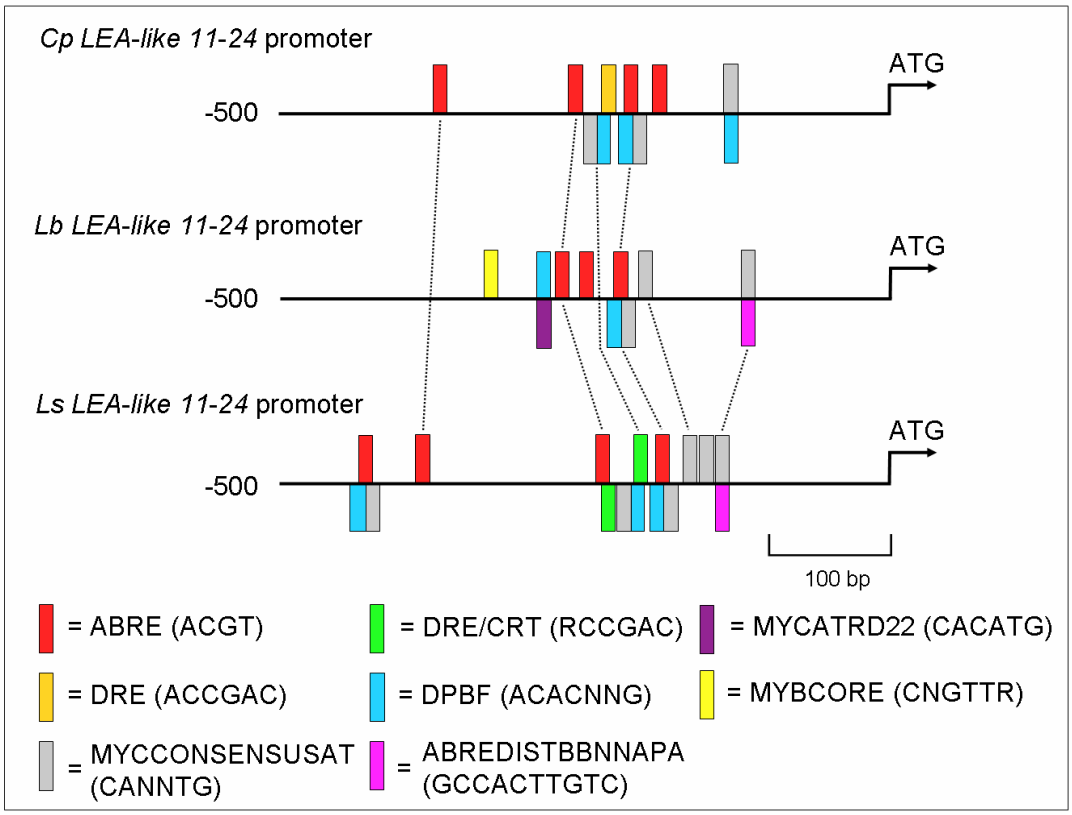


Figure 17. Distribution and conservation of putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression among the 500 bp *LEA-like 11-24* promoter sequences from *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Conserved *cis*-acting regulatory elements found in the promoter sequences are linked together with dotted lines.

3.4.2.3. Sequence and structural analysis of *LEA 3-06* promoter sequences

The alignment of *LEA 3-06* promoter sequences was done with fragments of 589 bp (Figure 18). The percentage of sequence identity between the *Cp LEA 3-06* promoter and *Lb LEA 3-06* promoter regions was 42%. The *Cp LEA 3-06* promoter and *Ls LEA 3-06* promoter regions possessed 46% sequence identity. An identity of 41% was found between the *Lb LEA 3-06* promoter and *Ls LEA 3-06* promoter sequences. Comparison showed that some of the identified *cis*-regulatory elements were conserved between the promoters (Figure 19).

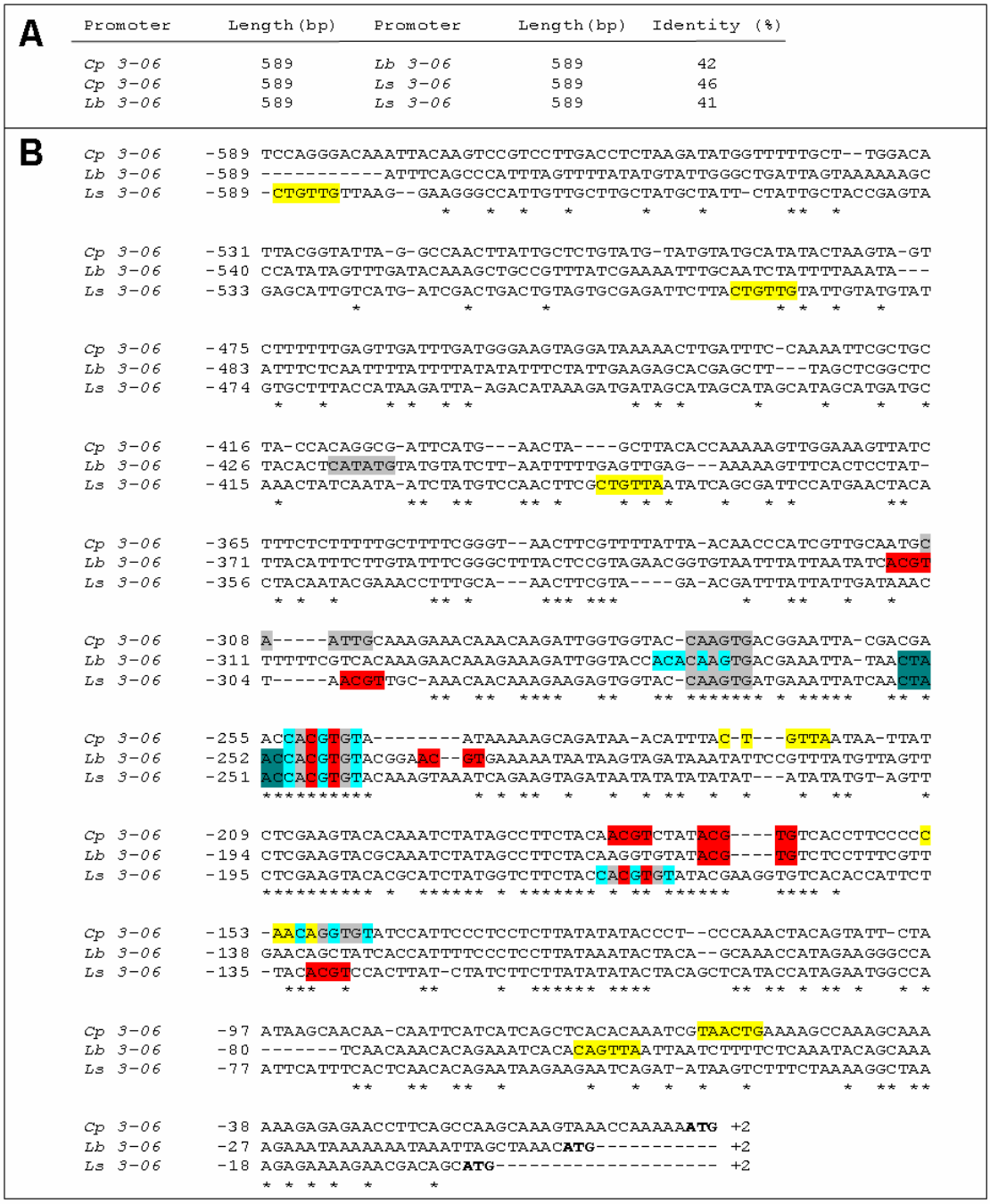


Figure 18. Sequence alignments between 589 bp *LEA 3-06* promoter regions from *C. plantagineum*, *L. brevidens* and *L. subracemos*a. (A) Nucleotide sequence identity between promoter regions. (B) Alignment data showing identities and putative *cis*-acting regulatory elements involved in ABA- and dehydration-responsive gene expression. Asterisks indicate nucleotides which are identical between all three promoter regions. Putative *cis*-acting regulatory elements are displayed in colored boxes: ACGT-containing ABRE elements are highlighted in red boxes, MYBATRD22 motif (CTAACCA) is displayed in bluish green, MYCCONSENSUSAT sequences (CANNTG) are shown in grey boxes, MYBCORE sequence (CNGTTR) is displayed in yellow and DPBF-core sequences (ACACNNG) are shown in blue boxes, respectively. Overlapping *cis*-regulatory elements are indicated with mixed colored boxes. The ATG start codons are shown in bold characters.

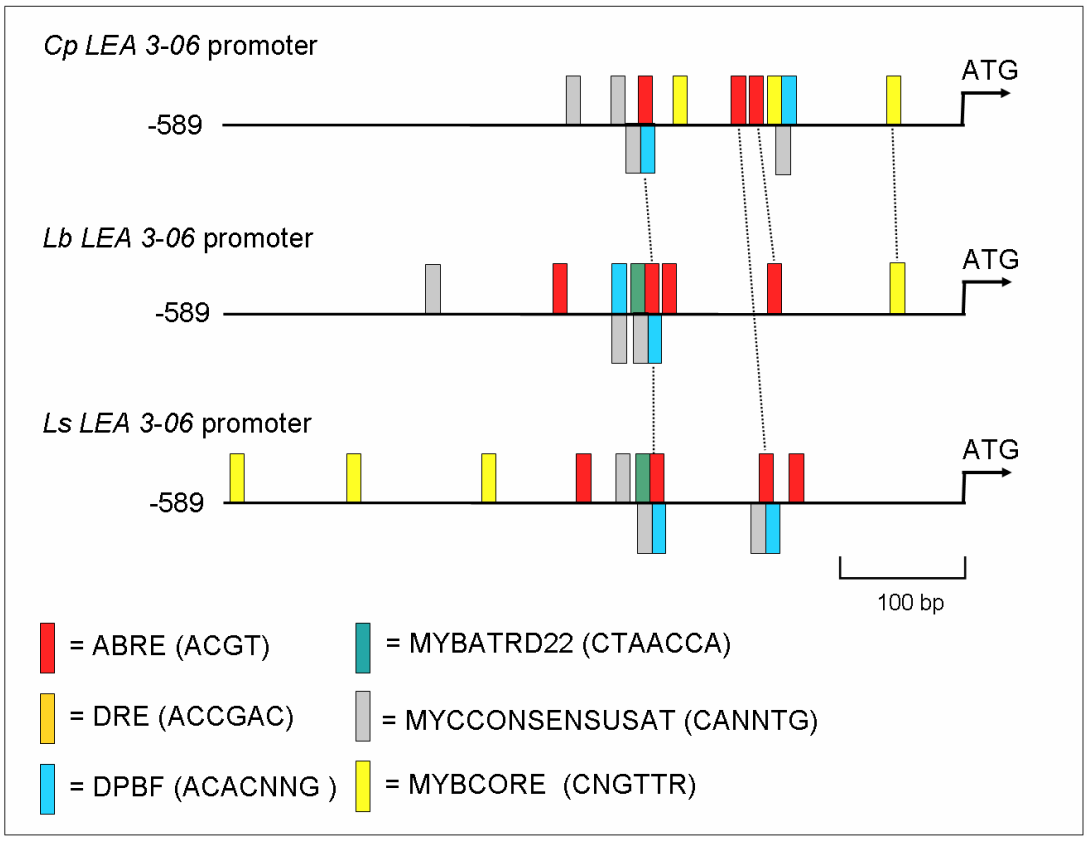


Figure 19. Distribution and conservation of putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression among 589 bp *LEA 3-06* promoter sequences from *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Conserved *cis*-acting regulatory elements found in the promoter sequences are linked together with dotted lines.

3.4.2.4. Sequence and structural analysis of *DSP22* promoter sequences

Sequence alignments of *DSP22* promoters were performed with 671 bp fragments (Figure 20). The alignment of the *Cp DSP22* promoter and *Lb DSP22* promoter regions revealed a sequence identity of 14% (Figure 10). The *Cp DSP22* promoter and *Ls DSP22* promoter regions exhibited an identity of only 7%. Sequence identity between the *Lb DSP22* promoter and *Ls DSP22* promoter regions was even lower (4%). Even though there was only a low degree of sequence identity between the different *DSP22* promoter regions, a few conserved *cis*-acting regulatory elements were found among the promoters (Figure 21).

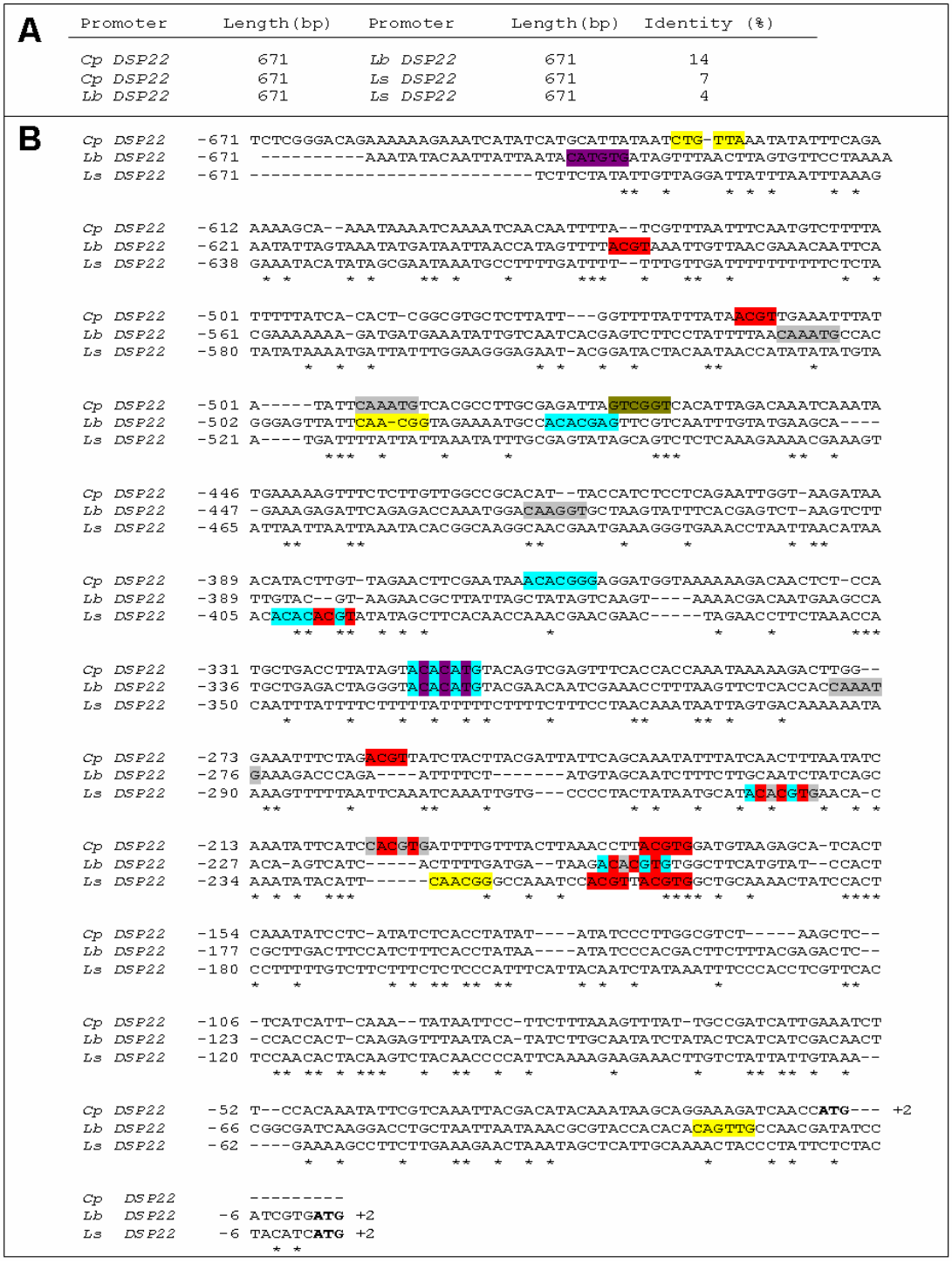


Figure 20. Sequence alignments between 671 bp *DSP22* promoter regions from *C. plantagineum*, *L. brevidens* and *L. subracemos*a. (A) Nucleotide sequence identity between promoter regions. (B) Alignment data showing identities and putative *cis*-acting regulatory elements involved in ABA- and dehydration-responsive gene expression. Asterisks indicate nucleotides which are identical between all three promoter regions. Putative *cis*-acting regulatory elements are displayed in colored boxes: ACGT-containing ABRE elements are highlighted in red boxes, MYCATRD22 motif (CACATG) is displayed in purple, MYCCONSENSUSAT sequences (CANNTG) are shown in grey boxes, MYBCORE sequence (CNGTTR) is displayed in yellow, DPBF-core sequences (ACACNNG) are shown in blue boxes and DRE2COREZMRAB17 element (ACCGAC) is indicated in dark yellow, respectively. Overlapping *cis*-regulatory elements are indicated with mixed colored boxes. The ATG start codons are shown in bold characters.

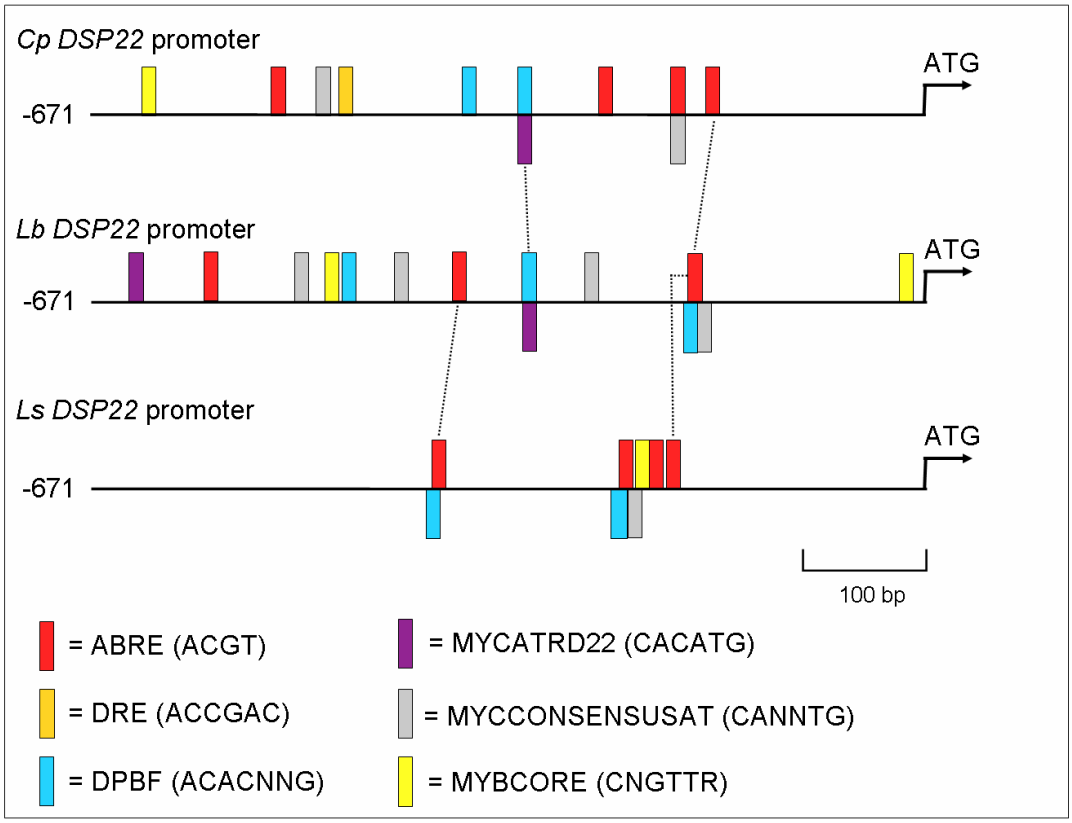


Figure 21. Distribution and conservation of putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression among 671 bp *DSP22* promoter sequences from *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Conserved *cis*-acting regulatory elements found in the promoter sequences are linked together with dotted lines.

3.4.2.5. Sequence and structural analysis of *LEA 27-45* promoter sequences

Fragments of, respectively, 629 bp and 1046 bp were taken for the alignment of the *Cp LEA 27-45* and *Lb LEA 27-45* promoter sequences (Figure 22). A nucleotide identity of 33% was found between the *Cp LEA 27-45* and *Lb LEA 27-45* promoters.

However, the comparative analysis of the upstream regions of *LEA 27-45* genes revealed a conservation over a longer stretch of the genome. In both *C. plantagineum* and *L. brevidens*, a *xyloglucan endotransglycosylase* gene was found upstream of the *LEA 27-45* gene. Comparison showed that the stretch of DNA sequence between the end of *xyloglucan endotransglycosylase* gene and the start of the *LEA 27-45* gene was longer in *L. brevidens* (1046 bp) than in *C. plantagineum* (629 bp). Further analysis revealed that the putative *Lb LEA 27-45* promoter region contained a 411 bp insertion.

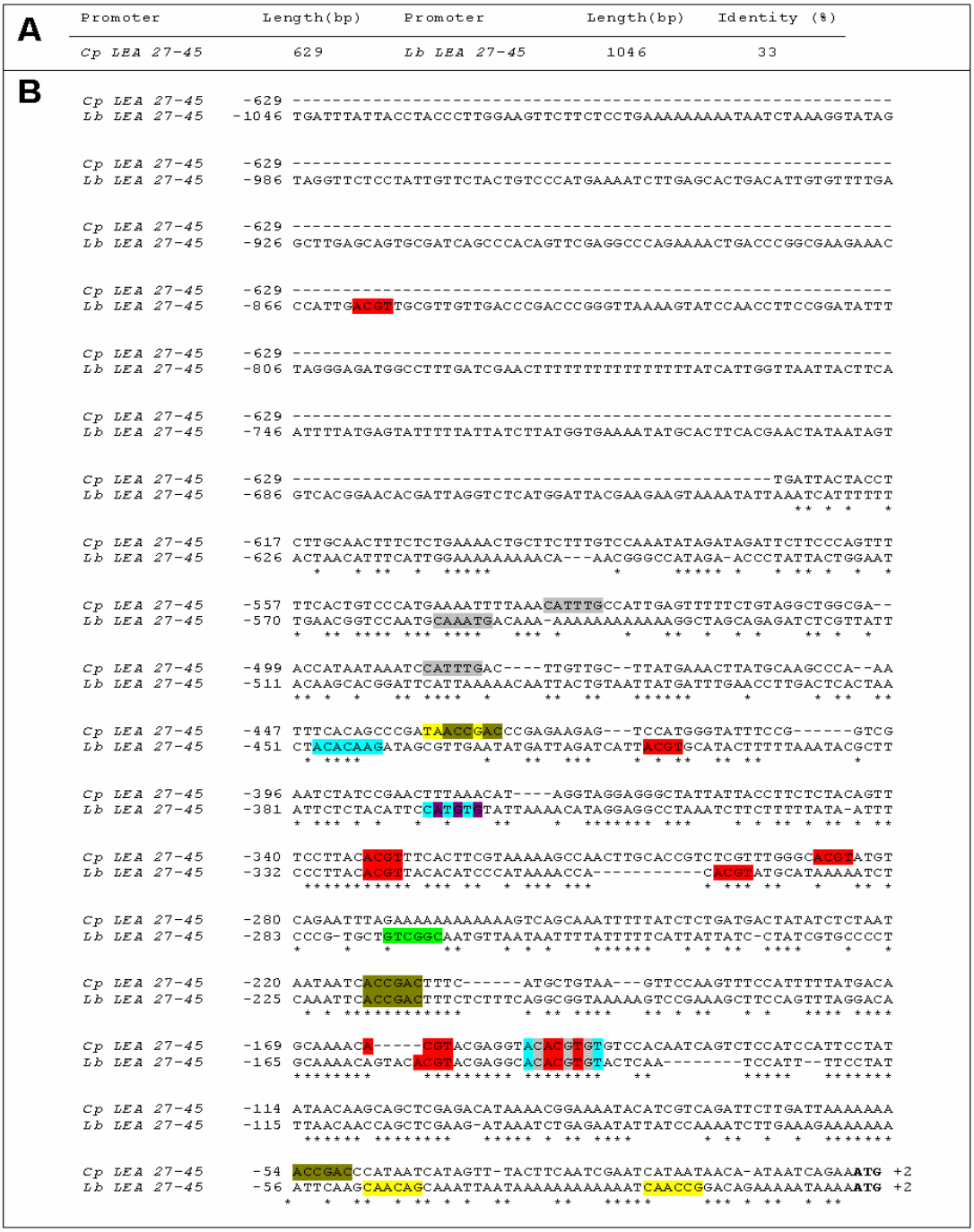


Figure 22. Sequence alignments between the 629 bp *LEA-like 27-45* promoter region from *C. plantagineum* and the 1046 bp *LEA-like 27-45* promoter region from *L. brevidens*. (A) Nucleotide sequence identity between promoter regions.
(B) Alignment data showing identities and putative *cis*-acting regulatory elements involved in ABA- and dehydration-responsive gene expression. Asterisks indicate nucleotides which are identical between all three promoter regions. Putative *cis*-acting regulatory elements are displayed in colored boxes: ACGT-containing ABRE elements are highlighted in red boxes, MYCATRD22 motif (CACATG) is displayed in purple, MYCCONSENSUSAT sequences (CANNTG) are shown in grey boxes, MYBCORE sequence (CNGTTR) is displayed in yellow, DPBF-core sequences (ACACNNG) are shown in blue boxes, DRE2COREZMRAB17 element (ACCGAC) is indicated in dark yellow and DRE/CRT motifs (RCCGAC) are displayed in green, respectively. Overlapping *cis*-regulatory elements are indicated with mixed colored boxes. The ATG start codons are shown in bold characters.

Alignments of the *Cp LEA 27-45* and *Lb LEA 27-45* promoters showed that promoter regions from approximately nucleotide position -370 to the transcription start site contained various conserved sequence elements. For example, a DRE motif and several ABRE motifs were well conserved in the *LEA 27-45* promoters of *C. plantagineum* and *L. brevidens*. The promoter sequences downstream of position -370 showed less similarities and this is caused by the insertion in the *Lb LEA 27-45* promoter.

When the promoter alignment was done, excluding the insertion sequence in the *Lb LEA 27-45* promoter, 58% of sequence identity between the *Cp LEA 27-45* and *Lb LEA 27-45* promoter regions was obtained (Figure S6, Supplemental data).

Distribution and conservation of putative *cis*-acting regulatory elements in the *Cp LEA 27-45* and *Lb LEA 27-45* promoter regions, both including and excluding the DNA insertion in the *Lb LEA 27-45* promoter is shown in Figures 23.

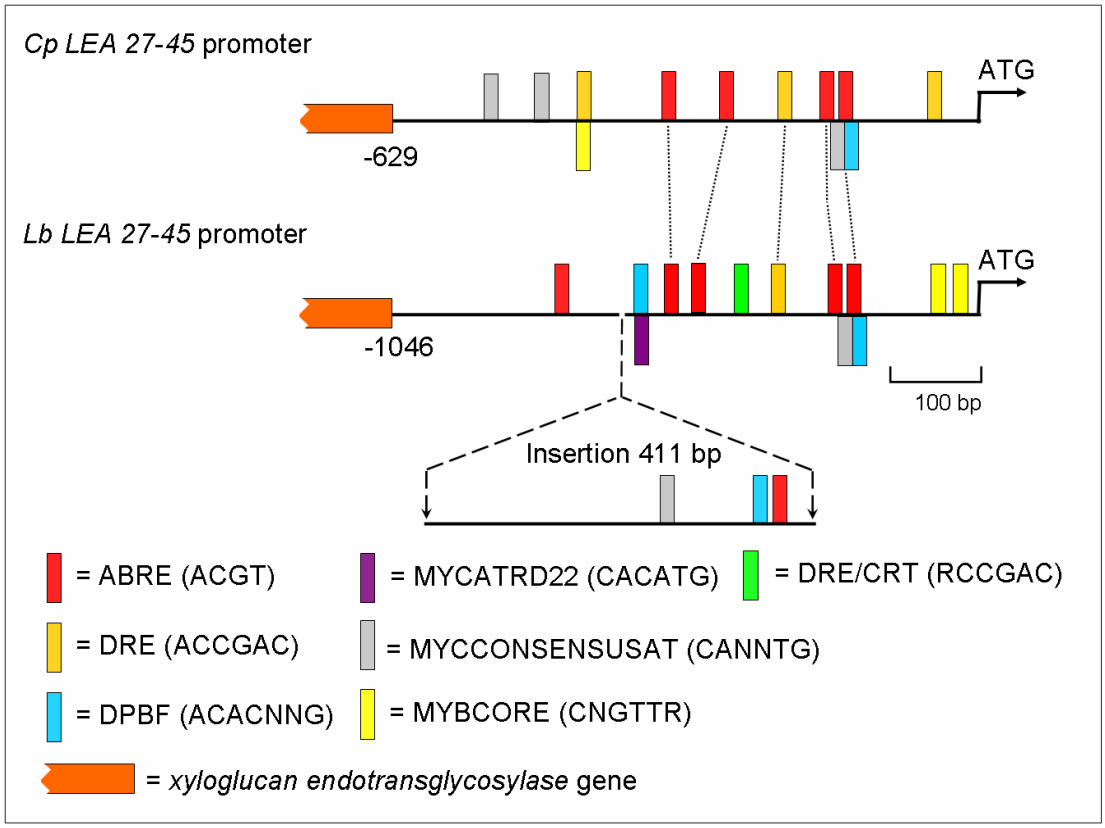


Figure 23. Distribution and conservation of putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression among the 629 bp *LEA 27-45* promoter sequences from *C. plantagineum*, and the 1046 bp *LEA 27-45* promoter sequences from *L. brevidens*. The 411 bp insertion in the *Lb LEA-like 27-45* promoter is displayed. Conserved *cis*-acting regulatory elements found in the promoter sequences are linked together with dotted lines.

The 411 bp insertion within the *Lb LEA 27-45* promoter contained an open reading frame. However, BLAST analysis revealed that the deduced amino acid sequence of this open reading frame did not has an obvious similarity to proteins in the searched database.

It was examined whether the insertion sequence present in the *Lb LEA 27-45* promoter was a transposable-like element. If this insertion sequence has features of a transposable element, it is expected that the genome carries multiple copies of the insertion. Southern blot analysis was performed to determine the copy number of the insertion sequence in the *L. brevidens* genome. Genomic DNA was linearized by digestion with *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I or *Xho*I, respectively, and probed with a 326 bp PCR fragment of the insertion sequence (Figure 24).

The Southern blot analysis showed that the insertion sequence was unique in the *L. brevidens* genome. In each lane only one band was visible, demonstrating that the genomic DNA carries only one copy of the insertion sequence. Thereby, these results indicate that the insertion sequence present in the *Lb LEA 27-45* promoter is a unique DNA fragment and not a transposable-like element.

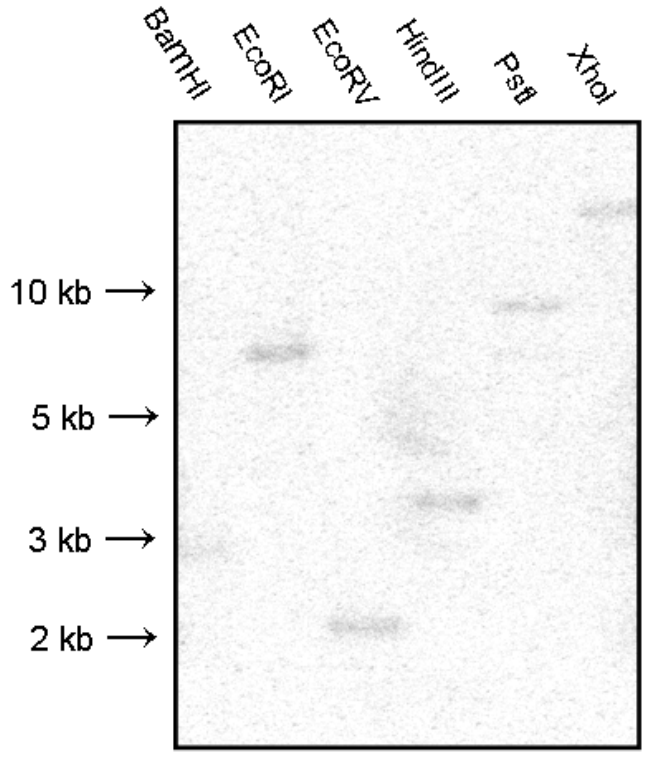


Figure 24. Southern blot against the 411bp insertion sequence present in the *Lb LEA 27-45* promoter. *L. brevidens* genomic DNA samples were digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I and *Xho*I, respectively. A 326bp fragment of the insertion sequence was used as a probe.

3.5. Analysis of expression profile of the *LEA-like 11-24* gene in *C. plantagineum*, *L. brevidens* and *L. subracemosa*

It was chosen to study the expression of the *LEA-like 11-24* gene in *C. plantagineum*, *L. brevidens* and *L. subracemosa* in more detail. RNA gel blot analysis was performed to compare the expression patterns of the *LEA-like 11-24* gene in *C. plantagineum*, *L. brevidens* and *L. subracemosa* leaf tissue. The expression was analyzed in untreated non-stressed leaf tissue and under dehydration and rehydration conditions. In *L. subracemosa LEA-like 11-24* expression was not analyzed in rehydrated leaves, since this species is not desiccation tolerant (Figures 11 and 12). Expression in leaf tissue was analyzed under the following conditions: untreated (control), after 24 hours dehydration, after 48 hours dehydration and first dehydrated for 48 hours and subsequently rehydrated for 24 hours. Total RNA was extracted from leaves and was probed with fragments of the *LEA-like 11-24* gene.

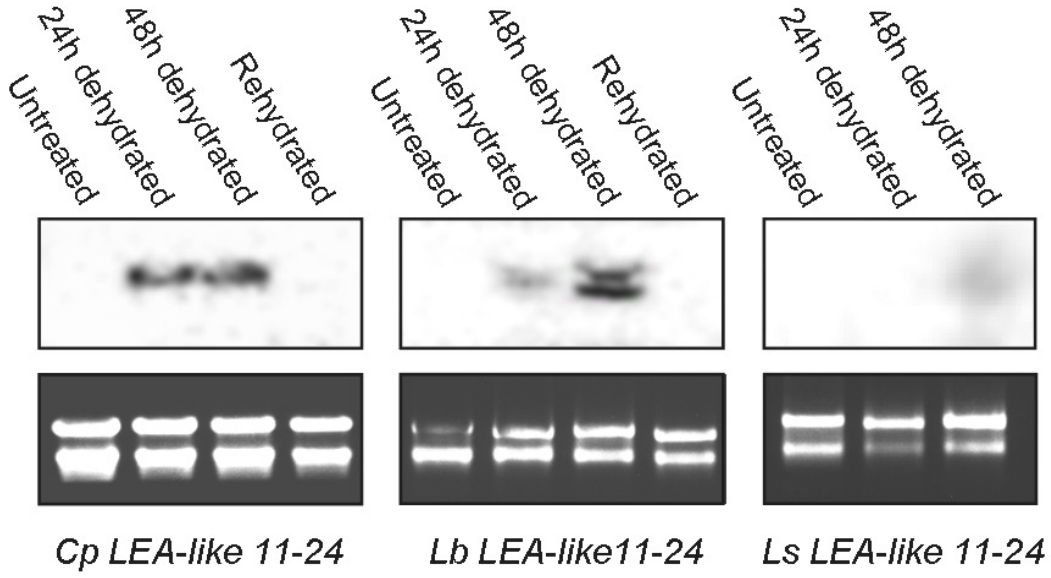


Figure 25. RNA gel blot analysis showing expression patterns of *LEA-like 11-24* genes during a dehydration/rehydration cylce in *C. plantagineum* (*Cp LEA-like 11-24*) and *L. brevidens* (*Lb LEA-like 11-24*) and dehydration in *L. subracemosa* (*Ls LEA-like 11-24*). Total RNA was extracted from the following tissues: untreated leaves, leaves after 24 hours of dehydration, leaves after 48 hours of dehydration and leaves dehydrated for 48 hours and subsequently rehydrated for 24 hours. RNA was probed with DNA fragments corresponding to the relevant *LEA-like 11-24* genes. Below EtBr stained agarore gels are displayed to check quality and equal loading of RNA.

RNA gel blot analysis showed that the *LEA-like 11-24* gene was not transcribed in untreated leaf tissue (Figure 25). However, *LEA-like 11-24* transcripts were observed in

both *C. plantagineum* and *L. brevidens* leaf tissue that was dehydrated for either 24h or 48h. When dried *C. plantagineum* and *L. brevidens* plants were rehydrated again the transcript disappeared. In *L.* subracemosa leaf tissue only a very weak signal was detected after 48 hours of dehydration. This RNA gel blot analysis showed that in *C. plantagineum* and *L. brevidens*, *LEA-like 11-24* expression was induced by dehydration and down-regulated when plants were rehydrated. However, based on the intensity of bands observed in lanes of 24 hours dehydrated samples, the transcription of the *LEA-like 11-24* gene appears to be induced more rapidly in *C. plantagineum* than in *L. brevidens*. These results demonstrate that in *C. plantagineum*, *L. brevidens* and, in lesser degree, also in *L. subracemosa*, transcription of the *LEA-like 11-24* gene is regulated by dehydration.

To study the effect of ABA and osmotic stress on *LEA-like 11-24* expression, leaves of *C. plantagineum*, *L. brevidens* and *L. subracemosa* were treated either for 48 hours with 100 µM ABA or with 0.8 M mannitol. As control, leaves were incubated for 48 hours in water. After these treatments, total RNA was isolated from the leaves and *LEA-like 11-24* expression was studied by performing a RNA gel blot analysis (Figure 26).

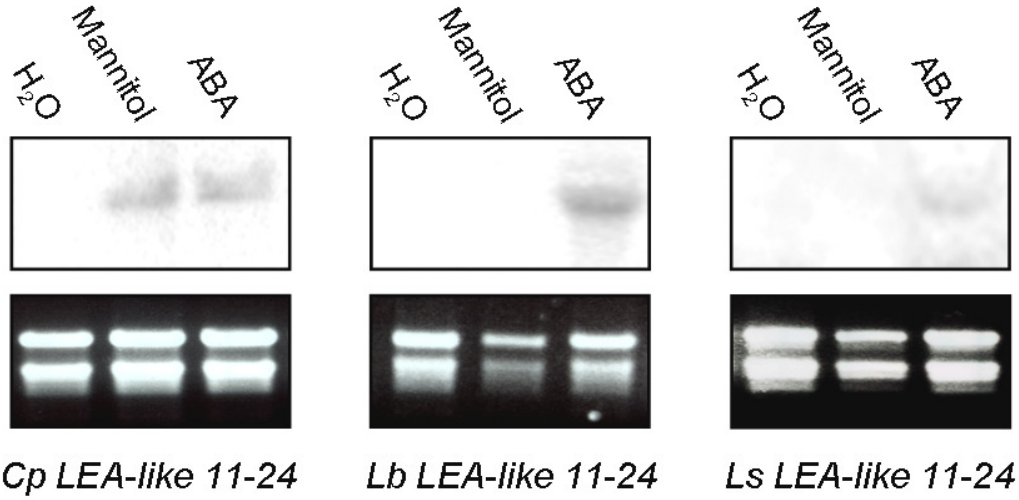


Figure 26. RNA gel blot showing expression of *LEA-like 11-24* genes in response to water, mannitol or ABA treatment in *C. plantagineum* (*Cp LEA-like 11-24*), *L. brevidens* (*Lb LEA-like 11-24*) and *L. subracemosa* (*Ls LEA-like 11-24*) leaves. Total RNA was extracted from leaves treated for 48 hours with water, 0.8 M mannitol or 100 µM ABA, respectively. RNA was probed with DNA fragments corresponding to the relevant *LEA-like 11-24* genes. Below EtBr stained agarose gels are displayed to check quality and equal loading of RNA.

The *LEA-like 11-24* transcript was detected in *C. plantagineum* leaves treated with either mannitol or ABA. In *L. brevidens* leaf tissue only a band was obtained after treatment with ABA, but no expression was found after mannitol treatment. Likewise,

in *L. subracemosa* a very faint band was detected after ABA treatment and again no expression was observed following mannitol treatment. No *LEA-like 11-24* expression was found in any of the leaf tissues treated with water.

3.6. Functional analysis of *LEA-like 11-24* promoter activity in leaf tissue

The *LEA-like 11-24* promoter regions were selected for further characterization and functional analysis, since the expression analysis of the *LEA-like 11-24* gene revealed difference in the patterns of *LEA-like 11-24* gene expression between *C. plantagineum*, *L. brevidens* and *L. subracemosa* (Figures 25 and 26). These results imply that the expression of the *LEA-like 11-24* gene of is regulated differentially between these plant species. To study regulatory mechanisms of ABA- and dehydration-induced *LEA-like 11-24* expression in *C. plantagineum*, *L. brevidens* and *L. subracemosa*, the activity of putative *LEA-like 11-24* promoters was analyzed in a transient expression assay.

First, *LEA-like 11-24* promoter regions from the three plant species were selected. Nucleotide sequences of the 5´-upstream regions of the LEA-like *11-24* gene of approximately 300 bp from *C. plantagineum* (from -309 bp to -2 bp), *L. brevidens* (from -323 bp to -2 bp) and *L. subracemosa* (from -278 bp to -2 bp) were amplified and subsequently isolated. These promoter regions were carefully chosen, because they showed high sequence similarity with each other and contained several putative *cis*-acting regulatory elements known to be involved in ABA- and dehydration-induced gene expression (Figure 28). The *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments harbored three ACGT-containing ABREs, named ACGT box 1 to 3. The *Ls LEA-like 11-24* promoter fragment contained two ACGT-containing ABREs, termed ACGT box and ACGT box 2. The *LEA-like 11-24* promoter fragment from *C. plantagineum* harbored a DRE element. In addition, two ABREs were conserved between all three promoters. The *Cp LEA-like* ACGT box 2, *Lb LEA-like* ACGT box 1 and *Ls LEA-like* ACGT box 1 were conserved in the *LEA-like 11-24* promoter fragments. Furthermore, *Cp LEA-like* ACGT box 3, *Lb LEA-like* ACGT box 3 and *Ls LEA-like* ACGT box 2 were conserved among the different promoters fragments (Figure 28).

In a previous study, it was demonstrated by Velasco *et al*. (1998) that the first 667 nucleotides of the *LEA-like 11-24* promoter from *C. plantagineum* held all elements required for ABA- and dehydration-induced promoter activity. Therefore, it was very likely that the chosen promoter fragments contained sufficient *cis*-regulatory elements to respond to ABA and dehydration. Furthermore, the relatively short length of the

promoter fragments could help to generate a functional minimal promoter, which is stress-inducible and tissue independent.

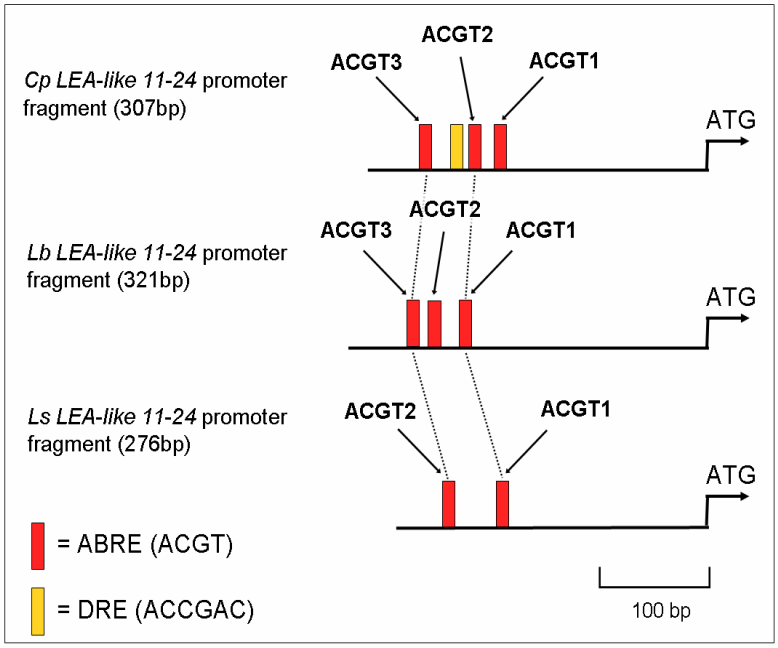


Figure 28. *LEA-like 11-24* promoter fragments from *C. plantagineum*, *L. brevidens* and *L. subracemosa* used for transient expression assay. Only ACGT-containing ABRE elements and the DRE element within the promoter fragments are displayed. The ACGT-containing ABREs present in the different *LEA-like 11-24* promoter fragments are numbered. Conserved *cis*-acting regulatory elements are linked together with dotted lines.

The sequences directly flanking the *cis*-acting regulatory element might be critical for the function of the element. Therefore, sequences immediately flanking the conserved ACGT motifs within *LEA-like 11-24* promoter fragments of *C. plantagineum*, *L. brevidens* and *L. subracemosa* were analyzed (Figure 29). The conserved ACGT motifs, including 6 nucleotides of 5'-flanking sequence and 6 nucleotides of 3'-flanking sequence were aligned.

Analysis of flanking sequences revealed that regions directly flanking *Cp LEA-like* ACGT box 2, *Lb LEA-like* ACGT box 1 and *Ls LEA-like* ACGT box 1 were almost identical and very well conserved between all three promoter fragments. The sequences flanking *Lb LEA-like* ACGT box 3 and *Ls LEA-like* ACGT box 3 were very similar. However, these flanking regions showed less similarity to the flanking sequences of *Cp LEA-like* ACGT box 3. Nevertheless, some specific nucleotides directly flanking the 5'-side and 3'-side of these ACGT motifs showed conservation, especially between *Cp LEA-like* ACGT box 3 and *Lb LEA-like* ACGT box 3.

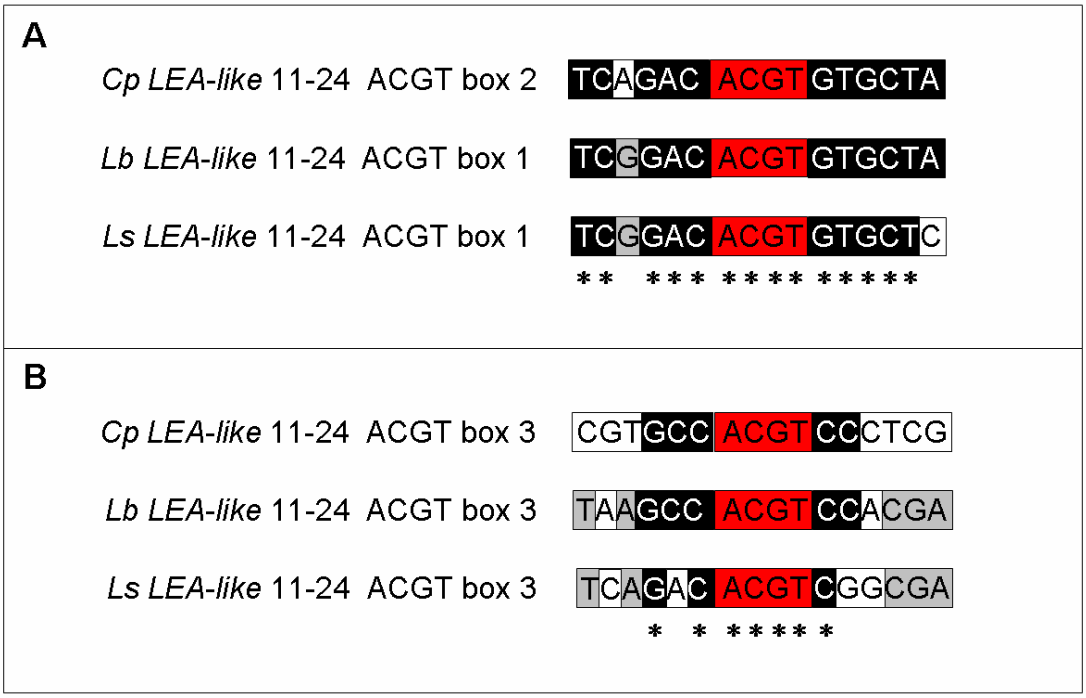


Figure 29. Analysis of sequences flanking the conserved ACGT motifs within *LEA-like 11-24* promoter fragments of *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Alignments were performed with conserved ACGT boxes, including 6 nucleotides of 5'-flanking sequence and 6 nucleotides of 3'-flanking sequence. (A) Alignment of *Cp LEA-like 11-24* ACGT box 2, *Lb LEA-like 11-24* ACGT box 1 and *Ls LEA-like 11-24* ACGT box 1. (B) Alignment of *Cp LEA-like 11-24* ACGT box 3, *Lb LEA-like 11-24* ACGT box 3 and *Ls LEA-like 11-24* ACGT box 3. The ACGT core sequence is displayed in a red box. Nucleotides identical to nucleotides in the flanking regions of ACGT boxes present in the *Cp LEA-like 11-24* promoter fragment are indicated in black boxes. Nucleotides that are identical between the flanking sequences of ACGT boxes within *Lb LEA-like 11-24* and *Ls LEA-like 11-24* promoter fragments are shown in grey colored boxes. Nucleotides identical between all three sequences are indicated by asterisks.

The selected putative *LEA-like 11-24* promoter fragments from *C. plantagineum* (307 bp), *L. brevidens* (321 bp) and *L. subracemosa* (276 bp) were fused to the *GUS* reporter gene.

3.6.1. Characterization of promoter activity in a transient expression assay

To conduct a functional promoter analysis, the chimeric *LEA-like 11-24* promoter::*GUS* fusion constructs were introduced in leaf cells of the same species (homologous transformation) via a particle bombardment. In order to test the efficiency for each bombardment, the *LEA-like 11-24* promoter::*GUS* constructs were co-bombarded with a CaMV 35S::*GFP* construct. This GFP expression construct was used as an internal control. GUS expression was normalized to the expression of the *GFP* reporter gene. Bombarded leaves were treated for 48 hours with either 100 µM ABA, 0.8 M mannitol or water. The transiently transformed leaves were then analyzed for GFP and GUS expression, respectively. The GFP expression was analyzed 24 hours after the bombardment and GUS expression was monitored after 48 hours (Figure 30). The activity of the three *LEA-like 11-24* promoter fragments was assessed by determining the number of GUS spots and GFP expressing cells in bombarded leaves and then calculating the percentage of promoter activity compared to the activity of the CaMV35S promoter (% GUS/GFP). The exact number of GFP expressing cells and GUS spots from each transient expression assay can be found in the Supplemental data section (Table S8 to Table S24).

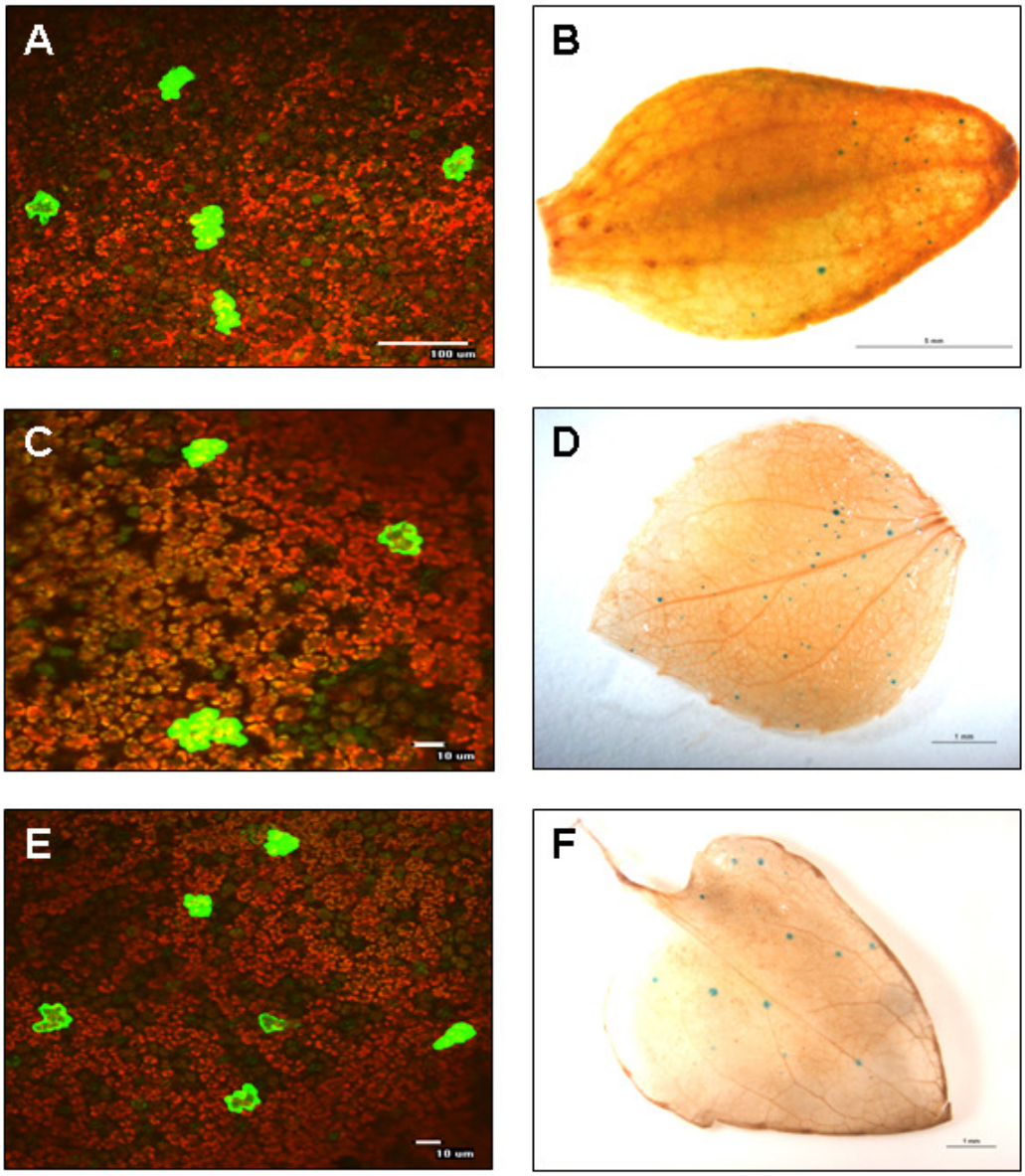


Figure 30. Examples of GFP and GUS expression in transiently transformed leaves of *C. plantagineum*, *L. brevidens* and *L. subracemosa*. (A) Section of a *C. plantagineum* leaf, showing five epidermal leaf cells expressing GFP. The bright green signal is GFP and the red signal is autofluorescence from chloroplasts. (B) GUS expression observed in a leaf of *C. plantagineum* treated with ABA. Blue spots indicate GUS expression. (C) Section of a *L. brevidens* leaf, showing three epidermal leaf cells expressing GFP. (D) GUS expression observed in a leaf of *L. brevidens* treated with ABA (E) Section of a *L. subracemosa* leaf, showing six epidermal leaf cells expressing GFP. (F) GUS expression observed in a leaf of *L. subracemosa* treated with ABA

3.6.2. Analysis of *LEA-like* 11-24 promoter activity in leaf tissue

The activity of the *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments increased after treatment with either ABA or mannitol when compared to the water-treated control leaves (Figure 31). The *LEA-like 11-24* promoter fragment of *L. subracemosa* showed only a very weak promoter activity after treatment with either ABA or mannitol. Here, the levels of activity after ABA or mannitol treatments were almost at the same level as the water-treated control leaves.

The highest activity in response to either ABA or osmotic stress of all three tested *LEA-like 11-24* promoter fragments was found for the *Cp LEA-like 11-24* promoter fragment. The activity of the *Cp LEA-like 11-24* promoter fragment was about 6–fold higher than that of the *Lb LEA-like 11-24* promoter fragment after ABA treatment and about 4-fold higher under osmotic stress conditions.

These transient expression assays demonstrate that the *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments is both functional and responds to ABA and water stress. Whereas, the *Ls LEA-like 11-24* promoter fragment was unable to respond to ABA and osmotic stress

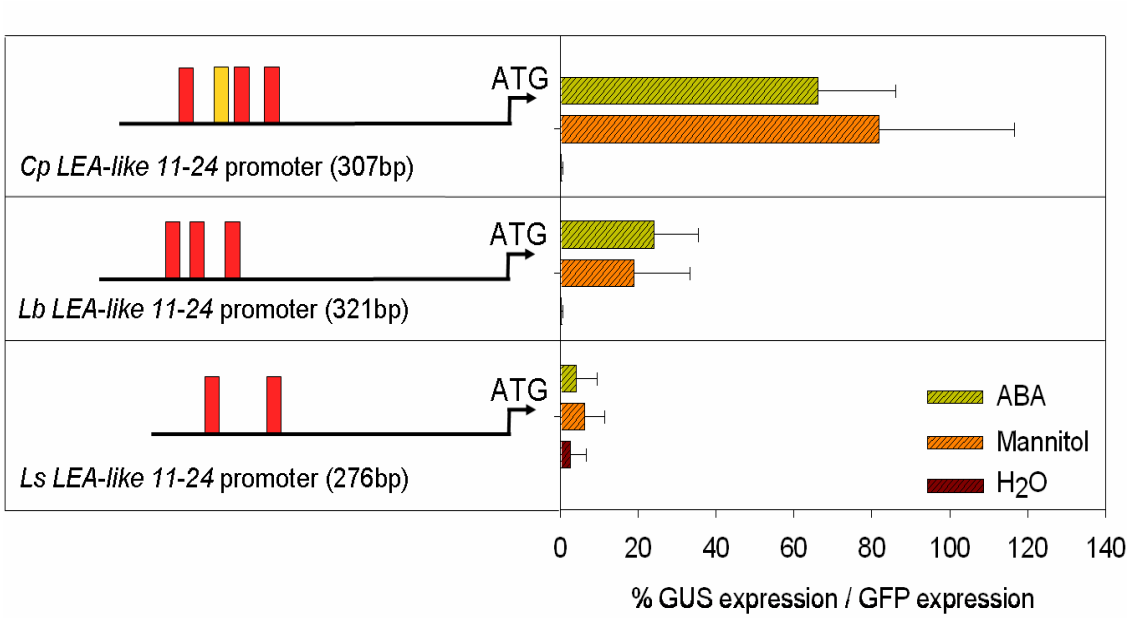


Figure 31. Relative promoter activity of the putative *LEA-like 11-24* promoter fragments from *C. plantagineum*, *L. brevidens* and *L. subracemosa* in a transient expression assay. Promoter activity was measured after treatment with ABA, mannitol or water. Activity is expressed as percentage of *LEA-like 11-24* promoter activity compared to the CaMV35S promoter (% GUS/GFP). Bars are mean values of seven independent experiments for each treatment (n = 7) and error bars indicate SD of the mean.

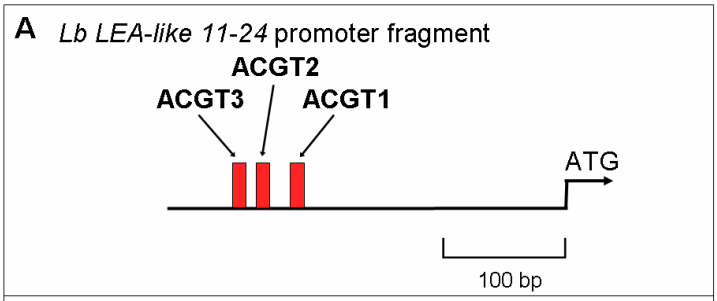
3.7. Identification of functional *cis*-acting regulatory elements involved in ABA- and dehydration-induced *LEA-like 11-24* promoter activity

Now that it was clear that the *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments, which were used in the transient expression assays, contained sufficient *cis*-acting regulatory elements to stimulate activity after ABA or mannitol treatment, the next step was to identify which of the *cis*-elements were actually involved in ABA- and dehydration-responsive *LEA-like 11-24* expression in *C. plantagineum* and *L. brevidens*. A site-directed mutagenesis approach was used to identify the critical *cis*-acting elements in ABA- and dehydration-responsive expression of the *LEA-like 11-24* gene. Side-directed mutagenesis was performed with the purpose to disturb the functionality of the *cis*-acting regulatory elements important for ABA- and dehydration-inducible promoter activity.

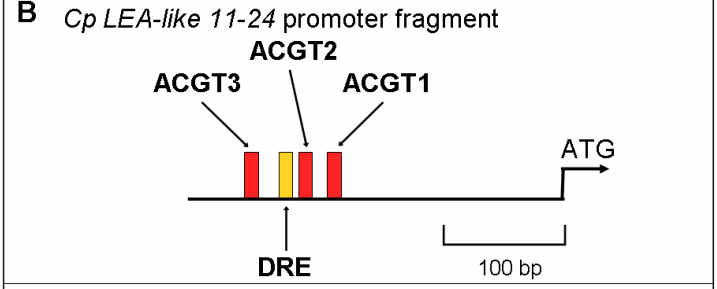
The *Lb LEA-like 11-24* promoter fragment held three ACGT-containing ABREs: ACGT1, ACGT2, ACGT3, respectively. The roles of ACGT1 and ACGT3 in ABA- and dehydration-mediated expression were investigated, because these two ABREs were conserved between the *LEA-like 11-24* promoter fragments of all three species (Figure 28). Site-specific mutations were made in ACGT1 and ACGT3 boxes of the *Lb LEA-like 11-24* promoter fragment (Figure 32).

The *Cp LEA-like 11-24* promoter fragment also harbored three ACGT-containing ABREs. In addition, the *Cp LEA-like 11-24* promoter fragment contained a DRE motif. Site-specific mutations were introduced into the three ACGT-containing ABREs and the DRE motif (Figure 32)

The ACGT-motif of ABREs was mutated into ATAT and the GTCGGT-motif of DRE was changed into GATGGT. Single mutations or different combinations of mutations were generated within the *LEA-like 11-24* promoter fragments of *C. plantagineum* and *L. brevidens* (Figure 32). The effect of the different mutations on promoter activity was determined.



Mutations
Lb 11-24 mutation ACGT1
Lb 11-24 mutation ACGT3



Mutations
Cp 11-24 mutation ACGT1
Cp 11-24 mutation ACGT2
Cp 11-24 mutation ACGT3
Cp 11-24 mutations ACGT1 & ACGT3
Cp 11-24 mutations ACGT1 & ACGT2
Cp 11-24 mutations ACGT2 & ACGT3
Cp 11-24 mutations ACGT1 & ACGT2 & ACGT3
Cp 11-24 mutation DRE
Cp 11-24 mutations ACGT1 & ACGT2 & ACGT3 & DRE

Figure 32. Site-directed mutations introduced within the *LEA-like 11-24* promoter fragments from *L. brevidens* and *C. plantagineum*.
(A) Single mutations gener-ated into ACGT-containing ABRE elements; ACGT1 and ACGT3 within the *Lb LEA-like 11-24* promoter fragment.
(B) Single or combinations of mutations introduced into ACGT-containing ABRE elements; ACGT1, ACGT2 and ACGT3, respectively, and DRE element within the *Cp LEA-like 11-24* promoter fragment.
The ACGT-containing ABRE elements were mutated into ATAT. The GTCGGT motif of the DRE element was changed into GATGGT.

3.8. Effects of site-specific mutations on *C. plantagineum LEA-like 11-24* promoter activity

The *Cp LEA-like 11-24* promoter fragment harbors three ACGT-containing ABREs and one DRE motif and. Mutations were generated in all these elements to examine whether these *cis*-acting regulatory elements are important for ABA- and dehydration-induced promoter activity. When mutations were introduced into ACGT1, ACGT2, ACGT3 and DRE motifs, the activity of *Cp LEA-like 11-24* promoter fragment was completely abolished under both ABA and mannitol treatments (Figure 33). These results indicate that the *Cp LEA-like 11-24* promoter fragment is sufficient and does not contain other *cis*-acting regulatory elements, besides the ACGT1, ACGT2, ACGT3 and DRE motifs, that are needed for ABA- and dehydration-responsiveness of the

promoter. The next step was to investigate the role of each of these *cis*-acting regulatory elements in ABA- and dehydration-induced activation of the of the *Cp LEA-like 11-24* promoter fragment.

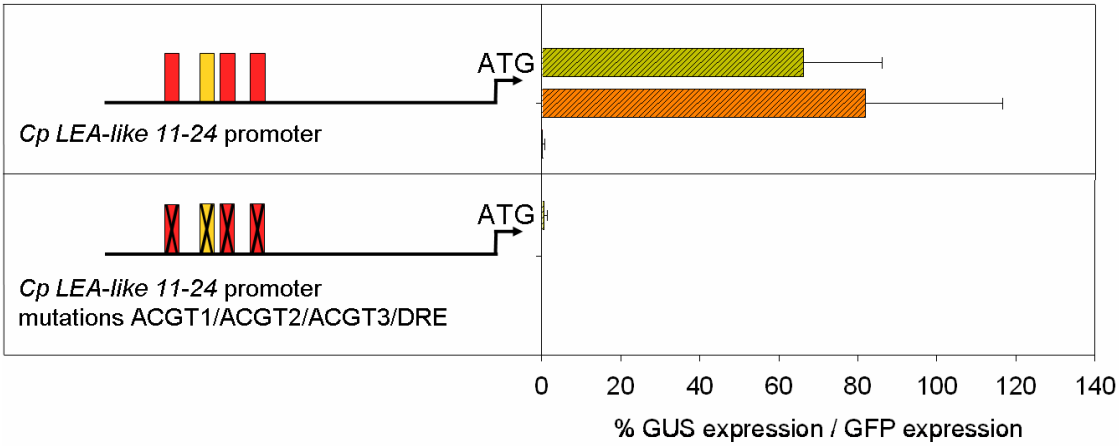


Figure 33. Relative promoter activity of *Cp LEA-like 11-24* promoter and *Cp LEA-like 11-24* promoter mutations ACGT1/ACGT2/ACGT3/DRE fragments measured in transient expression assays. Promoter activity was measured after treatment with ABA, mannitol or water. Activity is shown as percentage of *LEA-like 11-24* promoter activity compared to the CaMV35S promoter (% GUS/GFP). Data from *Cp LEA-like 11-24* promoter represent the means with SD of seven independent experiments for each treatment (n = 7). Results from the *Cp LEA-like 11-24* promoter mutations ACGT1/ACGT2/ACGT3/DRE fragment are mean values with SD of four independent experiments for each treatment (n = 4).

A major difference between the *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments is the presence of a DRE motif in the *LEA-like 11-24* promoter fragment of *C. plantagineum*. This DRE motif could be responsible for the difference in activity that was found between the *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments (Figure 31). Therefore, the role of this DRE motif in ABA- and dehydration-responsive expression was investigated.

Mutations were generated in all three ACGT-containing ABRE motifs present in the *Cp LEA-like 11-24* promoter fragment, ACGT1, ACGT2 and ACGT3, respectively. The resulting promoter fragment was able to confer an ABA and mannitol response (Figure 34). However, the activity of the *Cp LEA-like 11-24* promoter fragment harboring the three mutated ACGT-containing ABREs motifs was lower than the activity of the non-mutated *Cp LEA-like 11-24* promoter fragment. When all three ACGT boxes were mutated the activity after ABA treatment was reduced by 81% (Table 7). Mutagenesis of ACGT1, ACGT2 and ACGT3 reduced promoter activity

after mannitol treatment by 76% (Table 7). Nevertheless, these findings imply that the DRE motif is both involved in ABA- and dehydration-responsiveness of the *Cp LEA-like 11-24* promoter. To further clarify the role of the DRE motif in ABA- and dehydration-responsive expression, a mutation was introduced in the DRE box and the effect of this mutation on the *Cp LEA-like 11-24* promoter activity was examined (Figure 34). Mutagenesis of the DRE box led to a decrease in ABA-responsiveness (44%) and reduced mannitol-induced activity by 68% when compared to the non-mutated *Cp LEA-like 11-24* promoter fragment (Table 7). These results demonstrate that the DRE motif is required for both ABA- and dehydration-responsiveness of the *Cp LEA-like 11-24* promoter.

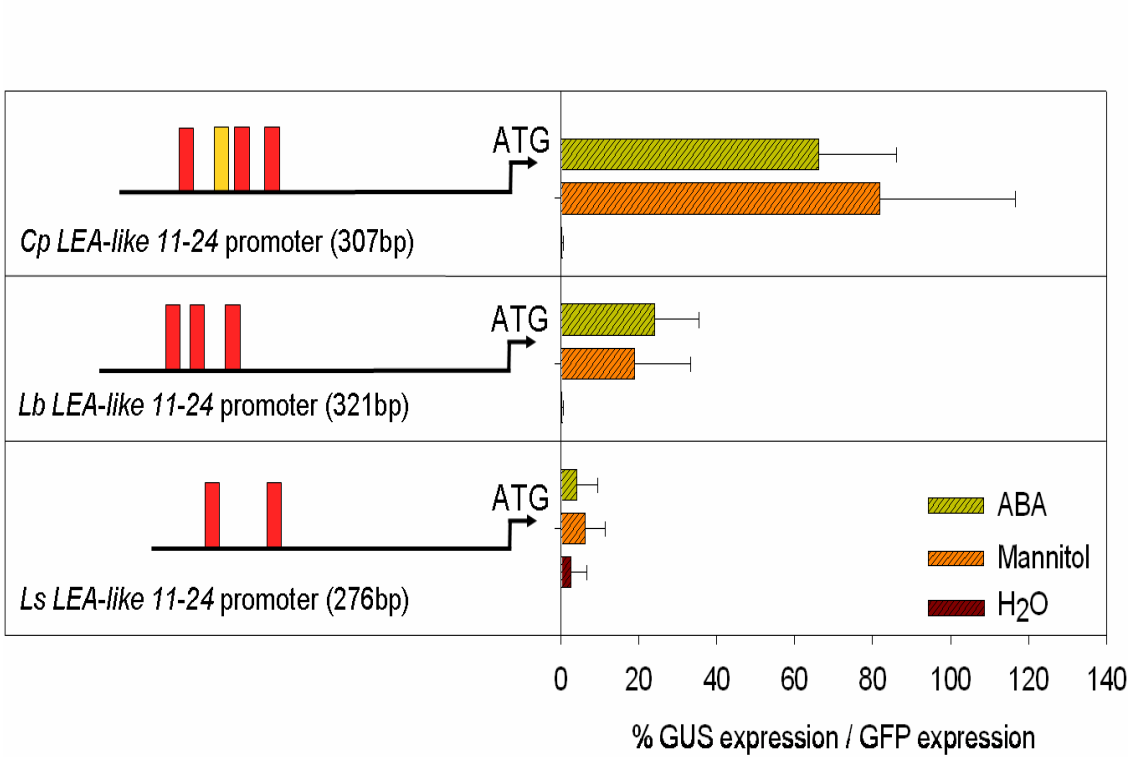


Figure 34. Relative promoter activity of *Cp LEA-like 11-24* promoter, *Cp LEA-like 11-24* promoter mutations ACGT1/ACGT2/ACGT3 and *Cp LEA-like 11-24* promoter mutation DRE fragments. Promoter activity was measured in transient expression assay after treatment with ABA, mannitol or water. Activity is shown as percentage of *LEA-like 11-24* promoter activity compared to the CaMV35S promoter (% GUS/GFP). Data from *Cp LEA-like 11-24* promoter represent the means with SD of seven independent experiments for each treatment (n = 7). Results from *Cp LEA-like 11-24* promoter mutations ACGT1/ACGT2/ACGT3 and *Cp LEA-like 11-24* promoter mutation DRE fragments are mean values with SD of four independent experiments for each treatment (n = 4).

The mutagenesis of the three ACGT-containing ABRE motifs in *Cp LEA-like 11-24* promoter fragment reduced the promoter activity after both ABA and osmotic treatment (Figure 34). Consequently, the individual roles of the ACGT1, ACGT2 and ACGT3

boxes in ABA- and mannitol-inducible *Cp LEA-like 11-24* promoter activity were studied. Mutations were created in the ACGT1, ACGT 2 and ACGT3 boxes of the *Cp LEA-like 11-24* promoter fragment, respectively. Different combinations of mutations were used to elucidate the function of each of the three ACGT-containing ABREs.

Site-directed mutagenesis of ACGT1 resulted in a small increase of ABA or dehydration-responsiveness when compared to the non-mutated promoter fragment (Figure 35). ABA-induced activity of the *Cp LEA-like 11-24* promoter mutation ACGT1 fragment was 13% higher than the activity of the non-mutated *Cp LEA-like 11-24* promoter fragment (Table 7). Furthermore, mutagenesis of ACGT1 mutation led to a 15% increase of promoter activity following mannitol treatment. Even some promoter activity was observed under the water conditions. These results suggest that ACGT1 could be involved in the repression of *Cp LEA-like 11-24* promoter activity under non-stressed conditions.

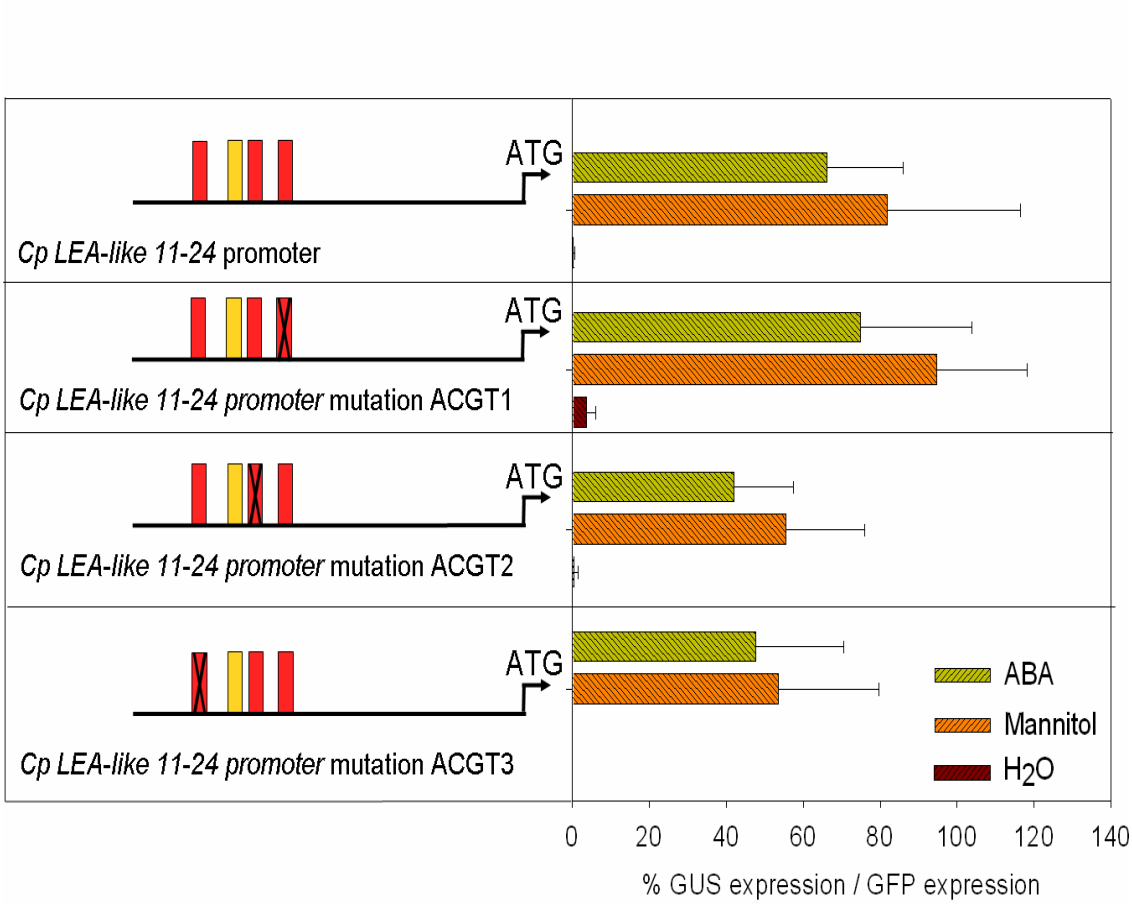


Figure 35. Relative promoter activity of the putative *LEA-like 11-24* promoter fragments from *C. plantagineum*, *L. brevidens* and *L. subracemosa* in transient expression assay after treatment with ABA, mannitol or water. Activity is expressed as percentage of *LEA-like 11-24* promoter activity compared to the CaMV35S promoter (% GUS/GFP). Data from *Cp LEA-like 11-24* promoter represent the means with SD of seven independent experiments for each treatment (n = 7). Results from different mutated *Cp LEA-like 11-24* promoters fragments are mean values with SD of four independent experiments for each treatment (n = 4).

The mutagenesis of either ACGT2 or ACGT3 led to a reduction in promoter activity under ABA and osmotic stress. The activity of the *Cp LEA-like 11-24* promoter mutation ACGT2 fragment was 37% lower after ABA treatment and decreased 32% following treatment with mannitol (Table 7). The ACGT3 mutation caused 28% reduction in ABA-induced activity and showed 35% lower activity under osmotic stress (Table 7).

To further study the roles of the three ACGT-containing ABREs present in the *Cp LEA-like 11-24* promoter fragment, different combinations of mutations were analyzed (Figure 36).

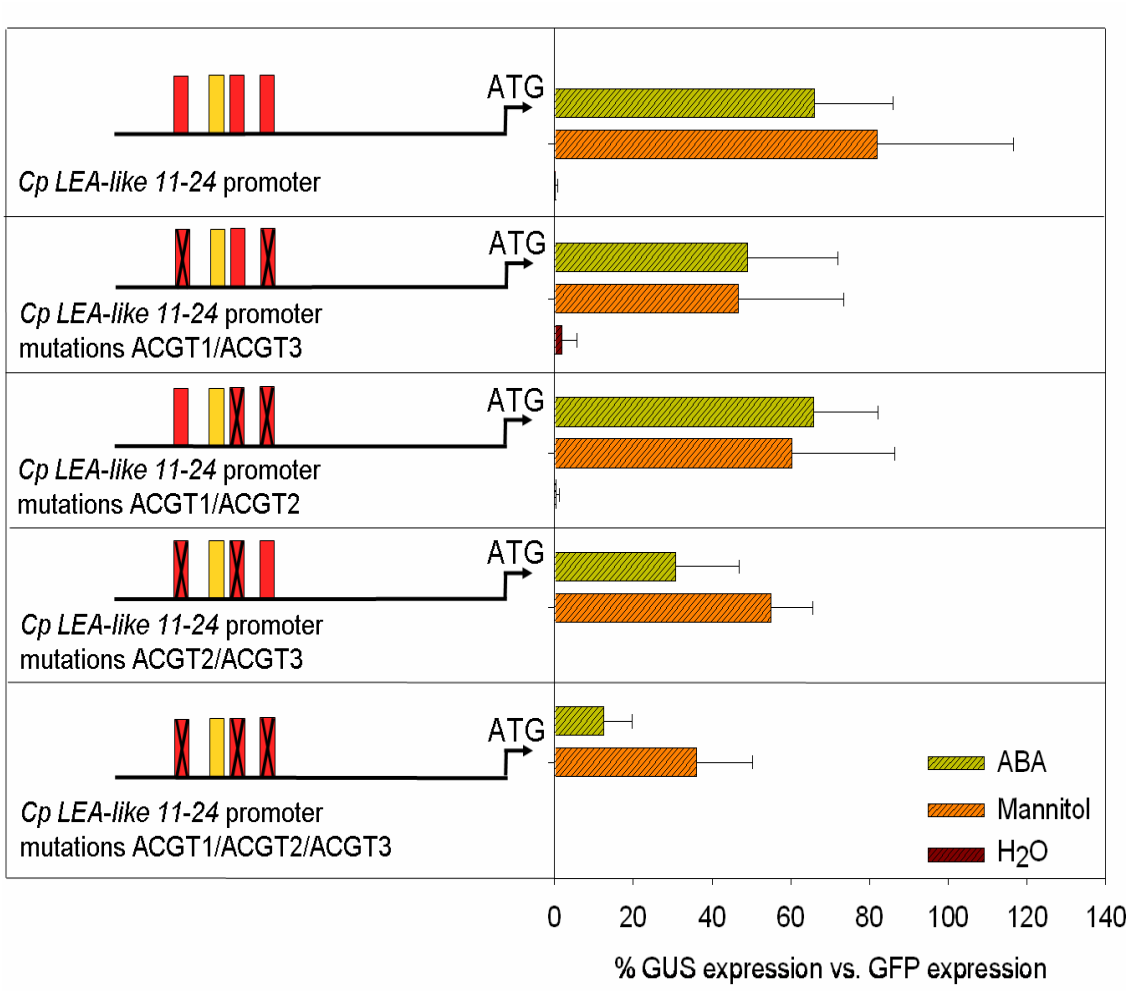


Figure 36. Relative promoter activity of *Cp LEA-like 11-24* promoter, *Cp LEA-like 11-24* promoter mutations ACGT1/ACGT3, *Cp LEA-like 11-24* promoter mutations ACGT1/ACGT2, *Cp LEA-like 11-24* promoter mutations ACGT2/ACGT3 and *Cp LEA-like 11-24* promoter mutations ACGT1/ACGT2/ACGT3 fragments. Promoter activity in transient expression assay was measured after treatment with ABA, mannitol or water. Activity is shown as percentage of *LEA-like 11-24* promoter activity compared to the CaMV35S promoter (% GUS/GFP). Data from *Cp LEA-like 11-24* promoter represent the means with SD of seven independent experiments for each treatment (n = 7). Results from different mutations of the *Cp LEA-like 11-24* promoter fragments are mean values with SD of four independent experiments for each treatment (n = 4).

Mutagenesis of both ACGT1 and ACGT2 in the same promoter fragment resulted in a 36% decrease of ABA-induced activity and a 43% decrease after mannitol treatment compared to the non-mutated promoter fragment (Table 7). Surprisingly, the mutation of both ACGT1 and ACGT3 had no effect at all on activity after ABA treatment and had only a negative effect on dehydration-responsiveness. These mutations gave rise to a 26% reduction of activity following mannitol treatment (Table 7). Mutating both ACGT2 and ACGT3 inhibited activity after ABA treatment by 54% and activity under osmotic stress was decreased by 33% (Table 7).

However, the mutagenesis of all three ACGT-containing ABRE motifs led to a stronger reduction in ABA-responsiveness than when only one or combinations of ACGT boxes were mutated (Table 7). When all three ACGT boxes were mutated the activity was dramatically reduced after ABA treatment (81%). Hence, these results indicate that all three ACGT boxes are involved in ABA- and dehydration-induced promoter activity.

Table 7. Effects of mutations on activity of the *Cp LEA-like 11-24* promoter fragment.

Mutations	Treatment	
	ABA	mannitol
non-mutated	100%	100%
ACGT1	113%	115%
ACGT2	63%	68%
ACGT3	72%	65%
ACGT1/ACGT3	100%	74%
ACGT1/ACGT2	74%	57%
ACGT2/ACGT3	46%	67%
ACGT1/ACGT2/ACGT3	19%	44%
DRE	56%	32%
ACGT1/ACGT2/ACGT3/DRE	0%	0%

Promoter activity measured after ABA and mannitol treatment. Activity of the mutated *Cp LEA-like 11-24* promoter fragments is expressed as percentage of activity compared to the non-mutated *Cp LEA-like 11-24* promoter fragment.

3.9. Effects of site-specific mutations on *L. brevidens LEA-like 11-24* promoter activity

The activity of the mutated *L. brevidens LEA-like 11-24* promoter constructs are shown after treatment with ABA , mannitol or water in Figure 37.

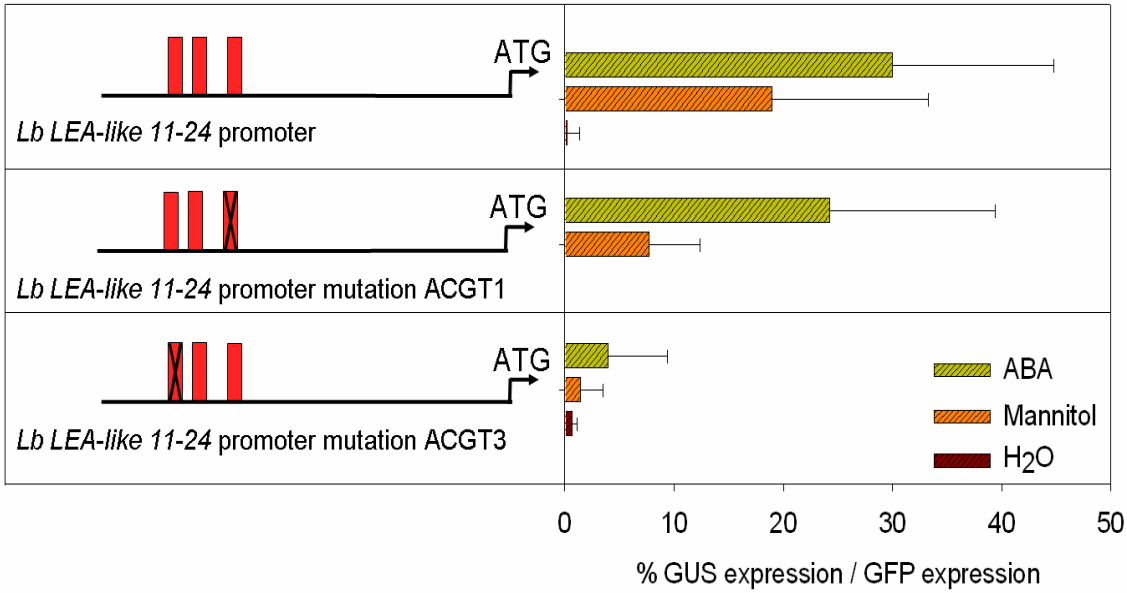


Figure 37. Relative promoter activity of mutagenized *Lb LEA-like 11-24* promoter fragments in transient expression assay after treatment with ABA, mannitol or water. Activity is shown as percentage level of promoter activity compared to the CaMV35S promoter (% GUS/GFP). Data from *Lb LEA-like 11-24* promoter represent the means with SD of seven independent experiments for each treatment (n = 7). Results from *Lb LEA-like 11-24* mutation ACGT1 and mutation ACGT3 promoters are mean values with SD of four independent experiments for each treatment (n = 4).

Mutagenesis of ACGT1 did only cause a slight decrease in ABA-responsiveness (19%) compared to the non-mutated *Lb LEA-like 11-24* promoter fragment (Table 8). In contrast, the mutation in ACGT3 resulted in a much stronger reduction in ABA-induced activity (87%) (Table 8). Mutagenesis of either ACGT1 or ACGT3 had a pronounced effect on promoter activity under osmotic stress. The ACGT1 mutation led to 59% decrease in water stress-induced promoter activity and mutagenesis of ACGT3 resulted in an even stronger a reduction (96%) (Table 8).

These results indicate that ACGT3 plays a key role in ABA-induced promoter activity. Dehydration-induced activity of the *Lb LEA-like 11-24* promoter fragment depends on both ACGT1 and ACGT3.

Table 8. Effects of mutations on activity of the *Lb LEA-like 11-24* promoter fragment.

Mutations	Treatment	
	ABA	mannitol
non-mutated	100%	100%
ACGT1	81%	41%
ACGT3	13%	4%

The table shows promoter activity measured after ABA and mannitol treatment. Activity of the mutated *Lb LEA-like 11-24* promoter fragments is expressed as percentage of activity compared to the non-mutated *Lb LEA-like 11-24* promoter fragment.

3.10. Functional analysis of *LEA-like 11-24* promoter activity in heterologous leaf tissue

As previously demonstrated, the *Ls LEA-like 11-24* promoter fragment did not show activity after ABA or mannitol treatment. The *Cp LEA-like 11-24* promoter fragment was transformed into *L. subracemosa* leaves, to test whether transcription factors essential for regulating ABA- and dehydration-induced promoter activity are absent in *L. subracemosa* leaves or if the DNA sequence of the *Ls LEA-like 11-24* promoter fragment accounts for the low promoter activity. In addition, the activity of the *Ls LEA-like 11-24* promoter fragment in *C. plantagineum* leaf tissue was examined. With this approach, the activity of the *Cp LEA-like 11-24* and *Ls LEA-like 11-24* promoter fragments in different *trans*-regulatory environments was investigated (Figure 38).

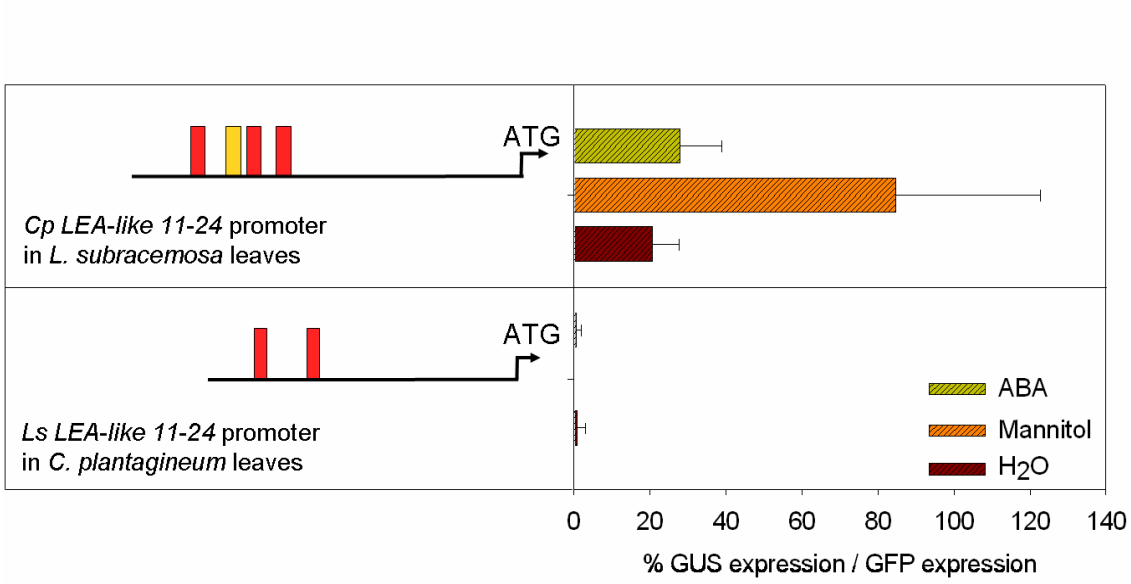


Figure 38. Relative promoter activity of the *Cp LEA-like 11-24* promoter fragments *L. subracemosa* leaf tissue and *Ls LEA-like 11-24* promoter fragment in *C. plantagineum* leaf tissue. Promoter activity in transient expression assay was measured after treatment with ABA, mannitol or water. Activity is shown as percentage of *LEA-like 11-24* promoter activity compared to the CaMV35S promoter (% GUS/GFP). Bars are means with SD of four independent experiments for each treatment (n = 4).

The *Cp LEA-like 11-24* promoter fragment showed activity following ABA and mannitol treatment in *L. subracemosa* leaves. Surprisingly, activity was even found in the water treated control leaves. However, no activity was detected when the *Ls LEA-like 11-24* promoter fragment was transformed in *C. plantagineum* leaf tissue. These results indicate that the *trans*-acting environment in *L. subracemosa* leaves is able to induce promoter activity in response to ABA and mannitol treatments.

3.11. Effect of *Ls LEA-like 11-24* promoter length on activity

The 276 bp *Ls LEA-like 11-24* promoter fragment probably lacked the required *cis*-acting regulatory elements to induce an ABA or dehydration response. In order to determine whether essential *cis*-acting elements were absent in the *Ls LEA-like 11-24* promoter fragment, a 488 bp *Ls LEA-like 11-24* promoter fragment was amplified. In comparison with the 276 bp *Ls LEA-like 11-24* promoter fragment, the 488 bp *Ls LEA-like 11-24* fragment held two additional ACGT-containing ABREs (Figure 39). The extended *Ls LEA-like 11-24* promoter fragment was fused to the *GUS* reporter gene and subsequently the activity of this longer promoter was analyzed.

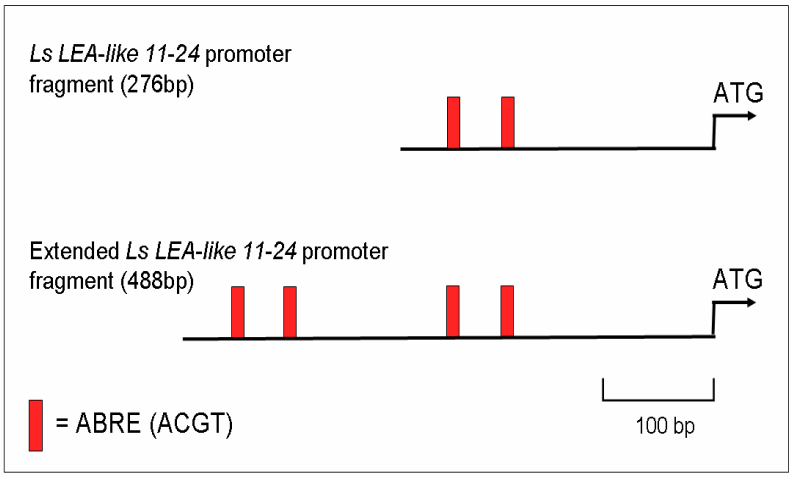


Figure 39. Distribution of ABREs within the 276bp and 488bp *Ls LEA-like 11-24* promoter fragments. The *Ls LEA-like 11-24* promoter fragment of 488bp contained two additional ACGT-containing ABREs.

In contrast to the shorter *Ls LEA-like 11-24* promoter fragment, the longer *Ls LEA-like 11-24* promoter fragment was able to induce activity after ABA or mannitol treatment in *L. subracemosa* leaves (Figure 40). Compared to the shorter *Ls LEA-like 11-24* promoter fragment, the longer *Ls LEA-like 11-24* promoter fragment showed approximately an 8-fold increase in activity under ABA and osmotic stress. This experiment reveals that the longer *Ls LEA-like 11-24* promoter fragment was functional and contained enough *cis*-acting regulatory elements to conduct ABA and dehydration responses. These results indicate that at least one of the additional ABREs was important for ABA- and mannitol-induced activity.

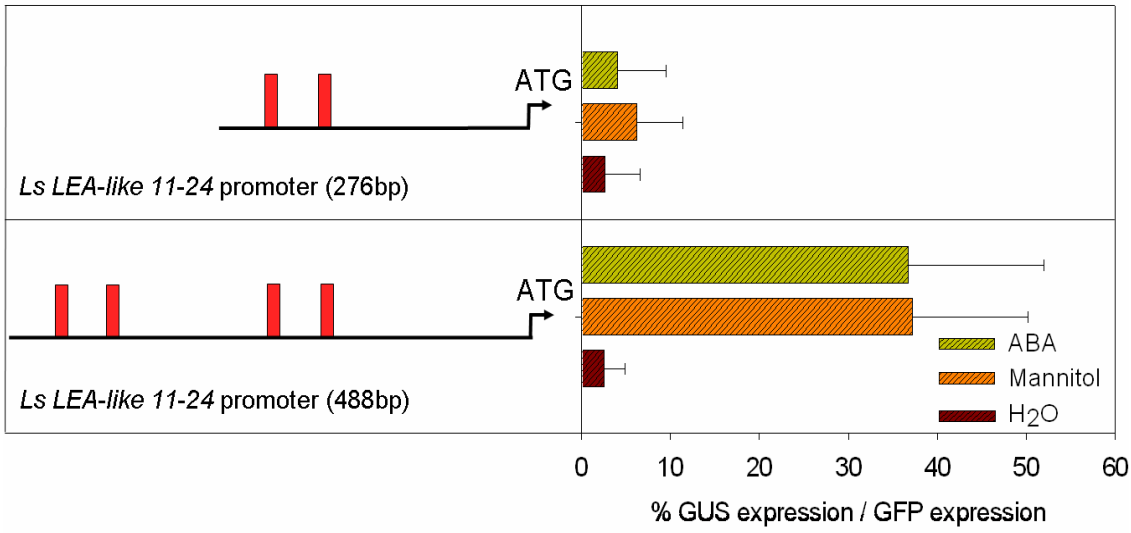


Figure 40. Relative promoter activity of the 276 bp and 488 bp *Ls LEA-like 11-24* promoter fragments in transient expression assay. Promoter activity in transient expression assay was measured after treatment with ABA, mannitol or water. Activity is expressed as percentage of *LEA-like 11-24* promoter activity compared to the CaMV35S promoter (% GUS/GFP). Bars are means with SD of four independent experiments for each treatment (n = 4).

3.12. Identification of proteins interacting with the *C. plantagineum LEA-like 11-24* promoter

A yeast one-hybrid system (Y1HS) was performed to identify potential transcription factors, which bind to the *Cp LEA-like 11-24* promoter fragment. The interactions between DNA-binding proteins (prey) and the promoter sequence (bait) were investigated with the Y1HS. Therefore, appropriate bait- and prey-plasmids were required. The bait plasmid (pSK1) contained the *Cp LEA-like 11-24* promoter fragment of interest and the prey plasmid (pPC86) carried a library of cDNAs, encoding many different proteins. These proteins were subsequently screened for their ability to bind the promoter-bait. The plasmid pSK1 held a *LEU2* marker gene and a *GAL* minimal promoter, which was fused to the *HIS3* reporter gene. The pSK1 (11-24) bait plasmid was constructed in the following manner.

A fragment of 60 bp in length from the *Cp LEA-like 11-24* promoter was selected as bait (Figure 41). This promoter fragment contained the ABREs; ACGT1 and ACGT2, and the DRE motif. Multiple copies of the selected promoter fragment were arranged in sequence, because the position of the *cis*-acting regulatory elements in relation to the ATG start codon could affect the ability to bind transcription factors and to stimulate expression. In total five copies of the promoter fragment were cloned back-to-back upstream of the *GAL* promoter (pGAL) into the plasmid pSK1. In this way the positions of the *cis*-acting regulatory elements in respect to the ATG start codon were variable.

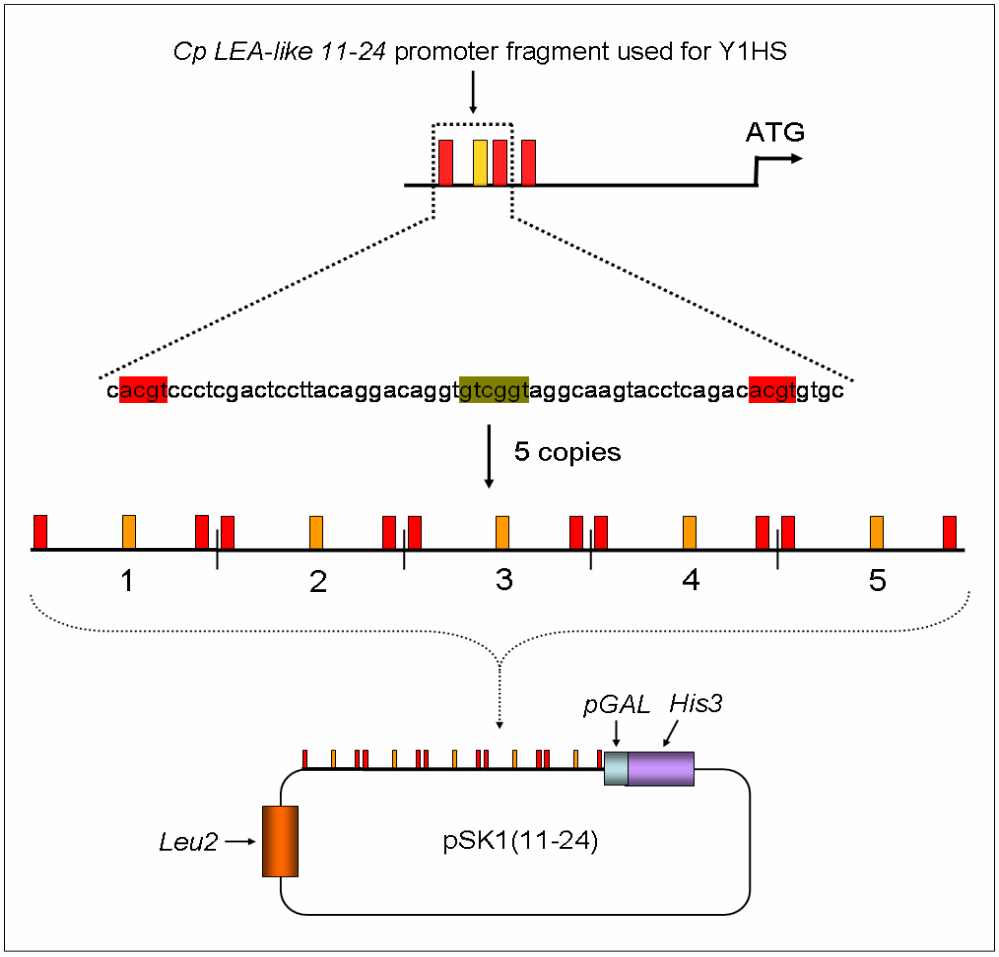


Figure 41. Strategy for the construction of the pSK1 (11-24) bait plasmid. Five copies of a 60 bp *Cp LEA-like 11-24* promoter fragment, containing the ACGT1, ACGT2, and DRE motifs are cloned in front of the *GAL* promoter (pGAL) into the pSK1 plasmid.

The pPC86 prey plasmid employed in the Y1HS was described by Ditzer and Bartels (2006). The library proteins that were used as prey, derived from a cDNA library constructed from partially dehydrated *C. plantagineum* leaf tissue (Hilbricht *et al.*, 2002). The cDNA fragments from this library had been fused to the *GAL4* activation domain (AD) into the pPC86 plasmid, which also contained the *TRP1* marker gene.

The strategy for performing the Y1HS is described briefly below and illustrated in Figure 42. The plasmids pSK1 (11-24) and pPC86, containing, respectively, the *LEU2* and *TRP1* marker genes were introduced into the yeast. After transformation, each yeast cell was expressing a different cDNA-GAL4 AD fusion protein. When one of the cDNA-GAL4 AD fusion proteins was able to interact with the *LEA-like 11-24* promoter bait, the GAL4 AD domain subsequently activated the transcription of the

HIS3 gene. This means that positive yeast clones were able to grow on medium lacking leucine, tryptophan and histidine.

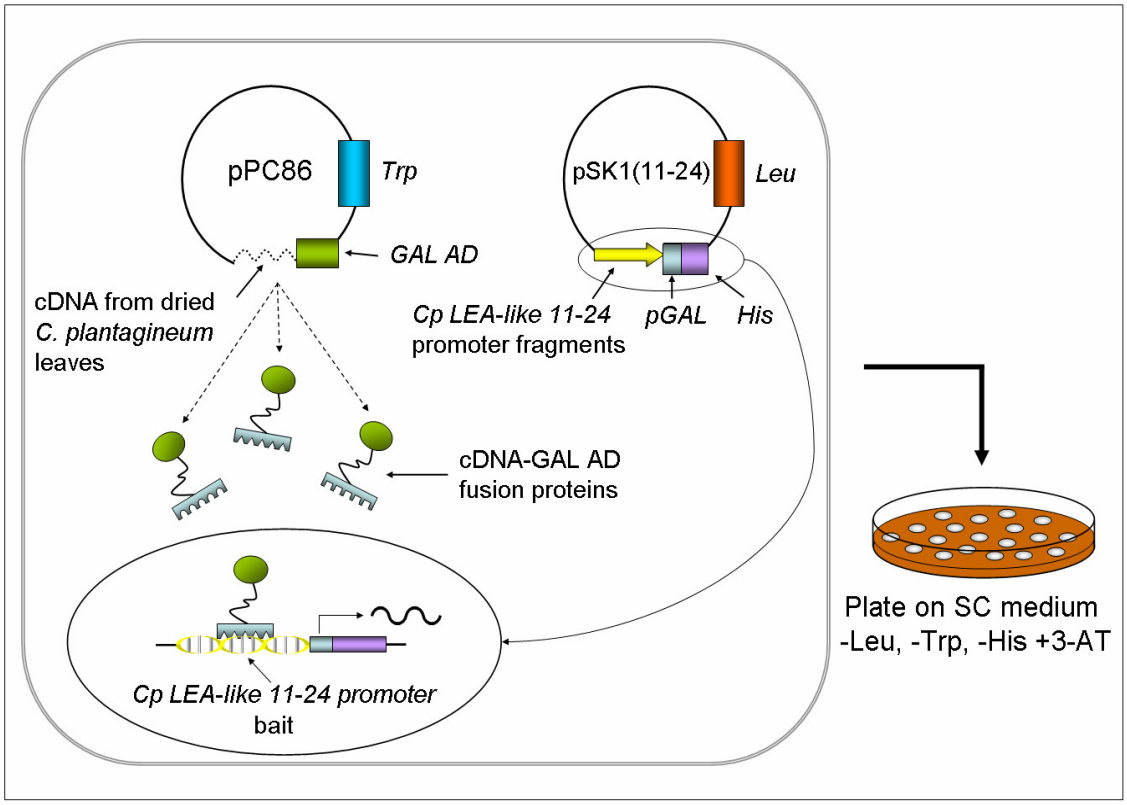


Figure 42. Illustration showing the principle of the Y1HS.

First, the pSK1 (11-24) plasmid was transformed into the yeast. Transformed yeast was plated on SC leu$^-$ media (Figure 43A). Subsequently, plasmid pPC86 without cDNA insert was introduced into yeast cells carrying the pSK1 (11-24) plasmid and transformed yeast was grown on SC trp$^-$ plates (Figure 43B).

Since, the expression of the *HIS3* gene has a tendency to be leaky due to self-activation and this could lead to the detection of false positive clones. Therefore, it was important to inhibit this background growth. The chemical 3-AT was used to inhibit the HIS3 gene product. Thus, a suitable concentration of 3-AT could suppress background growth of the yeast on medium lacking histidine. Only yeast clones, which have substantial increased *HIS3* expression, are able to grow on medium supplemented with the appropriate concentration of 3-AT.

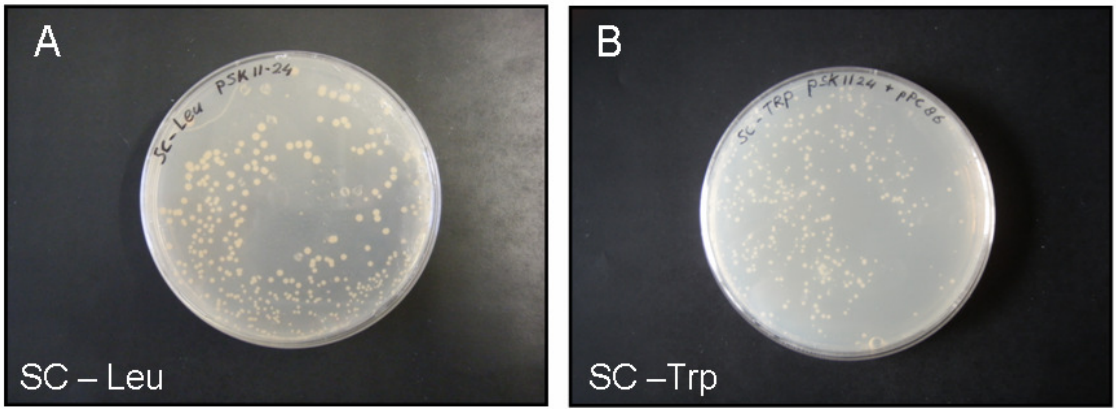


Figure 43. Selection plates showing colonies of yeast transformed with (A) plasmid pSK1 (11-24) and subsequently transformed with (B) plasmids pSK1 (11-24) and pPC86 (without cDNA).

Yeast transformants carrying the plasmids pSK1 (11-24) and pPC86 (without cDNA) were plated on SC leu$^-$ trp$^-$ his$^-$ medium plates containing different concentrations of 3-AT: 0 mM, 2.5 mM, 5 mM, 10 mM, 20 mM, 40 mM, 60 mM and 100 mM, respectively. The ability of yeast cells carrying the plasmids pSK1 (11-24) and pPC86 (without cDNA) to grow on these selection plates was analyzed.

The yeast only grew on selection plates without supplemented 3-AT. No yeast colonies were detected on medium plates containing 2.5 mM or higher concentrations of 3-AT. Hence, the supplementation of 2.5 mM 3-AT to the growth medium was sufficient to suppress background growth.

Next, pPC86 bait plasmids carrying cDNAs from dried *C. plantagineum* leaves were introduced into yeast harboring the pSK1 (11-24) plasmid. Transformed yeast cells were spread on SC leu$^-$ trp$^-$ his$^-$ plates containing 2.5 mM 3-AT. After transformation, 23 colonies were obtained (Y1HS clones 1 to 23). These colonies were picked and re-streaked on a fresh SC leu$^-$ trp$^-$ his$^-$ selection plate, supplemented with 5 mM of 3-AT. Growth of the clones was examined. All clones, with the exception of Y1HS clone number 10 were able to re-grow on these selection plates (Figure 44).

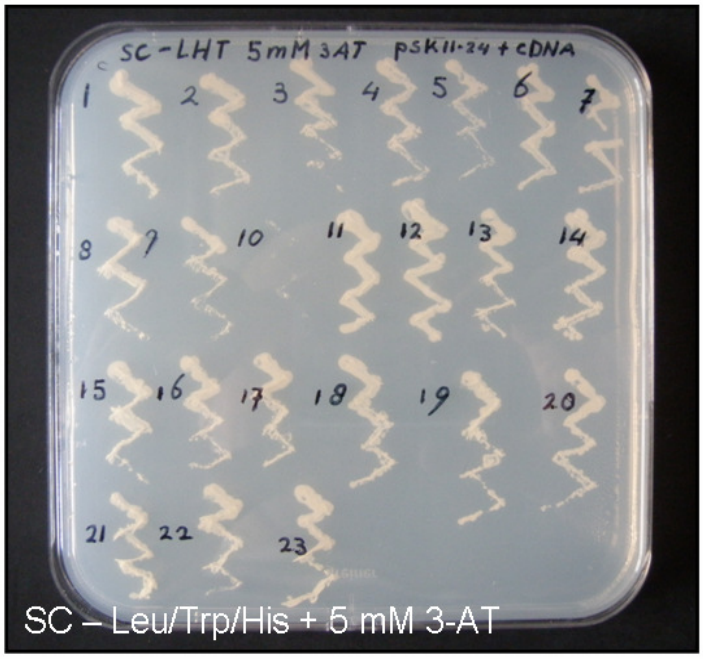


Figure 44. Growth of positive Y1HS clones on SC leu$^-$ trp$^-$ his$^-$ medium plate containing 5 mM 3-AT. All clones, except Y1HS clone nr. 10 showed re-growth on this selection plate.

The pPC86 plasmid containing the cDNA insert was recovered from the 22 positive clones and subsequently sequenced. However, no satisfactory sequencing results were obtained when plasmids were directly sequenced after extraction from yeast. Therefore, the recovered pPC86 plasmids were transformed into *E. coli*, isolated and then sequenced. With this approach the cDNA inserts of Y1HS clones 18 and 19 were successfully sequenced.
BLAST analysis revealed the cDNA sequences from both these clones were identical with the bZIP1 DNA sequence from *C. plantagineum* (data not shown).

3.12.1. Verification of interaction between bZIP1 and *C. plantagineum LEA-like 11-24* promoter

To control whether the results obtained for Y1HS were reliable, the interaction between bZIP1 protein and the *Cp LEA-like 11-24* promoter was verified. The pPC86 plasmids extracted from Y1HS clones 18 and 19 were retransformed into the yeast, harboring the pSK1 (11-24) vector. Yeast carrying pSK1 (11-24) alone or together with pPC86 (without cDNA) were used as negative controls.
For each transformation, colonies were obtained. Several of these yeast clones were picked and re-grown on different selection media plates (Figure 45).

Growth was observed on SC leu$^-$ trp$^-$ his$^-$ medium plates containing 3-AT concentrations of up to 40 mM. This indicates that the interaction between the bZIP1 protein and the LEA-like 11-24 promoter fragment is relatively strong. As expected, yeast cells bearing the plasmid pSK1 (11-24) grew only on SC leu$^-$ medium and the yeast containing both pSK1 (11-24) and pPC86 (without cDNA) was only able to grow on SC leu$^-$ and SC trp$^-$ medium plates. These results verify the ability of the *C. plantagineum* bZIP1 protein to interact with the 60 bp *Cp LEA-like 11-24* promoter fragment.

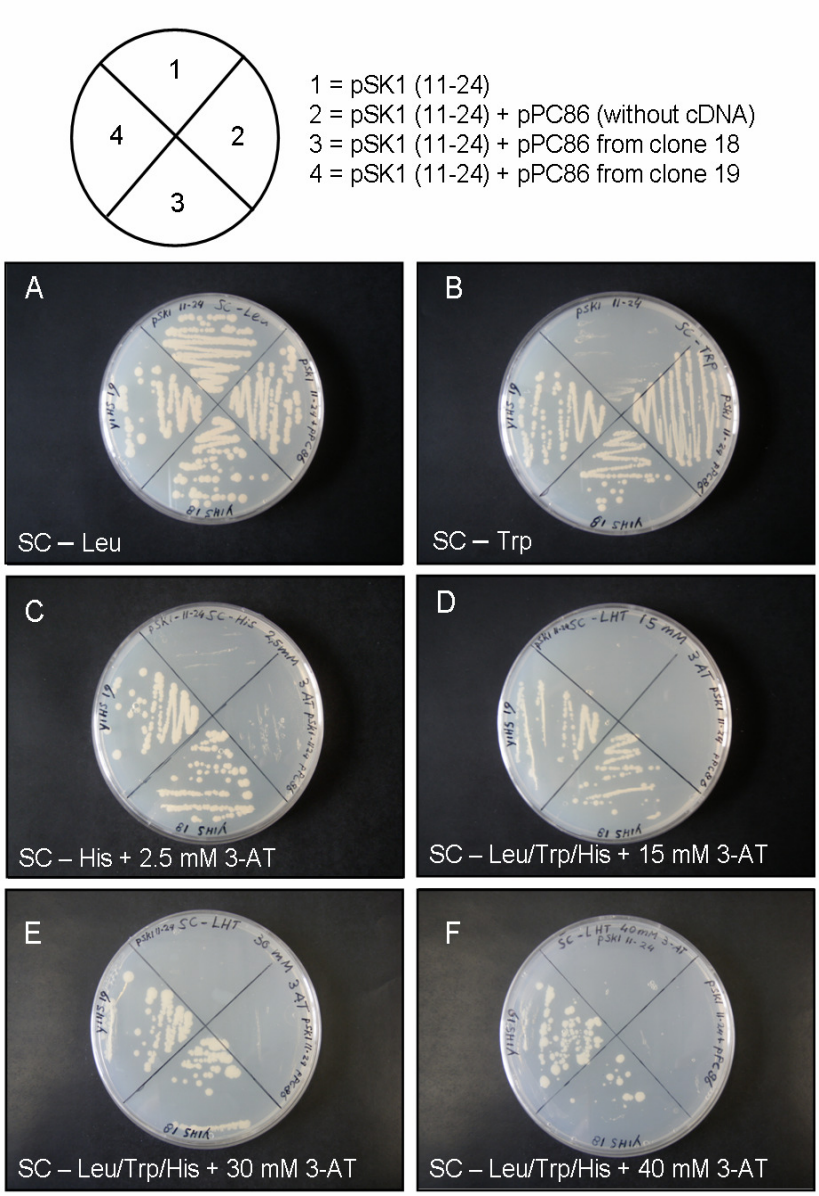


Figure 45. Analysis of growth of yeast clones. Yeast clones: (1) pSK1 (11-24), (2) pSK1 (11-24) + pPC86 (without cDNA), (3) pSK1 + pPC86 isolated from Y1HS clone 18 and (4) pSK1 + pPC86 isolated from Y1HS clone 19. Clones were grown on the following selection plates: (A) SC leu$^-$, (B) SC trp$^-$, (C) SC leu$^-$ trp$^-$ his$^-$, containing 2.5 mM 3-AT, (D) SC leu$^-$ trp$^-$ his$^-$ containing 15 mM 3-AT, (E) SC leu$^-$ trp$^-$ his$^-$ containing 30 mM 3-AT and (F) SC leu$^-$ trp$^-$ his$^-$ containing 40 mM 3-AT.

3.13. Sequence similarity between the *C. plantagineum* bZIP1 protein and other bZIP proteins

An EST sequence encoding a bZIP protein was identified in the EST collection of *L. subracemosa* (Table S2, Supplemental data). BLAST analysis further revealed that *C. plantagineum* bZIP1 protein showed homology to the bZIP2 protein of *C. plantagineum* and the transcription factor-like protein bZIP53 of *A. thaliana*. Sequence similarity between the *C. plantagineum* bZIP1 protein (*Cp*bZIP1), the *C. plantagineum* bZIP2 protein (*Cp*bZIP2), the *L. subracemosa* bZIP protein (*Ls*bZIP) and the *A. thaliana* bZIP53 protein (*At*bZIP53) was investigated (Figure 46).

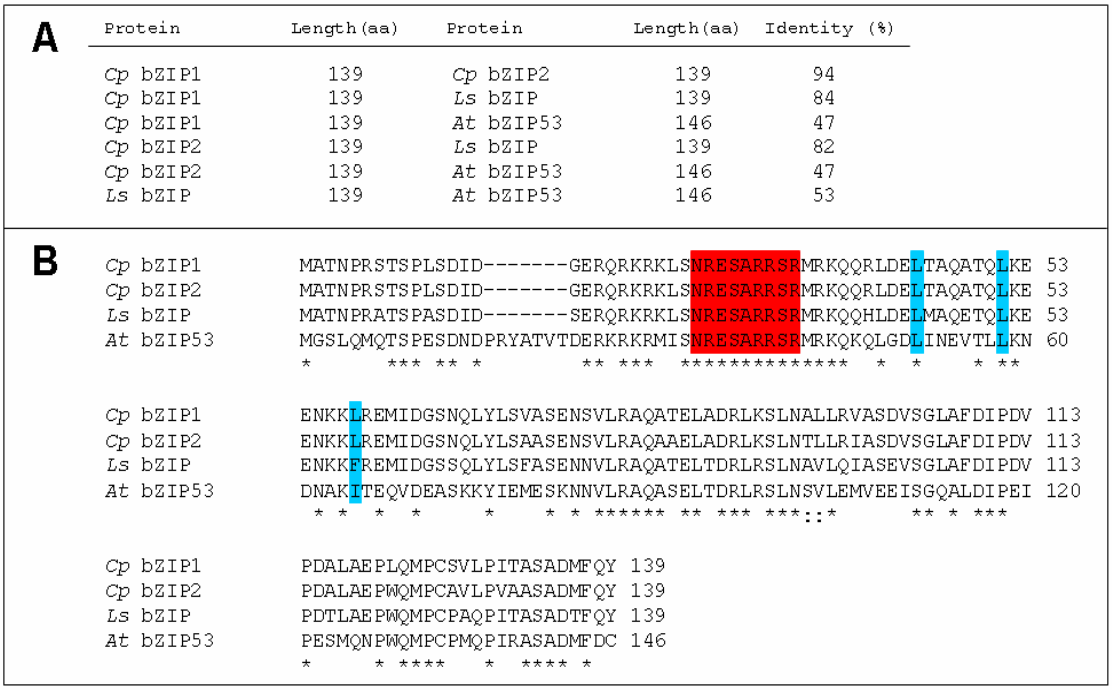


Figure 46. Protein sequence alignments between the *C. plantagineum* bZIP1 protein (*Cp*bZIP1), *C. plantagineum* bZIP2 protein (*Cp*bZIP2), *L. subracemosa* bZIP protein (*Ls*bZIP) and *A. thaliana* bZIP53 protein (*At*bZIP53). (A) Amino acid sequence identity between different bZIP proteins. (B) Alignment of bZIP protein sequences. The conserved amino acids residues in motif important for DNA binding of bZIP domains are indicated in red and the typical repeat of leucines are indicated in blue. In the *L. subracemosa* bZIP protein and the *A. thaliana* bZIP53 protein the leucines of the last leucine repeat were replaced by phenylalanine (F) and isoleucine (I), respectively. Asterisks indicate amino acids which are identical between all four bZIP proteins.

The highest protein similarity was found between the two bZIP proteins of *C. plantagineum*. The *C. plantagineum* bZIP1 and bZIP2 proteins shared 94% sequence identity. High sequence similarity was also observed between the *C. plantagineum* bZIP1 protein and the bZIP of the closely related species *L. subracemosa*, the *C. plantagineum* bZIP1 protein exhibited 84% amino acid identity to the bZIP protein of

L. subracemosa. The amino acid identity between bZIP1 protein of *C. plantagineum* and bZIP53 of *A. thaliana* was 47%.
All four analyzed bZIP protein sequences contained typical structural features of plant bZIP proteins (Jacoby *et al*., 2002). The amino acid residues in the motif important for DNA binding of the bZIP domain were identical between all four bZIP proteins (Figure 46). Furthermore, the hydrophobic leucine zipper region of the bZIP proteins harbored the typical leucine residues that was repeated every seventh amino acid. In the *L. subracemosa* bZIP protein and the *A. thaliana* bZIP53 protein, the leucine residues of the last leucine repeat were substituted by phenylalanine or isoleucine, respectively. However, the substitution of leucine by other hydrophobic amino acid residues often occurs in bZIP proteins (Jacoby *et al*., 2002).

3.13. Analysis of subcellular localization of the *Cp*bZIP1 protein in *C. plantagineum* leaf tissue

In order to determine the subcellular location of the bZIP1 protein in *C. plantagineum*, leaves of *C. plantagineum* were bombarded with a CaMV 35S::*CpbZIP1*::*GFP* fusion construct. As control, leaves were transiently transformed with a vector containing a CaMV 35S::*GFP* construct. All transformed leaves were incubated in water. The GFP signal was analyzed 24 hours after the bombardment (Figure 47).
The GFP signal in the control leaves was observed all over the transformed leaf epidermal cell. Whereas, in leaf cells transformed with the CaMV 35S::*CpbZIP1*::*GFP* construct, the GFP signal was only observed in the nucleus.

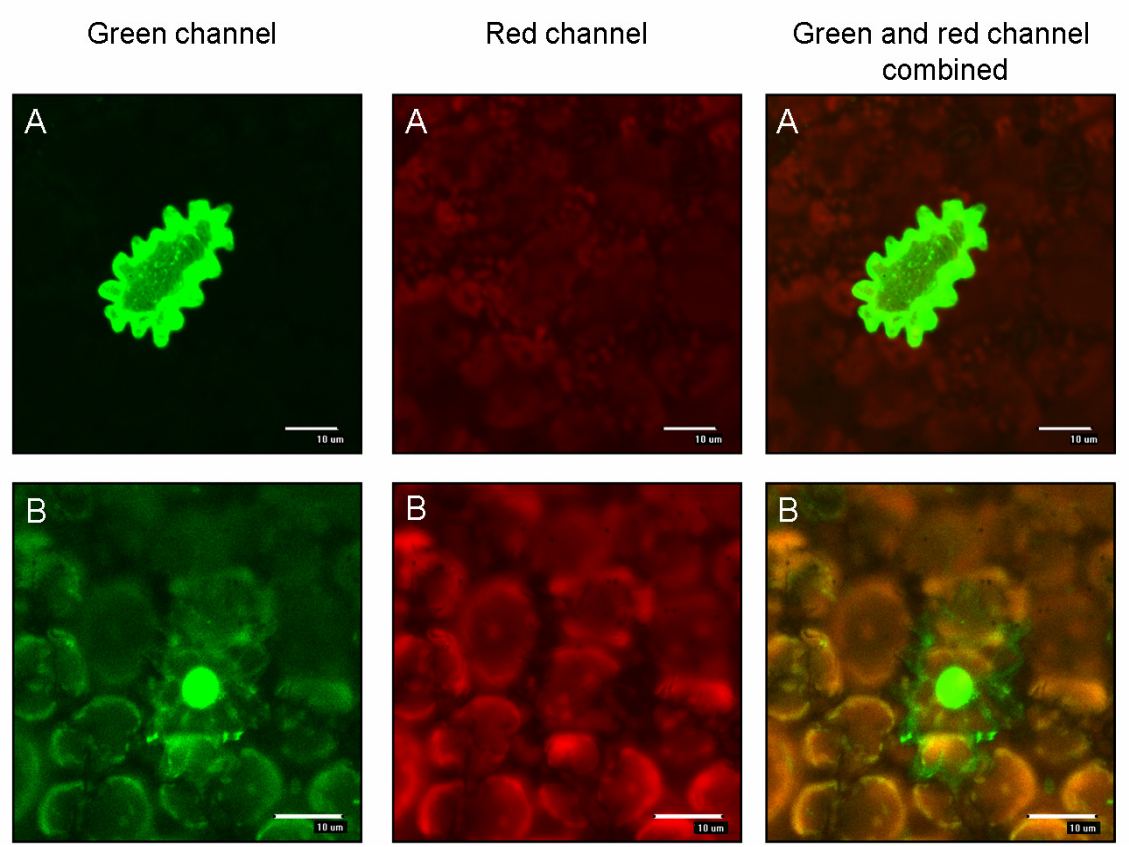


Figure 47. Confocal microscopy images of an epidermal leaf cells transformed with (A) CaMV 35S::*GFP* construct or (B) CaMV 35S::*CpbZIP1*::*GFP* fusion construct. The bright green signal is GFP and the red signal is autofluorescence of chloroplasts.

3.14. Analysis of *CpbZIP1* expression in *C. plantagineum* leaves in response to ABA and mannitol treatments.

To examine whether exogenous ABA and osmotic treatments had an effect on the expression pattern of the *bZIP1* gene in *C. plantagineum* leaves, a RT-PCR approach was employed. First-strand cDNA synthesis was performed with total RNA, which was extracted from *C. plantagineum* leaves treated with either ABA, mannitol or water. Leaves were treated for 48 hours with 100 µM ABA or 0.8 M mannitol and control leaves were incubated in water for 48 hours. Subsequently, RT-PCR amplifications were performed and the abundance of different cDNA products was analyzed.

As positive control the expression of the *Cp LEA-like 11-24* gene was analyzed. RNA gel blot analysis demonstrated that in *C. plantagineum* leaves, the expression of *Cp LEA-like 11-24* was induced by treatment with ABA or mannitol (Figure 26). The expression of the *transketolase 3* (*TKT3*) gene was analyzed as internal control. It was demonstrated by Bernacchia *et al.* (1995) that the transcript *TKT3* is constitutively

expressed in untreated, dried or rehydrated leaf tissue of *C. plantagineum*. PCR products of *bZIP1* and *LEA-like 11-24* transcripts were compared to the transcript level of *TKT3* to control that the same amount of cDNA template was used for each RT-PCR reaction. Reaction conditions for all RT-PCR amplifications were identical. Amplified PCR products were visualized on an EtBr-stained agarose gel (Figure 48).
PCR products of the correct sizes were found and expression was detected for all genes analyzed. Transcript abundance was determined by analyzing the intensity of the bands on the agarose gel by eye. Transcript abundances after ABA and mannitol treatments were compared to the expression levels after treatment with water to investigate whether treatments with ABA or mannitol had an effect on gene expression.

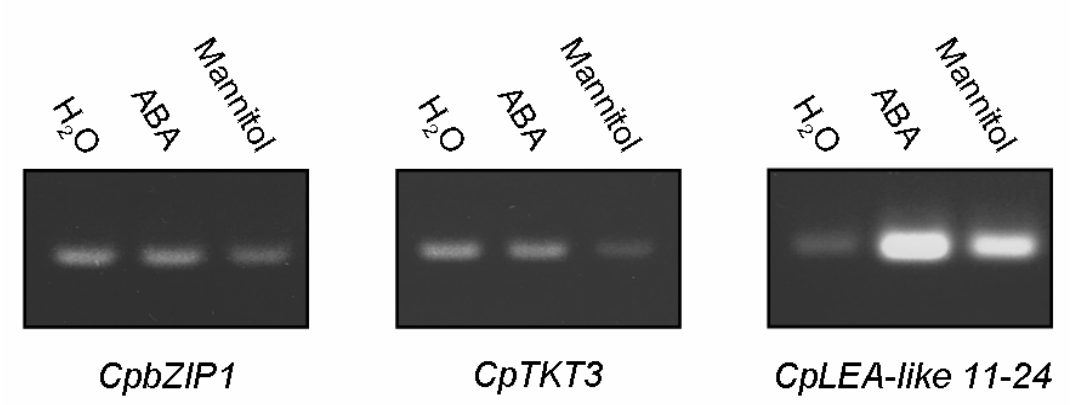


Figure 48. RT-PCR analysis of *bZIP1* expression in *C. plantagineum* leaves treated with water, ABA and mannitol. The cDNA templates were amplified with specific primers and all RT-PCR reactions were performed under the conditions. The *TKT3* gene was used as internal control. The expression pattern of the *LEA-like 11-24* gene is shown as positive control.

The intensity of bands of *bZIP1* PCR products was similar after each treatment, indicating that ABA or mannitol treatments did not enhance *bZIP1* expression in *C. plantagineum* leaf tissue. Furthermore, it appears that the transcript levels of *bZIP1* under different conditions were similar to the transcript levels of the internal control *TKT3* and this suggests that the *bZIP1* gene is constitutively expressed under all the tested conditions. It must be mentioned that it seems that the mannitol treated samples contained less cDNA than the samples that were treated with ABA or water, however, this did not affect the results of the RT-PCR analysis. Analysis of the *LEA-like 11-24* expression showed that the transcripts levels of *LEA-like 11-24* in *C. plantagineum* leaves were induced by treatment with ABA or mannitol as compared to water treated control leaves. These findings demonstrate that differences in gene expression in response to ABA or mannitol could be detected with this RT-PCR assay. This RT-PCR analysis reveals that *bZIP1* expression is constitutive and was not induced in *C. plantagineum* leaves by exogenous ABA or osmotic treatment.

3.15. Effect of *Cp*bZIP1 on transactivtion of *C. plantagineum LEA-like 11-24* promoter

To examine the ability of *Cp*bZIP1 protein to enhance the activity of the *Cp LEA-like 11-24* promoter, a transactivation assay was performed. A plasmid containing the bZIP1 coding sequence under the control of the *CaMV 35S* promoter was used in the transactivation assay. This CaMV 35S::*Cp*bZIP1 construct was co-bombarded with *Cp LEA-like 11-24* promoter::*GUS* and CaMV 35S::*GFP* vectors into *C. plantagineum* leaves. Transactivation experiments were carried out with the following DNA concentration ratios of CaMV 35S::*Cp*bZIP1 construct to *Cp LEA-like 11-24* promoter::*GUS* construct: 1:1 and 4:1. The activity of *Cp LEA-like 11-24* promoter was determined as described in paragraph 3.6.1.

The levels of *Cp LEA-like 11-24* promoter activity co-expressed with *CpbZIP1* were similar in comparison with that of the *Cp LEA-like 11-24* promoter alone (data not shown). These results indicate that the transient expression of *CpbZIP1* is not sufficient to enhance *Cp LEA-like 11-24* promoter activity in *C. plantagineum* leaves.

4. Discussion

4.1. The dehydration-responsive transcriptomes of *L. brevidens* and *L. subracemosa* display distinct differences

The main objective of this study was to analyze the transcriptional regulation of genes involved in desiccation tolerance in closely related plant species with varying desiccation tolerance. It is assumed that differences in transcriptional regulation between desiccation tolerant and desiccation sensitive plants might account for the difference in desiccation tolerance. A comparative approach was employed to detect differences in gene expression between the closely related species *L. brevidens* and *L. subracemosa*, which differ in desiccation tolerance.

4.1.1. Transcripts with desiccation-related functions are more abundant in *L. brevidens* than in *L. subracemosa*

Comparison of the *L. brevidens* and *L. subracemosa* EST collections revealed notable differences (Figure 13). The most striking difference between the two dehydration-responsive transcriptomes of *L. brevidens* and *L. subracemosa* was the variance in the number of transcripts that were classified into the functional group "Desiccation-related". The *L. brevidens* transcriptome displayed a higher percentage of transcripts related with desiccation tolerance (30%) than the transcriptome of *L. subracemosa*, where 17% of the annotated transcripts accounted for desiccation-related transcripts. The diversity in the abundance of desiccation-related transcripts between transcriptomes of *L. brevidens* and *L. subracemosa* implies that the expression of desiccation-related genes is differentially regulated between the desiccation tolerant plant *L. brevidens* and the desiccation sensitive plant *L. subracemosa*.

4.1.2. Difference in the number of transcripts encoding LEA proteins between *L. brevidens* and *L. subracemosa*

Within the functional group "Desiccation-related" also transcripts encoding LEA or LEA-like proteins were classified. LEA or LEA-like transcripts comprised 24% of all annotated ESTs in the *L. brevidens* EST collection and covered 16% of annotated ESTs from *L. subracemosa*. Hence, the *L. brevidens* transcriptome contains a higher

proportion of LEA or LEA-like transcripts. This finding strongly suggests an important role for LEA proteins in the acquisition of desiccation tolerance in *L. brevidens*.

4.1.3. The *DSP22* gene is abundantly expressed in *L. brevidens*

Another prominent difference between the dehydration-responsive transcriptomes of *L. brevidens* and *L. subracemosa* was the number of transcripts coding for the DSP22 protein. In the EST collection of *L. brevidens*, 16% of all annotated transcripts showed similarity to the DSP22 protein of *C. plantagineum*. In EST collection of *L. subracemosa*, only 1% of the annotated transcripts encoded the DSP22 protein. The DSP22 protein possibly plays an important role in desiccation tolerance in *L. brevidens*. The ELIP-like protein DSP22 is presumed to protect photosynthetic structures during desiccation (Alamillo and Bartels, 2001). The desiccation tolerant *L. brevidens* is homoiochlorophyllous and retains its chlorophyll during desiccation (Phillips *et al*., 2008). Hence, in *L. brevidens*, the DSP22 protein is most likely involved in the protection of the photosynthetic apparatus during desiccation. The chlorophyll also absorbs light during dehydration and this probably causes the formation of reactive oxygen species. The DSP22 protein probably has a role in the prevention of oxidative stress damage caused by reactive oxygen species.

4.1.4. Differences in the numbers of transcripts associated with signal transduction and disease and defense reactions between *L. brevidens* and *L. subracemosa*

Other distinctions between the dehydration-responsive transcriptomes of *L. brevidens* and *L. subracemosa* were observed. The number of transcripts involved in “Disease and defense” and “Signal transduction” were different between the EST collections of *L. brevidens* and *L. subracemosa*. The functional groups “Signal transduction” and “Disease and defense” made up, respectively, 12% and 13% of the total annotated ESTs of *L. subracemosa*, while in *L. brevidens* these groups comprised, respectively, 6% and 7%. The divergence in desiccation tolerance between *L. brevidens* and *L. subracemosa* might explain the differences in the abundance of transcripts involved in both “Signal transduction” and “Disease and defense” events. Leaves of *L. brevidens* and *L. subracemosa* were partially dehydrated for the construction of the dehydration-responsive EST collections. Probably, the applied dehydration treatment was experienced as stressful by *L. subracemosa*, since this plant does not possess the ability to survive desiccation of vegetative tissues. Therefore, *L. subracemosa* probably tried to overcome the water stress effects trough the activation of different types of

protection processes, including those involved in disease- and defense-related processes. Signal transduction pathways were probably stimulated to activate these protection mechanisms. Thus, it seems that desiccation tolerant plant *L. brevidens* induces a specialized gene expression program that offers protection against desiccation and that the desiccation sensitive plant *L. subracemosa* induces a more generalized stress response to overcome dehydration stress.

4.1.5. The dehydration-responsive transcriptome of *L. brevidens* contains a high number of transcripts with unknown function

A relatively large proportion of all analyzed *L. brevidens* EST sequences (35%) could not be annotated. Fifty-six percent of these non-annotated ESTs showed similarity to hypothetical or uncharacterized proteins. The remaining 44% of the non-annotated *L. brevidens* transcripts did not display similarity to sequences present in the searched databases. These high numbers of transcripts of unknown function may imply that novel gene products, which are involved in mechanisms of desiccation tolerance, could be discovered from the *L. brevidens* EST collection.
The sizes of the *L. brevidens* and *L. subracemosa* EST collections were relatively small. The addition of more EST sequences would definitely help to obtain a better coverage of the *L. brevidens* and *L. subracemosa* transcriptomes. The comparison between the dehydration-responsive transcriptomes of *L. brevidens* and *L. subracemosa* may be improved by increasing the number of transcripts in the EST collections. On the other hand, the small size of the EST collections helped to identify the most important transcripts involved in dehydration responses. The major classes of desiccation-related transcripts (e.g. transcripts encoding LEA proteins) were detected in the EST collections of *L. brevidens* and *L. subracemosa*. This indicates that these transcripts have an important function in dehydration responses.

4.2. The expression of genes involved in desiccation tolerance is differentially regulated between desiccation tolerant and desiccation sensitive plant species

Comparison of the dehydration-responsive transcriptomes of *L. brevidens* and *L. subrecemosa* indicated that the expression of genes associated with desiccation tolerance is differentially regulated between these two plant species. Similar results were found when dehydration-responsive transcriptomes of the resurrection plant *S. lepidophylla* and the desiccation sensitive plant, *Selaginella moellendorffii* were compared (Iturriaga *et al*., 2006). Iturriaga *et al*. (2006) showed that the dehydration-

responsive EST collection of the resurrection plant *S. lepidophylla* contained a higher number of EST sequences involved in stress responses (e.g. LEA proteins) than that of the closely related, but desiccation sensitive plant, *S. moellendorffii*. Thus, it seems that dehydration-responsive genes are regulated differentially between desiccation sensitive and desiccation tolerant plants species. This hypothesis is supported by the fact that the ability to survive desiccation is often found in embryos of desiccation sensitive plants. Orthodox seeds of many types of desiccation sensitive plants are able to survive desiccation (Hoekstra *et al*., 2001). This means that desiccation sensitive plants that produce orthodox seeds must contain all genes necessary for the acquisition of desiccation tolerance. Thus, transcriptional regulation of genes involved in mechanisms of desiccation tolerance appears to play a crucial role in the ability to withstand desiccation.

4.3. Transcripts encoding LEA proteins are abundant in the dehydration-responsive transcriptomes of the resurrection plants *L. brevidens* and *S. lepidophylla*

The dehydration-responsive transcriptome of *L. brevidens* was compared to that of the resurrection plant *S. lepidophylla* to analyze similarities and differences in dehydration-responsive gene expression between different species of resurrection plants. Comparison of the transcriptome of *L. brevidens* with the outcome of the transcriptome study of *S. lepidophylla* performed by Iturriaga *et al*. (2006), showed that in both these resurrection plants EST sequences encoding LEA proteins were highly abundant. This finding underlines the important function that LEA proteins play in the acquisition of desiccation tolerance.

The crucial role of LEA proteins in desiccation tolerance is further supported by a transcriptome study that was performed in the resurrection plant *Xerophyta humilis* (Collett *et al*., 2004). Collet *et al*. (2004) monitored changes in the transcriptome of *X. humilis* during a dehydration-rehydration cycle and found that the expression of 55 genes was up-regulated in response to dehydration. Classification of these dehydration-up-regulated genes into functional groups revealed that genes coding for LEA proteins accounted for the majority of dehydration-up-regulated genes. LEA proteins made up 31% of the dehydration-up-regulated genes in *X. humilis*.

However, LEA proteins seem not only to be involved in protection mechanisms during desiccation, but probably also play a role in the recovery of desiccated tissue upon rehydration. In a study by Oliver *et al*. (2004), the rehydration transcriptome of the desiccation tolerant plant *Totula ruralis* was analyzed and it was found that transcripts

encoding LEA proteins were most abundant within the rehydration cDNA library of *T. ruralis*. Consequently, Oliver *et al*. (2004) speculated that LEA proteins are also involved in the recovery of desiccated tissue of *T. ruralis* after rehydration. However, it was found that in the same plant the expression patterns of *LEA* genes could vary between different tissues and under different conditions. In *Arabidopsis*, it has been shown that different *LEA* genes had different expression patterns (Hundertmark and Hincha, 2008). This indicates that at least in *Arabidopsis*, the expression of different *LEA* genes is differentially regulated.

The comparison between the transcriptomes of *L. brevidens* and *S. lepidophylla* revealed also some differences. A noticeable difference between these EST collections was the difference in the number of transcripts involved in photosynthesis. The collection of *S. lepidophylla* contained various transcripts encoding proteins involved in photosynthesis (17%). In *L. brevidens*, only a few ESTs were associated with photosynthesis and these were classified into the functional group "Energy", which made up 7% of total annotated ESTs (Figure 13). In contrast to *L. brevidens*, the resurrection plant *S. lepidophylla* is poikilchlorophyllous and its photosynthetic apparatus is dismantled during desiccation (Iturriaga *et al*., 2006). Difference in the degree of desiccation stress might explain why the number of ESTs with photosynthetic functions is higher in *S. lepidophylla*. Microphyll fronds of *S. lepidophylla* were only dehydrated for two and half hours to a relative water content of 35%, whereas *L. brevidens* plants were dehydrated for a period of three weeks. It is likely that the photosynthetic apparatus still showed some activity in the partially dehydrated leaves of *S. lepidophylla* and therefore many transcripts involved in photosynthesis were detected. It is generally assumed that genes involved in photosynthesis are down-regulated during desiccation. In the resurrection plant *X. humilis,* it was shown that most dehydration-down-regulated genes were associated with photosynthesis and metabolism (Collett *et al*., 2004). Furthermore, in *C. plantagineum*, it was demonstrated that of the expression of a gene encoding a small subunit of Rubisco, an enzyme that has a role in photosynthesis, was down-regulated in response to dehydration (Bernacchia *et al*., 1996).

Interestingly, the *L. brevidens* EST collection contained only a small number of transcripts that encoded transcription factors (Table S1, Supplemental data). In contrast, several bZIP, MYB-related and other transcriptions factors were identified among the EST sequences of *S. lepidophylla* (Iturriaga *et al*., 2006). The difference in the degree of desiccation between leaves of *L. brevidens* and *S. lepidophylla* might cause the variance in transcription factor abundance between *L. brevidens* and *S. lepidophylla*. Most likely the transcription machinery was inactivated by the dehydration in *L.*

brevidens leaves, whereas the transcription machinery was still active in leaves of *S. lepidophylla*. The differences in transcription factor abundance might also be due to differences in genome complexity between *L. brevidens* and *S. lepidophylla*. It is assumed that the genome of *L. brevidens* is more complex than that of the club-moss *S. lepidophylla*. For this reason, it is very likely that *L. brevidens* has a more diverse and larger transcriptome than *S. lepidophylla*. Therefore, transcripts encoding transcription factors might be less abundant in the transcriptome of *L. brevidens*.

4.4. Lack of relationship between the sequence conservation in promoter and protein coding regions of dehydration-responsive genes of *C. plantagineum*, *L. brevidens* and *L. subracemosa*

The protein coding regions of the dehydration-responsive genes *LEA 6-19*, *LEA-like 11-24*, *LEA 3-06* and *DSP22* of *C. plantagineum*, *L. brevidens* and *L. subracemosa* showed an amino acid sequence identity of at least 49% (Table 9). The amino acid sequences of the *L. brevidens* and *L. subracemosa* proteins were predicted from sequences that were derived from the EST collections. As seen in Table 9, there is no consistent link between sequence conservation in promoter regions and protein coding regions of dehydration-responsive genes of *C. plantagineum*, *L. brevidens* and *L. subracemosa*. It is not clear whether a general relationship between promoter and protein conservation truly exists. A study in humans and mouse showed that there was only a very weak correlation between promoter conservation and protein conservation of human and mouse orthologous genes (Chiba *et al*., 2008).

Alignment of promoter regions of the dehydration-responsive genes *LEA 6-19*, *LEA-like 11-24*, *LEA 3-06* and *DSP22* of *C. plantagineum*, *L. brevidens* and *L. subracemosa* revealed that some of these promoters share high sequence similarity with each other (Table 9). The *LEA-6-19* promoter fragments of *C. plantagineum* and *L. brevidens* and *LEA-like 11-24* and *LEA 3-06* promoter fragments of all three plant species displayed a relatively high degree of overall sequence identity. It is assumed that mutations within non-coding DNA sequences can sometimes accumulate rapidly (Ludwig, 2002). Therefore, high sequence similarity between promoter regions of different species could indicate functional conservation of important promoter elements.

Instead of analyzing overall sequence similarities between promoters, it is more effective to search for common structural features among promoter regions to investigate a possible relationship between promoter conservation and function. It is known that functionally important regulatory regions within promoters tend to remain conserved (De Meaux, 2006; Ludwig, 2002). Conserved sequence domains in

promoters of closely related species are likely to be preserved by evolution, because these motifs have an essential function in gene expression regulation.

Table 9. Sequence similarities between proteins and promoters from *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

Proteins:	Percentage amino acid identity		
	Cp - Lb	*Cp - Ls*	*Lb - Ls*
LEA 6-19	68	49	48
LEA-Like 11-24	46	51	50
LEA 3-06	67	49	56
DSP22	71	56	53
Promoters:	**Percentage nucleotide identity**		
	Cp - Lb	*Cp - Ls*	*Lb - Ls*
LEA 6-19	51	10	10
LEA-Like 11-24	51	53	53
LEA 3-06	42	46	41
DSP22	14	7	4

Note: The LEA 27-45 proteins and promoter fragments are not included in this table, because the *Lb LEA 27-45* promoter fragment harbored an insertion.

4.5. Promoter regions of desiccation-responsive genes of *C. plantagineum*, *L. brevidens* and *L. subracemosa* share structural similarities and conserved *cis*-acting regulatory elements

A comparative approach was conducted to detect structural similarities and conserved *cis*-acting regulatory elements between promoter regions of dehydration-responsive genes of *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

Comparative promoter analysis demonstrated that multiple *cis*-acting regulatory elements, which have been characterized to participate in ABA- and dehydration-mediated gene expression, were conserved between promoter regions of *C. plantagineum*, *L. brevidens* and *L. subracemosa* (Figures 14 to 23). Several of the major *cis*-acting regulatory elements involved in dehydration-responsive gene expression, such as ABRE and DRE motifs, showed conservation between promoter regions of these plant species. The presence of conserved ABA- and dehydration-responsive elements in the promoters suggests that the analyzed promoters are indeed involved in desiccation-induced gene expression. Thus, it appears that these promoters are functionally conserved between *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

More similarities in promoter architecture between promoters were detected. For example, the distribution and spacing of several of the identified putative *cis*-acting regulatory element within the promoter regions was sometimes similar. Furthermore, it was found that the *xyloglucan endotransglycosylase* gene was located upstream of both the *C. plantagineum* and *L. brevidens LEA 27-45* promoters. This finding indicates that gene order is conserved between the desiccation tolerant species *C. plantagineum* and *L. brevidens*. In addition, comparison of the promoter regions of the *LEA 27-45* gene promoter analysis showed that the *Lb LEA 27-45* promoter harbored a 411 bp insertion. Southern blot analysis demonstrated that this insertion occurred as a single copy in the *L. brevidens* genome. Therefore, this insertion is probably not a transposon-like element. Most transposable elements are present in high copy numbers in plant genomes (Feschotte *et al*., 2002). However, transposable-like elements that have very low copy numbers have also been found in plants (Konieczny *et al*., 1991; Johns *et al*., 1985). It is believed that transposable elements are involved in regulatory evolution, because the insertion of a transposable element alters the promoter architecture when inserted into a promoter region (Kidwell and Lisch, 1997). Even though it appears that the insertion within the *Lb LEA 27-45* promoter is not a transposable-like element, it is very likely that the insertion has an effect on the regulation of the *LEA 27-45* gene in *L. brevidens*.

The comparative promoter analysis did not reveal any novel putative *cis*-acting regulatory elements in the promoters. Footprinting analysis may be used to identify novel transcription factor binding sites in the promoters (Busk and Pages, 2002). Sequences within the promoter that are protected from treatment with DNase indicate putative binding sites of proteins. Furthermore, the promoter analysis did not uncover any specific promoter features that were exclusively conserved between the promoters of the desiccation tolerant plant species. Therefore, it is presumed that functional variation between the promoters of *C. plantagineum*, *L. brevidens* and *L. subracemosa* is due to differences in promoter architecture and variability of the *trans*-acting environment.

4.6. Comparative functional analysis of a dehydration- and ABA-responsive promoter: development of a transient expression assay

The activity of the *LEA-like 11-24* promoter fragments was characterized by functional promoter analysis. It was chosen to test the relative activity of the different *LEA-like 11-24* promoter fragments in a transient expression assay. In this way the activity of a high number of different promoter constructs could be investigated in a short time. The

production of stably transformed plants was not an option due to time constraints. The transformation methods available for stably transforming *C. plantagineum* and *L. brevidens* are very time consuming and complex to perform (Furini *et al*., 1994; Smith-Espinoza *et al*., 2007).

The transient expression assay that was used in this study proved to be an efficient and relatively rapid method for analyzing promoter activity *in vivo*. The different *LEA-like 11-24* promoter::*GUS* constructs were introduced into leaf tissue via a gold particle bombardment. Other studies had already shown that transient transformation by particle bombardment is a suitable method for measuring promoter activity in different plants species (Endo *et al*., 2007; Basu *et al*., 2003). Furthermore, the transient expression assay allowed the analysis of promoter activity in both homologous and heterologous plant tissue.

The evaluation method that was used for determining the relative activity of the different *LEA-like 11-24* promoter fragments was based on a method developed by Schenk *et al*. (1998). The *LEA-like 11-24* promoter activity was standardized to the activity of the CaMV 35S promoter to measure the effectiveness of each bombardment. Promoter activity analysis demonstrated that this evaluation method was a suitable method for determining the relative promoter activity in transiently transformed leaves of *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

4.6.1. Possible improvements of the transient expression assay

Functional analysis of *LEA-like 11-24* promoter activity demonstrated that the transient expression assay employed in this study was sensitive enough to detect differences in promoter activity between species and different treatments. Furthermore, the promoter activity that was measured for the *C. plantagineum LEA-like 11-24* promoter fragment were in agreement with the results obtained by Velasco *et al*. (1998). In the study by Velasco *et al*. (1998), it was shown that the activity of the *LEA-like 11-24* promoter of *C. plantagineum* could be induced by ABA and dehydration.

However, sometimes large standard deviations were observed, indicating high variability between measurements. High variability in promoter activity measurements might be the result of age- and size-related differences between the transiently transformed leaves. Although plants were grown under equal conditions and plants of the same size and age were used for each particle bombardment, it was not always possible to select only leaves of an identical size and age. The osmotic stress treatment of the bombarded leaves might also affect the accuracy of the promoter activity measurements. Treatment with mannitol caused the cells in the leaf to shrink due to

water loss. The small size of leaf cells made it often hard to distinguish GFP expressing cells from the neighboring cells in mannitol treated leaves. In addition, endogenous fluorescence of certain cellular compounds (e.g. cell walls) made the detection of GFP expressing cells sometimes difficult. Another difficulty was the determination of the exact number of GUS spots present on the surface of the bombarded leaves. Due to diffusion of GUS reaction products, closely localized GUS spots might be recognized as one large GUS spot.

The expression of *GFP* and *GUS* genes might be measured more accurately with a specific quantification assay and this could possibly solve problems with the detection of these reporter genes. Assays that provide accurate quantification of GFP and GUS proteins in plants have been developed (Robic *et al*., 2009; Jefferson *et al*., 1987). However, the GFP and GUS levels in the bombarded leaves are probably too low for accurate quantification. For instance, results in our laboratory have shown that the GUS protein could not be quantified accurately in leaves that were transiently transformed by particle bombardment.

Optionally, the functional promoter analysis might be improved by changing the transient expression system. For example, promoter activity can be measured in a transient expression system that uses protoplasts (Ohkama-Ohtsu *et al*., 2008). However, from experience in our laboratory it is known that it is very difficult to extract protoplasts from *C. plantagineum*. In addition, protoplast isolation methods for *L. brevidens* and *L. subracemosa* have not been established yet.

4.7. Desiccation-responsive *LEA-like 11-24* expression is regulated differently in *C. plantagineum*, *L. brevidens* and *L. subracemosa*

The functional promoter analysis revealed that the strength of the *LEA-like 11-24* promoter fragments of *C. plantagineum*, *L. brevidens* and *L. subracemosa* was variable in the transient expression assay. Considerable differences in promoter activity were detected between the *LEA-like 11-24* promoter fragments of these three closely-related plant species (Figure 31).

The *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments were both functional and inducible by exogenous ABA and mannitol treatments. However, the levels of activity of the *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments in response to the different treatments were different. The strongest promoter activity was observed for the *Cp LEA-like 11-24* promoter fragment. The activity of the *Cp LEA-like 11-24* promoter fragment was about 6-fold higher than that of the *Lb LEA-like 11-24* promoter fragment after ABA treatment and about 4-fold higher after treatment

with mannitol (Figure 31). Furthermore, it was shown that the 276 bp *Ls LEA-like 11-24* promoter fragment was not functional and did not respond to treatment with either ABA or mannitol (Figure 31).

Results obtained for the *Cp LEA-like 11-24* promoter fragment demonstrated that the length of this promoter could be shortened to at least 307 bp to retain full activity. Velasco *et al*. (1998) reported that a 667 bp *LEA-like 11-24* promoter fragment of *C. plantagineum* contained the required *cis*-acting regulatory elements for ABA- and dehydration-induced promoter activity. Hence, in this current work, it was shown that the first 307 bp of the *C. plantagineum LEA-like 11-24* promoter were already sufficient for ABA- and dehydration-responsiveness. Therefore, this 307 bp *Cp LEA-like 11-24* promoter fragment can perhaps be used as an ABA- and dehydration-responsive minimal promoter in future.

The relative activities of the *LEA-like 11-24* promoter fragments, which were measured in the transient expression assay, were in agreement with the *LEA-like 11-24* expression patterns that were observed in dehydrated leaves of *C. plantagineum*, *L. brevidens* and *L. subracemosa* (Figure 25). RNA blot analysis showed that *LEA-like 11-24* transcript levels increased more rapidly in response to dehydration in *C. plantagineum* leaves than in leaves of *L. brevidens* and *L. subracemosa*. The variability in *LEA-like 11-24* promoter strength could explain the differences in the *LEA-like 11-24* expression profiles between *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Taken all these results together, it can be concluded that there are differences in the regulation of *LEA-like 11-24* gene expression between *C. plantagineum*, *L. brevidens* and *L. subracemosa* during dehydration.

One remark about the RNA analysis of *L. brevidens* must be made. Two bands were observed on the RNA blot of *L. brevidens* (Figure 25). Alternative splicing of the *L. brevidens LEA-like 11-24* transcript might explain the occurrence of two bands on the RNA blot of *L. brevidens*. Another explanation for this observation might be that different isoforms of *LEA-like 11-24* are expressed in *L. brevidens*.

4.7.1. Differences in *LEA-like 11-24* promoter architecture account for variability in promoter activity between *C. plantagineum*, *L. brevidens* and *L. subracemosa*

It was demonstrated by functional promoter analysis that the *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments of, respectively, 307 bp and 321 bp contained sufficient *cis*-acting regulatory elements to confer ABA and dehydration inducibility. Nevertheless, clear differences in promoter activity between the *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments were observed. It is assumed that these

differences in promoter activity are due to structural differences between the promoter fragments.

The 276 bp *Ls LEA-like 11-24* promoter fragment did not display induced activity under ABA and osmotic stress. However, the elongation of the *Ls LEA-like 11-24* promoter fragment from 276 bp to 488 bp was sufficient to generate a functional and ABA- and mannitol-inducible promoter fragment (Figure 40). The 488 bp *Ls LEA-like 11-24* promoter fragment, which harbored two additional ACGT-containing ABREs, was capable of conferring an ABA and osmotic stress response. This implies that at least one of these two additional ABREs present in the 488 bp *Ls LEA-like 11-24* promoter fragment is involved in ABA- and mannitol-induced activity. These results indicate that differences in promoter architecture account for variation in promoter activity between the *Cp LEA-like 11-24*, *Lb LEA-like 11-24* and *Ls LEA-like 11-24* promoter fragments.

4.8. The DRE motif accounts for differences in activity between *LEA-like 11-24* promoter fragments of *C. plantagineum* and *L. brevidens*

The major structural difference between the *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments is the presence of a DRE motif in the *LEA-like 11-24* promoter fragment of *C. plantagineum*.

Mutagenesis of the DRE motif reduced ABA- and dehydration-responsiveness of the *Cp LEA-like 11-24* promoter fragment (Figure 34). This finding provides evidence that the DRE motif plays a role in both dehydration- and ABA-induced *Cp LEA-like 11-24* promoter activity. Furthermore, mutagenesis of only two of the three ACGT boxes present in the *Cp LEA-like 11-24* promoter fragment did not abolish promoter activity. These results showed that one single intact ACGT-containing ABRE and a DRE motif were sufficient to induce the activity of the *Cp LEA-like 11-24* promoter fragment with ABA. It is known that an ABRE motif cannot function independently. A single ABRE motif requires the presence of other *cis*-acting elements or coupling elements for ABA-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005; Shen and Ho, 1995). For example, the promoter of the *Arabidopsis RD29B* gene requires at least two ABREs motifs for the ABA-responsive expression (Uno *et al*., 2000). It is known that interaction between ABRE and DRE motifs occurs and that the DRE motif has a role in ABA-induced gene expression (Narusaka, *et al*., 2003). The *Arabidopsis RD29A* gene was shown to be inducible by ABA and the promoter of this gene contained both a single ABRE and DRE motif (Knight and Knight, 2001). Thus, the combination of a single ABRE motif and a single DRE motif is sufficient to confer an ABA-response.

The conclusion that the DRE motif was involved in both ABA- and dehydration-induced *Cp LEA-like 11-24* promoter activity was further supported by the fact that the *Cp LEA-like 11-24* promoter fragment that was harboring only an intact DRE motif was still responsive to both ABA and osmotic stress (Figure 34). The mutagenesis of all three ACGT boxes did also not completely abolish the activity of the *Cp LEA-like 11-24* promoter fragment. All these results provide evidence that the DRE motif plays a role in both ABA- and dehydration-induced *Cp LEA-like 11-24* promoter activity. It is suggested that the DRE motif is the key *cis*-acting regulatory element that controls ABA- and dehydration-responsive expression of the *LEA-like 11-24* gene in *C. plantagineum.*

It was already mentioned that the mutagenesis of the DRE box led to reduction in *Cp LEA-like 11-24* promoter activity in response to both ABA and mannitol treatments. The activity of the *Cp LEA-like 11-24* mutation DRE promoter fragment is compared with the activities of the non-mutated *LEA-like 11-24* promoter fragments of *C. plantagineum* and *L. brevidens* (Figure 49). This comparison reveals that the levels of activity of the *Cp LEA like 11-24* mutation DRE promoter fragment and the non-mutated *Lb LEA-like 11-24* promoter fragment were similar. Therefore, it is assumed that the DRE motif is responsible for differences in activity between the *Cp LEA-like 11-24* promoter fragment and the *Lb LEA-like 11-24* promoter fragment.

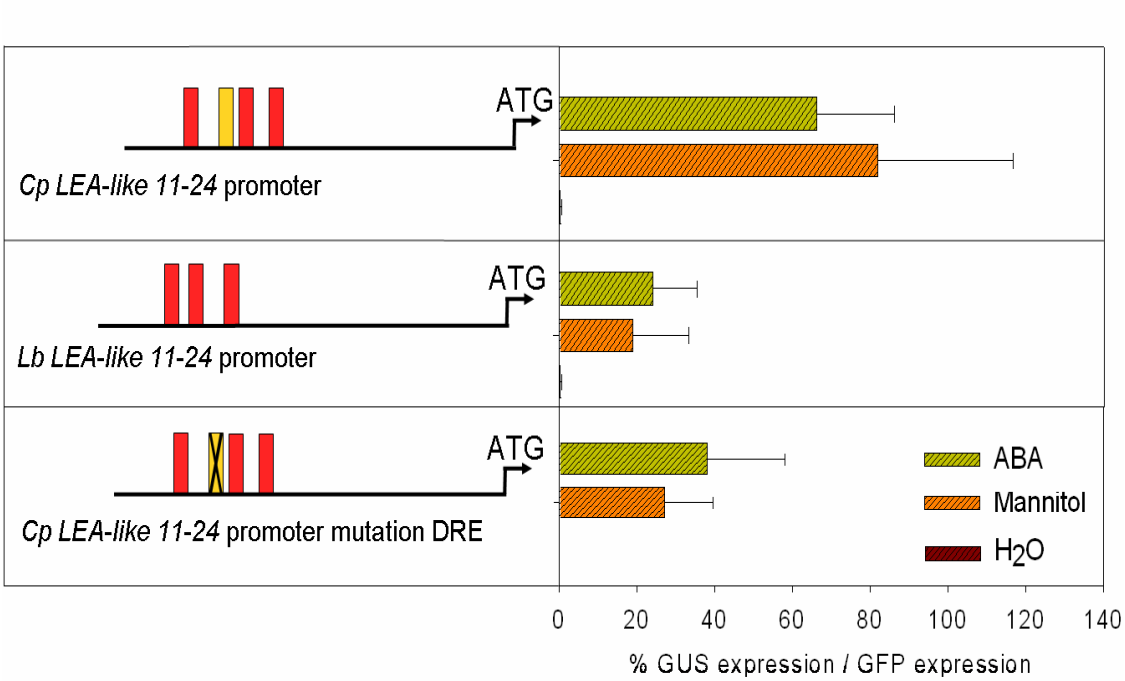


Figure 49. Comparison of relative promoter activity of the *Cp LEA-like 11-24* promoter, *Lb LEA-like 11-24* promoter and *Cp LEA-like 11-24* mutation DRE promoter fragments after treatment with ABA, mannitol or water. Activity is shown as percentage of *LEA-like 11-24* promoter activity compared to the CaMV35S promoter (% GUS/GFP). Data from *Cp LEA-like 11-24* and *Lb LEA-like 11-24* promoter fragments represent the means with SD of seven independent experiments for each treatment (n = 7). Data from the *Cp LEA-like 11-24* mutation DRE promoters fragment are mean values with SD of four independent experiments for each treatment (n = 4).

4.9. The ACGT1, ACGT2, ACGT3 and DRE motifs are governing ABA- and mannitol-responsiveness of the *Cp LEA-like 11-24* promoter fragment

Mutagenesis of the ACGT1, ACGT2, ACGT3 and DRE motifs in the *Cp LEA-like 11-24* promoter fragment resulted in a complete loss of activity in response to treatment with ABA or mannitol (Table 7). These results demonstrated that, besides the ACGT1, ACGT2, ACGT3 and DRE motifs, no other *cis*-acting regulatory elements are involved in ABA- and osmotic stress-induced activity of the *Cp LEA-like 11-24* promoter fragment.

Site-directed mutagenesis analysis revealed that the ACGT1, ACGT2 and ACGT3 boxes are all participating in ABA- and mannitol-responsive activity of the *LEA-like 11-24* promoter fragment of *C. plantagineum*. Mutagenesis of all three ACGT boxes present in the *Cp LEA-like 11-24* promoter fragment resulted in a larger reduction in activity than when combinations of mutations were introduced in only two of the three ACGT boxes (Table 7). The role of each of the ACGT boxes in ABA- and dehydration-

induced promoter activity was investigated and the function of ACGT2 and ACGT3 boxes appeared to be similar. The mutagenesis of either ACGT2 or ACGT3 had very similar effects on the activity of the *Cp LEA-like 11-24* promoter fragment in response to ABA and mannitol (Table 7). Compared to the effects of mutagenesis either the ACGT2 or ACGT3 box alone, the reduction in ABA-responsive activity of the *Cp LEA-like 11-24* promoter was additive when mutations were introduced in both the ACGT2 and ACGT3 box (Table 7).The exact role of the ACGT1 box in the regulation of *LEA-like 11-24* gene expression in *C. plantagineum* could not be clarified. Mutagenesis of the ACGT1 box resulted in elevation of *Cp LEA-like 11-24* promoter activity in response to both ABA and osmotic stress conditions (Table 7). Even some promoter activity was observed under well watered control conditions. These results imply that the *Cp LEA-like 11-24* promoter was depressed by the presence of the mutation in the ACGT1 box. Therefore, it is suggested that the ACGT1 motif might also play a role in the repression of the *Cp LEA-like 11-24* promoter fragment activity. Transcriptional repressors that bind to *cis*-acting regulatory elements that contain the ACGT-core sequence have been reported. It has been shown that *Catharanthus roseus* G-box binding factors (Crgbf), Crgbf1 and Crgbf2, respectively, preferentially bound to the G-box *cis*-acting regulatory element (CACGTG) in the promoter of the *strictosidine synthase* gene. Subsequently, it was demonstrated by transient expression experiments that Crgbf1 and Crgbf2 transcription factors were acting as transcriptional repressors and down-regulated the expression of the *strictosidine synthase* gene (Siberil *et al*., 2001).

So, it appears that the ACGT1 box functions in both induction and repression of the *Cp LEA-like 11-24* promoter activity. One explanation for the double role of the ACGT1 motif could be that the transcription factor that interacts with the ACGT1 motif acts as both activator and repressor of *LEA-like 11-24* transcription. Transcription factors that function as both transcriptional activator and repressor have been described. For example, the VP1 protein of maize was shown to have activator or repressor functions. The VP1 transcriptional activator is required for ABA-induced expression of maturation-specific genes in seeds. However, it has also been shown that VP1 can act as repressor of *α-amylase* gene expression (Hoecker *et al*., 1995). Another explanation for the activation and repression function of ACGT1 could be that the transcription factor that binds to the ACGT1 box is dependent on post-translational modification or on the interaction with other proteins to stimulate *Cp LEA-like 11-24* promoter activity. It is known that transcription factors can interact with each other in order to enhance gene expression (Singh, 1998).

4.10. The ACGT3 box is the key regulator of ABA- and dehydration-induced *Lb LEA-like 11-24* promoter activity

The mutation that was introduced into the ACGT3 box of the *Lb LEA-like 11-24* promoter fragment dramatically reduced the promoter activity in response to both ABA and mannitol (Table 8). Even though, the *Lb LEA-like 11-24* mutation ACGT3 promoter fragment still contained two intact ACGT-containing ABREs, this mutated promoter fragment was almost not responding to treatment with either ABA or mannitol. Mutagenesis of the ACGT1 box also reduced ABA- and mannitol-induced activity of the *Lb LEA-like 11-24* promoter fragment (Table 8). However, the influence of the ACGT1 mutation on *Lb LEA-like 11-24* promoter activity was not as strong as the effect of the mutation in the ACGT3 box. Therefore, it can be concluded that the ACGT3 box is the key regulator of ABA- and dehydration-induced *Lb LEA-like 11-24* promoter activity.
The function of ACGT2 box was not examined, however, based on the results obtained for the different mutated *Lb LEA-like 11-24* promoter fragments, it is likely that this motif is also involved in ABA-induced promoter activity. For instance, the *Lb LEA-like 11-24* mutation ACGT1 promoter fragment was still able to respond to ABA and as mentioned earlier, an ABRE motif cannot function independently. A single ABRE motif requires other *cis*-acting elements, for example other ABREs, to function (Yamaguchi-Shinozaki and Shinozaki, 2005).

4.11. ABRE motifs in the *LEA-like 11-24* promoters of *C. plantagineum* and *L. brevidens* show functional conservation

Functionally important *cis*-acting regulatory elements are often evolutionarily conserved among related species due to functional constraints (Ludwig, 2002). Functional evolution of *cis*-acting regulatory elements in the *LEA-like 11-24* promoters of *C. plantagineum*, *L. brevidens* and *L. subracemosa* was investigated.
Comparative promoter analysis revealed that the *Cp LEA-like 11-24* ACGT box 2, the *Lb LEA-like 11-24* ACGT box 1 and the *Ls LEA-like 11-24* ACGT box 1 were conserved between the LEA-like 11-24 promoters of *C. plantagineum*, *L. brevidens* and *L. subracemosa* (Figure 28). Furthermore, *Cp LEA-like 11-24* ACGT box 3, *Lb LEA-like 11-24* ACGT box 3 and *Ls LEA-like 11-24* ACGT box 2 were also found to be conserved among the three *LEA-like 11-24* promoters. Analysis showed that nucleotides directly flanking the ACGT-core of the conserved ABREs were very similar between the different promoters (Figure 29). It is known that the nucleotides

flanking the ACGT-core of the ABRE motif are important for the functionality of this *cis*-acting element (Hattori *et al*., 2002). Functional promoter analysis clarified that the conserved ACGT-containing ABRE motifs in the *LEA-like 11-24* promoter fragments of *C. plantagineum* and *L. brevidens* all mediated ABA- and mannitol-induced promoter activity. Thus, these ABRE motifs are both structurally and functionally conserved between the *LEA-like 11-24* promoters of *C. plantagineum* and *L. brevidens*.

4.12. The *C. plantagineum* bZIP1 protein interacts with the *Cp LEA-like 11-24* promoter

The *C. plantagineum* bZIP1 protein was identified to interact with a 60 bp fragment of the *Cp LEA-like 11-24* promoter in the Y1HS. The bZIP1 protein from *C. plantagineum* was described for the first time by Ditzer and Bartels (2006). Ditzer and Bartels (2006) demonstrated that the *Cp*bZIP1 protein bound to the promoter of the *C. plantagineum* LEA group 4 gene, *C2*.

The interaction between the *Cp*bZIP1 protein and the *Cp LEA-like 11-24* promoter fragment was verified by a retransformation assay. Evaluation of this retransformation assay revealed that positive yeast clones were able to grow on selective SC medium with a 3-AT concentration of up to 40 mM. The ability of the retransformed yeast clones to grow on selective medium with such a high concentration of 3-AT indicates that the interaction between the *Cp*bZIP1 protein and the *LEA-like 11-24* promoter is relatively strong. One should keep in mind that the background growth of the yeast was already inhibited when selective medium plates were supplemented with 2.5 mM of 3-AT. For comparison, in the study by Ditzer and Bartels (2006), growth of retransformed yeast clones was tested on selective medium plates containing 2.5 mM of 3-AT.

The 60 bp *LEA-like 11-24* promoter fragment that was employed for the Y1HS harbored the ACGT2 and ACGT3 boxes and the DRE motif (Figure 41). The results from the Y1HS did not clarify to which motif the *Cp*bZIP1 protein was binding. However, the *Cp*bZIP1 protein is most likely to interact with either one or with both ACGT boxes present in 60 bp *Cp LEA-like 11-24* promoter fragment. It is known that bZIP transcription factors preferentially bind to *cis*-acting elements that contain the ACGT sequence (Jacoby et al., 2002). An electrophoretic mobility shift assay (EMSA) might be applied to identify the specific binding site of *Cp*bZIP1 protein in the 60 bp *LEA-like 11-24* promoter fragment of *C. plantagineum*. EMSA is a powerful tool to study specific interaction between transcription factors and a promoter (Zhong *et al*., 2007; Ditzer and Bartels, 2006; Schwechheimer *et al*., 1998).

The specific binding site of the *Cp*bZIP1 protein may be determined by using a site-directed mutagenesis approach in combination with the EMSA technique. The introduction of a site-directed mutation in a specific *cis*-acting regulatory motif present in the 60 bp *Cp LEA-like 11-24* promoter fragment may disturb the ability of the *Cp*bZIP1 protein to specifically interact with this *cis*-acting regulatory element.

4.13. Putative functions of the *C. plantagineum* bZIP1 protein

The ability of the *Cp*bZIP1 protein to interact with different promoters, namely the *LEA-like 11-24* and *LEA C2* promoters, indicates that the *Cp*bZIP1 protein has a prominent role in the transcriptional regulation of genes encoding LEA and LEA-like proteins in *C. plantagineum*.

The *Cp*bZIP1 protein is probably involved in the transcriptional regulation of many genes in *C. plantagineum*. Therefore, it is interesting to investigate whether the *Cp*bZIP1 protein interacts with the promoters of other desiccation-responsive genes. Promoters that are bound by the *Cp*bZIP1 protein can be identified by chromatin immunoprecipitation (ChIP). ChIP is a powerful method to study interactions between transcription factors and DNA *in vivo* (Dryer and Covey, 2006; Lee *et al*., 2006). The protein of interest binds the DNA at specific binding sites. Subsequently, the protein is cross-linked to the DNA and the DNA is fragmented into small pieces. The cross-linked protein-DNA complexes are immunoprecipitated using specific antibodies against the protein of interest and then recovered DNA fragment is analyzed.

In *A. thaliana*, bZIP transcription factors have been associated with the regulation of various biological processes, like pathogen defense, light and stress signaling, flower development and seed maturation (Jacoby *et al*., 2002). BLAST analysis revealed that the *Cp*bZIP1 protein showed high sequence similarity to the bZIP53 protein sequence of *A. thaliana* (Figure 46). Based on structural and functional features the bZIP proteins of *Arabidopsis* were classified into ten different groups (Jacoby *et al*., 2002). The *At*bZIP53 protein is a member of Group S. The *At*bZIP53 transcription factor has been identified as transcriptional regulator of *Arabidopsis* seed maturation genes (Alonso *et al*., 2009). Thus, it appears that the *At*bZIP53 protein is involved in the acquisition of desiccation tolerance in *A. thaliana* seeds. An *in vitro* method determined that the AtbZIP53 protein is binding to the G-box motif (Alonso *et al*., 2009). This finding further supports the hypothesis that the *Cp*bZIP1 protein plays an important role in the regulation of desiccation-responsive gene expression in *C. plantagineum*. However, the exact functions of the *Cp*bZIP1 protein still remain to be resolved.

In this current study, different methods were employed to asses the role of the *Cp*bZIP1 protein in the regulation of *LEA-like 11-24* gene expression in *C. plantagineum*. The subcellular localization of the *Cp*bZIP1 protein was investigated and the *CpbZIP1* expression profile was studied. Furthermore, the ability of *Cp*bZIP1 protein to activate the *LEA-like 11-24* promoter activity in *C. plantagineum* was analyzed.

4.13.1. The *Cp*bZIP protein is nuclear localized in epidermal leaf cells of *C. plantagineum*

The subcellular localization of the *Cp*bZIP1 protein was studied by utilizing a GFP fusion protein. Tagging transcription factors with GFP has been shown to be a suitable approach for determining the subcellular localization of transcription factors *in vivo* (Peng *et al*., 2009; Hu *et al*., 2008; Baumann and Reyes, 1999). It was shown that the *Cp*bZIP1 protein was localized in the nucleus of *C. plantagineum* epidermal leaf cells (Figure 47). Previously, it was reported that the *Cp*bZIP1 protein was localized in the nucleus of guard cells of *C. plantagineum* leaves (Ditzer and Bartels, 2006). The nuclear localization of the *Cp*bZIP1 protein in both guard cells and epidermal leaf cells indicates that the *Cp*bZIP1 protein is functioning in the nucleus.

4.13.2. The *CpbZIP1* gene is constitutively expressed in *C. plantagineum* leaves

The expression pattern of the *CpbZIP1* gene in response to ABA and osmotic stress was studied. RT-PCR analysis demonstrated that the expression of *CpbZIP1* gene was not upregulated in response to exogenous ABA or osmotic stress treatment in *C. plantagineum* leaves. The RT-PCR analysis showed that the expression of the *LEA-like 11-24* gene was induced by treatment with ABA or mannitol, demonstrating that the applied RT-PCR assay was able to detect differences in gene expression in response to the employed treatments. Nevertheless, it may still be possible that the changes in expression levels of the *CpbZIP1* gene in response to the treatments were too low to detect with the employed RT-PCR method. Quantitative real-time PCR analysis provides a sensitive method for the detection, quantification and comparison of RNA levels (Bustin *et al*., 2005). Some quantitative real-time PCR reactions were conducted to analyze the *bZIP1* expression pattern in *C. plantagineum* leaves in response to ABA and osmotic stress. Preliminary results from this quantitative real-time PCR analysis were consistent with the data from the RT-PCR analysis and confirmed that *CpbZIP1* expression was not enhanced by treatments with ABA or mannitol (data not shown). Ditzer and Bartels (2006) demonstrated earlier that the expression of the *CpbZIP1* gene

was not induced in leaf, root and callus tissue of *C. plantagineum* by ABA or dehydration. Thus, it appears that the *bZIP1* gene is constitutively expressed in *C. plantagineum***.** Therefore, it is assumed that the mechanisms regulating desiccation-induced expression of the *LEA-like 11-24* gene in *C. plantagineum* are not dependent on enhanced transcription of the *CpbZIP1* gene. This hypothesis is supported by the observation that treatments with either ABA or mannitol did not change the transcript levels of *CpbZIP1,* but elevated the *LEA-like 11-24* transcript levels in *C. plantagineum* leaves (Figure 48).

4.13.3. Co-expression of *CpbZIP1* does not enhance the activity of the *LEA-like 11-24* promoter fragment in *C. plantagineum*

The ability of the *Cp*bZIP1 protein to stimulate the promoter activity of the *LEA-like 11-24* promoter fragment of *C. plantagineum* was tested in a transactivation assay. Transient co-expression of *Cp*bZIP1 protein did not alter the levels of *Cp LEA-like 11-24* promoter activity in response to either ABA, mannitol or water treatment. The levels of *Cp LEA-like 11-24* promoter activity that was co-expressed with *CpbZIP1* were similar in comparison to those of the *Cp LEA-like 11-24* promoter alone. The results from the transactivation assay suggest that transient expression of *CpbZIP1* on its own is insufficient to enhance the activity of the *Cp LEA-like 11-24* promoter fragment. Several reasons may explain why co-expression of the *Cp*bZIP1 protein did not enhance *Cp LEA-like 11-24* promoter activity. Perhaps the *Cp*bZIP requires post-translational modifications to become functional. Possibly the *Cp*bZIP1 protein depends on interaction with another proteins or elements to induce promoter activity. Although not very likely, it might even be possible that the *Cp*bZIP1 protein does not function as transcriptional activator.

4.14. Post-translational modifications may regulate the activity of the *Cp*bZIP1 protein

As mentioned above, the functionality of the *Cp*bZIP1 protein might be dependent on post-translational modifications, such as phosphorylation or dimer formation. It is known that a specific group of bZIP transcription factors (group A) from *Arabidopsis* alter their phosphorylation state in response to ABA or abiotic stress (Schutze *et al*., 2008). It may be that *Cp*bZIP1 protein requires phosphorylation for activation. Methods for analyzing protein phosphorylation in *C. plantagineum* have been developed (Röhrig *et al*., 2008).

Furthermore, bZIP transcription factors are known to form dimers and bind DNA either as homo- and heterodimers (Jacoby *et al*., 2002; Ellenberger *et al*., 1992). It was reported by Elhert *et al*. (2006) that the *At*bZIP53 protein was able to form heterodimers with different members of group C bZIP proteins from *A. thaliana*. It was further demonstrated that co-expression of *At*bZIP53 with bZIP heterodimerization partners enhanced transactivation properties of the *At*bZIP53 protein (Weltmeier *et al*., 2006). Heterodimerization of *At*bZIP53 with bZIP members of group C led to transcriptional activation. In addition, it has also been shown that heterodimerization increased the DNA binding activity of *At*bZIP53 (Alonso *et al*., 2009). The DNA binding activity of the *At*bZIP53 transcription factor was enhanced by the formation of heterodimers between *At*bZIP53 and *At*bZIP10 or *At*bZIP25.

Therefore, the *Cp*bZIP1 protein may require heterodimerization with another protein to become functionally active. Protein-protein interaction can be studied *in vivo* with the yeast two hybrid system (Causier and Davies, 2002). Therefore, the yeast two hybrid system is a suitable technique to identify putative interaction partners of the *Cp*bZIP1 protein. The principle of the yeast 2 hybrid system is similar to that of the Y1HS. However, instead of a DNA fragment, a protein of interest is used as bait. When a potential interaction partner of the *Cp*bZIP1 protein is discovered, it may be investigated whether the two proteins are targeted together to the *Cp LEA-like 11-24* promoter. The targeting of the proteins can be studied by using the serial ChIP method (Xie and Grotewold, 2008). The serial ChIP technology makes use of two specific antibodies, one for each of the proteins. However, a problem that may arise with the identification of an interaction partner of *Cp*bZIP1, is that the *Cp*bZIP1 protein interacts with itself and is dependent on auto-activation.

The role of the *CpbZIP1* protein in the regulation of *Cp LEA-like 11-24* promoter activity may be studied further by using the antisense technology. The antisense technology is as a useful tool for analyzing to the roles of transcription factors (Schwechheimer *et al*., 1998). Transient expression of *CpbZIP1* in an antisense orientation may suppress the translation of the *CpZIP1* gene. The antisense transcript pairs with the complementary mRNA strand and block the translation of the mRNA into protein. In this way, the amount of *Cp*bZIP1 protein in *C. plantagineum* leaves might be reduced. The *Cp LEA-like 11-24* promoter fragment and an expression vector carrying the *CpbZIP1* sequence in an antisense orientation can be introduced into *C. plantagineum* leaf tissue via a co-bombardment. The effect of suppressed *CpbZIP1* translation on *Cp LEA-like 11-24* promoter activity can subsequently be analyzed using the transient expression assay. However, a problem of the antisense approach might be

that the translation of a whole group of bZIP proteins is inhibited, because bZIP members of the same group have similar sequences.

4.15. Transcriptional regulation of *LEA-like 11-24* gene expression in response to dehydration in the resurrection plant *C. plantagineum*

Based on the results obtained in this work, a model for transcriptional regulation of the *C. plantagineum LEA-like 11-24* gene in response to dehydration is proposed (Figure 50).

The *Cp LEA-like 11-24* promoter fragment was responsive to treatment with ABA or mannitol. This means that the activity of the *LEA-like 11-24* promoter of *C. plantagineum* is regulated by an ABA-dependent and an ABA-independent pathway. Functional promoter analysis revealed that all three ACGT-containing ABRE motifs present in the *Cp LEA-like* promoter fragment have a role in ABA-responsive promoter activity. It was determined by using the Y1HS method that the *Cp*bZIP1 protein was able to interact with a 60 bp fragment of the *Cp LEA-like 11-24* promoter. It was not resolved to which *cis*-acting regulatory element the *Cp*bZIP1 protein binds. Most likely the *Cp*bZIP1 protein will interact with one of the ACGT motifs present in the *Cp LEA-like 11-24* promoter. It is known that bZIP transcription factors bind to motifs, which contain an ACGT sequence (Jacoby *et al*., 2002). This suggests that the *Cp*bZIP1 protein is involved in ABA-mediated promoter activity. It is not clear how the *Cp*bZIP1 protein functions. The *Cp*bZIP1 protein might function as monomer, homodimer or heterodimer. However, a putative interacting partner for the *Cp*bZIP1 protein has not yet been discovered. Furthermore, the *Cp*bZIP1 protein might require phosphorylation to become active.

It was shown by functional promoter analysis that the DRE motif plays a role in ABA-independent activity of the *Cp LEA-like 11-24* promoter. Transcription factors that bind to the DRE motif present in the *Cp LEA-like 11-24* promoter were not identified. Probably, a member of the DREB protein family will interact with the DRE motif. Members of the DREB protein family are transcription factors that specifically interact with DRE motifs (Yamaguchi-Shinozaki and Shinozaki, 2005; Liu *et al*., 1998). The DRE motif in the *Cp LEA-like 11-24* promoter was shown to be also involved in ABA-induced *Cp LEA-like 11-24* promoter activity. This means that cross-talk between the ABA-independent and the ABA-dependent pathways that regulate the expression of the *LEA-like 11-24* gene exists. It is assumed that the DRE motif might function as a coupling element for the ABRE motifs in the ABA-dependent pathway.

Upon dehydration stress transcription factors specifically interact with the *cis*-acting regulatory elements present within the promoter region of the *LEA-like 11-24* gene. The binding of transcriptional activators leads to the recruitment of the RNA polymerase complex to the promoter region. Subsequently, the RNA polymerase complex binds to the TATA box and initiates the transcription of the *LEA-like 11-24* gene.

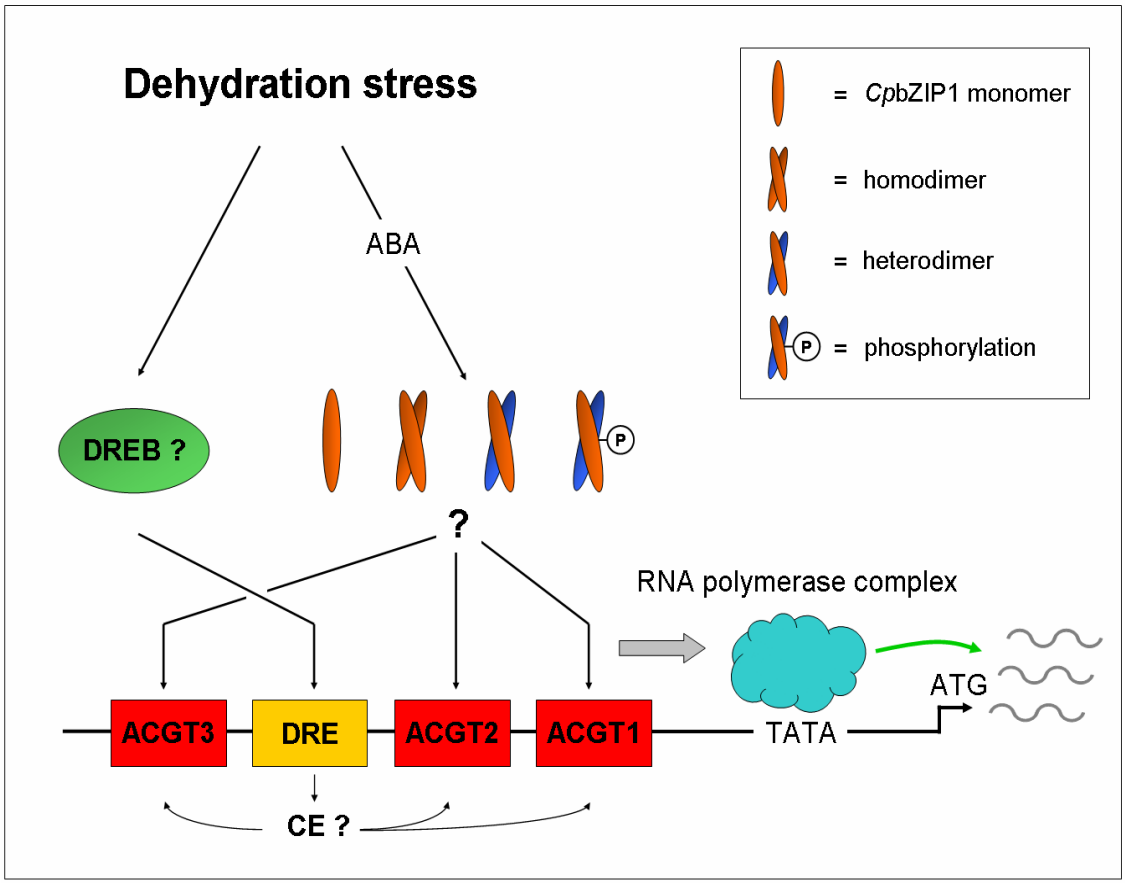


Figure 50. Model describing the transcriptional regulation of the *LEA-like 11-24* gene in response to dehydration in *C. plantagineum*. Upon dehydration stress the ABA-independent and ABA-dependent pathways are activated. It is believed that DRE binding (DREB) proteins are involved in the ABA-independent pathway and interact with the DRE motif. The *Cp*bZIP1 protein is assumed to participate in the ABA-dependent pathway and interacts with one or more of the ACGT motifs present in the *LEA-like 11-24* promoter. The *Cp*bZIP1 protein might function either as monomer, homodimer or heterodimer. In addition, the *Cp*bZIP1 protein may require phosphorylation to become activated. The binding of transcriptional activators to the *LEA-like 11-24* promoter leads to the recruitment the RNA polymerase complex to the promoter region. The RNA polymerase complex binds to the TATA box and transcription of the *LEA-like 11-24* gene is initiated. Cross-talk between the ABA-independent and ABA-dependent pathways occurs. The DRE motif probably functions as coupling element (CE) for the ACGT motifs.

One should be aware that the model, which is proposed in this study, is a simplified version of reality. The mechanisms regulating the *LEA-like 11-24* gene expression in *C. plantagineum* are probably much more complex than described in the model.

Presumably, many other *cis*-acting regulatory elements, which are located outside the analyzed 307 bp *Cp LEA-like 11-24* promoter fragment, are involved in the regulation of the *Cp LEA-like 11-24* promoter. In addition, various other transcription factors are likely to play a role in the activation and suppression of *LEA-like 11-24* gene expression in *C. plantagineum*. The *trans*-acting environment has strong influence on the activity of the *LEA-like 11-24* promoter. It was shown that a change of *trans*-acting background affected the activity of the *Cp LEA-like 11-24* promoter fragment. When the *Cp LEA-like 11-24* promoter fragment was transiently expressed in *L. subracemosa* leaves, where the promoter experienced a different *trans*-acting environment, the activity of the promoter was different than when it was expressed in homologous leaf tissue (Figure 38). Several homeodomain leucine zipper proteins, which are putative transcription factors, have been identified in *C. plantagineum* (Deng *et al*., 2006; Deng *et al*., 2002). Perhaps some of these homeodomain leucine zipper proteins might participate in transcriptional regulation of the *LEA-like 11-24* gene.

Moreover, transcriptional regulation is not only controlled by *cis*- and *trans*-acting factors. Various other mechanisms are known to be involved in transcriptional regulation. For example, DNA methylation and histone modifications are know to regulate gene activity (Fuks, 2005; Tariq and Paszkowski, 2004). DNA methylation and histone modifications affect chromatin structure and this has an effect on gene expression activity. Processes like DNA methylation and histone modification are also likely to control the transcriptional regulation of *LEA-like 11-24* gene. Furthermore, sugars might also be involved in the regulation of *LEA-like 11-24* gene expression. Sucrose was shown to repress the translation of a bZIP transcription factor in *Arabidopsis* (Wiese *et al*., 2005). In addition, it is known that microRNAs (miRNAs) are able to regulate gene expression post-transcriptionally. These short non-coding miRNAs bind to 3'-untranslated region of the complementary target mRNAs and inhibit the translation of the mRNA (Cannell *et al*., 2008; Jackson and Standart, 2007). It is very probable that miRNA-mediated repression of gene expression also plays a role in the regulation of *LEA-like 11-24* expression. Thus, many mechanisms are believed to be involved in the regulation of dehydration-responsive expression of the *LEA-like 11-24* gene in *C. plantagineum*.

4.16. Future perspectives

The results obtained in this study provided valuable information about dehydration-responsive gene regulation in closely related plant species that differ in their ability to tolerate desiccation. The transcriptome comparison between *L. brevidens* and *L. subracemosa* revealed differences in the expression of genes putatively involved in desiccation tolerance, such as genes encoding LEA proteins. These findings strongly suggest that dehydration-responsive gene expression is regulated differently in these closely related plant species. Differential gene expression might account for the variation in desiccation tolerance between *L. brevidens* and *L. subracemosa*.

Functional promoter analysis showed that structural differences contributed to functional variation in promoter regions of *LEA-like 11-24* genes of *C. plantagineum*, *L. brevidens* and *L. subracemosa*. However, no promoter elements unique to resurrection plants could be discovered. Therefore, it may be sensible to expand the promoter study in order to identify unique structures in the promoters of resurrection plants, although it may be that resurrection plants do not have unique *cis*-acting regulatory elements. In addition, it might be interesting to compare promoters of homologues *LEA* genes, which are expressed in vegetative tissues of resurrection plants and orthodox seeds of desiccation sensitive plants.

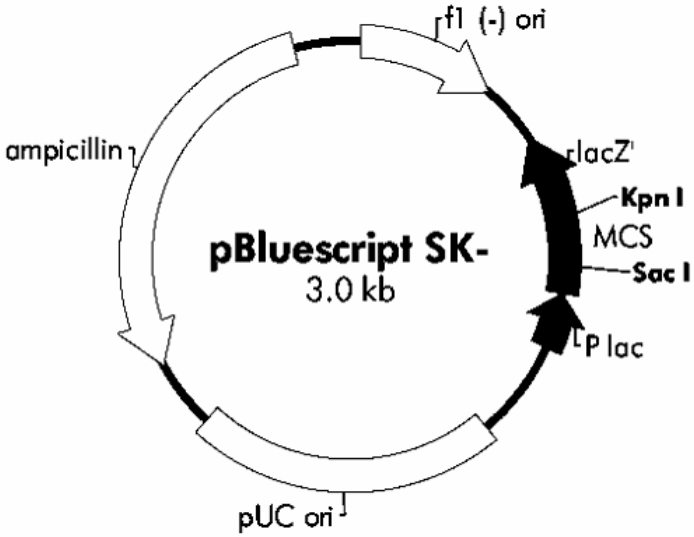
Furthermore, more knowledge is needed concerning the *trans*-acting factors involved in regulation of dehydration-responsive gene expression. The ability of the bZIP1 protein of *C. plantagineum* to interact with the *LEA-like 11-24* and *LEA* C2 promoters indicates that this protein plays an important role in the regulation of dehydration-responsive gene expression. However, further analyzes is required to elucidate the function of the *Cp*bZIP1 protein in relation to dehydration-responsive gene expression. The post-translational regulation of the *Cp*bZIP1 protein should be investigated. Additionally, overexpression and antisense techniques may be employed to gain more information about the function of the *Cp*bZIP1 protein. For these purposes, stable transformed *C. plantagineum* plants might be generated. It is also important to investigate other mechanisms of gene regulation in resurrection plants, such as histone modification and DNA methylation.

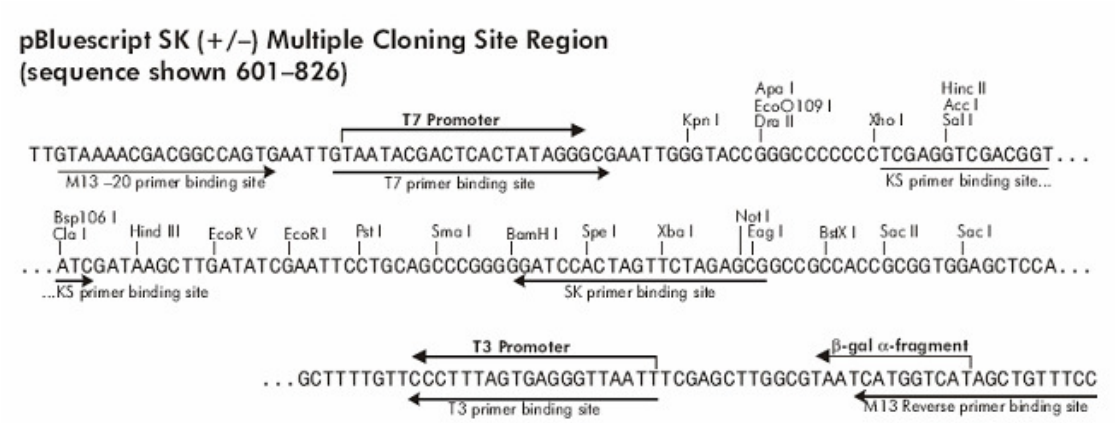
Desiccation tolerance is a complex trait and is controlled by the expression of many genes. Understanding the regulation of genes involved in desiccation tolerance in resurrection plants may contribute to the development of more dehydration tolerant crops.

5. Supplemental data

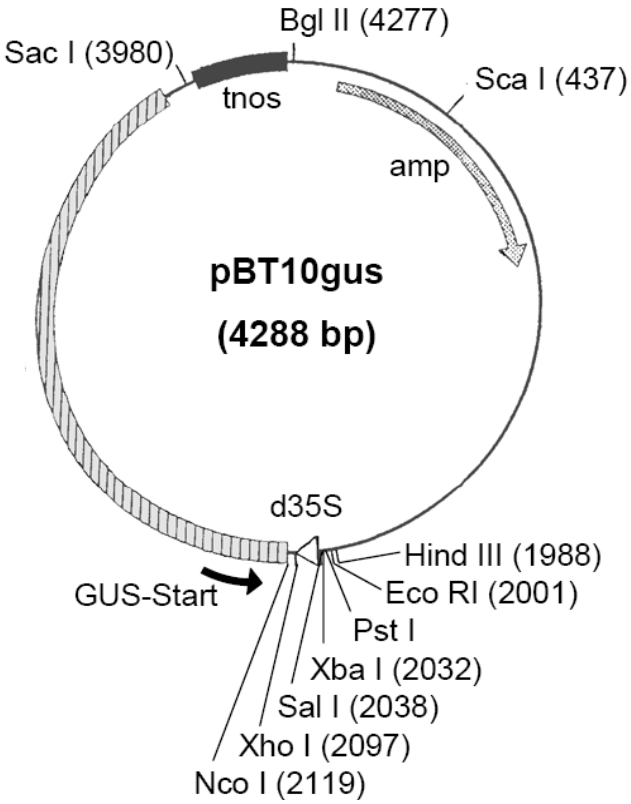
5.1. Vector maps

5.1.1. pBluescript® SK(-) vector map with restriction enzyme digestion sites

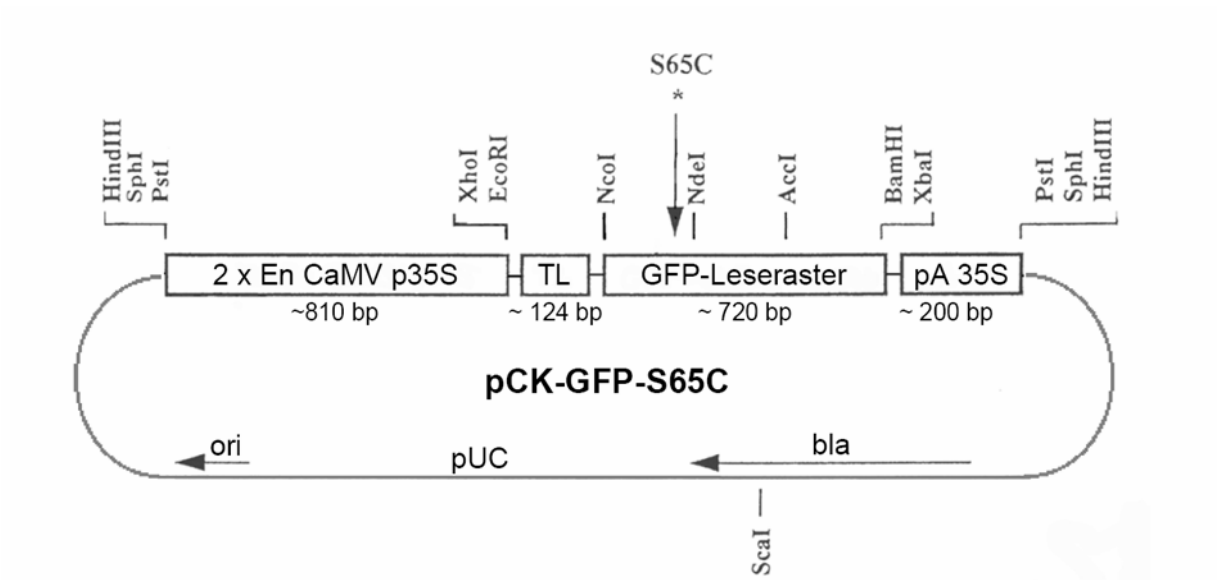




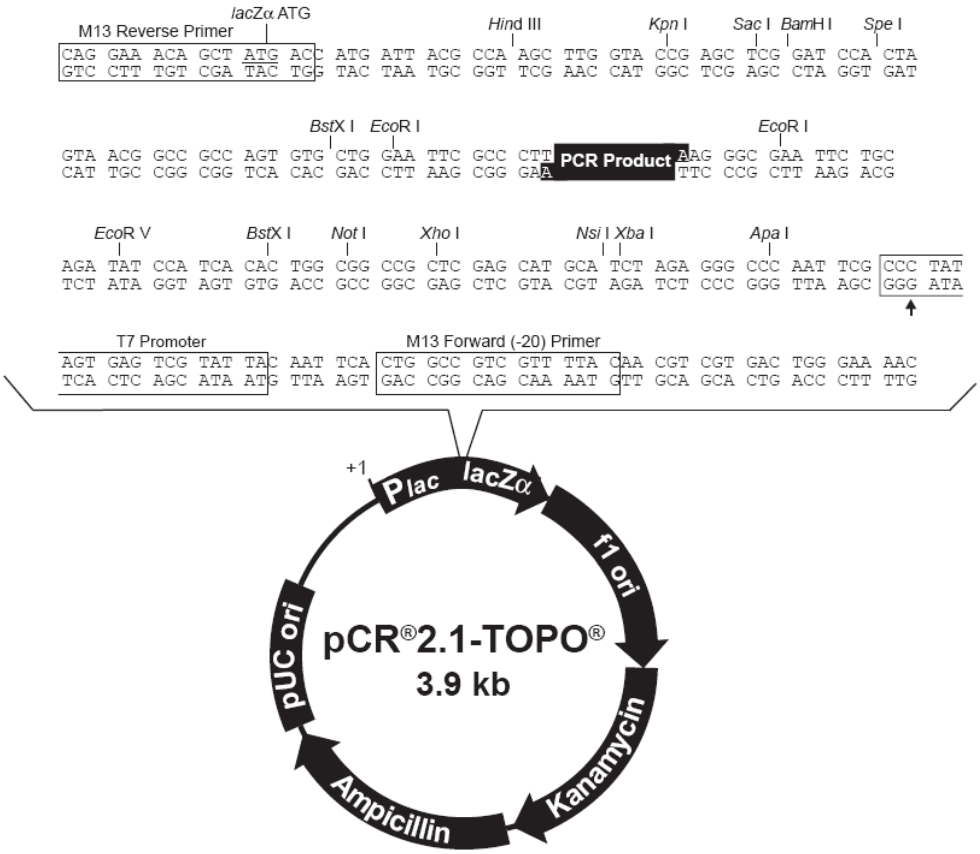
5.1.2. pBT10 GUS vector map with restriction enzyme digestion sites



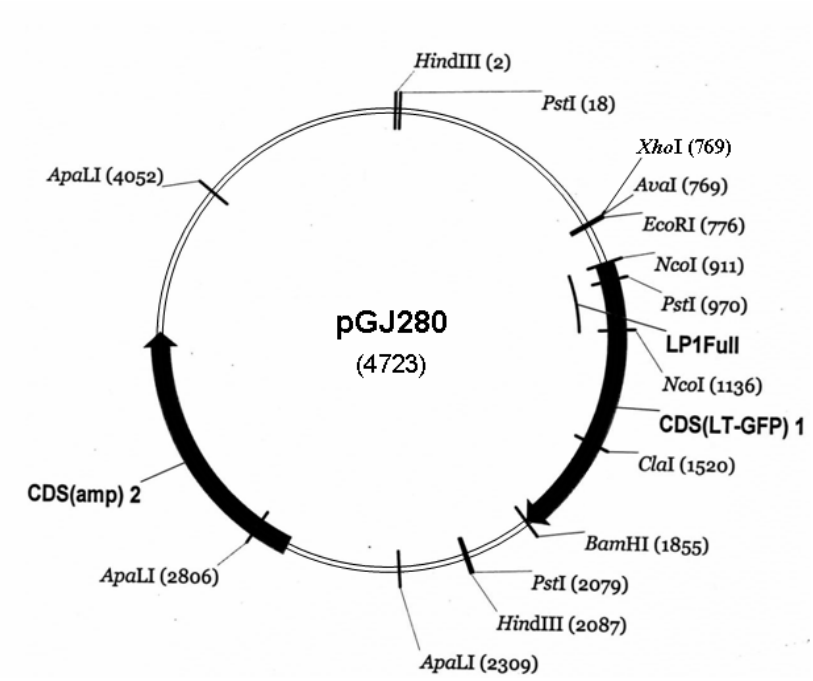
5.1.3. pCK-GFP-S65C vector map with restriction enzyme digestion sites



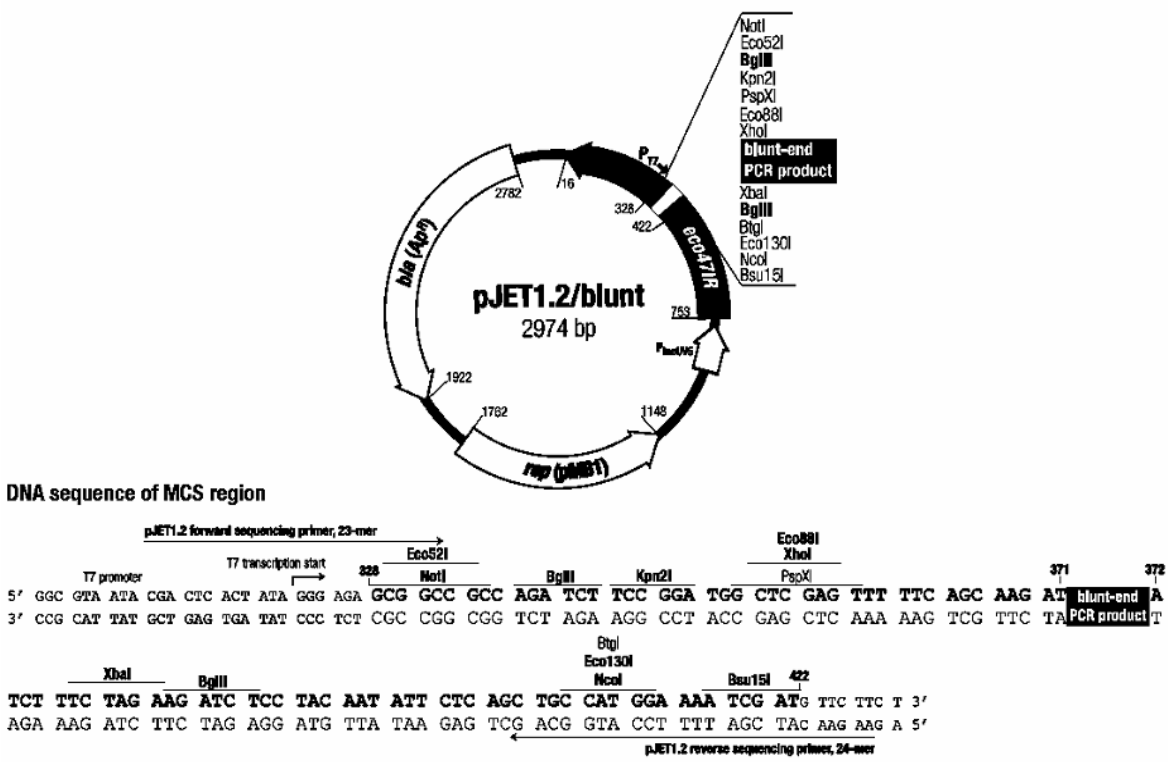
5.1.4. pCR2.1®-TOPO® vector map with restriction enzyme digestion sites



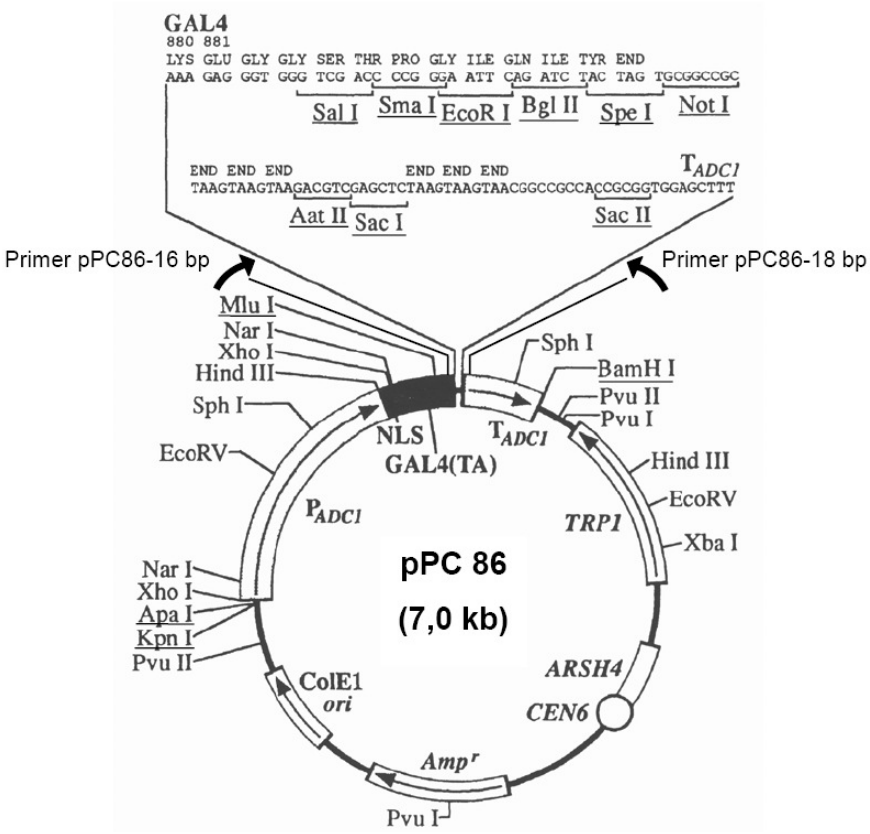
5.1.5. pGJ280 vector map with restriction enzyme digestion sites



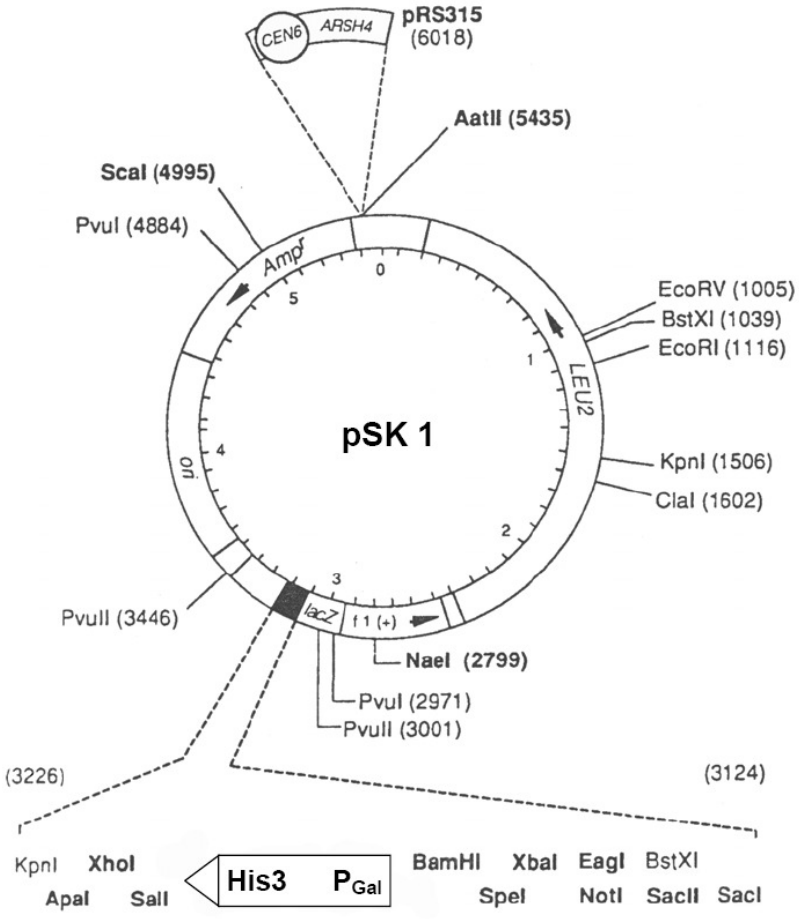
5.1.6. pJET1.2 vector map with restriction enzyme digestion sites



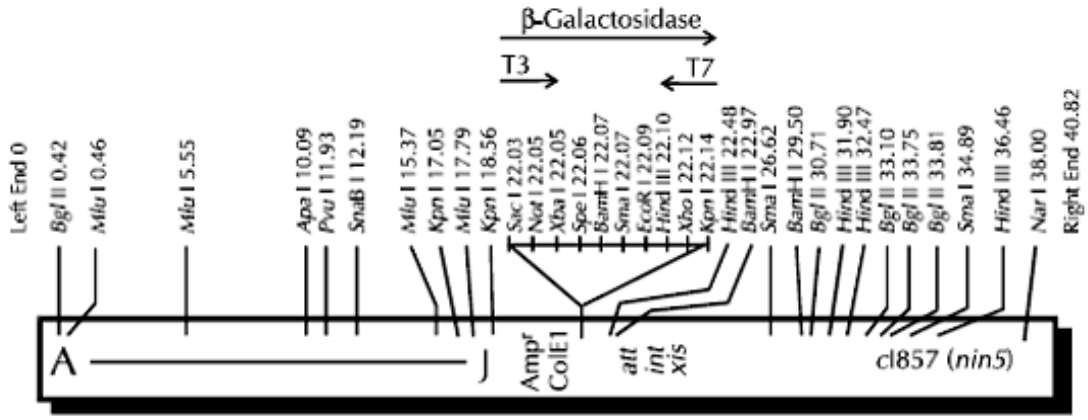
5.1.7. pPC86 vector map with restriction enzyme digestion sites



5.1.8. pSK1 vector map with restriction enzyme digestion sites



5.1.9. Uni-ZAP® XR vector map with restriction enzyme digestion sites



5.2. EST collections of *L. brevidens* and *L. subracemosa*

Table S1. *L. brevidens* EST collection

Target protein	Organism	Functional catagory	E-value
MTD1	*Medicago truncatula*	Cell growth / division	9E-09
Gag-Pol	*Ipomoea batatas*	Cell growth / division	2E-50
Seed maturation protein PM21	*Glycine max*	Cell growth / division	8E-08
Kinesin heavy chain-like protein	*Solanum tuberosum*	Cell structure	3E-90
Membrane protein-like	*Oryza sativa (japonica cultivar-group)*	Cell structure	1E-57
Cell-wall invertase	*Lycopersicon esculentum*	Cell structure	9E-39
Putative glucan synthase	*Arabidopsis thaliana*	Cell structure	4E-40
11 kDa LEA protein	*Helianthus annuus*	Desiccation related	3E-19
11 kDa LEA protein	*Helianthus annuus*	Desiccation related	3E-19
11 kDa LEA protein	*Helianthus annuus*	Desiccation related	2E-12
Dehydrin putative	*Arabidopsis thaliana*	Desiccation related	1E-06
Dehydrin-like protein Dh2	*Boea crassifolia*	Desiccation related	1E-10
Desiccation-related protein (CDeT11-24)	*Craterostigma plantagineum*	Desiccation related	5E-10
Desiccation-related protein (CDeT11-24)	*Craterostigma plantagineum*	Desiccation related	3E-05
Desiccation-related protein (CDeT11-24)	*Craterostigma plantagineum*	Desiccation related	6E-09
Dessication-related protein (PCC13-62)	*Craterostigma plantagineum*	Desiccation related	1E-88
Desiccation-related protein (CDeT27-04)	*Craterostigma plantagineum*	Desiccation related	4E-04
Desiccation-related protein (CDeT27-04)	*Craterostigma plantagineum*	Desiccation related	4E-04
Dessication-related protein (CDeT27-45)	*Craterostigma plantagineum*	Desiccation related	2E-74
Desiccation-related protein (CDeT3-06)	*Craterostigma plantagineum*	Desiccation related	7E-45
Dessication-related protein (CDeT3-06)	*Craterostigma plantagineum*	Desiccation related	4E-56
Dessication-related protein (CDeT3-06)	*Craterostigma plantagineum*	Desiccation related	5E-66
Dessication-related protein (CDeT3-06)	*Craterostigma plantagineum*	Desiccation related	1E-04
Dessication-related protein (CDeT3-06)	*Craterostigma plantagineum*	Desiccation related	3E-01
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	4E-01
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	6E-03
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	6E-03
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	8E-02
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	2E-04
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	5E-03
Dessication-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	1E-01
Dessication-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	2E-02
Dessication-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	2E-37
Desiccation-stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	7E-59
Desiccation stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	1E-64
Desiccation-stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	4E-69
Desiccation-stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	6E-50
Desiccation-stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	1E-63
Desiccation-stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	1E-63
Desiccation-stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	1E-63
Desiccation-stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	2E-62
Desiccation-stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	8E-64
Desiccation-stress protein DSP22	*Craterostigma plantagineum*	Desiccation related	2E-10
Desiccation-stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	9E-62
Dessication-related protein	*Craterostigma plantagineum*	Desiccation related	6E-65
Dessication-related protein	*Craterostigma plantagineum*	Desiccation related	7E-04
Dessication-related protein	*Craterostigma plantagineum*	Desiccation related	3E-01
Dessication-related protein	*Craterostigma plantagineum*	Desiccation related	9E-02
Dessication-related protein	*Craterostigma plantagineum*	Desiccation related	6E-61
Dessication-related protein	*Craterostigma plantagineum*	Desiccation related	4E-66
Dessication-related protein	*Craterostigma plantagineum*	Desiccation related	1E-44
DHN1	*Solanum tuberosum*	Desiccation related	2E-14
Early-responsive to dehydration 7	*Arabidopsis thaliana*	Desiccation related	5E-45
LEA protein	*Catharanthus roseus*	Desiccation related	2E-38
LEA protein	*Catharanthus roseus*	Desiccation related	5E-32
LEA protein	*Catharanthus roseus*	Desiccation related	1E-22
LEA protein Dc3	*Daucus carota*	Desiccation related	7E-31
LEA protein Dc3	*Daucus carota*	Desiccation related	4E-31
LEA protein Dc3	*Daucus carota*	Desiccation related	4E-26
LEA protein	*Isatis tinctoria*	Desiccation related	5E-36
LEA protein 5	*Nicotiana tabacum*	Desiccation related	8E-14
LEA protein	*Glycine max*	Desiccation related	2E-04
LEA protein	*Glycine max*	Desiccation related	6E-04
LEA protein	*Cicer arietinum*	Desiccation related	4E-30
LEA protein	*Cicer arietinum*	Desiccation related	2E-36
LEA protein	*Cicer arietinum*	Desiccation related	1E-58

LEA protein	*Cicer arietinum*	Desiccation related	1E-36
LEA protein	*Glycine max*	Desiccation related	4E-04
LEA1-like protein	*Capsicum annuum*	Desiccation related	4E-40
LEA1P	*Daucus carota*	Desiccation related	2E-36
LEA1P	*Daucus carota*	Desiccation related	2E-32
LEA1P	*Daucus carota*	Desiccation related	2E-18
Lophopyrum elongatum dehydrin-/LEA group 2-like protein	*Oryza sativa (japonica cultivar-group)*	Desiccation related	4E-02
Putative group 3 LEA protein	*Oryza sativa (japonica cultivar-group)*	Desiccation related	5E-02
Putative dessication-related protein	*Arabidopsis thaliana*	Desiccation related	2E-36
LEA protein	*Glycine max*	Desiccation related	6E-04
Pathogenesis-related protein 10	*Vitis pseudoreticulata*	Disease / defense	5E-34
Major allergen Mal d 1.0501	*Malus x domestica*	Disease / defense	3E-32
Major allergen Mal d 1.0501	*Malus x domestica*	Disease / defense	1E-32
Major allergen Mal d 1.0501	*Malus x domestica*	Disease / defense	1E-32
Aldehyde dehydrogenase	*Craterostigma plantagineum*	Disease / defense	2E-45
Pathogenesis-related protein PR-3 type	*Sambucus nigra*	Disease / defense	9E-75
Major allergen Mal d 1.0501	*Malus x domestica*	Disease / defense	1E-32
ALDH5F1	*Arabidopsis thaliana*	Disease / defense	1E-56
Putative cold regulated protein	*Oryza sativa (japonica cultivar-group)*	Disease / defense	3E-53
Putative DnaJ protein	*Camellia sinensis*	Disease / defense	5E-35
Pathogenesis-related protein 5-1	*Helianthus annuus*	Disease / defense	1E-80
Chitinase	*Chenopodium amaranticolor*	Disease / defense	9E-77
Thaumatin-like protein	*Sambucus nigra*	Disease / defense	2E-25
Beta-13 glucanase	*Pisum sativum*	Disease / defense	9E-80
MLO1	*Lycopersicon esculentum*	Disease / defense	4E-12
Beta-13-glucanase	*Olea europaea*	Disease / defense	2E-40
At1g20030	*Arabidopsis thaliana*	Disease / defense	2E-15
Pyruvate kinase isozyme A chloroplast precursor	*Ricinus communis*	Energy	6E-138
Small blue copper protein Bcp1	*Boea crassifolia*	Energy	5E-12
Glucose-6-phosphate isomerase	*Solanum tuberosum*	Energy	8E-27
Citrate synthase mitochondrial precursor	*Daucus carota*	Energy	1E-65
Citrate synthase mitochondrail precursor	*Citrus junos*	Energy	1E-120
Senescence-associated protein-like	*Oryza sativa (japonica cultivar-group)*	Energy	3E-22
Seed imbibition protein	*Hordeum vulgare subsp*	Energy	2E-92
Seed imbibition protein	*Hordeum vulgare subsp. vulgare*	Energy	7E-25
Seed imbibition protein	*Hordeum vulgare subsp. vulgare*	Energy	4E-42
CPRD2	*Vigna unguiculata*	Energy	8E-81
CPRD2	*Vigna unguiculata*	Energy	5E-70
Glyceraldehyde 3-phosphate dehydrogenase	*Craterostigma plantagineum*	Energy	2E-132
Glyceraldehyde 3-phosphate dehydrogenase	*Craterostigma plantagineum*	Energy	3E-124
Small blue copper protein Bcp1	*Boea crassifolia*	Energy	5E-12
Citrate synthase mitochondrial precursor	*Daucus carota*	Energy	1E-160
Glyceraldehyde-3-phosphate dehydrogenase	*Nicotiana tabacum*	Energy	2E-118
glyceraldehyde 3-phosphate dehydrogenase	*Craterostigma plantagineum*	Energy	3E-52
Syntaxin-like protein	*Solanum tuberosum*	Intracellular traffic	1E-51
Protein translocase/ protein transporter	*Arabidopsis thaliana*	Intracellular traffic	8E-17
Putative nonsense-mediated mRNA decay protein	*Arabidopsis thaliana*	Intracellular traffic	2E-16
Voltage-Dependent Anion Channel Porins	*Lotus corniculatus var. japonicus*	Intracellular trafic	1E-71
Mitochondrial energy transfer protein	*Solanum tuberosum*	Intracellular trafic	2E-95
Avr9/Cf-9 rapidly elicited protein 197	*Nicotiana tabacum*	Intracellular traffic	8E-24
SYP71 (SYNTAXIN OF PLANTS 71)	*Arabidopsis thaliana*	Intracellular traffic	6E-97
Putative xyloglucanase inhibitor	*Solanum tuberosum*	Metabolism	2E-63
Glutathione reductase chloroplast precursor	*Spinacia oleracea*	Metabolism	4E-117
ThiF family protein	*Arabidopsis thaliana*	Metabolism	2E-103
Glucosyltransferase-like protein	*Arabidopsis thaliana*	Metabolism	5E+07
Squalene monooxygenase 2	*Medicago truncatula*	Metabolism	6E-23
Sucrose synthase	*Craterostigma plantagineum*	Metabolism	8E-161
Patatin-like protein 3	*Nicotiana tabacum*	Metabolism	5E-43
Catalytic/ hydrolase	*Arabidopsis thaliana*	Metabolism	6E-34
Sucrose synthase 1	*Craterostigma plantagineum*	Metabolism	2E-85
Lactoylglutathione lyase	*Arabidopsis thaliana*	Metabolism	1E-67
UDP-glucose glucosyltransferase	*Rhodiola sachalinensis*	Metabolism	4E-28
Fatty acid multifunctional protein 2	*Arabidopsis thaliana*	Metabolism	6E-52
Glutathione reductase	*Zinnia elegans*	Metabolism	4E-123
Geranyl diphosphate synthase	*Vitis vinifera*	Metabolism	7E-10
Geranyl diphosphat synthase	*Quercus robur*	Metabolism	5E-27
Aminoalcoholphosphotransferase	*Pimpinella brachycarpa*	Metabolism	4E-80
Aminoalcoholphosphotransferase	*Brassica rapa*	Metabolism	4E-08
Sucrose synthase	*Craterostigma plantagineum*	Metabolism	3E-45
S-adenosylmethionine-dependent methyltransferase	*Arabidopsis thaliana*	Metabolism	1E-93
Putative esterase	*Cicer arietinum*	Metabolism	4E-59
ENSANGP00000023872	*Anopheles gambiae str. PEST*	Metabolism	2E-80
NFU1	*Arabidopsis thaliana*	Metabolism	2E-41
Alcohol dehydrogenase	*Petunia x hybrida*	Metabolism	3E-100

Alcohol dehydrogenase	*Arabidopsis thaliana*	Metabolism	7E-75
Putative myo-inositol oxygenase	*Oryza sativa (japonica cultivar-group)*	Metabolism	2E-36
Digalactosyldiacylglycerol synthase 1	*Lotus corniculatus var.japonicus*	Metabolism	3E-81
Catalytic/ hydrolase	*Arabidopsis thaliana*	Metabolism	5E-35
Hydrolase-like	*Oryza sativa (japonica cultivar-group)*	Metabolism	3E-06
Putative Sec61	*Oryza sativa (japonica cultivar-group)*	Protein destination and storage	4E-103
SEC (SECRET AGENT)	*Arabidopsis thaliana*	Protein destination and storage	7E-02
ADP-ribosylation factor	*Hyacinthus orientalis*	Protein destination and storage	5E-75
Polyubiquitin	*Pinus sylvestris*	Protein destination and storage	2E-145
Putative ubiquitin protein	*Arabidopsis thaliana*	Protein destination and storage	2E-39
Ring-box protein-like	*Arabidopsis thaliana*	Protein destination and storage	2E-55
E3 ubiquitin ligase Prototypical U-box domain protein 14	*Arabidopsis thaliana*	Protein destination and storage	3E-11
Miraculin precursor	*Synsepalum dulcificum*	Protein destination and storage	3E-52
Miraculin precursor	*Synsepalum dulcificum*	Protein destination and storage	2E-51
CGI-58 protein -like	*Oryza sativa (japonica cultivar-group*	Protein destination and storage	7E-05
Putative ubiquitin-conjugating enzyme 16	*Arabidopsis thaliana*	Protein destination and storage	1E-74
RBX1	*Arabidopsis thaliana*	Protein destination and storage	4E-55
Ring-box protein-like	*Arabidopsis thaliana*	Protein destination and storage	2E-55
At3g49060/T2J13_100	*Arabidopsis thaliana*	Protein destination and storage	4E-24
GDP dissociation inhibitor	*Nicotiana tabacum*	Protein destination and storage	4E-69
Ring-box protein-like	*Arabidopsis thaliana*	Protein destination and storage	2E-55
Clathrin propeller N-terminal; Protein prenyltransferase	*Medicago truncatula*	Protein destination and storage	1E-120
Protein binding / ubiquitin-protein ligase/ zinc ion binding	*Arabidopsis thaliana*	Protein destination and storage	1E-70
Ubiquitin fusion-degradation protein-like	*Solanum tuberosum*	Protein destination and storage	4E-108
OSJNBa0023J03.11	*Oryza sativa (japonica cultivar-group)*	Protein destination and storage	3E-46
Ubiquitin-conjugating enzyme E2-17 kDa	*Solanum tuberosum*	Protein destination and storage	2E-82
GDP dissociation inhibitor	*Nicotiana tabacum*	Protein destination and storage	5E-66
Avr9/Cf-9 rapidly elicited protein 36	*Nicotiana tabacum*	Protein destination and storage	1E-35
Putative WD-repeat protein 12	*Oryza sativa (japonica cultivar-group)*	Protein destination and storage	3E-45
Protein binding / ubiquitin-protein ligase/ zinc ion binding	*Arabidopsis thaliana*	Protein destination and storage	1E-46
Protein translocase	*Arabidopsis thaliana*	Protein destination and storage	9E-37
60S acidic ribosomal protein P1 (L12)	*Zea mays*	Protein synthesis	1E-22
Structural constituent of ribosome	*Arabidopsis thaliana*	Protein synthesis	3E-03
Putative 60S acidic ribosomal protein P1	*Arabidopsis thaliana*	Protein synthesis	1E-05
Elongation factor 1-alpha	*Aureobasidium pullulans*	Protein synthesis	3E-31
Translation elongation factor EF-2 subunit	*Aspergillus fumigatus Af293*	Protein synthesis	4E-45
Cytochrome p450 isoform PM17	*Mentha x piperita*	Secondary Metabolism	2E-60
Putative lung alpha/beta hydrolase protein 1	*Oryza sativa (japonica cultivar-group)*	Secondary Metabolism	7E-67
UDP-glucuronic acid:anthocyanin glucuronosyltransferase	*Bellis perennis*	Secondary Metabolism	3E-15
UDP-glucuronic acid:anthocyanin glucuronosyltransferase	*Bellis perennis*	Secondary Metabolism	2E-06
Cinnamyl alcohol dehydrogenase	*Eucommia ulmoides*	Secondary Metabolism	3E-04
Isochorismate synthase	*Catharanthus roseus*	Secondary Metabolism	6E-81
Isochorismate synthase protein	*Nicotiana tabacum*	Secundary metablolism	6E-94
GTP-binding protein yptV3	*Volvox carteri*	Signal transduction	8E-06
Calmodulin NtCaM13	*Nicotiana tabacum*	Signal transduction	2E-46
Calmodulin-like-domain protein kinase CPK2	*Cucurbita maxima*	Signal transduction	2E-21
Protein phosphatase-2C; PP2C	*Mesembryanthemum crystallinum*	Signal transduction	2E-74
Protein kinase family protein	*Arabidopsis thaliana*	Signal transduction	2E-14
Protein kinase MK6	*Mesembryanthemum crystallinum*	Signal transduction	4E-20
FUS6 (FUSCA 6)	*Arabidopsis thaliana*	Signal transduction	2E-58
Protein kinase CK2 alpha subunit	*Nicotiana tabacum*	Signal transduction	2E-96
ATP binding / protein serine/threonine kinase	*Arabidopsis thaliana*	Signal transduction	5E-24
Protein phosphatase-2C; PP2C	*Mesembryanthemum crystallinum*	Signal transduction	6E-70
Protein kinase; U box	*Medicago truncatula*	Signal transduction	2E-58
Ser/Thr protein kinase	*Lotus corniculatus var. japonicus*	Signal transduction	2E-124
Calcium-binding protein	*Olea europaea*	Signal transduction	2E-35
ESD4 cysteine-type peptidase	*Arabidopsis thaliana*	Transcription	9E-74
At2g43970/F6E13.10	*Oryza sativa (japonica cultivar-group)*	Transcription	2E-02
HB-1; transcription factor	*Arabidopsis thaliana*	Transcription	1E-20
Putative WRKY family transcription factor	*Arabidopsis thaliana*	Transcription	2E-74
Heat stress transcription factor A3	*Lycopersicon peruvianum*	Transcription	3E-20
Auxin response factor-like protein	*Mangifera indica*	Transcription	3E-39
DNA binding / zinc ion binding	*Arabidopsis thaliana*	Transcription	3E-20
Pre-mRNA splicing factor-like protein	*Solanum tuberosum*	Transcription	6E-78
Nucleic acid binding	*Arabidopsis thaliana*	Transcription	2E-97
Nam-like protein 4	*Petunia x hybrida*	Transcription	7E-69
Transcription regulator	*Arabidopsis thaliana*	Transcription	2E-45
At2g43970/F6E13.10	*Oryza sativa (japonica cultivar-group)*	Transcription	2E-02
Nucleic acid binding protein	*Oryza sativa (japonica cultivar-group)*	Transcription	2E-37
SPF1 protein	*Ipomoea batatas*	Transcription	6E-53
Nucleic acid binding NABP	*Medicago truncatula*	Transcription	4E-37
Nucleotide binding	*Arabidopsis thaliana*	Transcription	4E-101
OSJNBa0070O11.6	*Oryza sativa (japonica cultivar-group)*	Transcription	2E-57
Yippee-like protein	*Solanum tuberosum*	Transcription	1E-54

GIF2 (GRF1-INTERACTING FACTOR 2)	*Arabidopsis thaliana*	Transcription	2E-22
PDR-type ABC transporter 2	*Nicotiana tabacum*	Transporters	3E-131
Putative adenine nucleotide translocase	*Castanea sativa*	Transporters	7E-07
Vacuolar H(+)-ATPase subunit-like protein	*Arabidopsis thaliana*	Transporters	1E-111
Putative proline transporter 1	*Oryza sativa (japonica cultivar-group)*	Transporters	3E-13
Vacuolar H(+)-ATPase subunit-like protein	*Arabidopsis thaliana*	Transporters	6E-115
Proline/glycine betaine transporter	*Atriplex hortensis*	Transporters	1E-70
Protein transporter	*Arabidopsis thaliana*	Transporters	4E-16
Plasma membrane H+ ATPase	*Oryza sativa (japonica cultivar-group*	Transporters	3E-74
At1g55690	*Arabidopsis thaliana*	Transporters	4E-22
At1g55690	*Arabidopsis thaliana*	Transporters	4E-22
AAA-type ATPase like protein	*Arabidopsis thaliana*	Transporters	1E-54
Hexose transporter	*Lycopersicon esculentum*	Transporters	7E-70
Putative permease 1	*Lycopersicon esculentum*	Transporters	1E-100
Transporter	*Arabidopsis thaliana*	Transporters	9E-83
Protein translocase/ protein transporter	*Arabidopsis thaliana*	Transporters	9E-37
Aquaporin 1	*Nicotiana tabacum*	Transporters	5E-22
Metal ion transporter	*Arabidopsis thaliana*	Transporters	3E-59
ACA10; calcium-transporting ATPase/ calmodulin binding	*Arabidopsis thaliana*	Transporters	8E-38
Seed-specific metallothionein-like protein	*Sesamum indicum*	Transporters	5E-32
At5g07330	*Arabidopsis thaliana*	Unclear classification	4E-12
B2 protein	*Daucus carota*	Unclear classification	2E-66
Blight-associated protein p12 precursor	*Citrus jambhiri*	Unclear classification	1E-17
Blight-associated protein p12 precursor	*Citrus jambhiri*	Unclear classification	4E-16
EDGP precursor	*Daucus carota*	Unclear classification	2E-43
Expressed protein	*Arabidopsis thaliana*	Unclear classification	5E-05
Fiber protein	*Hyacinthus orientalis*	Unclear classification	2E-02
Hypothetical Protein	*Arabidopsis thaliana*	Unclear classification	1E-03
Hypothetical protein	*Arabidopsis thaliana*	Unclear classification	5E-04
hypothetical protein	*Plantago major*	Unclear classification	2E-07
hypothetical protein	*Plasmodium falciparum 3D7*	Unclear classification	8E-02
hypothetical protein	*Arabidopsis thaliana*	Unclear classification	3E-100
Hypothetical protein	*Gibberella zeae PH-1*	Unclear classification	2E-74
Hypothetical protein	*Oryza sativa (japonica cultivar-group*	Unclear classification	2E-24
Hypothetical protein	*Arabidopsis thaliana*	Unclear classification	3E-67
Hypothetical protein	*Arabidopsis thaliana*	Unclear classification	8E-35
Hypothetical protein	*Plantago major*	Unclear classification	2E-07
Hypothetical protein	*Plantago major*	Unclear classification	2E-07
Hypothetical protein	*Arabidopsis thaliana*	Unclear classification	1E-07
Hypothetical protein	*Arabidopsis thaliana*	Unclear classification	9E-15
Hypothetical protein DDB0185771	*Dictyostelium discoideum*	Unclear classification	6E-01
Hypothetical protein DDB0219979	*Dictyostelium discoideum*	Unclear classification	4E-18
Hypothetical protein MG03930.4	*Magnaporthe grisea 70-15*	Unclear classification	3E-20
Hypothetical protein PB105099.00.0	*Plasmodium berghei*	Unclear classification	2E-07
Hypothetical protein PB105099.00.0	*Plasmodium berghei*	Unclear classification	1E-06
Little protein 1	*Oryza sativa (japonica cultivar-group)*	Unclear classification	2E-23
Little protein 1	*Oryza sativa (japonica cultivar-group)*	Unclear classification	2E-23
Little protein 1	*Oryza sativa (japonica cultivar-group)*	Unclear classification	5E-23
Protein of Unknown function DUF567	*Medicago truncatula*	Unclear classification	8E-57
Putative protein	*Arabidopsis thaliana*	Unclear classification	5E-02
Putative protein	*Arabidopsis thaliana*	Unclear classification	1E-51
Putative protein	*Arabidopsis thaliana*	Unclear classification	3E-38
Putative protein	*Arabidopsis thaliana*	Unclear classification	7E-02
Related to spore coat protein SP96 precursor	*Neurospora crassa*	Unclear classification	6E-20
Toma2A	*Arabidopsis thaliana*	Unclear classification	4E-29
Unknown protein	*Arabidopsis thaliana*	Unclear classification	3E-51
Unknown protein	*Arabidopsis thaliana*	Unclear classification	1E-47
Unknown protein	*Arabidopsis thaliana*	Unclear classification	2E-09
Unknown protein	*Oryza sativa (japonica cultivar-group)*	Unclear classification	6E-03
Unknown protein	*Arabidopsis thaliana*	Unclear classification	3E-35
Unknown protein	*Arabidopsis thaliana*	Unclear classification	2E-10
Unknown protein	*Arabidopsis thaliana*	Unclear classification	5E-11
Unknown protein	*Arabidopsis thaliana*	Unclear classification	4E-79
Unknown protein	*Arabidopsis thaliana*	Unclear classification	7E-47
Unknown protein	*Oryza sativa (japonica cultivar-group)*	Unclear classification	4E-02
Unknown protein	*Oryza sativa (japonica cultivar-group)*	Unclear classification	6E-78
Unknown protein	*Arabidopsis thaliana*	Unclear classification	2E-30
Unknown protein	*Arabidopsis thaliana*	Unclear classification	4E-11
Unknown protein	*Arabidopsis thaliana*	Unclear classification	5E-11
Unknown protein	*Arabidopsis thaliana*	Unclear classification	7E-26
Unknown protein	*Arabidopsis thaliana*	Unclear classification	8E-68
Unknown protein	*Arabidopsis thaliana*	Unclear classification	2E-10
Unknown protein	*Arabidopsis thaliana*	Unclear classification	2E-50
Unknown protein	*Arabidopsis thaliana*	Unclear classification	6E-10

Unknown protein	*Arabidopsis thaliana*	Unclear classification	3E-13
Unknown protein	*Oryza sativa (japonica cultivar-group*	Unclear classification	6E-48
Unknown protein	*Arabidopsis thaliana*	Unclear classification	5E-26
Unknown protein	*Arabidopsis thaliana*	Unclear classification	1E-11
Unknown protein	*Arabidopsis thaliana*	Unclear classification	1E-77
Unknown protein	*Arabidopsis thaliana*	Unclear classification	1E-12
Unknown protein	*Arabidopsis thaliana*	Unclear classification	4E-51
Unknown protein	*Arabidopsis thaliana*	Unclear classification	3E-30
Unknown protein	*Oryza sativa (japonica cultivar-group)*	Unclear classification	5E-30
Unknown protein	*Arabidopsis thaliana*	Unclear classification	5E-14
Unknown protein	*Oryza sativa (japonica cultivar-group)*	Unclear classification	3E-09
Unnamed protein product	*Arabidopsis thaiana*	Unclear classification	8E-39
Unnamed protein product	*Arabidopsis thaliana*	Unclear classification	3E-64
Unnamed protein product	*Arabidopsis thaliana*	Unclear classification	5E-48
Unnamed protein product	*Arabidopsis thaliana*	Unclear classification	2E-47
Putative protein	*Arabidopsis thaliana*	Unclear classification	2E-29
No match		Unclassified	
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Table S2. *L. subracemosa* EST collection

Target protein	Organism	Functional category	E-value
Seed maturation protein PM41	*Glycine max*	Cell growth / division	4E-19
Seed maturation protein PM27	*Glycine max*	Cell growth / division	6E -8
Seed maturation protein PM41	*Glycine max*	Cell growth / division	4E-19
LYTB-like protein precursor-like	*Solanum tuberosum*	Cell structure	3E-102
Plastid fibrillin 2	*Coffea canephora*	Cell structure	5E-73
Plastid-lipid-associated protein	*Ricinus communis*	Cell structure	1E-77
Plastid-lipid-associated protein	*Ricinus communis*	Cell structure	8E-15
Plastid-lipid-associated protein	*Ricinus communis*	Cell structure	5E-83
Dehydrin	*Nicotiana tabacum*	Desiccation related	5E-6
Dehydrin	*Glycine max*	Desiccation related	4E-4
Dehydrin	*Boea crassifolia*	Desiccation related	3E-7
Dehydrin 1	*Pinus pinaster*	Desiccation related	2 E-5
Dehydrin 1	*Pinus pinaster*	Desiccation related	2E-5
Dehydrin-like protein	*Picea abies*	Desiccation related	7E-8
Dehydrin-like protein	*Picea abies*	Desiccation related	4E-9
Dehydrin-like protein	*Picea glauca*	Desiccation related	2E-8
Dehydrin-like protein	*Picea abies*	Desiccation related	1 E-7
Dehydrin-like protein	*Picea abies*	Desiccation related	4E-8
Dehydrin-like protein	*Capsicum annuum*	Desiccation related	5E-10
Desiccation stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	1E-48
Desiccation stress protein DSP-22	*Craterostigma plantagineum*	Desiccation related	2E-48
Desiccation-related protein (CDet11-24)	*Craterostigma plantagineum*	Desiccation related	3E-20
Desiccation-related protein (CDet11-24)	*Craterostigma plantagineum*	Desiccation related	2E-33
Desiccation-related protein (CDet11-24)	*Craterostigma plantagineum*	Desiccation related	1E-46
Desiccation-related protein (CDeT3-06)	*Craterostigma plantagineum*	Desiccation related	1E-45
Desiccation-related protein (CDeT3-06)	*Craterostigma plantagineum*	Desiccation related	1E-45
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	2E-3
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	5E-2
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	2E-3
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	6E-2
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	2E-3
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	1E-1
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	1E-1
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	5E-2
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	3E-2
Desiccation-related protein (CDeT6-19)	*Craterostigma plantagineum*	Desiccation related	3E-2
Late embryogenesis abundant protein	*Catharanthus roseus*	Desiccation related	1E-15
Late embryongenesis abundant protein	*Glycine max*	Desiccation related	8E-37
PTP-1	*Craterostigma plantagineum*	Desiccation related	4E-5
1-cys peroxiredoxin	*Xerophyta viscosa*	Disease / defense	1E-81
Beta-13-glucanase	*Coffea arabica x Coffea canephora*	Disease / defense	1E-73
Beta-13-glucanase	*Coffea arabica x Coffea canephora*	Disease / defense	1E-75
Beta-13-glucanase basic	*Coffea arabica x Coffea canephora*	Disease / defense	9E-51
Beta-13-glucanase basic	*Coffea arabica x Coffea canephora*	Disease / defense	2E-87
Beta-13-glucanase basic	*Coffea arabica x Coffea canephora*	Disease / defense	3E-87
Beta-13-glucanase basic	*Coffea arabica x Coffea canephora*	Disease / defense	2E-87
Disease resistance-responsive family protein	*Arabidopsis thaliana*	Disease / defense	6E-38
DnaJ protein	*Hevea brasilienis*	Disease / defense	6E-94
Endochitinase PR4	*Zea mays*	Disease / defense	1E-76
Heat shock factor	*Boea hygrometrica*	Disease / defense	3E-19
Heat shock factor	*Boea hygrometrica*	Disease / defense	1E-47
Heat shock protein 70 cognate	*Salix gilgiana*	Disease / defense	1E-100
Manganese superoxide dismutase	*Camellia sinensis*	Disease / defense	4E-36
Osmotin	*Piper colubrinum*	Disease / defense	1E-99
Osmotin-like	*Theobroma cacao*	Disease / defense	4E-56
Pathogen-related protein STH-2	*Salvia miltiorrhiza*	Disease / defense	2E-13
Putative laccase	*Rosa hybrid cultivar*	Disease / defense	3E-89
Putative laccase	*Rosa hybrid cultivar*	Disease / defense	1E-86
Putative stress-responsive protein	*Tamarix androssowii*	Disease / defense	3E-6
Small rubber particle protein	*Hevea brasilienis*	Disease / defense	5E-24
Stress responsive protein	*Zea mays*	Disease / defense	4E-43
Thaumatin-like protein	*Actinidia deliciosa*	Disease / defense	6E-75
Beta-amylase 1	*Nicotiana langsdorffii x Nicotiana sanderae*	Energy	1E-105
Dicyanin	*Lycopersicon esculentum*	Energy	8E-29
Monodehydroascorbate reductase putative	*Ricinus communis*	Energy	2E-136
Reticuline oxidase precursor putative	*Ricinus communis*	Energy	1E-93
Reticuline oxidase precursor putative	*Ricinus communis*	Energy	8E-68
Fructose-bisphosphate aldolase-like	*Solanum tuberosum*	Energy	4E-138
NADH dehydrogenase subunit 4L	*Vitis vinifera*	Energy	7E-29
Protein disulfide isomerase-like protein	*Glycine max*	Energy	1E-59
Syntaxin putative	*Ricinus communis*	Intracellular traffic	3E-69
Acetamidase putative	*Ricinus communis*	Metabolism	8E-117

Alpha-amylase	*Plantago major*	Metabolism	1E-85
Aminomethyltransferase	*Arabidopsis thaliana*	Metabolism	3E-46
Anthranilate N-benzoyltransferase protein putative	*Ricinus communis*	Metabolism	2E-73
Basic cellulase	*Citrus sinensis*	Metabolism	4E-55
Beta-amylase	*Prunus armeniaca*	Metabolism	4E-102
Catalytic	*Arabidopsis thaliana*	Metabolism	4E-97
Cystathionine beta-lyase putative	*Ricinus communis*	Metabolism	2E-68
Esterase/lipase/thioesterase family protein	*Arabidopsis thaliana*	Metabolism	2E-47
Esterase/lipase/thioesterase family protein	*Rheum australe*	Metabolism	3E-107
Ethanolamine-phosphate cytidylyltransferase putative	*Ricinus communis*	Metabolism	3E-100
Galactinol synthase 1	*Verbascum phoeniceum*	Metabolism	3E-143
GDH2	*Actinidia chinensis*	Metabolism	9E-128
Glucan endo-13-beta-glucosidase precursor putative	*Ricinus communis*	Metabolism	8E-68
Glutamate decarboxylase	*Nicotiana tabacum*	Metabolism	2E-51
Glutamate dehydrogenase	*Brassica napus*	Metabolism	2E-112
Glycosyl hydrolase family-like protein	*Salvia miltiorrhiza*	Metabolism	7E-32
Lipid transfer protein; glossy1 homolog	*Arabidopsis thaliana*	Metabolism	5E-41
Myo-inositol 1-phosphate synthase	*Sesamum indicum*	Metabolism	3E-79
Myo-inositol 1-phosphate synthase	*Sesamum indicum*	Metabolism	2E-138
Phosphatidylcholine transfer protein putative	*Ricinus communis*	Metabolism	5E-36
Phosphoglycerate dehydrogenase putative	*Ricinus communis*	Metabolism	6E-56
Putative acyl-ACP thioesterase B	*Capsicum annuum*	Metabolism	1E-66
Putative aldo/keto reductase 2	*Salvia miltiorrhiza*	Metabolism	5E-123
Putative esterase	*Nicotiana tabacum*	Metabolism	1E-92
Putative pyruvate dehydrogenase E1 beta subunit	*Capsicum annuum*	Metabolism	1E-81
Pyruvate dehydrogenase2	*Zea mays*	Metabolism	2E-118
UDP-glucose:sesaminol 2'-O-glucoside-O-glucosyltransferase	*Sesamum indicum*	Metabolism	4E-59
UDP-glucuronic acid decarboxylase 2	*Populus tomentosa*	Metabolism	3E-71
26S proteasome AAA-ATPase subunit RPT4a-like protein	*Solanum tuberosum*	Protein destination and storage	6E-159
26S proteasome regulatory subunit rpn1 putative	*Ricinus communis*	Protein destination and storage	1E-142
Cysteine protease	*Aster tripolium*	Protein destination and storage	3E-96
Cysteine protease	*Aster tripolium*	Protein destination and storage	3E-103
EIN3-binding F-box protein 2	*Lycopersicon esculentum*	Protein destination and storage	1E-92
F-box family protein	*Arabidopsis thaliana*	Protein destination and storage	5E-28
F-box family protein	*Populus trichocarpa*	Protein destination and storage	4E-44
Fimbriata-associated protein	*Antirrhinum majus*	Protein destination and storage	8E-65
Precursor of protein cell division protease ftsh-like protein	*Populus trichocarpa*	Protein destination and storage	3E-46
PREDICTED: similar to Zinc finger RING-type	*Vitis vinifera*	Protein destination and storage	4E-21
Proteasome beta subunit	*Petunia x hybrida*	Protein destination and storage	7E-113
Ring finger protein putative	*Ricinus communis*	Protein destination and storage	6E-50
Rnf5 putative	*Ricinus communis*	Protein destination and storage	3E-61
Similar to putative polyubiquitin (UBQ10)	*Vitis vinifera*	Protein destination and storage	1E-32
Subtilisin-like protease	*Solanum lycopersicum*	Protein destination and storage	7E-48
Ubiquitin	*Arabidopsis thaliana*	Protein destination and storage	2E-93
Ubiquitin	*Arabidopsis thaliana*	Protein destination and storage	1E-70
Ubiquitin	*Medicago truncatula*	Protein destination and storage	4E-106
Zinc finger (C3HC4-type RING finger) family protein	*Arabidopsis thaliana*	Protein destination and storage	1E-33
F-box family protein	*Arabidopsis thaliana*	Protein destination and storage	6E-41
Elongation factor 1 gamma-like protein	*Nicotiana tabacum*	Protein synthesis	E-86
Putative ribosome recycling factor	*Arabidopsis thaliana*	Protein synthesis	1E-49
Putative translation initiation factor eIF-1A-like	*Solanum tuberosum*	Protein synthesis	3E-52
Allene oxide cyclase	*Nicotiana tabacum*	Secondary metabolism	1E-78
CYP81B2v2	*Nicotiana tabacum*	Secondary metabolism	1E-99
Gytochrome P450-like TBP protein	*Lilium longiflorum*	Secondary metabolism	6E-48
Glutathione reductase	*Picrorhiza kurrooa*	Secondary metabolism	3E-76
Leucoanthocyanidin dioxygenase putative	*Ricinus communis*	Secondary metabolism	2E-102
14-3-3-like protein	*Helianthus annuus*	Signal transduction	4E-124
Ankyrin protein kinase putative	*Arabidopsis thaliana*	Signal transduction	4E-70
Calcineurin B-like interacting protein kinase	*Solanum lycopersicum*	Signal transduction	1E-130
CBL-interacting serine/threonine-protein kinase putative	*Ricinus communis*	Signal transduction	5E-69
CDPK-related protein kinase	*Gossypium hirsutum*	Signal transduction	9E-53
G protein beta subunit	*Nicotiana plumbaginifolia*	Signal transduction	4E-140
Inositol-tetrakisphosphate 1-kinase putative	*Ricinus communis*	Signal transduction	1E-82
MLO-like protein 17	*Vitis vinifera*	Signal transduction	4E-69
Protein phosphatase 2C	*Lycopersicon esculentum*	Signal transduction	1E-19
Protein phosphatase 2c putative	*Ricinus communis*	Signal transduction	1E-46
Protein phosphatase 2c putative	*Ricinus communis*	Signal transduction	7E-37
Protein phosphatase-2c putative	*Ricinus communis*	Signal transduction	6E-24
Protein phosphatase-related	*Arabidopsis thaliana*	Signal transduction	6E-36
Putative protein kinase	*Arabidopsis thaliana*	Signal transduction	2E-36
Receptor protein kinase CLAVATA1 precursor putative	*Ricinus communis*	Signal transduction	6E-101
Rho-GTPase-activating protein-related	*Arabidopsis thaliana*	Signal transduction	1E-50
Serine/threonine protein phosphatase putative	*Ricinus communis*	Signal transduction	3E-59
Serine-threonine protein kinase plant-type putative	*Ricinus communis*	Signal transduction	2E-32
SOS2-like protein kinase	*Glycine max*	Signal transduction	8E-44
Wall-associated kinase putative	*Ricinus communis*	Signal transduction	3E-108

WD-40 repeat family protein	*Arabidopsis thaliana*	Signal transduction	1E-80
ANAC017; transcription factor	*Arabidopsis thaliana*	Transcription	4E-42
AREB-like protein	*Lycopersicon esculentum*	Transcription	3E-15
BZIP1 protein	*Craterostigma plantagineum*	Transcription	3E-58
DEAD BOX RNA helicase RH15 [Arabidopsis thaliana]	*Arabidopsis thaliana*	Transcription	2E-101
DNA binding / transcription factor	*Arabidopsis thaliana*	Transcription	4E-20
ERF4_TOBAC Ethylene-responsive transcription factor 4	*Nicotiana tabacum*	Transcription	8E-24
Ethylene-responsive transcription factor 3	*Nicotiana tabacum*	Transcription	5E-26
FUS-interacting serine-arginine-rich protein 1 putative	*Ricinus communis*	Transcription	5E-33
Histone H1.1 putative	*Ricinus communis*	Transcription	2E-15
I-box binding factor	*Lycopersicon esculentum*	Transcription	6E-63
NAC domain-containing protein putative	*Ricinus communis*	Transcription	6E-74
Putative glycine-rich RNA binding protein 3	*Catharanthus roseus*	Transcription	1E-32
SIN3 component histone deacetylase complex	*Populus trichocarpa*	Transcription	2E-49
Splicing endonuclease positive effector sen1 putative	*Ricinus communis*	Transcription	7E-138
Transcription factor putative	*Ricinus communis*	Transcription	2E-4
Transcription initiation factor ia putative	*Ricinus communis*	Transcription	5E-44
Transducin family protein	*Arabidopsis thaliana*	Transcription	4E-109
Zinc-finger DNA binding protein	*Petunia x hybrida*	Transcription	2E-27
ABC transporter family protein	*Populus trichocarpa*	Transporters	9E-83
ABC transporter homolog	*Populus nigra*	Transporters	1E-118
ADPATP carrier protein putative	*Ricinus communis*	Transporters	6E-97
Atpob1 putative	*Ricinus communis*	Transporters	3E-96
Carbohydrate transporter putative	*Ricinus communis*	Transporters	1E-72
GDP-mannose transporter	*Arabidopsis thaliana*	Transporters	1E-21
Glucose-6-phosphate/phosphate translocator 1	*Ricinus communis*	Transporters	2E-122
Hexose transporter pGlT	*Olea europaea*	Transporters	1E-118
Mitochondrial phosphate transporter	*Glycine max*	Transporters	3E-28
Putative ADPATP carrier-like protein	*Trifolium pratense*	Transporters	4E-68
Ara4-interacting protein putative	*Ricinus communis*	Unclear classification	
ATP binding	*Arabidopsis thaliana*	Unclear classification	
Conserved hypothetical protein	*Ricinus communis*	Unclear classification	
Hypothetical protein	*Vitis vinifera*	Unclear classification	
Hypothetical protein	*Ricinus communis*	Unclear classification	
Hypothetical protein	*Vitis vinifera*	Unclear classification	
Iron-sulfer cluster scaffold protein NFS1	*Eucalyptus grandis*	Unclear classification	
Predicted protein	*Populus trichocarpa*	Unclear classification	
Predicted protein	*Populus trichocarpa*	Unclear classification	
UP-9A	*Arabidopsis thaliana*	Unclear classification	
Unknown protein	*Arabidopsis thaliana*	Unclear classification	
Unknown protein	*Arabidopsis thaliana*	Unclear classification	
Unknown protein	*Arabidopsis thaliana*	Unclear classification	
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Unknown protein	*Arabidopsis thaliana*	Unclear classification	
Unknown protein	*Arabidopsis thaliana*	Unclear classification	
Unknown protein	*Arabidopsis thaliana*	Unclear classification	
Unknown protein	*Vitis vinifera*	Unclear classification	
Unnamed protein product	*Nicotiana tabacum*	Unclear classification	
No match		Unclassified	
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5.3. Protein alignment analysis

A

Protein	Length(aa)	Protein	Length(aa)	Identity (%)
Cp LEA 6-19	155	*Lb* LEA 6-19	144	68
Cp LEA 6-19	155	*Ls* LEA 6-19	185	49
Lb LEA 6-19	144	*Ls* LEA 6-19	185	48

B

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Cp LEA 6-19     MAQFGGEKYGGRHTDEYGNPIQQGAGAHRGGGIMGGGQQ--AGQHGTTGVLGHGTAGQ-- 56
Lb LEA 6-19     MAQFGGEKYGGRQTDEFGNPIHHGAGEH--GGILGGQQ------HGATTGLGHGAT---- 48
Ls LEA 6-19     MASYQ-NRPGGQATDEYGNPIQQQYDEY--GNPMGGGGYGTGGGGGATGGQGYGTGGQGY 57
                **       **  *** ****          *   **          * *   * *

Cp LEA 6-19     ------HGTTGGGLGHGT----AGTGGAL-----GGQHRRSG---------SSSSSSSSE 92
Lb LEA 6-19     -------GATG--IGHGT----TATGGGLT----GGQHRRSG---------SSSSSSSSE 82
Ls LEA 6-19     GSGGQGYGTGGQGYGTGTGTEGFGTGGGARHHGQEQLHKESGGGLGGMLHRSGSGSSSSS 117
                       *  *   * **      ***          *  **          * * ****

Cp LEA 6-19     SDGEGGRRKKGMKDKMKEKLP----GGHGTTTDQQQYG-TAATHGQA----QQHEKKGIM 143
Lb LEA 6-19     DDGEGGRRKKGMKDKIKEKMPAAAAGGNGNTTATQDH-----QYGQG----GHEEKKGIM 133
Ls LEA 6-19     DDGQGGRRKKGITQKIKEKLP----GHHDQSGQAQAMGGMGSGYDAGGYGGEHHEKKGMM 173
                 ** *******   * *** *    *        *                   **** *

Cp LEA 6-19     DKIKEKLPGGQH 155
Lb LEA 6-19     DKIKEKLPGGH- 144
Ls LEA 6-19     DKIKEKLPGGGR 185
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Figure S1. Protein sequence alignments between the *C. plantagineum* LEA 6-19 protein (*Cp* LEA 6-19), *L. brevidens* LEA 6-19 protein (*Lb* LEA 6-19) and *L. subracemosa* LEA 6-19 protein (*Ls* LEA 6-19). (A) Amino acid sequence identity between the LEA 6-19 proteins. (B) Alignment of LEA 6-19 protein sequences. Asterisks indicate amino acids which are identical between all three LEA 6-19 proteins.

A

Protein	Length(aa)	Protein	Length(aa)	Identity (%)
Cp LEA 3-06	201	*Lb* LEA 3-06	193	67
Cp LEA 3-06	201	*Ls* LEA 3-06	248	49
Lb LEA 3-06	193	Ls LEA 3-06	248	56

B

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Cp LEA 3-06     MEAMSFAARSTVLSISKS-FPKNNSPTYLTLRPKFSRVRFTTVASQSQGRQQ-------- 51
Lb LEA 3-06     MEAMSFVRSPVVLNLSKS-LPRNQSPTYLNLRSKPSRVRFISAASQSEGRQQ-------- 51
Ls LEA 3-06     MEAMIFAKS-TVLNLSKSSFPKNN-PTYLNLRPKFSKVRFTSAGSHPQGQRRQATSRDYA 58
                **** *     **  ***  * *  **** ** * * ***    *   *

Cp LEA 3-06     --------------------VSENAEDAKKKFSETTDSLKHKTSEATDSASHKANG---- 87
Lb LEA 3-06     --------------------VSENTEQAKQKFSDVTNSVKHKTNEATDTASDKAH----- 86
Ls LEA 3-06     SFSTDDSNKRRQAMNQVIDRASENAVEAKRKASDAAESMKHKTSEATPTASEKAKDTAHE 118
                                  ***   ** * *    * **** ***  ** **

Cp LEA 3-06     -------AARETNDKAKETYNAASGKAGELKDKTQEGAENVREKAMDAGNDAMEKTRNAG 140
Lb LEA 3-06     ----------EGNAKAKQTYDDASGKANELKDKTQEGMENAREKTMEAGQEAKENTKAAG 136
Ls LEA 3-06     MDEKAKDTAHEVNEKAKQTYGAVSEKAGDMKEKTKEGMENVKEKTMETGYEAKEKTKAAG 178
                         * * *** **   * **   * ** ** **  ** *  *  * * *  **

Cp LEA 3-06     ERVADGVSNVGQNVKENVMGAGEKVKEFAEDVKDTVMGKSEEVKNQAE-----HETK--- 192
Lb LEA 3-06     EKVADGVGNLGEKAKETVMGAGEKVKETAENIKDTVVGKSEDVKHDVE-----HESK--- 188
Ls LEA 3-06     QTVAETVEGIGEKAKETVKGAWDKAKETTGKIKETVVGKSEDVKHDADNLVEDHVRKPMR 238
                  **  *   *   ** * **  * **     * ** **** **         *  *

Cp LEA 3-06     --KRSTSTNYF 201
Lb LEA 3-06     --KRDWN---- 193
Ls LEA 3-06     EAENESKKKY- 248
```

Figure S2. Protein sequence alignments between the *C. plantagineum* LEA 3-06 protein (*Cp* LEA 3-06), *L. brevidens* LEA 3-06 protein (*Lb* LEA 3-06) and *L. subracemosa* LEA 3-06 protein (*Ls* LEA 3-06). (A) Amino acid sequence identity between the LEA 3-06 proteins. (B) Alignment of LEA 3-06 protein sequences. Asterisks indicate amino acids which are identical between all three LEA 3-06 proteins.

A

Protein	Length(aa)	Protein	Length(aa)	Identity (%)
Cp DSP22	199	*Lb* DSP22	202	71
Cp DSP22	199	*Ls* DSP22	193	56
Lb DSP22	202	*Ls* DSP22	193	53

B

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Cp DSP22          MASSTCYATIPAMSCRGQSTITRFGPNNLFLGKQSYELPLMRRNAKFTVRSMREDN---E 57
Lb DSP22          MSSATCYASSVMPSALVTSCGGRSSTTRLFGGRPNNLLPLKRNNSKFTLRSMKEDLGEKM 60
Ls DSP22          -MAASCNAMLSVSCGINQTTLTRVGPKNMLFGPSSRALPLRR-VAKFTVRSMSEEE---E 55
                       * *              *        *     *** *   *** *** *

Cp DSP22          KEEQQQQKQQQTHDGGPDLTPNRTEVTTKRTVDLFSFDGLAPERINGRSAMIGFVAAVGV 117
Lb DSP22          KEELLGANKEQSHDGGPGLTPNRTEVKTKRTTDLFSFDGFAPERINGRSAMIGFVAAIAV 120
Ls DSP22          KEKVAGSGRS---DPLPKPSPPPKPKATTSFTDVFSFSGPAPERINGRLAMIGFAAAVAV 112
                  **           *  *   *      *    * *** * ******** ***** **  *

Cp DSP22          ELATGRDVFSQVFNGGVMWFLLTSAVLVLATLIPIYRGLSPEAKNNGFWNSDAEIWNGRF 177
Lb DSP22          ELASGEDVLSQVFNGGIKWFLLTSAILSLATLIPIYRGLSPESKNNGFWTSDAEIWNGRA 180
Ls DSP22          ELASGQDLLSQVQNGGVQWFLGTSALLSVASLIPIFRGVAVESKSDGFMTSDAELWNGRF 172
                  *** * *  *** ***  *** *** *  * **** **   * *  **  **** ****

Cp DSP22          AMIGLVALAFTEYVKGGPLINV 199
Lb DSP22          AMIGLVALAFTEYVKGGPLINV 202
Ls DSP22          AMIGLVALAFTEYLKGSALIV- 193
                  ************* **  **
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Figure S3. Protein sequence alignments between the *C. plantagineum* DSP22 protein (*Cp* DSP22), *L. brevidens* DSP22 protein (*Lb* DSP22) and *L. subracemosa* DSP22 protein (*Ls* DSP22). (A) Amino acid sequence identity between the DSP22 proteins. (B) Alignment of DSP22 protein sequences. Asterisks indicate amino acids which are identical between all three DSP22 proteins.

A

Protein	Length(aa)	Protein	Length(aa)	Identity (%)
Cp LEA 27-45	151	*Lb* LEA 27-45	151	93

B

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Cp LEA 27-45      MAQLMNKAKNFVAEKVANVEKPKASVEDVDLKDVGRHGITYLTRICVENPYSASIPVGEI 60
Lb LEA 27-45      MAQLVEKAKNFVAEKVANVEKPKASVDDVDLKDVGRHGITYLAKICVENPYGASIPVGEI 60
                  ****  ****************** ***************  ******* ********

Cp LEA 27-45      KYTLKSAGRVIVSGNIPDPGSLKGNDKTMLEPAIKVPHSALVSLIKDIGADMDIDYVLEL 120
Lb LEA 27-45      KYTLKSAGRVIVSGNIADPGSLKGNDKTMLEPAIKVPHSALVSLIKDVGADMDIDYVLEL 120
                  *************** ***************************** ************

Cp LEA 27-45      GLVVDLPVIGNFTIPLSHKGEMKLPGLSDIF 151
Lb LEA 27-45      GLVVDLPVIGNFTIPLSHKGEMKLPTFSDIF 151
                  ************************  ****
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Figure S4. Protein sequence alignments between the *C. plantagineum* LEA 27-45 protein (*Cp* LEA 27-45) and the *L. brevidens* LEA 27-45 protein (*Lb* LEA 27-45). (A) Amino acid sequence identity between the LEA 27-45 proteins. (B) Alignment of LEA 27-45 protein sequences. Asterisks indicate amino acids which are identical between the two LEA 27-45 proteins.

A

Protein	Length(aa)	Protein	Length(aa)	Identity (%)
Cp LEA-like 11-24	422	*Lb* LEA-like 11-24	449	46
Cp LEA-like 11-24	422	*Ls* LEA-like 11-24	329	38
Lb LEA-like 11-24	449	*Ls* LEA-like 11-24	329	44

B

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Cp LEA-like 11-24  MESQLHRPTEQ----------EVMEGQTADHGEKKSMLAKVKEKAKKLKGSINKKHGSSQ 50
Lb LEA-like 11-24  MESQMHRPVEQ----------ETVEGQ-GEQGGKMSVLKKVKEKAKKLKGSIKKHGSNSG 49
Ls LEA-like 11-24  MESQMHRPSEQEHPQHVASDNEMVEDQ-AEHGEKKSMLKKVKDKAKKIKGTIKKHGLGQD 59
                   **** *** **          *  * *    * * * * *** **** ** * *

Cp LEA-like 11-24  DDDADNDEEINTSPAVHGAP---------GTSPPPPTQGGEYGGLSE-RDVNIPHPLAST 100
Lb LEA-like 11-24  QEEDGSDEEMDTSPAVHGAP---------GMTPPP-TQGGDLEKPAD-AGG-----FTSL 93
Ls LEA-like 11-24  QE----DEEIKTNPAVHGPPPGRVPPVITGTNPPPMQGGFNLEKPTDTREDRYDHHSDNV 115
                         ***  * ***** *         *  ***   *

Cp LEA-like 11-24  QANLD---------KPADVTDASRELQVPPPVP-------ETTPEVSDKG--------LT 136
Lb LEA-like 11-24  KGDGG---------VDTPPQAAEREFNFPKHED-------VSPTDYQTR---------- 126
Ls LEA-like 11-24  KGGGGEYTSLREKGVTSPPQDMETEFNFPKPEKNEPEMKNITKPDVKTETSDITPSDYQT 175
                                  *   *

Cp LEA-like 11-24  EDLGSNAGQGVKESDVDSLTQGLKGVNYGGDDSNPLSGQEHQTISDE------PKSLPGQ 190
Lb LEA-like 11-24  -DVDS-SGQ-----DVGSLTQGLKDMNVGGDESKAVP-----EVQEQ------PRSTPAA 168
Ls LEA-like 11-24  RDVDS-TGQGVKEADVGSLTQGLKKVSVG-DESKPLPGEEQPPSYSGSHGQFAPQSTP-- 231
                    *  *  **     ** *******    * * *                    * * *

Cp LEA-like 11-24  GND--LPQSHPS-SEDEPKKFDANDQPQSMPQDT-ITGKLSSVPAVIIDRAAAAKNVVAS 246
Lb LEA-like 11-24  AEETHLPQSHPVPAEDEPKKYDPN-RPDSTPQDTTYIGKITSVPAVIVDKAAAAKNVVAS 227
Ls LEA-like 11-24  TKESLLPQSHPL-PQDEPKRYDPN-HPDSMPXDT-ITGKISSXSAVIVDKAAXAXNVXAX 288
                        ******    ****  * *  * * * **   **  *  *** * ** * ** *

Cp LEA-like 11-24  KLGYGG-----------------------------------------------SQAQES-- 258
Lb LEA-like 11-24  KLGYGANNQAQEPTTTPDVVGGGGAATQQKKPLTETAAEYKNMVAEKLGYGASKAQESVD 287
Ls LEA-like 11-24  KXGYGGX----------------------------------------------TQNLXT-- 301
                   * ***

Cp LEA-like 11-24  -AADAGAAQQKKPLTETAAEYKNLVAEKLTPVYEKVAGAGSTVTSKVWGSGGTTAGEQTQ 317
Lb LEA-like 11-24  VGGDGGATQQKKPLTETAAEYKNMVAEKLAPVYGKVSGAGTGVISRVRGTG---AGEQTQ 344
Ls LEA-like 11-24  ------VXLTKKXFTXXPXNXK--------PGCGKXSXPGAP------------------ 329
                           **  *      *        *   *    *

Cp LEA-like 11-24  GGEGVVDGGGAASNKGVFTKDYLSEKLKPGDEDKALSQAIMEKLQLS-KKPAVEGGAGDE 376
Lb LEA-like 11-24  GGDEAAKG------------SYLSEKLKPGDEDKALSKAITEKLQLTGNKPTAD----ES 388
Ls LEA-like 11-24  ------------------------------------------------------------

Cp LEA-like 11-24  TKASESSPGVVGTIKGAVGSLIGG----GNKSSGAESAAAADEQTQALGE---------- 422
Lb LEA-like 11-24  KAATEASPGVVGSIKGVVGSLLGGKSNNGSESAGGEQPQSLGSEGIAAREGADVVEPTAE 448
Ls LEA-like 11-24  ------------------------------------------------------------


Cp LEA-like 11-24  -
Lb LEA-like 11-24  Q 449
Ls LEA-like 11-24  -
```

Figure S5. Protein sequence alignments between the *C. plantagineum* LEA-like 11-24 protein (*Cp* LEA-like 11-24), *L. brevidens* LEA-like 11-24 protein (*Lb* LEA-like 11-24) and *L. subracemosa* LEA-like 11-24 protein (*Ls* LEA-like 11-24). (A) Amino acid sequence identity between the LEA-like 11-24 proteins. (B) Alignment of LEA-like 11-24 protein sequences. Asterisks indicate amino acids which are identical between all three LEA-like 11-24 proteins.

5.4. *Cis*-acting regulatory elements in promoter fragments

Table S3. Putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression within *LEA 6-19* promoter sequences regions from *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

Promoter	*Cis*-element	Sequence	Position	Reference
Cp LEA 6-19 (971 bp)	ABRELATERD1	ACGTG	-174, -213, -387, -430	Simpson *et al.*, 2003
	ACGTATERD1	ACGT	-387, -629	Simpson *et al.*, 2003
	MYCCONSENSUSAT	CANNTG	-16, -34, -65, -124, -213, -280, -933, -942, -954	Abe *et al.*, 2003
	DRE/CRT	RCCGAC	-235	Dubouzet *et al.*, 2003
	DPBFCOREDCDC3	ACACNNG	-388, -427, -648, -590	Kim *et al.*, 1997
Lb LEA 6-19 (1029 bp)	ABRELATERD1	ACGTG	-136, -178, -399, -476	Simpson *et al.*, 2003
	ACGTATERD1	ACGT	-505	Simpson *et al.*, 2003
	MYBCORE	CNGTTR	-248, -674	Luscher and Eiseman, 1990
	MYCCONSENSUSAT	CANNTG	-178, -248, -316, -340, -476, -677, -702, -751	Abe *et al.*, 2003
	DRE/CRT	RCCGAC	-197	Dubouzet *et al.*, 2003
	DPBFCOREDCDC3	ACACNNG	-476	Kim *et al.*, 1997
Ls LEA 6-19 (931 bp)	ABRELATERD1	ACGTG	-207, -248, -354, -547, -775	Simpson *et al.*, 2003
	ACGTATERD1	ACGT	-655	Simpson *et al.*, 2003
	MYBCORE	CNGTTR	-127, -319	Luscher and Eiseman, 2000
	MYCCONSENSUSAT	CANNTG	-130, -247, -353, -370, -497, -722, -745	Abe *et al.*, 2003
	DRE2COREZMRAB17	ACCGAC	-311	Busk *et al.*, 1997
	DRE/CRT	RCCGAC	-264	Dubouzet *et al.*, 2003
	DPBFCOREDCDC3	ACACNNG	-247, -352, -376, -544, -721	Kim *et al.*, 1997

These c*is*-acting regulatory elements were identified using the PLACE database. Table shows: sequence motif (where N is any nucleotide and R is adenine or guanine), position and reference.

Table S4. Putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression within *LEA-like 11-24* promoter sequences from *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

Promoter	*Cis*-element	Sequence	Position	Reference
Cp LEA-like 11-24	ACGTCBOX	GACGTC	-517	Foster *et al.*, 1994
(1613 bp)	ABRELATERD1	ACGTG	-179, -202, -253, -544, -577, 790, -1405	Simpson *et al.*, 2003
	ACGTATERD1	ACGT	-367, -569, -746, -854	Simpson *et al.*, 2003
	MYBCORE	CNGTTR	-813	Luscher and Eiseman, 2000
	MYCCONSENSUSAT	CANNTG	-123, -201, -228, -543, -576, -875	Abe *et al.*, 2003
	DRE2COREZMRAB17	ACCGAC	-223	Busk *et al.*, 1997
	DPBFCOREDCDC3	ACACNNG	-200, -227, -123, -543, -1348	Kim *et al.*, 1997
Lb LEA-like 11-24	ABRELATERD1	ACGTG	-215, -241, -264, -684, -923, -1272	Simpson *et al.*, 2003
(1415 bp)	ACGTATERD1	ACGT	-988, -1221, -1243, -1263	Simpson *et al.*, 2003
	ABREDISTBBNNAPA	GCCACTTGTC	-136	Ezcurra *et al.*, 1999
	MYBCORE	CNGTTR	-320, -613	Luscher and Eiseman, 2000
	MYCATRD22	CACATG	-276, -1363	Abe *et al.*, 1997
	MYCCONSENSUSAT	CANNTG	-138, -197, -214, -523, -683, -876	Abe *et al.*, 2003
	DPBFCOREDCDC3	ACACNNG	-213, -275, -682	Kim *et al.*, 1997
Ls LEA-like 11-24	ACGTCBOX	GACGTC	-377	Foster *et al.*, 1994
(500 bp)	ABRELATERD1	ACGTG	-178, -228, -433	Simpson *et al.*, 2003
	ABREDISTBBNNAPA	GCCACTTGTC	-101	Ezcurra *et al.*, 1999
	MYCCONSENSUSAT	CANNTG	-103, -117, -160, -177, -204, -432	Abe *et al.*, 2003
	DRE/CRT	RCCGAC	-199, -224	Dubouzet *et al.*, 2003
	DPBFCOREDCDC3	ACACNNG	-176, -203, -431	Kim *et al.*, 1997

These c*is*-acting regulatory elements were identified using the PLACE database. Table shows: sequence motif (where N is any nucleotide and R is adenine or guanine), position and reference.

Table S5. Putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression within *LEA 3-06* promoter sequences from *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

Promoter	*Cis*-element	Sequence	Position	Reference
Cp LEA 3-06	ABRELATERD1	ACGTG	-171, -197	Simpson *et al.*, 2003
(1310 bp)	ACGTATERD1	ACGT	-174, -1142	Simpson *et al.*, 2003
	MYBCORE	CNGTTR	-53, -148, -217, -600, -645	Luscher and Eiseman, 1990
	MYCCONSENSUSAT	CANNTG	-145, -247, -270, -303, -615	Abe *et al.*, 2003
	DRE1COREZMRAB17	ACCGAGA	-1032	Busk *et al.*, 1997
	DPBFCOREDCDC3	ACACNNG	-144, -246, -615	Kim *et al.*, 1997
Lb LEA 3-06	ABRELATERD1	ACGTG	-150, -233, -245, -311	Simpson *et al.*, 2003
(893 bp)	ACGTATERD1	ACGT	-612, -853	Simpson *et al.*, 2003
	MYBATRD22	CTAACCA	-248	Abe *et al.*, 1997
	MYBCORE	CNGTTR	-53	Luscher and Eiseman, 2000
	MYCCONSENSUSAT	CANNTG	-244, -267, -414	Abe et al., 2003
	DPBFCOREDCDC3	ACACNNG	-243, -269	Kim *et al.*, 1997
Ls LEA 3-06	ABRELATERD1	ACGTG	-128, -160, -244	Simpson *et al.*, 2003
(589 bp)	ACGTATERD1	ACGT	-298	Simpson *et al.*, 2003
	MYBATRD22	CTAACCA	-247	Abe *et al.*, 1997
	MYBCORE	CNGTTR	-380, -486, -583	Luscher and Eiseman, 2000
	MYCCONSENSUSAT	CANNTG	-159, -243, -267	Abe *et al.*, 2003
	DPBFCOREDCDC3	ACACNNG	-158, -242	Kim *et al.*, 1997

These c*is*-acting regulatory elements were identified using the PLACE database. Table shows: sequence motif (where N is any nucleotide and R is adenine or guanine), position and reference.

Table S6. Putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression within *DSP22* promoter sequences from *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

Promoter	*Cis*-element	Sequence	Position	Reference
Cp DSP22	ABRELATERD1	ACGTG	-171, -197	Simpson *et al.*, 2003
(672 bp)	ACGTATERD1	ACGT	-258, -511	Simpson *et al.*, 2003
	MYCATRD22	CACATG	-309	Abe *et al.*, 1997
	MYBCORE	CNGTTR	-625	Luscher and Eiseman 1990
	MYCCONSENSUSAT	CANNTG	-196, -490	Abe *et al.*, 2003
	DRE2COREZMRAB17	ACCGAC	-466	Busk *et al.*, 1997
	DPBFCOREDCDC3	ACACNNG	-309, -357, -541	Kim *et al.*, 1997
Lb DSP22	ABRELATERD1	ACGTG	-196	Simpson *et al.*, 2003
(671 bp)	ACGTATERD1	ACGT	-381, -583	Simpson *et al.*, 2003
	MYCATRD22	CACATG	-314, -647	Abe *et al.*, 1997
	MYBCORE	CNGTTR	-18, -486	Luscher and Eiseman, 2000
	MYCCONSENSUSAT	CANNTG	-195, -275, -424, -506	Abe et al., 2003
	DPBFCOREDCDC3	ACACNNG	-194, -314, -468	Kim *et al.*, 1997
Ls DSP22	ABRELATERD1	ACGTG	-204, -240, -395, -198	Simpson *et al.*, 2003
(980 bp)				
	ACGTATERD1	ACGT	-655	Simpson *et al.*, 2003
	MYBCORE	CNGTTR	-217, -854	Luscher and Eiseman, 2000
	MYCCONSENSUSAT	CANNTG	-240	Abe et al., 2003
	DPBFCOREDCDC3	ACACNNG	-239, -396	Kim *et al.*, 1997

These c*is*-acting regulatory elements were identified using the PLACE database. Table shows: sequence motif (where N is any nucleotide and R is A or G), position and reference

Table S7. Putative *cis*-acting regulatory elements associated with ABA- and dehydration-mediated gene expression within *LEA 27-45* promoter sequences from *C. plantagineum* and *L. brevidens*.

Promoter	*Cis*-element	Sequence	Position	Reference
Cp LEA 27-45 (629 bp)	ABRELATERD1	ACGTG	-145, -158, -284, -329	Simpson *et al.*, 2003
	MYBCORE	CNGTTR	-428	Luscher and Eiseman 1990
	MYCCONSENSUSAT	CANNTG	-144, -480, -526	Abe *et al.*, 2003
	DRE2COREZMRAB17	ACCGAC	-48, -207, -426	Busk *et al.*, 1997
	DPBFCOREDCDC3	ACACNNG	-142	Kim *et al.*, 1997
Lb LEA 27-45 (1046 bp)	ABRELATERD1	ACGTG	-136, -149, -297, -321, -411	Simpson *et al.*, 2003
	ACGTATERD1	ACGT	-856	Simpson *et al.*, 2003
	MYCATRD22	CACATG	-372	Abe *et al.*, 1997
	MYBCORE	CNGTTR	-15, -43	Luscher and Eiseman, 2000
	MYCCONSENSUSAT	CANNTG	-135, -550	Abe *et al.*, 2003
	DRE2COREZMRAB17	ACCGAC	-212	Busk *et al.*, 1997
	DRE/CRT	(A/G)CCGAC	-269	Dubouzet *et al.*, 2003
	DPBFCOREDCDC3	ACACNNG	-134, -371, -442	Kim *et al.*, 1997

These c*is*-acting regulatory elements were identified using the PLACE database. Table shows: sequence motif (where N is any nucleotide and R is A or G), position and reference.

5.5. Alignment of *LEA 27-45* promoters regions from *C. plantagineum* and *L. brevidens*

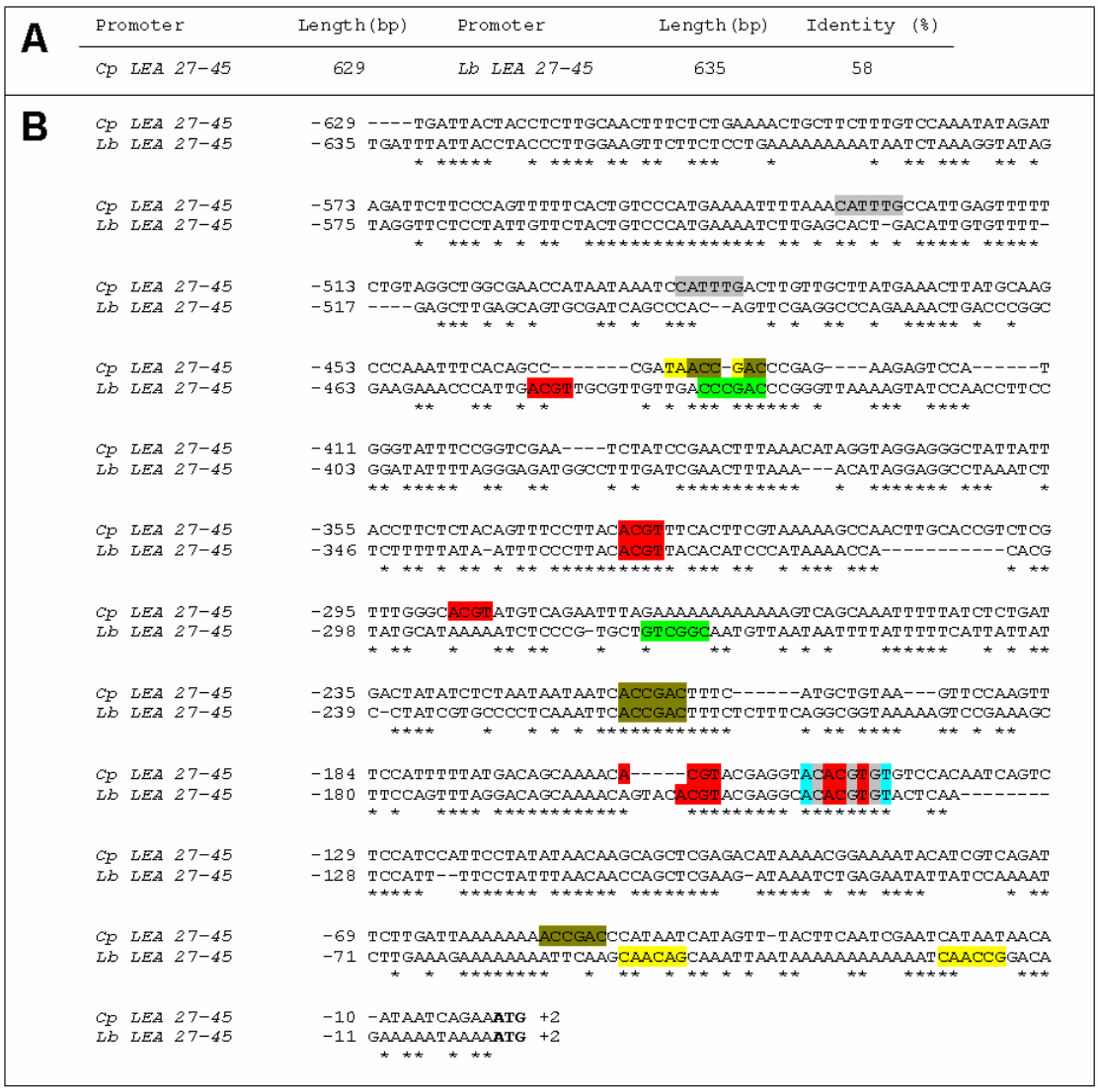

Figure S6. Sequence alignments between the 629 bp *LEA 27-45* promoter region from *C. plantagineum* and the 635 bp *LEA 27-45* promoter region from *L. brevidens*, lacking the DNA insertion sequence. (A) Nucleotide sequence identity between promoter regions.
(B) Alignment data showing identities and putative *cis*-acting regulatory elements involved in ABA- and dehydration-responsive gene expression. Asterisks indicate nucleotides which are identical between all three promoter regions. Putative *cis*-acting regulatory elements are displayed in colored boxes: ACGT-containing ABRE elements are highlighted in red boxes, MYCATRD22 motif (CACATG) is displayed in purple, MYCCONSENSUSAT sequences (CANNTG) are shown in grey boxes, MYBCORE sequence (CNGTTR) is displayed in yellow, DPBF-core sequences (ACACNNG) are shown in blue boxes, DRE2COREZMRAB17 element (ACCGAC) is indicated in dark yellow and DRE/CRT motifs (RCCGAC) are displayed in green, respectively. Overlapping *cis*-regulatory elements are indicated with mixed colored boxes. The ATG start codons are shown in bold characters.

5.6. Results from transient expression assays

Tables S8 to S21 show the number of GFP expressing cells and GUS spots obtained for each transformation. Transformed leaves were treated with 100 µM ABA, 0.8 M mannitol or with water. For each treatment either seven or four transformations were performed. For each treatment, the average values of *LEA-like 11-24* promoter activity in transient expression assays was compared to the average activity of the CaMV 35S promoter (% GUS spots / GFP spots).

Table S8. Results of biolistic transformations of *Cp LEA-like 11-24* promoter::*GUS* and CaMV35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Transformation	Number of GFP spots	Number of GUS spots
1	24	14
2	194	198
3	264	197
4	129	90
5	64	44
6	69	29
7	70	33
Treatment: 0.8 M mannitol		
Transformation	Number of GFP spots	Number of GUS spots
1	61	66
2	30	34
3	10	3
4	50	25
5	182	119
6	55	47
7	106	128
Treatment: H_2O		
Transformation	Number of GFP spots	Number of GUS spots
1	67	0
2	21	0
3	25	0
4	11	0
5	31	0
6	3	0
7	72	1
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	66,10	19,94
mannitol	81,87	34,64
H_2O	0,20	0,52

Table S9. Results of biolistic transformations of *Lb LEA-like 11-24* promoter::*GUS* and CaMV 35S::*GFP* constructs into *L. brevidens* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	77	16
2	113	24
3	21	4
4	24	13
5	162	26
6	554	186
7	172	78
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	81	7
2	16	1
3	28	7
4	67	12
5	314	112
6	63	1
7	32	12
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	36	0
2	90	1
3	57	0
4	191	0
5	97	0
6	183	1
7	78	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	30,03	14,77
mannitol	18,94	14,32
H_2O	0,16	0,42

Table S10. Results of biolistic transformations of *Ls LEA-like 11-24* promoter::*GUS* and CaMV 35S::*GFP* constructs into *L. subracemosa* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	100	1
2	178	2
3	324	24
4	116	5
5	467	69
6	73	0
7	31	0
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	40	0
2	69	0
3	39	2
4	102	15
5	144	8
6	101	9
7	56	5
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	116	0
2	59	7
3	35	1
4	34	0
5	42	0
6	78	0
7	436	4
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	4,09	5,43
mannitol	6,18	5,25
H_2O	2,66	3,99

Table S11. Results of biolistic transformations of *Lb LEA-like 11-24* mutation 1 promoter::*GUS* and CaMV 35S::*GFP* constructs into *L. brevidens* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	296	65
2	69	9
3	123	57
4	134	21
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	153	19
2	105	3
3	83	4
4	92	10
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	81	0
2	47	0
3	109	0
4	113	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	24,25	15,19
mannitol	7,74	4,62
H_2O	0	0

Table S12. Results of biolistic transformations of *Lb LEA-like 11-24* mutation 2 promoter::*GUS* and CaMV 35S::*GFP* constructs into *L. brevidens* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	338	2
2	128	11
3	60	0
4	108	7
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	92	1
2	29	0
3	45	2
4	89	0
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	169	0
2	86	0
3	94	0
4	75	2
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	3,92	5,46
mannitol	1,38	2,10
H_2O	0,67	1,19

Table S13. Results of biolistic transformations of *Cp LEA-like 11-24* mutation 1 promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	71	71
2	33	16
3	137	70
4	36	36
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	104	108
2	45	39
3	51	58
4	40	26
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	40	2
2	41	2
3	37	0
4	60	3
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	74,89	29,01
mannitol	94,71	23,53
H_2O	3,72	2,48

Table S14. Results of biolistic transformations of *Cp LEA-like 11-24* mutation 2 promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	142	91
2	353	127
3	64	18
4	91	36
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	49	19
2	165	125
3	167	62
4	229	162
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	44	0
2	16	0
3	46	1
4	120	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	41,94	15,52
mannitol	55,60	20,49
H_2O	0,54	1,09

Table S15. Results of biolistic transformations of *Cp LEA-like 11-24* mutation 3 promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	126	75
2	195	31
3	47	32
4	38	18
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	106	59
2	213	73
3	78	27
4	193	173
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	20	0
2	61	0
3	13	0
4	58	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	47,72	22,85
mannitol	53,55	26,06
H_2O	0	0

Table S16. Results of biolistic transformations of *Cp LEA-like 11-24* mutations 1 and 2 promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	205	60
2	233	171
3	133	85
4	37	11
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	188	163
2	89	28
3	50	19
4	115	35
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	18	0
2	14	0
3	13	1
4	29	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	49,07	22,93
mannitol	46,65	26,91
H_2O	1,92	3,85

Table S17. Results of biolistic transformations of *Cp LEA-like 11-24* mutations 1 and 3 promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	26	21
2	62	49
3	25	13
4	58	30
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	52	32
2	30	10
3	71	36
4	151	144
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	110	0
2	36	0
3	64	1
4	157	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	65,88	16,20
mannitol	60,24	26,14
H_2O	0,39	0,78

Table S18. Results of biolistic transformations of *Cp LEA-like 11-24* mutations 2 and 3 promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	66	10
2	56	21
3	171	86
4	111	22
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	59	34
2	87	60
3	97	44
4	102	49
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	45	0
2	23	0
3	13	0
4	28	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	30,69	16,23
mannitol	55,00	10,70
H_2O	0	0

Table S19. Results of biolistic transformations of *Cp LEA-like 11-24* mutations 1 & 2 & 3 promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	66	7
2	97	3
3	148	29
4	78	13
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	124	61
2	236	97
3	87	14
4	126	48
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	33	0
2	12	0
3	12	0
4	20	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	12,49	7,30
mannitol	36,12	14,15
H_2O	0	0

Table S20. Results of biolistic transformations of *Cp LEA-like 11-24* mutation DRE promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	20	6
2	28	18
3	50	9
4	19	7
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	77	33
2	222	33
3	46	9
4	83	24
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	26	0
2	14	0
3	17	0
4	58	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	37,28	19,61
mannitol	26,55	12,34
H_2O	0	0

Table S21. Results of biolistic transformations of *Cp LEA-like 11-24* mutations 1 & 2 & 3 & DRE promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	51	1
2	82	0
3	41	0
4	27	0
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	64	0
2	107	0
3	36	0
4	17	0
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	23	0
2	35	0
3	21	0
4	23	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	0,49	0,98
mannitol	0	0
H_2O	0	0

Table S22. Results of biolistic transformations of *Cp LEA-like 11-24* promoter::*GUS* and CaMV 35S::*GFP* constructs into *L. subracemosa* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	267	103
2	205	37
3	83	30
4	242	46
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	74	61
2	55	58
3	28	35
4	160	63
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	200	43
2	74	8
3	179	48
4	118	28
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	27,94	10,93
mannitol	84,69	38,11
H_2O	20,71	6,95

Table S23. Results of biolistic transformations of *Ls LEA-like 11-24* promoter::*GUS* and CaMV 35S::*GFP* constructs into *C. plantagineum* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	38	1
2	38	0
3	44	0
4	39	0
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	47	0
2	57	0
3	50	0
4	229	0
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	24	0
2	74	0
3	75	3
4	40	0
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	0,66	1,32
mannitol	0	0
H_2O	1,00	2,00

Table S24. Results of biolistic transformations of *Ls LEA-like 11-24* longer promoter::*GUS* and CaMV 35S::*GFP* constructs into *L. subracemosa* leaves.

Treatment: 100 µM ABA		
Experiment	Number of GFP spots	Number of GUS spots
1	61	25
2	119	67
3	186	42
4	174	47
Treatment: 0.8 M mannitol		
Experiment	Number of GFP spots	Number of GUS spots
1	33	10
2	55	21
3	91	50
4	67	17
Treatment: H_2O		
Experiment	Number of GFP spots	Number of GUS spots
1	67	2
2	317	6
3	19	0
4	218	12
Treatment	% GUS spots / GFP spots	Standard deviation
ABA	36,72	15,23
mannitol	37,20	12,95
H_2O	2,60	2,30

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Summary

The closely related resurrection plants *C. plantagineum* and *L. brevidens* have the rare ability to withstand desiccation of their vegetative tissues. The species *L. subracemosa* is a close relative of *C. plantagineum* and *L. brevidens*, but cannot tolerate desiccation of its vegetative tissues. This variability in desiccation tolerance was used to assess differences in the transcriptional regulation of desiccation-responsive genes between *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Comparative analysis of the dehydration-responsive transcriptomes of *L. brevidens* and *L. subracemosa* revealed that *L. brevidens* expressed more transcripts associated with desiccation tolerance. This finding indicates that the expression of dehydration-responsive genes is regulated differentially between the desiccation tolerant plant *L. brevidens* and the desiccation sensitive plant *L. subracemosa*. Transcript encoding LEA proteins were highly abundant in the dehydration-responsive transcriptome of *L. brevidens*.

A promoter study was conducted to gain more understanding of the transcriptional regulation of dehydration-responsive genes in *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Promoter regions of five dehydration-responsive genes were analyzed: *LEA 6-19*, *LEA-like 11-24*, *LEA 3-06*, *LEA 27-45* and *DSP22*, respectively. Putative *cis*-acting regulatory elements known to mediate ABA- and dehydration-responses were detected in promoter regions of all genes. Comparison of promoter regions revealed that several of the detected *cis*-acting regulatory elements were conserved among the promoters of *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Expression analysis showed that the *LEA-like 11-24* gene was differentially expressed in *C. plantagineum*, *L. brevidens* and *L. subracemosa* in response to different stress treatments. The activity of *LEA-like 11-24* promoter fragments of *C. plantagineum*, *L. brevidens* and *L. subracemosa* was analyzed in a transient expression assay. Functional promoter analysis demonstrated differences in promoter activity between *LEA-like 11-24* promoter fragments of *C. plantagineum*, *L. brevidens* and *L. subracemosa*. It was shown that variation in promoter architecture accounts for differences in *LEA-like 11-24* promoter activity between *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

A mutagenesis approach was employed to identify *cis*-acting regulatory elements essential for promoter activity. *Cis*-acting regulatory elements critical for ABA- and

dehydration-responsive promoter activity were detected in *LEA-like 11-24* promoter fragments of *C. plantagineum* and *L. brevidens*. Furthermore, it was demonstrated that specific *cis*-acting regulatory elements were structurally and functionally conserved among the *LEA-like 11-24* promoters of *C. plantagineum* and *L. brevidens*.

The yeast one hybrid system showed that the bZIP1 protein of *C. plantagineum* was able to interact with the *C. plantagineum LEA-like 11-24* promoter. This finding indicates that the *C. plantagineum* bZIP1 protein is involved in transcriptional regulation of the *LEA-like 11-24* gene. It was shown that the *C. plantagineum* bZIP1 protein was nuclear localized in epidermal leaf cells of *C. plantagineum*. Expression analysis revealed that the *bZIP1* gene was constitutively expressed in *C. plantagineum* leaf tissue. Although co-expression of *bZIP1* did not enhance the promoter activity of the *LEA-like 11-24* promoter fragment in *C. plantagineum* leaves, it was proposed that the bZIP1 protein is involved, but not sufficient for the activation of *LEA-like 11-24* gene expression in *C. plantagineum*. This study provided more insight in the regulation of desiccation-responsive genes in the closely related species *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

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