Promoter regulation of the *Craterostigma plantagineum Cp LEA-like 11-24* gene and functional characterization of its transcription factor (CpbZIP1)

Dissertation

Zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

Vorgelegt von

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aus

Mianeh, Iran

Bonn, 2014

For my father and my mother

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-WilhelMS-Universität Bonn

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Tag der mündlichen Prüfung: 18.08.2014

Erscheinungsjahr:18.08.2014

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ERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass ich für meine Promotion keine anderen als die angegebenen Hilfsmittel benutzt habe, und dass die inhaltlich und wörtlich aus anderen Werken entnommenen Stellen und Zitate als solche gekennzeichnet sind.

Saeedeh Ataei

Acknowledgements

Many people have helped me to complete this work. First of all, I would like to thank my supervisor Professor Dr. Dorothea Bartels for all her hard work teaching me to be a proper scientist. Her scientific suggestions and guidance helped me with my research. In addition, my deepest appreciation for the time and effort she spends for reading and commenting my thesis and manuscript.

I am grateful to Dr. Naim Stiti who helped me to start the laboratory work at the beginning of my project. I would like to express my special thanks to Dr. Tagnon Dègbédji Missihoun and Dr. Dinakar Challabathula for their useful recommendations during the lab work and writing the thesis. They were always ready to reply my questions even after they left the laboratory. Many thanks to Dr. Ilona Juszczak for correcting my thesis. She was nice and friendly even though she was new in our laboratory.

I am grateful to Dr. Bekir Ulker who helped me and gave me a valuable advice at my first arrival to Bonn.

I appreciate Christine Marikar for her assistance in solving the administrative issues.

I would like to thank Christiane Buchhold for her useful recommendations in plants growth and organizing lab requirements. I believe she is the best technician I have ever seen. She was always friendly and ready to help in a best and short way.

I would like to say thank you to all my friends in lab. For sure without your help and friendly atmosphere I will not able to finish my PhD study.

My great and voluble thank goes to my husband Faraham Ahmadzadeh. Thank him for always believing in me and encouraging me to go forward. He supported me emotionally and spiritually during my PhD study.

My last but not least thank goes to my parents, particularly my mother who kindly encourage us to go forward even after my father passed away. She was a mother and a father for me. I appreciate her mind.

I would like to extend my great thank to my lovely sisters Fariba, Leila and Neda. They always encourage and support me emotionally.

Contents

1. Introduction

1.1 Drought stress and climate change

Drought stress adversely affects plant growth and productivity. Many studies have been done to estimate the impact and severity of damages in different parts of the world (Wullschleger et al. 2002; Averyt et al. 2013). To date, global warming and climate change are important risks for crop production and impacting food supplies thereby affecting people health. As per the reports from the World Bank (2007) in sub-Sahara Africa climate changes severely affect the crop production. With regard to the growing World's population, it seems to be necessary to increase the yield of crops. FAO (2008) reported that number of hungers due to insufficient food and un-accessibility to food have been increased from 90 million in 1970 to 225 million in 2008 and will reach to 325 million until 2015. Climate change is directly threatening water resources and affects the global scarcity of water supplies in the coming decades.

It is obvious that crop plants have to compete for water access. Currently, about 70% of the world's fresh water consumed is used for the irrigation of crops (UNESCO, 2006). Drought can have a substantial impact on the ecosystem and agriculture of the affected region. Drought is one of the main factors, damaging the cellular structure and inhibiting the normal physiological activities (Bray et al. 2000; Zhu 2002; Bartels and Sunkar 2005). Therefore, to cope with the progressive climate change and consequently drought, it is necessary to investigate how plants can survive under adverse environmental conditions.

1.2 Three strategies of plants in response to water stress

Plants have developed multiple strategies to cope with water stress in the cells. There are three strategies in adaptation of plant cells to water scarcity, namely avoidance, resistance and tolerance (Le and McQueen-Mason 2000). Desiccation avoidance is characteristic for the annual plants, which produce the seeds in a favorable condition and finish the life cycle before facing the dry season and water deficit. On the other hand, desiccation resistant plants withstand drought conditions due to adapted morphological or physiological changes, such as increasing the water uptake by decreasing the water potential in the cells, closing the stomata and reduction of leaf surface, either in leaf size or covering the surface with special structures such as waxes or hairs to avoid transpiration.

The third strategy that plants use is desiccation tolerance. Small group of angiosperm plants have an extraordinary ability to keep the water up to 10% and regain a biologically functional state after rehydration. These plants are so called desiccation tolerant/resurrection plants (Gaff 1971; Bartels et al. 2005). Losing around 90% of water content is equivalent to a water potential of -100 MPa (Alpert 2005), which is a severe stress to the plant and stops enzymatic reactions and metabolic pathways (Billil and Potts 2002). These plants are fundamentally different than other drought-surviving plants such as glycophytes or succulents. Desiccation tolerance is frequently acquired in seeds of vascular plants or pollen grains, but it is rare in vegetative tissues. Desiccation tolerance is a complex multi-genic and multi-factorial process. The combination of genetic, metabolic, antioxidants and structural stabilization systems are involved in the protection mechanisms (Bartels and Salamini 2001; Moore et al. 2009).

1.3 Three closely related species of Linderniaceae family are experimental model plants to study molecular basis of desiccation tolerance

One model plant for studying the desiccation tolerance mechanisms is the resurrection plant *Craterostigma plantagineum* Hochst (Bartels et al. 1990; Piatkowski et al. 1990; Bartels et al. 2006; Farrant and Moore 2011). It is an endemic species in arid areas of South Africa and belongs to the Linderniaceae family. Several drought tolerant species have been reported in this family (Fischer 1992). The family includes also another species that can withstand drought, namely *Lindernia brevidens* Skan (Phillips et al. 2008). This plant is endemic to mountain forest of costal Africa, where no drought period is experienced. Recovery was observed in this plant after extreme dehydration under laboratory conditions, even though it is endemic in the tropical rain forests of East Africa (Phillips et al. 2008). For comparative analysis, a desiccation sensitive member of the Linderniaceae family, *Lindernia subracemosa,* has been introduced (Phillips et al. 2008). The phylogenetic relationship between these three species and other members of Linderniaceae family has been analyzed using sequences of the chloroplast maturase gene (*MATK*) (Rahmanzadeh et al. 2005). The close relationship of these three species is shown in Fig. 1-1. Three species *C. plantagineum*, *L. brevidens* and *L. subracemosa* are widely used in extensive comparative analyses to understand the drought tolerance mechanisms. For instance,

recently functional promoter analysis of desiccation-induced gene (*LEA-like 11-24*) was compared between the three species and elucidated a step in regulatory mechanism of desiccation tolerance (van den Dries et al. 2011).

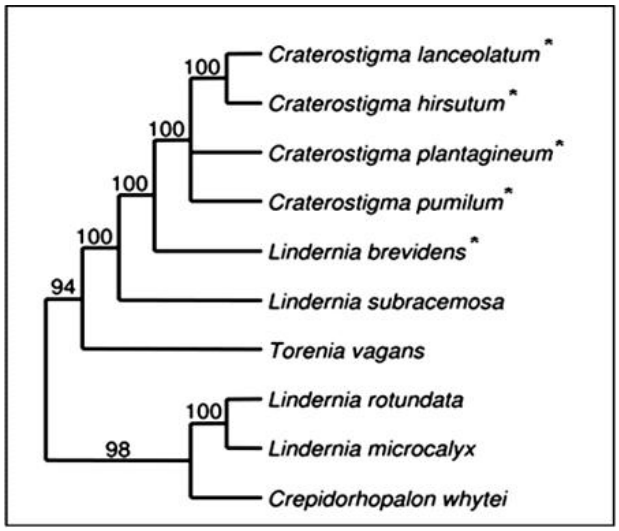


Fig. 1-1: Phylogenetic tree showing relationship among selected members of the Linderniaceae family. It was constructed based on *MATK* gene sequences. The desiccation-tolerant species are marked with an asterisk. The phylogenetic tree in this form was published by Phillips et al. (2008), but the data are derived from the study of Rahmanzadeh et al. (2005) (see also van den Dries 2010).

1.4 Protection mechanisms in response to dehydration

Drought and dehydration stress affects almost all plant functions including growth, development and productivity (Nakashima et al. 2009). It causes severe metabolic and mechanical changes of plant cells (Bray 1993). It mediate the increase of concentration of cellular content due to decreased water content, thereby causes unwanted molecular interactions, macromolecular denaturation and alteration of biochemical activities (Gechev et al. 2012). Desiccation tolerant plants have the capability to cope with severe water deficit (Bartels and Sunkar 2005). A numbers of investigations have afforded to understand the mechanism involved in desiccation tolerance (Phillips et al. 2002; Deng et al. 2006). These plants have complex protective mechanisms to protect the macromolecules or cell organelles from stress-mediated injury. Some of well studied resurrection plants are *Craterostigma plantagineum*, *Lindernia brevidens*, *Haberlea rhodopensis*, *Xerophyta viscosa*, *Boea hygrometrica*, *Sporobolus stapfianus, Ramonda*

nathaliae, Ramonda serbica (Ingle et al. 2007; Rodriguez et al. 2010; Oliver et al. 2011; Dinakar et al. 2013; Gechev et al. 2013; Rakić et al. 2014).

1.4.1 LEA proteins and association with desiccation tolerance

More than two decades ago, LEA (late embryogenesis abundant) proteins which are abundantly expressed during the late phase of embryo development were discovered in cotton seeds (Galau et al. 1986) and later they were also discovered in vegetative tissues of plants under dehydration suggesting the adaptation role during desiccation (Bartels et a. 1990; Ramanjulu and Bartels 2002; Hundertmark and Hincha 2008). These proteins show common feature of high hydrophilicity, unordered structure, thermal stability (Battaglia et al. 2008) and become more structured during dehydration (Wise and Tunnacliffe 2004). Despite extensive studies, the exact biochemical function of LEA proteins is not fully understood (Bartels and Salamini 2001). LEA proteins have been divided into at least 6 groups based on sequence similarities and biochemical properties (Battaglia et al 2008). These proteins are localized in different cellular compartments such as chloroplast, cytosol, mitochondria and nuclei (Dinakar et al. 2013). In most angiosperms, seeds face extreme dehydration condition at this developmental stage. On the other hand, the expression of LEA proteins during seed development abundantly induced (Bartels, 2005; Velasco *et al*., 1998). Therefore, a association of LEA proteins with desiccation and acquisition of tolerance has been suggested (Cuming 1999). Many protective functions have been proposed for LEA proteins in different studies. For instance, LEA proteins act against DNA damages, stabilize other proteins or enzymes, cytoskeleton filaments, maintain cellular structural organization, prevent ion crystallization during dehydration and act as chaperones were reported (Baker et al. 1988; Robert et al. 1993; Wise and Tunnacliffe 2004; Tunnacliffe and Wise 2007). In addition, a role in preventing aggregation of proteins by providing a water hydration ‘’shell’’ has been proposed for LEA proteins (Goyal et al. 2005; Reyes et al. 2005).

Several of the genes induced by desiccation have been cloned and characterized as LEA proteins in *C. plantagineum*. The transcript levels of many *LEA* genes encoding LEA proteins from group 4 (*CpC2*), group 2 LEA (the dehydrin, *Cp LEA 6-19*), group 5 LEA (*CDeT27-45*) were increased in *C. plantagineum* leaves upon dehydration or osmotic treatments (Bartels et al.

1990; Piatkowski et al. 1990; Michel et al. 1994; Velasco et al. 1998; Ditzer et al. 2006). The *Cp LEA-like 11-24* is one of these stress inducible genes in *C. plantagineum* that has been extensively studied on the transcript and protein level as well as promoter region (Velasco et al. 1998, Bartels and et al. 1990; Bartels 2005; Facchinelli 2009; van den Dries et al. 2011). Recently, the biochemical properties and structure of the *Cp LEA-like 11-24* protein were analyzed. It protects citrate synthase and lactate dehydrogenase against damaging effects caused by desiccation *in vitro* (Petersen et al. 2012). RD29A (responsive to dehydration 29 A) and RD29B from *Arabidopsis* are two close homologue of *LEA like 11-24* gene (Velasco et al. 1998), which the expression is induced under ABA, dehydration and salt stress (Yamaguchi-Shinozaki and Shinozaki 1993; Nakashima et al. 2006). These proteins share similar features with other LEA proteins like hydrophilicity in response to water stress (Yamaguchi-Shinozaki and Shinozaki 1993).

1.4.2 Carbohydrate

Accumulation of carbohydrates is a major response during desiccation in many resurrection plants (Dinakar et al. 2012). While sucrose accumulates in most plants as a product of photosynthesis, *C. plantagineum* has the capability to accumulate 2-octulose in the leaves and convert to sucrose during desiccation (Bianchi et al. 1991, Norwood et al. 2000). Following dehydration, sucrose accumulation progressively increases, but the accumulation of 2-octulose decreases. Together with conversion of 2-octulose to sucrose, sucrose synthase and sucrose phosphate synthase are also upregulated (Ingram et al. 1997, Ramanjulu and Bartels 2002). It suggests that 2-octulose has a role in the carbon storage under dehydration condition, as *C. plantagineum* plants do not accumulate starch (Bianchi et al. 1991). However, the exact metabolic pathway of 2-octulose synthesis from sucrose or the conversion of sucrose into 2-octulose is not clear. In addition, the expression of two classes of transketolases were increased during rehydration in *C. plantagineum* (Bernacchia et al. 1995). Recently, the involvement of transketolase in the conversion of sucrose into 2-octulose has been proposed based on enzymatic assays and gene expression analyses (Willige et al. 2009).

Also accumulation of sucrose, raffinose and trehalose (Ingram and Bartels 1996) which are believed to function as osmoprotectant in protecting against oxidative stress, has been reported in dehydration conditions (Nishizawa et al. 2008). Most metabolic changes in these plants during dehydration associate with sugar metabolism (Dinakar et al. 2012). Some sugars (e.g. raffinose) act as storage of carbohydrates and are remobilized to produce sucrose during dehydration (Norwood et al. 1993). Some other sugars (such as sucrose and trehalose) play osmoprotectant role for stabilizing membrane structure. Apart from protective role, they can act as signaling molecule in regulating metabolic pathways. For instance, trehalose and trehalose-6-phosphate are the important metabolic regulators which control the carbohydrate status, growth and energy level in the cell (Smeekens et al. 2010).

For example, desiccation-tolerant *Sporobolus stapfianus* contains higher levels of osmolytes and nitrogen metabolites compared to the concentration of energy-associated-metabolites of desiccation-sensitive *Sporobolus pyramidalis* (Moore et al. 2009; Dinakar et al. 2012). Thus, accumulation of sucrose and glucose in specific locations of resurrection plants under dehydration was proposed as stabilizing the chloroplast and tonoplast membranes (Martinelli et al. 2008). It is proposed that sucrose may form glasses phases like solid liquid. Formation of intracellular glass may protect phospholipid bilayers against dehydration-mediated injury (Crowe et al. 1992). Interaction of LEA proteins and sugars has been suggested in another study to form a thigh hydrogen-bonding network, thus stabilize the cellular structure (Wolkers et al. 2001).

1.4.3 Compatible solutes (proline)

Many compatible solutes are produced in plants during dehydration (Verslues and Sharma 2010). Compatible solutes are small molecules that are accumulated in the cells under stress without any toxic effect. They stabilize the cell membranes and proteins in response to stress (Hare and Cress 1997; Yancey 2005; Verslues and Sharma 2010). Compatible solutes are naturally synthesized in some plants and serve as protectants to cope with stresses. Several kinds of compatible solutes such as sugars (mannitol, trehalose and sucrose), proline, glycine betaine, spermine and spermidine are believed to function as osmotica and protect the membranes and

proteins (Delauney and Verma 1993; Nuccio et al. 1999). Among the compatible solutes, proline has long been known as an osmoprotectant that accumulates in plants under water stress (Hare and Cress 1997; Yancey 2005). It is widely distributed in all organisms (Maggio et al. 2002). It plays an important role as source of energy, carbon and nitrogen compounds, hydroxyl radical scavenger and plasma membrane protector (Hare and Cress 1997; Nakashima et al. 1998; Verslues and Sharma 2010). Proline accumulation is thought to have an adaptive role in response to salinity, drought, high and low temperatures, pathogen attack, nutrient deficiency and UV irradiation (Verbruggen and Hermans 2008). It can translocate from other parts of plant to the stressed tissues. Proline biosynthesis occurs in the cytosol in the plastids while catabolism takes place in mitochondria (Verslues and Sharma 2010). Proline is synthesized from glutamate by two enzymes, namely pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), whereas proline dehydrogenase (ProDH) and pyrroline-5-carboxylate dehydrogenase (P5CDH) are involved in its catabolism to glutamate (Fig. 1-2) (Deuschle et al. 2004, Verslues and Sharma 2010). The main pathway for proline synthesis during osmotic stress is the glutamate pathway (Fig. 1-2) through pyrroline-5-carboxylate synthetase (PC5S) enzyme. However, the ornithine pathway in young *Arabidopsis* plants seems to contribute as well (Roosens et al. 1998).

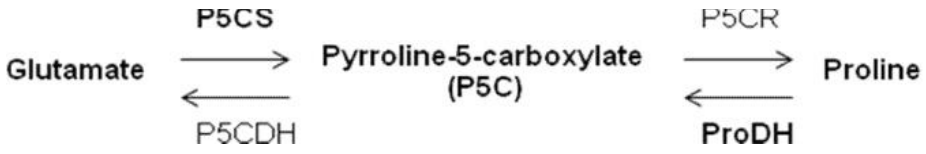


Fig. 1-2: The main proline metabolism and catabolism pathway. Proline is produced by regulation of P5C synthetase (P5CS) and P5C reductase (P5CR) enzymes in cytosol and plastids. It is catabolised to Glutamate by proline dehydrogenase (ProDH) enzyme and P5C dehydrogenase (P5CDH) in mitochondria.

The PC5S enzyme has been proposed to participate in protein-protein interaction or to maintain the protein structure (Verslues and Sharma 2010). It contains a leucine zipper region in each domain. Transcript accumulation of *PC5S1* is strongly induced under salt, dehydration and cold stress (Yoshiba et al. 1999). Transcriptional up-regulation of *PC5S1* is in positive correlation with the proline level under dehydration (Yoshiba et al. 1995) indicating the involvement of

P5CS1 in the biosynthesis of proline from glutamate (Kreps et al. 2002). The knockout mutants of *P5CS1* show hypersensitivity to salt or water deficit (Sharma and Verslues 2010). In contrast, *PC5S2* has a minor role under stress conditions as no transcript accumulated (Szekely et al. 2008). Tobacco overexpressing *P5CS1* showed more tolerance to salt stress (Kishor et al. 1995). Studies on exogenous abscisic acid (ABA) treatment and ABA-deficient mutants have shown that proline accumulation under stress can be partially regulated by ABA, thus ABA-dependent and -independent regulation of proline has been proposed (Yoshiba et al. 1995; Savoure et al. 1997; Verslues and Bray 2006; Sharma and Verslues 2010).

1.4.4 Role of ABA in abiotic stress

The plant hormone, Abscisic acid (ABA) regulates several important aspects in growth and development including seed dormancy and germination, embryo maturation as well as responses to environmental stresses (Bewley, 1997; Fujita et al. 2011, Finkelstein 2013). Different abiotic stresses such as drought, cold and salinity induce ABA synthesis, therefore, ABA is considered as plant stress hormone (Swamy et al. 1999). ABA plays crucial role in response to abiotic stress. ABA acts as endogenous messenger to activate plant responses to abiotic stresses (Christmann et al. 2006). Initial physiological changes under mild water deficit mediate rapid changes in ABA concentrations (Ramanjulu and Bartels 2002). The accumulation of ABA mediates the stomatal closure and activates the induction of many stress inducible genes (Webb et al. 2001). Consequently, it regulates the induction of genes associated with stress such as LEA proteins, osmoprotectant, carbohydrate metabolism and transcription factors and kinases (Webb et al. 2001; Kirch et al. 2001; Bartels and Sunkar 2005; Ditzer et al. 2006; Kempa et al. 2008; Fujita et al. 2011). Therefore, accumulation of ABA is associated with stress and application of exogenous ABA is used to mimic the dehydration responses (Yamaguchi-Shinozaki and Shinozaki 2006). For instance, i*n vitro* callus tissues of *C. plantagineum* are not drought tolerant, unless ABA treated tissues (Bartels and Salamini 2001). Therefore, ABA has a critical role in desiccation tolerance of *C. plantagineum* through induction of ABA-responsive genes.

1.4.5 Regulation of stress and ABA-inducible genes

The regulation of ABA-mediated genes is very complex and both positive and negative transcriptional regulations have been reported (Deng et al. 2006; Hilbricht et al. 2002). Ca^{+2} - dependent signaling has been proposed to regulate the expression of ABA biosynthetic genes. It is believed that calcium ions as secondary messengers are important in ABA signal transduction pathways (Webb et al. 2001). Presences of ABA can change the sytosolic concentration of calcium. Many proteins in ABA perception and ABA transduction have been found. The best characterized and the most convincing candidates as ABA receptors are a family of soluble proteins named PYR/PYL/PCAR (pyrabactin resistant/PYR-like/regulatory component of ABA receptor) which establish the beginning of a core ABA signaling pathway (Ma et al. 2009; Finkelstein et al. 2013). ABA binds to a PYR/PYL/PCAR protein and changes the conformation into a stable complex and inactivate the PP2C thereby, activating the SNRK2s protein kinase (SNF1 related kinase) through removal of activating phosphates (Klinger et al. 2010). In this pathway SNRK2s proteins are required for activation of transcription factors and act as positive regulator, PP2Cs act as negative regulator. In addition, several protein phosphorylation cascades have been found that are involved in ABA signalling.

1.4.5.1 ABA-induced genes are bind to ABRE *cis*-element

The promoter region has fundamental role in controlling the gene expression. There are specific motifs named *cis*-elements in the promoter region of genes, which mediates the expression of gene in response to different stimuli. Depends on the stress responsibility of gene different *cis*-acting elements can be found in the promoter. For example, dehydration, pathogen defense, hypoosmolarity. Therefore, there are two different signaling pathways in the regulation of stress inducible genes, ABA-dependent pathway and ABA-independent pathway (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2005; Fujita et al. 2011). Each pathway contains specific transcription factors binding to a particular motif in the promoter sequence.

One of the important *cis*-elements in the promoter of ABA-inducible genes is the ACGT-core motif containing a 8-10 base pair long sequence (PYACGTGGC) (Fujita et al. 2011) and is named ABA responsive element (ABRE) (Hattori et al. 2002). The flanking sequence of ACGT

motif is important for the functionality of the *cis*-acting elements (Shinozaki et al. 2003). A conserved sequence of CACGTG is named G-box which is recognized by ABA-regulated transcription factors. Three transcription factors namely MYB, MYC and ABF (ABA binding factor) proteins are associated with ABA responsiveness and bind to the MYB, MYC and ABRE recognition sites, respectively (Abe et al. 2003; Shinozaki et al. 2005).

The proteins that are binding to the ABRE sites called ABRE binding factors. These factors are bZIP (basic leucine zipper proteins) protein that bind to the ABRE motif and activate ABA-dependent gene expression (Choi et al., 2000). All ABRE elements work in close cooperation with other ABA-response regulatory elements such as MYC and MYB (Iwasaki et al. 1995). In general, repeated copies of ABREs or one ABRE and one coupling element (CE) in the promoter are required to confer ABA-mediated induction of gene expression (Shen and Ho 1995, Skriver et al. 1991, Uno et al. 2000).

In the promoter of dehydration-responsive genes (such as *RD29A*, *RD29B*, *Cp LEA-like 11-24*), there is another *cis*-element named dehydration responsive element named DRE/C-repeat (CRT) (Shinozaki *et al*., 2003; van den Dries et al. 2011). It has been reported that the expression of genes in response to salt, dehydration and cold is regulated by this motif in the promoter (Yamaguchi-Shinozaki and Shinozaki, 1994; van den Dries et al. 2011). DRE motif in the promoter of *RD29A* contains 9 bp long conserve sequence and special transcription factors bind to this motif. DREB1A (DRE-binding protein) and DREB2A are two major transcription factors regulating dehydration, salt and cold response and specifically bind to DRE motif in the promoter (Liu et al. 1998).

Expression of many stress inducible genes increase upon dehydration in *C. plantagineum,* (Bartels and Sunkar 2005). Core promoter of an ABA-inducible gene of *C. plantagineum*, *CpC2* from group 4 LEA gene has also been reported to have at least 2 ABRE elements (Ditzer et al. 2006). In the case of LEA-2 (*Cp LEA 6-19*) dehydrin, presence of 3 ABRE related motif in the promoter region was not enough to enhanced the promoter activity in response to ABA using different promoter fragments. The activity of promoter enhanced in the presence of full promoter. This indicated that some promoter sequences which leads to increase of promoter activity may locate on the largest promoter fragment (Michel et al. 1994).

1.4.5.2 bZIP transcription factors

The transcription factor basic leucine zipper (bZIP) family is found in all eukaryotes and is one of the largest families of transcription factors in the plant kingdom with many different functions. These proteins integrate in many biological processes, which are crucial for the function of organism (Deppmann et al. 2006). In animals, bZIP proteins are required for development of organs such as liver, heart, bone and fat (Wang et al. 1992; Eferl et al. 1999). In adult animals, bZIP proteins are also involved in essential processes such as circadian clock, metabolism, learning and memory (Deppmann et al. 2006). In plants, bZIP factors are essential for organ development, cell elongation and morphogenesis, seed development, flower maturation, osmotic stress, sugar signaling, carbon metabolism and starvation (Chuang et al.1999; Hai et al. 1999; Jakoby et al. 2002; Alonso et al. 2009; Weltmeier et al. 2009; Dietrich et al. 2011; Satoh et al. 2004). The bZIP protein structurally consists of two domains. A basic region contains 16 basic amino acid residues which are connected to the major groove of the target DNA. A heptad leucine zipper region contains hydrophobic amino acids in the C-terminal part of the protein responsible for dimerization of bZIPs (Fig. 1-3). The leucine zipper region regulates the expression of the target genes through dimerization, which occurs through the amphipathic coiled-coil leucine zipper domain (Hai et al. 1999). The *Arabidopsis* genome contains 75 bZIP genes (Jakoby 2002). The number of bZIP genes in *Arabidopsis* is four times more than in yeast (Jakoby 2002; Riechmann et al. 2000, Amoutzias et al. 2008). So far, less is known about bZIP transcription factors in plants than observed for those in humans and yeast.

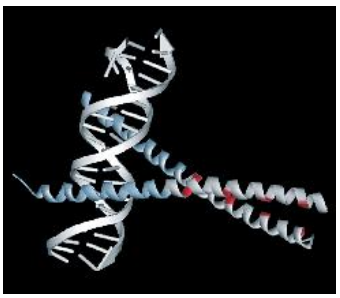


Fig. 1-3: Shematic interaction of bZIP transcription factor with DNA. bZIP proteins bind DNA as dimer in a major groove of DNA. The DNA binding domain and the leucine zipper region are shown in the picture (Jakoby et al. 2002).

According to sequence similarities of the basic region and additional conserved motifs bZIPs can be divided into 10 groups from A to I and one small group, the so-called S-class (Jakoby 2002) (Fig. 1-4).

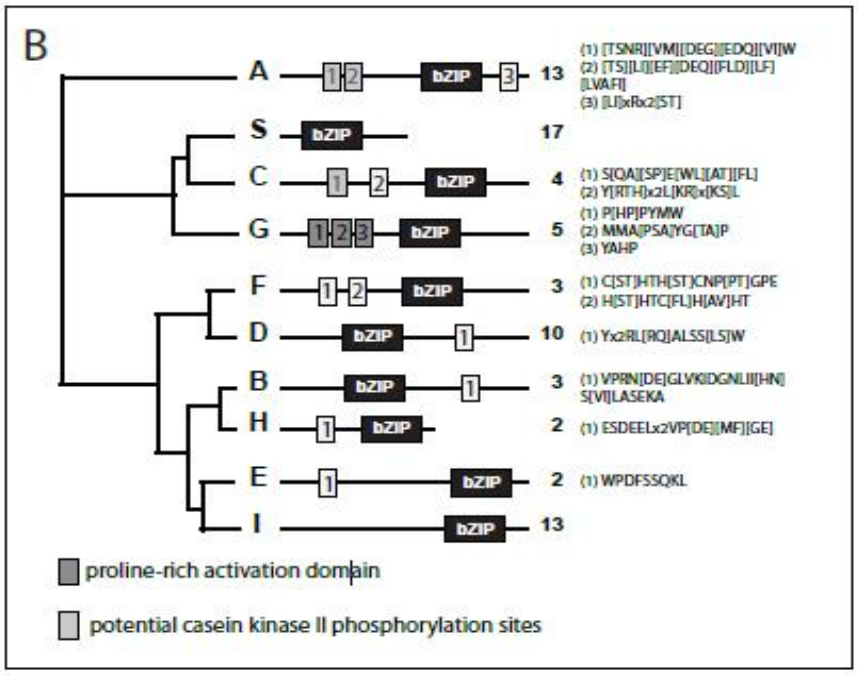


Fig. 1-4: Phylogeny of bZIP protein family in *Arabidopsis* (Hanssen 2009). AtbZIP proteins are divided in 10 major groups (A to I and S) (Jakoby 2002). The sequences of the group-specific domains are shown on the right after the number of proteins within each group.

Group A of bZIP transcription factors, is the most extensively analysed group of bZIP proteins in *Arabidopsis* plants. Members of this group are involved in ABA or stress signaling response to abiotic stresses. The abiotic stresses such as drought, salt and cold induce the ABF/AREB gene expression and trigger AREB phosphorylation. So far, all seven members of group A have been analyzed namely (*AtbZIP39*/*ABI5*, *AtbZIP36*/*ABF2*/*AREB1*, *AtbZIP38*/*ABF4*/*AREB2*, *AtbZIP66*/*AREB3*, *AtbZIP40*/*GBF4*, *AtbZIP35*/*ABF1* and *AtbZIP37*/*ABF3*) (Jakoby et al. 2002). Members of group B, have been analyzed recently. The proteins mediate salt and endoplasmic reticulum stress responses in *Arabidopsis* (Liu et al. 2007a, b). The members of group C, share structural features with maize Opaque2 and parsely CPFR2 (Jakoby et al. 2002). The proteins contain an extended leucine zipper, with up to nine heptad repeats. They are involved in regulation of seed storage proteins (Jakoby et al. 2002; Alonso et al. 2009; Dietrich et al. 2011).

AtbZIP10 and AtbZIP25 from *Arabidopsis* are the closest homologous of Opaque2 transcription factor which has been shown to regulate the expression of storage proteins (Alonso et al. 2009). Members of group D are linked with two different processes, namely defense against pathogen and development. For members of group E no functional data are available. It is known that they are similar to members of group I but differ from them with respect to the lysine residue in the position -10. The members of the group, G binding factors (GBF) are mainly involved in ultraviolet and blue light signal transduction. These proteins bind to the G-boxes present in the promoters of light responsive genes (Schindler 1992; Armstrong et al. 1992). Two members of group H are predicted to be involved in photo-morphogenesis. On the other hand, some members of this group are proposed to regulate the vascular development. Group S, this group is the largest bZIP group in *Arabidopsis* that has been shown to have multiple functions in response to different stresses. All members of bZIP contain at least one domain such as a proline-rich activation domain or a potential casein kinase *II* phosphorylation site except members of group S of *bZIP* transcription factor.

1.4.5.3 bZIP transcription factors belonging to S1-class bZIP

The S1-class of *bZIPs* consists of 5 subclasses: *AtbZIP1*, *AtbZIP2*, *AtbZIP11*, *AtbZIP44* and AtbZIP53. Proteins from this class are at low molecular weight and contain long zipper domains of 8-9 leucines (Rook et al. 1998; Wiese et al. 2004). This group of *bZIPs* contains an unusual long upstream open reading frame (uORF) in the 5' region, which is highly conserved between species (Weltmeier et al. 2009). This region encodes a small protein that has a function in controlling the sugar concentration in the cell called SIRT mechanism (Sucrose Induced Repression of Translation) (Rook et al. 1998; Wiese et al. 2004; Weltmeier et al. 2009). This class of bZIPs can be repressed at the translational level by sucrose through binding of the small protein to the upstream open reading frame (uORF) in the 5' region of transcript (Wiese et al. 2004; Weltmeier et al. 2009). Most of the S1-class bZIPs from *Arabidopsis* contain the long conserved uORF which encodes 41-42 amino acids (Table 1-1). Only *AtbZIP53* and *AtbZIP1* have shorter uORFs of 28 and 25 amino acids respectively (Table 1-1). Highest similarities are

found in the C-terminal part of these polypeptides. All bZIP proteins in this class are localized in the nucleus (Jakoby et al. 2002; Wiese et al. 2004; Alonso et al. 2009; Dietrich et al. 2011).

Table 1-1: 5'-UTR harbouring the uORF in the bZIP transcription factors from different plants (Wiese et al. 2004).

Plant	Gene Name	Accession Number	Intercistronic Spacer Length (nt)	SC-uORF Length (aa)
Arabidopsis thaliana (thale cress)	*ATB2/AtbZIP11*	At4g34590	255	42
Arabidopsis	*AtbZIP44*	At1g75390	164	41
Arabidopsis	*AtbZIP2*	At2g18160	116	41
Arabidopsis	*AtbZIP53*	At3g62420	182	28
Arabidopsis	*AtbZIP1*	At5g49450	**10**	25
Nicotiana tabaccum (tobacco)	*tbz17*	D63951	240	28
N. tabaccum	*tbzF*	AB032478	162	25
Zea mays (maize)	*mLIP15*	D26563	170	26
Z. mays	*OCSBF-1*	X6245.1	188	28
Antirrhinum majus (snapdragon)	*BZIP910*	Y13675.1	166	25
A. majus	*BZIP911*	Y13676.1	130	32
Oryza sativa (rice)	*glip19*	D63955	169	26
O. sativa	–	AAAA01011550	254	41
Glycine max (soybean)	*GmATB2*	AF532621	228	41
Petroselinum crispum (parsley)	*cprf6*	AJ292744	138	25
Lycopersicon esculentum (tomato)	–	BI207937	114	41
Medicago trunculata (barrel medic)	–	AC121244	198	42
Gossypium arboreum (tree cotton)	–	BG446720	89	41
Lotus japonicus	–	AP006137	115	28
Capsicum chinense (pepper)	*PPI1*	AF430372	188	41

1.4.5.4 bZIP transcription factors and dimerization capacity

Dimerization of transcription factors provide a wide range of combination of proteins for differential regulation of target genes via specific DNA binding capacities of dimers (Wiese et al. 2004). Forming heterodimer or homodimer has distinct effect on the regulation of target genes (Ehlert et al. 2006). Despite efforts to understand the structural specificity of coiled coil establishing a paired dimer, the mechanism of dimerization between two transcription factors is still unknown (Deng et al. 2008). However, Deppmann (2006) presented a comprehensive bZIP dimerization network of *Homo sapiens*, *Arabidopsis thaliana*, *Drosophila melanogaster* and *Saccharomyces cervisiae* by using a network mapping approach. He reported that animal bZIP factors extensively heterodimerize, whereas bZIPs in plants almost exclusively form homodimer networks by having long leucine zippers with asparagine which are located in the different heptads.

In *Arabidopsis*, S1-class bZIP proteins form heterodimers with C-class bZIP proteins. However, functional homodimer formation has also been reported for AtbZIP11. *In vivo* and *in vitro* heterodimerization of AtbZIP53 and two transcription factors of C-class AtbZIP10 and AtbZIP25 were shown to lead to a strong activation of seed-specific genes (Alonso et al. 2009). Heterodimerzation of bZIP transcription factors have been proposed to increase the affinity for binding to the target gene and stabilization of the dimer structure. Transient expression analyses have shown that five proteins in the S1-class namely, AtbZIP53, AtbZIP44, AtbZIP11, AtbZIP2, AtbZIP1 form different combinations that bind to the ACTCAT motif in the ProDH promoter region and activate the expression under different stress conditions such as high proline level, low energy and starvation (Satoh et al. 2004). Moreover, protein availability during developmental or environmental changes regulates the target gene expression (Weltmeier et al. 2006). Several members of S1 class AtbZIPs including AtbZIP1 and AtbZIP53 have been suggested in starvation responses and in amino acid metabolism.

1.5 Association of the desiccation tolerance in seeds and the vegetative tissues during evolution

Seed formation is divided into two phases, early and late embryogenesis. During early embryogenesis the morphology of embryo is formed, while late embryogenesis mediates accumulation of storage and seed desiccated proteins and dormancy (Goldberg et al. 1994). Not all of the seeds are able to cope with drought. They can be divided into two groups in terms of capability to withstand dehydration. The first group of seeds called orthodox seeds, is able to survive drying and freezing during ex-situ conservation (Bewley and Black 1994). Reduction of the metabolic rate to the lowest level at the time of (before/after) shedding (Farrant et al. 1997) has been shown in orthodox seeds. In addition, water content decrease to 5-10% in orthodox seeds (Manfre et al. 2009). This phenomenon appears in the seeds of most plant species (e.g. *Arabidopsis*). Another group of seeds called recalcitrant seeds lose their viability upon the weak drought stress or when the temperature is less than 10°C (e.g. Avicenna, avocado and mango). These plants are mostly endemic to humid tropic or subtropic area (Gaméné et al. 2004;

Pritchard et al. 2004; Berjak et al. 2008). In recalcitrant seeds, metabolic pathways and DNA replication are actively preceded (Boubriak et al. 2000, Faria et al. 2004).

Bryophytes which are the non-vascular land plants were the first plants moved from the water to the land (Mishler and churchil 1985) and the need to colonize the land caused evolutionary changes (Charron and Quatrano et al. 2009). Most likely angiosperms lost the drought tolerance possible in vegetative tissues during the evolution, as they were not required to be protected (Farrant and Moore 2011). It is believed that the genetic program for dehydration acquisition in the orthodox seeds has been derived from the ancestors of seed plants from gametophytic or sporophytic tissues of bryophytes/pteridophytes (Oliver et al. 2000). The emergence of LEA proteins are connected with the appearance of orthodox seeds (Cumming 1999). Although, the molecular signature of seed in vegetative tissue of resurrection plants has not been established convincingly, seed-specific protection strategy such as upregulation of LEA genes as well as cys-peroxiredoxin genes in the leaves give the strong evidence for acquisition of desiccation tolerance from the seeds (Illing et al. 2005).

Another evidence to prove the seed background of desiccation-induced genes in the leaves and roots of resurrection plants is research done in *Xerophyta humilis*. The expression profile of genes in *Xerophyta humilis* and *Arabidopsis thaliana* as desiccation-tolerant and -sensitive species, respectively were compared using cDNA and microarray technology (Walford 2008). The result revealed high similarity among the gene expression of seed, leaf and root of *X. Humilis*. Storage proteins, peroxiredoxins, *LEA* and *HSP* genes were the most abundantly induced genes in seeds and vegetative tissues. However, the respected genes are seed specific in *Arabidopsis* and are not expressed in the vegetative tissues. Therefore, it is convincing that desiccation tolerant species re-activate and recruit the seed development strategy in the vegetative tissue to protect the organs against damage caused by desiccation (Farant and Moore 2011).

1.6 Circadian clock system and association with stress

Circadian clocks are endogenous rhythms within 24 hours which constantly occur (Hanano et al. 2008; Marcolino-Gomes et al. 2014). Many photosynthesis related photosystem I and II genes

are known to be circadian clock rehgulated (Zhang and McClung 1996; Marcolino-Gomes et al. 2014). The genes associated with photosynthesis and photorespiration such as photosystem I and II reaction center proteins (Zhang and McClung 1996), the genes involved in carbon assimilation have a peak in afternoon (Harmer et al. 2000; McClung. 2001). Also nitrogen assimilation has been known to be clock regulated for decades (Cohen and Cumming 1974). It should be noticed that a number of genes involved in stress responses including cold and pathogen responses are also clock-regulated (Harmer et al. 2000; Schaffer et al. 2001).

Recently bioinformatic network analyses suggested that the *AtbZIP1* is regulated by the circadian clock (Gutierrez et al. 2008). It has been shown that the gene encoding circadian clock associated 1 (*CCA1*) protein binds to the promoter of the *AtbZIP1* gene (Gutierrez et al. 2008). Several reports have shown that the circadian clock affects stress responses in *Arabidopsis*. The correlation between plant response to abiotic stresses and circadian clock rhythms has been proposed in many studies (Bieniawska et al. 2008; Legnaioli et al. 2009; Wilkins et al. 2010). For instance, in *C. plantagineum* a chloroplastic desiccation inducible protein Dsp22 shows expression fluctuation in dark treatment (Alamillo and Bartels 1996). The homolog of Dsp22 protein in *Arabbidopsis* is an early light-inducible protein (*ELIP*) protein that is regulated by circadian clock (Grimm et al. 1989). Alamillo and Bartels (1996) proposed the involvement of circadian clock in regulation of *Dsp22*.

1.7 Promoter analyses to study mechanisms of desiccation tolerance in *C. plantagineum*

Functional promoter analyses among three closely related genes has been performed (van den Dries et a. 2011). *LEA-like 11-24* promoter from two desiccation tolerants and a desiccation sensitive species was investigated to understand the role of functional *cis*-elements in determining the transcript level of the *LEA like 11-24* genes. The importance of promoter region in stress responsibility of genes was investigated. The presence of a DRE motif in the promoter region has been identified as the key element for higher induction of *Cp LEA-like 11-24* gene from *C. plantagineum* in response to stresses (van den Dries et al. 2011).

1.7.1 Promoter architecture of *LEA like 11-24* in *C. plantagineum*, *L. brevidens* and *L. subracemosa*

Minimal stress inducible promoter fragments of the *LEA-like 11-24* gene were identified in *C. plantagineum* (307 bp), *L. brevidens* (321 bp) and *L. subracemosa* (488 bp) (van den Dries et al. 2011). A functional promoter analysis of these promoter fragments has shown that all three-promoter fragments can be induced by ABA and osmotic stresses (van den Dries et al. 2011). However, variation in the expression was observed between three species. A similar induction was found by ABA and osmotic stress as the full-length promoters (Velasco et al. 1998; van den Dries et al. 2011). These promoter fragments contain essential *cis*-acting regulatory elements that are involved in the regulation of dehydration-responsive gene expression (Guiltinan et al. 1990; Yamaguchi-Shinozaki et al. 1994; Hattori et al. 2002) such as ABA-responsive elements (ABREs) and drought responsive elements (DRE). Two ABREs are located at conserved positions in all three promoter fragments (ABRE1 and ABRE2 (Fig. 3-5). In the case of *C. plantagineum*, the promoter fragment also harbors one DRE element (van den Dries et al. 2011). The *LEA-like 11-24* promoter fragment of C. *plantagineum* is also inducible by ABA and osmotic stress in heterologous genetic backgrounds as shown for *A. thaliana* (Velasco et al. 1998) and *L. subracemosa* (van den Dries et al. 2011).

The transcript of the *LEA-like 11-24* gene accumulates in *C. plantagineum*, *L. brevidens* and *L. subracemosa* at different levels under dehydration conditions and after ABA treatment (van den Dries et al. 2011). Among them *L. subracemosa* shows the lowest expression level. Its association with differences in the promoter sequence of *LEA-like 11-24* in three species has been reported (van den Dries et al. 2011). Loss of function mutation in the *cis*-elements of the *LEA-like 11-24* promoter fragment of *C. plantagineum* was also identified which proves the critical role of DRE element in higher induction of the *LEA-like 11-24* promoter fragment of *C. plantagineum* under ABA and osmotic stress compared to *L. brevidens* and *L. subracemosa*. A DRE deletion reduced the promoter activity in both treatments to the level obtained for the *LEA-like 11-24* promoters of *L. brevidens* and *L. subracemosa* (van den Dries et al. 2011). While *C. plantagineum* showed the highest expression, *L. subracemosa* showed the weakest expression. This demonstrates that the expression level of transcripts make the difference

between desiccation-tolerance and sensitive ones as observed in *C. plantagineum* and *L. subracemosa,* respectively (van den Dries et al. 2011), although the effect of trans-regulation-environment of different plant species in the activity of *LEA-like 11-24* promoter is not known. Higher expression of the *LEA-like 11-24* transcript and protein in *C. plantagineum* than in *L. subracemosa* suggests that the promoter architecture is involved in the regulation of expression level of *LEA-like 11-24* gene.

1.7.2 Studying promoter function using transient transformation methods

In analyzing promoter functions transient transformation methods are a fast alternative compared to stably transformed plants and their results are not influenced by the chromosomal insertion position as reported for stable transformation (Yang et al. 2000). Transient transformation methods can be divided into physical, chemical and biological procedures. Physical methods include electroporation (Fromm et al. 1985) and particle bombardment (Ditzer et al. 2006; Ueki et al. 2009; van den Dries et al. 2011; Sparks and Jones 2014). Chemical methods consist of transformation by polyethylene glycol (Riazunnisa et al. 2007). Biological methods comprise *Agrobacterium tumefaciens*-mediated (Bevan 1984; Li et al. 2009; Ismagul et al. 2014; Xu et al. 2014) and virus-mediated transformation (Porta et al. 2002; Gelvin 2005) processes.

Depending on the research objectives, different transient expression studies including biolistic bombardment (Christou 1995; van den Dries et al. 2011; Sparks and Jones 2014; Liu et al. 2014), protoplast transfection (Michel et al. 1994; Sheen 2001; Ohkama-Ohtsu et al. 2008) or *Agrobacterium*-mediated transient assays (Yang et al. 2000; Li et al. 2009; Xu et al. 2014; Ismagul et al. 2014) are applicable. Among the transient transformation methods tobacco leaf infiltration (Marion et al. 2008), *Agrobacterium*-mediated transformation and particle bombardment are the most commonly used methods (Barampuram et al. 2011). *Agrobacterium tumefaciens*-mediated transformation has been extensively used since 1983 in plant molecular biology studies and for genetic improvement of crops (Lopez et al. 2004). The advantages of *Agrobacterium*-mediated transformation are the ability to transfer large size DNA fragments,

low copy number and high transformation efficiency (Ko and Korban 2004; Lopez et al. 2004; Takata and Eriksson 2012; Xu et al. 2014).

1.7.3 Critical factors in developing an *Agrobacterium*-mediated plant transformation protocol for Linderniaceae species

In the optimization of the *Agrobacterium*-mediated transient transformation for different plant species, many studies showed that one must take into consideration the age of plants (Armstead et al. 1987; Chabaud et al. 1988; Yang et al. 2000), growth stage of *Agrobacterium* cells (Song, 2001), concentration of the bacteria (Fillati et al. 1987) and duration of co-cultivation (Chabaud et al. 1988; Fillati et al. 1987).

Co-cultivation as a simplified infiltration method has been developed for *Arabidopsis* seedlings to transiently transform root epidermal cells with *A. rhizogenes* (Campanoni et al. 2003). Also the *Agrobacterium*-mediated co-cultivation method the so-called FAST assays (Fast Agro-mediated Seedling Transformation) was developed for *Arabidopsis* seedlings (Li et al. 2009). The method provides a useful and quick transient assay of test promoter activity or gene expression (Li et al. 2009). *Agrobacterium*-mediated transient transformation of leaf explants have also been described for other plant species, but it is not known how well these assays work in *C. plantagineum* and two *Lindernia* species. However, *Agrobacterium*-based stable transformation method of leaves has been developed in *C. plantagineum* successfully (Furini et al. 1994).

1.8 Objectives of the study

Analyzing the promoter function requires use of an effective transformation method. Transient transformation methods are suitable to analyze promoter function under various abiotic stresses. Transient transformation methods are fast and flexible, compared to generation of stably transformed lines and the results are not influenced by the chromosomal insertion position as reported for stable transformation. A transient transformation method, particle bombardment, which was used in previous studies to analyze the promoter activity in three Linderniaceae

species, was not suitable for quantitative measurement of promoter activity. Therefore, optimization of the method possessing capability to analyze the promoter activity accurately is one of the objectives of this study. In this study, two desiccation tolerant species, *C. plantagineum* and *L. brevidens,* and a desiccation sensitive species *L. subracemosa* have been used as experimental models.

- An *Agrobacterium*-mediated transient transformation method called "co-cultivation" was developed for three close related species. The important parameters such as leaf size, Silwet concentration, bacteria density and duration of co-cultivation, which have the key role in the functionality of the method, was optimized. Then activity of the minimal inducible promoter fragments of LEA-like 11-24 gene were compared among three species under ABA and osmotic stress treatments. Finally, the applicability of the co-cultivation method for Linderniaceae species has been discussed.
- Previous comparison of *LEA-like 11-24* transcript and protein accumulation from *C. plantagineum*, *L. brevidens* and *L. subracemosa* under dehydration and ABA treatment showed the highest expression level for *C. plantagineum* and the lowest for *L. subracemosa*. Differences in the promoter sequences have been proposed to explain this gene expression differences. Therefore, to understand the effect of trans-environmental regulation in the activity of *LEA-like 11-24* promoter fragments of *C. plantagineum*, *L. brevidens* and *L. subracemosa*, cross comparison has been performed using the co-cultivation method and the activity of each promoter fragments in homologous and heterologous background have been compared.
- On the other hand, *Cp LEA-like 11-24* promoter fragment has been shown to interact with CpbZIP1 proteins in the yeast-one-hybrid system. Therefore, the trans-activation of the *Cp LEA-like 11-24* promoter fragment by the *CpbZIP1* coding sequence under the control of CaMV-35S promoter has been investigated in a homologous background (in *C. plantagineum* leaves) using the co-cultivation method or heterologous background using stably transformed *Arabidopsis* plants.
- Furthermore, Protein sequences of *CpbZIP1* shows 47% similarity with a transcription factor *AtbZIP53* from *Arabidopsis* plants, which abundantly expressed during seed maturation.

Therefore, it was the objective to analyze the stress responsiveness of the *CpbZIP1* transcript in response to dehydration, ABA and salt stresses at different time points.

- In addition, a closely related homologue of *CpbZIP1* in *Arabidopsis*, *AtbZIP53*, functions in remobilization of carbon or nitrogen in starvation/darkness. Therefore, it would be interesting to know whether *CpbZIP1* has the same function in *C. plantagineum*. Thus, the hypothesis of Farrant and Moore 2011 that seed specific response genes of *A. thaliana* were re-activated in the vegetative tissue of desiccation tolerant plants could be proved. For this, the *Arabidopsis* transgenic plants ectopically expressing the *CpbZIP1* have been generated and some selective functional analyses were performed.

2. Materials and Methods

2.1 Materials

2.1.1 Plant Material

Wild type of *Arabidopsis thaliana* ecotype Col-0 was used in this study. All transgenic plants were generated based on the Col-0 ecotype. Transgenic lines containing *Cp LEA-like 11-24 GUS* (long promoter, 1.5 kbp, L-lines) had been generated (Velasco et al. 1998), while other transgenic lines such as G-lines (*Cp LEA-like 11-24 GUS* (short promoter, 307 kbp), GZ-lines (*Cp LEA-like 11-24::GUS* (short promoter) + 35S::CpbZIP1), LZ-lines (*Cp LEA-like 11-24::GUS* (long promoter) + 35S::CpbZIP1), S-lines (35S::CpbZIP1) were established in the Institute of Molecular Physiology and Biotechnology of Plants (IMBIO). *Craterostigma plantagineum* Hochst was collected in eastern Africa as described in Bartels et al. (1990) and maintained in IMBIO. *Lindernia brevidens* and *Lindernia subracemosa* were collected in the tropical rainforests of Taita Hills in Kenya (Philips et al. 2008) by Prof. E. Fischer (University of Koblenz, Germany) and cultivated in the botanical garden of the University of Bonn.

2.1.2 Buffers, solutions and media

All solutions and media were autoclaved for 20 min at 120ºC at 1.5 bars and kept at 4 ºC. In case of toxic or degradable solutions, filter serialization was applied.

Vitamin solution for plant media:	2 mg/ml glycine; 0.5 mg/ml niacin (Nicotine acid); 0.5 mg/ml pyridoxine-HCl; 0.1 mg/ml thiamine-HCl. A dilution of 1:1000 was Used and stored at 4°C
50X TAE (Tris-Acetate-EDTA):	2 M Tris base; 100 mM EDTA, pH 8.0; pH was adjusted with glacial acetic acid..
Ampicillin (made as stock solution):	100 mg of Ampicillin was dissolved in 1 ml water; filter sterilization was applied and stored at -20°C; (concentration of working solution: 1:1000 dilution).

Kanamycin (made as stock solution):	50 mg of kanamycin was dissolved in 1 ml water, filter sterilization was applied and stored at -20°C; (concentration of working solution: 1:1000 dilution).
Rifampicin (made as stock solution):	50 mg of Rifampicin was dissolved in 1 ml DMSO and stored at -20°C; working solution: 1:500 dilution of stock solution
1 X TE buffer:	10 mM Tris-HCl; 1mM EDTA (pH 8.0)
10 X DNA loading buffer (10 ml):	25 mg Bromophenol blue, 1 ml 1 X TAE; 25 mg Xylencyanol; 3 ml glycerol; 6 ml sterile water.
X-Gluc staining solution:	0.5 mg/ml X-Gluc dissolved in DMF (100 µl DMF per 10 mg X-Gluc); 0.1% (v/v) Triton X-100; 50 mM $NaHPO_4$ buffer, pH 7.2; 8 mM β-mercapto-ethanol; stored at -20°C.
RNase A (made as stock solution):	10 mg of RNase A was dissolved in 1 ml sterile water and stored in aliquots at -20°C.
Denaturing buffer:	0.5 M NaOH; 1.5 M NaCl; stored at room temperature.
Neutralizing buffer;	1.5 M NaCl; 1 M Tris; pH was adjusted to 8.0 with concentrated HCl.
20X SSC	0.3 M sodium citrate; 3 M NaCl; store at room temperature
10X MEN :	200 mM MOPS (3-(N-morpholino) propanesulfonic acid); 80 mM sodium acetate pH7.0. Then 0.5 M EDTA pH 8.0 was added to a final concentration of 10 mM and filter sterilised. It should be protected against direct exposure to light.
RNA-, DNA-blot washing buffer:	2X SSC (Saline sodium citrate); 0.1% (w/v) SDS.
100X Denhardt's solution:	2% (w/v) Ficoll-400; 2% (w/v) PVP 360000; 2% (w/v) BSA.

	Store in aliquots at -20°C.
LB-medium (per litre):	10 g peptone; 5 g yeast extract; 10 g NaCl; pH 7.5; 15 g bacto-agar was added to the liquid medium for solidification.
YEB (per liter):	5 g meat extract; 5 g peptone; 2 mM $MgSO_4$; 5 g sucrose; 1 g yeast extract; pH was adjusted to 7.0; 15 g bacto-agar was added to the liquid medium for solidification.
SOC (per litre):	10 mM NaCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$; 2% (w/v) trypton; 0.5% (w/v) selected yeast extract.
MS-medium (per litre):	4.6 g MS-salt; 1 ml vitamin mixture; 20 g sucrose, pH adjusted to 5.8; 8 g select-agar was added to the liquid medium for solidification.

2.1.3 Bacteria

2.1.3.1 *E. coli* DH10B (Lorrow and Jessee, 1990)

For all studies genotype: F-mcrA Ä(mrr-hsdRMS-mcrBC) 80d lacZÄM15 ÄlacX 74 endA1 recA1 deoR Ä(ara, leu)7697 araD139 galU galK nupG rpsL ë was used.

2.1.3.2 *Agrobacterium tumefaciens* GV3101/pmP90RK (Koncz and Shell 1986)

This strain was used for transient and stable transformations, either in wild-type *A. thaliana* or *C. plantagineum* plants.

2.1.4 Plasmid vectors

The plasmid vectors were kept at -20°C (IMBIO). The bacteria were frozen in 100% glycerol and stored at -80ºC. Plasmid vectors used in this study are listed below.

2.1.4.1 pJET1.2

The vector pJET1.2 (Fermentas, St. Leon-Rot, Germany) was used to clone PCR products as described by the manufacturer.

2.1.4.2 pBT10-GUS

The vector pBT10-GUS (Sprenger-Haussels and Weisshaar 2000) is an expression vector containing the coding sequence of the β-glucuronidase gene (GUS/*uidA*). The vector contains the β-lactamase gene which is ampicillin resistant. *Cp, Lb and Ls LEA-like 11-24::*GUS constructs (Van den Dries et al. 2011) were used for cloning the promoter fragments in the pBIN19 binary vector (Vector map see AppendixXXXXX).

2.1.4.3 pGJ280

This vector contains the GFP reporter gene (Green Fluorescent Protein), constructed by Dr G. Jach (Max-Planck-Institute, Cologne, Germany) which was expressed under the control of CaMV 35S promoter. It was used for the calculation of the relative activity of the promoter fragments in biolistic transformation (vector map see AppendixXXXXX).

2.1.4.4 pBIN19

The plasmid pBIN19 (Bevan 1984; Frisch et al. 1995) is a binary vector was used to transform *Agrobacterium tumefaciens* with either *LEA-like 11-24-GUS* construct from *C. plantagineum*, *L. brevidens*, *L: subracemosa* or CaMV35S-*CpbZIP1* or CaMV35S-GUS constructs. pBIN19 vector contains the *NPTII* gene encoding the neomycin phosphotransferase enzyme which confers the kanamycin resistance to *A. tumefaciens* cells and plants.

2.1.5 Primers (5‘-3‘) Sequences

All primers were synthesized by Sigma-Aldrich, dissolved in sterile water to reach 100 μM concentration and frozen at -20 ºC.

Table 2-1: List of primers used in this study.

Primer name	**Sequence** (5’ to 3’)
p35S-pROK2_fwd	CACTGACGTAAGGGATGACGC

pGJ280_fwd	ACG AATCTCAAGCAATCAAGCA
pGJ280_rev	TGTGCCCATTAACATCACCA
pBT10 left	TTAATGCAGCGGATCA
pBt10 right	TTTTGATTTCACGGGT
pBIN Direction Hind_fwd	AGCTATGACCATGATTACGCCAAG
pBIN Direction EcoRI_rev	CGATTAAGTTGGGTAACGCCAGG
GUS start_rev	GGTTGGGGTTTCTACAGGACG
GUS sense_ fwd	CGTCCTGTAGAAACCCCAACC
GUS_ rev	GATAGTCTGCCAGTTCAGTTCG
pROK-NPTII_fwd	TGGATTGCACGCAGGTTCTC
pROK-NPTII_rev	TGGGCGAAGAACTCCAGCAT
PA17 Hind MU_fwd	GAGGCAGAGGAAGCGCAAACTCTCCAACCGCGAATC
PA17 Hind MU_rev	GATTCGCGGTTGGAGAGTTTGCGCTTCCTCTGCCTC
RT Cp bZIP1_fwd	CAATTGTACCTCAGCGTTGC
RT Cp bZIP1_rev	CTCACATCAGAAGCAACTCGAA
Cp TKT3_fwd	GCAGAACCTTAAGAGGCCATC
Cp TKT3_rev	GGCACTCTCACCACCAAGAAC
Cp LEA-like 11-24_fwd	TCGGAAGACGAGCCTAAGAA
Cp LEA-like 11-24_rev	AGATGAAGACAAGGCGCTGT
At-ProDH1_fwd	ATGGCAACCCGTCTTCTCCG
At-ProDH1_rev	CGGAGAAGACGGGTTGCCAT
pJET_fwd	CGACTCACTATAGGGAGAGCGGC
pJET_rev	AAGAACATCGATTTTCCATGGCAG

2.1.6 Membranes, enzymes and markers

For RNA-blots the nylon membrane Hybond TM (Amersham Biosciences, Buckinghamshire, UK) was used. Restriction enzymes and buffers were provided by MBI-Fermentas (St. Leon-Rot, Germany), Roche/Boehringer (Mannheim, Germany, Sigma-Aldrich (Munich, Germany). The DNA ladder (1 kb) was from Invitrogen/GibcoBRL (Karlsruhe, Germany).

2.1.7 Kits

NucleoBond® Xtra Maxi Kit (Macherey-Nagel; Düren, Germany) was used for Midi-Maxi-prep of Plasmid DNA. PCR product or DNA fragments were cloned in pJET vector using the CloneJET^{TM} PCR cloning Kit (Fermentas; St. Leon-Rot, Germany). DNAs were purified from agarose gel with Qiaex II Gel Extraction Kit (Qiagen, Hilden, Germany) and NucleoSpin® Extract II (Machenery- Nagel, Düren, Germany). The Reverse Aid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas; Burlington, USA) was used for the RT–PCR cDNA synthesis. QuikChange® II Site-Directed Mutagenesis Kit (Stratagene; Heidelberg, Germany) was used to generate site-directed mutations. The Hexa Label^{TM} DNA Labeling Kit (Fermentas, Germany) was used for ^{32}P-labeling of DNA probes.

2.1.8 Instruments and other devices

The following equipment and devices were used according to the manufacturer's instructions:

- Elektroporation apparatus Gene Pulser II, (Bio-Rad, Hercules, USA).
- Typhoon Scanner (Amerscham Pharmacia Biotech).
- Electrophoresis power supplies, (Gibco BRL, Carlsbad, Canada).
- VersaFluorTM Fluorometer (Bio-rad, Munich, Germany).
- Confocal Laser Scanning Microscope ZE2000 with Laser D-eclipse C1, (Nikon, Düsseldorf, Germany).
- Gun Biolistic®: Particle Delivery System 1000/He System Bio-Rad (Hercules, USA).
- Spectrophotometer smartSpec 3000 (Bio-rad, Hercules, USA).
- T3-Thermocycler (Biometra, Göttingen, Germany).
- BioSpec-nano spectrophotometer (Shimadzu Biotech, Chiyoda-ku, Japan).
- pH meter (Mainz, Germany).
- Binocular microscope SMZ-800 (Nikon, Düsseldorf, Germany).
- Gas exchange and chlorophyll fluorescence analyser GFS-3000 (Hein walz, Germany).

2.1.9 Chemicals

Chemicals used during this project were purchased from the following companies:

- Amersham Buchler-Braunschweig, Boehringer-Manheim
- Merck VWR International, Darmstadt, Germany
- Qiagen, Hilden, Germany
- Pharmacia, Freiburg, Germany
- Machery Nagel, Düren, Germany
- Roth, Karlsruhe,Germany
- Biomol, Hamburg, Germany
- Stratagene Heidelberg, Germany

2.1.10 Software, programs and online tools

DNA sequencing reactions were performed by Macrogen DNA sequencing services (Seoul, Korea). DNA sequencing primers are listed in table 2-1. The Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify regions of sequence similarity between query sequences and sequences in the database. DNA sequence alignments were performed with APE or Vector NTI™ Suite Program ver. 10.0. DNA sequence were translated to protein sequences with the ExPASy translate tool. (http://www.expasy.ch/tools/dna.html). Program IMAGE J 1.45v as free software was used for measuring the root elongation in the seedlings.

2.2 Methods

2.2.1 Growth conditions

2.2.1.1 L. brevidens, L. subracemosa and C. plantagineum

Lindernia brevidens and *Lindernia subracemosa* were grown on MS medium containing 4.6 g MS-salt; 1 ml vitamin stock; 20 g sucrose pH adjusted to 5.8, 0.8% (w/v) select-agar (Invitrogen, Karlsruhe, Germany). *Craterostigma plantagineum* plants were grown, either *in*

vitro under sterile conditions on half-strength MS medium or in pots filled with clay granulate (Seramis;Masterfoods, Verden, Germany). All plants were maintained in climate-controlled shot day conditions at a day/night temperature of 22°C/18°C, 80 E/m2/s.

2.2.1.2 *Arabidopsis thaliana* L. cv Columbia

Arabidopsis thaliana plants were germinated and cultivated in growth chamber at a 8 h light/ 16 h dark and day/night temperature of 22°C. Transgenic lines were generated in Col-0 ecotype of *Arabidopsis thaliana* by floral dip method.Transgenic plants used in this work are listed bellow: Accession of *A thaliana* WT (Col 0), S-lines, S represents CaMV35S::CpbZIP1. G-lines, G represents *Cp LEA-like 11-24short prom::GUS* (307 bp) transformed in *A. thaliana*. GZ-lines, GZ represents *Cp LEA-like 11-24 short prom::GUS +35S::CpbZIP1* transformed in *A. thaliana*.. L-lines, L represents *Cp LEA-like 11-24 long prom::GUS* (1.5 kbp) transformed in *A. thaliana*. LZ-lines, LZ represents *Cp LEA-like 11-24* long prom*::GUS Arabidopsis transgenic* line *transformed with 35S::CpbZIP1*. S*L lines,S*L represents crossed lines of *Cp LEA-like 11-24::GUS* (16-11)* *35S::CpbZIP1* (S28). The number after represented lines assigned to independent transgenic lines in T2 generation.

2.2.2 Seed sterilization and cultivation

For analyses of the plants at the seedling stage, seeds washed for 2 minutes with 70% (v/v) ethanol followed by 10-12 minutes sterilization with the mixture of 7% (v/v) sodium hypochlorite (NaOCl) and 0.1% (w/v) SDS with regular inverting. Afterwards, the seeds were rinsed five times with sterile water and sown on MS-agar plates (Murashige and Skoog 1962). Transgenic seeds were selected in MS-agar plates supplemented with 50 μg/ml kanamycin and after 10 days, kanamycin-resistant ones were transferred either onto a new plates for further stress experiments or into the soil-pots and then subjected to the various stress treatments at a 4-5 week old.

2.2.3 Bacterial growth conditions

Agrobacterium tumefaciens strain (GV3101) was cultured on a solid YEB medium on plate or liquid culture supplemented with appropriate antibiotics under shaking (200-220 rpm) and in 28°C. *E. coli* strain (DH10B) was grown in LB (Luria-Bertani) medium containing 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl. The pH was adjusted to 7.0 and 1.5% (w/v) select-agar was added if required.

2.2.4 Bacterial glycerol stock preparation

A single colony of the bacteria was inoculated in 3 ml LB medium with appropriate antibiotics and grown overnight with shaking at 220 rpm and proper temperature. The day after, 500 µl of the bacteria culture was mixed with an equal volume of 100% autoclaved glycerol, frozen immediately in liquid nitrogen and placed at -80°C.

2.2.5 Plasmid purification

2.2.5.1 DNA plasmid purification in *E. coli*, mini- prep

Plasmid DNA was isolated from *E. coli* cells using the method of Sambrook et al. (1989). For plasmid preparation from *E. coli* cells, a single colony was inoculated in 3 ml LB medium containing appropriate selection markers and grown under constant shaker (220 rpm) overnight at 37°C. The bacteria pellet was harvested by centrifugation for 5 minutes at 6000 rpm and room temperature. The supernatant was removed and the pellet was resuspended in 200 µl solution I by vortexing for a few seconds and kept at room temperature for 5 min. Then 400 µl of solution II (lysis buffer) was added, the mixture was mixed by inverting the tube five times and incubated for 3 minutes without shaking. 300 µl of ice-cold solution III containing 3 M potassium acetate (PH 5.5) was added to the mixture and gently mixed in an inverted position for 10 seconds to obtain a viscous bacterial lysate. The tube was stored on ice for 5 minutes and ccentrifuged for 5 minute at 13,200 rpm and 4°C. The aqueous phase was transferred to the new tube and equal volume of phenol:chloroform (24:1) was added and mixed. The supernatant was transferred to a new tube after centrifugation for 10 min at 13200 rpm and 4°C. To obtain high quality plasmid

DNA, 0.1 volume 3 M sodium acetate (pH 5.2) and 0.8 volume of isopropanol were added and centrifuged for 20 min at 14000 rpm after 5min incubation on ice. Precipitated DNA was collected and washed twice with 1 ml 70% ethanol for 5min at 4°C. The air-dried DNA pellet was dissolved in 25 µl sterile miliQ water or Tris-HCl (pH 8).

Solution I: 50 mM Tris-HCl, 10 mM EDTA pH 8.0, 100 µg/ml RNase A; **Solution II:** 200mM NaOH, 1% SDS (always prepared freshly); **Solution III:** 3 M Potassium acetate (pH 5.5, adjusted with glacial acetic acid).

2.2.5.2 Plasmid DNA purification of *A. tumefaciens* cells

Plasmid DNA was extracted from *A. tumefaciens* according to Birnboim and Doly (1979). A single colony was cultured in 2 ml YEB medium containing appropriate selection markers (in this case kanamycin and rifampicin) and grown overnight at 220 rpm and 28°C. The culture was centrifuged at room temperature for 5 minutes at 6000 rpm. 400 µl of solution I was added and the bacteria pellet was resuspended. The mixture was incubated at room temprature for 10 min without shaking. Afterwards 800 µl of solution II was mixed with the suspension and further incubated at room temperature for 10 minutes. Following incubation 600 µl of 3 M sodium acetate (pH 5.2) and 120 µl of solution III were added to the suspension, slowly mixed and kept at -20°C for 15 min. The suspension was centrifuged for 10 min at 14000 rpm and 4°C. The supernatant was collected and divided into three Eppendorf tubes and 2 volume of cold absolute ethanol was added to each Eppendorf tube and kept at -80°C for 15 min. The aliquots were centrifuged for 10 minutes at 14000 rpm and 4°C and the pellet was resuspended in 1 ml ethanol 100%, 500 µl 0.3 M sodium acetate (pH 7.0) followed by incubation for 15 min at -80°C and centrifuged for 10 min at 14000 rpm and 4°C. Subsequently, the pellet was washed with 1 ml 70% (v/v) ethanol and dried at room temperature. Finally, 25-40 µl of 10 mM Tris-HCl (pH 8) containing 20 µg/ml of RNase A was added to the pellet and incubated at 37°C for 15 minutes. The concentration and quality of extracted DNA was tested in 0.8% agarose gel as well as nano-spectrophotometer. **Solution I:** 50 mM glucose; 10 mM EDTA; 25 mM Tris, pH 8.0; 4 mg/ml lysozyme; **Solution II:** 0.2 M NaOH; 1% (w/v) SDS (always prepared freshly); **Solution III:** 2 volume of solution II + 1 volume of phenol.

2.2.5.3 plasmid DNA purification in large scale, Maxi prep

In order to obtain higher amounts of plasmid DNA, the NucleoBond®Xtra Midi/Maxi plasmid DNA purification Kit (Macherey-Nagel, Düren,Germany) was used. The DNA extraction procedure was followed based on the protocol which was recommended by the manufacturer. It is briefly described in this part. This method is based on binding the plasmid DNA to the anion-exchange resin in a supplied columns and specific buffers for washing and neutralizing the plasmid DNA. A single *E. coli* cell was inoculated in 5 ml LB medium as pre-culture, containing the appropriate antibiotic, grown overnight at 220 rpm, 37°C. Next day, the pre-culture was added to the 250 ml of new LB medium containing appropriate antibiotic. The following day, the culture was centrifuged for 15 min at 15000 rpm, at 4°C and bacteria cells harvested. The pellet was suspended in 12 ml resuspension buffer containing RNase A (100 µg/ml) and lysed in 12 ml lysis buffer by inverting 5 times. Afterwards, chromosomal DNA and other cell components were precipitated by adding 12 ml neutralization buffer (2.8 M potassium acetate, pH 5.1). The NucleoBond® column was equilibrated with equilibration buffer and clarified lysate was loaded on the column and cell components removed via the NucleoBond® filter. Subsequently, the column was washed with 25 ml washing buffer and elute was collected by adding 15 ml elution buffer. To precipitate plasmid DNA, 10.5 ml isopropanol in room temperature was added to the mixture and centrifuged at 15000 g for 30 min at 4°C. The supernatant was discarded and the DNA pellet was washed with 5 ml 70% (v/v) ethanol. Air-dried pellet was resolved in an appropriate volume (150-200 µl) of TE buffer.

2.2.6 Nucleic acid extraction from the plant materials

2.2.6.1 Extraction of Genomic DNA from *C. plantagineum*

Genomic DNA was extracted from *Craterostigma* leaf tissues according to CTAB method (Murray and Thompson, 1980) with some modifications. Fresh young leaf tissues were frozen with liquid nitrogen and ground to a fine powder using a mortar and pestle. To extract genomic DNA, 0.5 g of polyvinylpyrrolidone which eliminate polysaccharides was added to 2 g of the plant materials. Afterwards, 8 ml of warm 2-ME/CTAB solution containing Cetyl Trimethyl

Ammonium Bromide (CTAB), 20 mM EDTA pH=8.0, 1.4 M NaCl, 100 mM Tris-HCl pH=8.0, 2% (v/v) β-Mercaptoethanol was added to the samples, thoroughly mixed by vortexing and placed for 1h at 65°C. Using 8 ml of chloroform-isoamylalcohol (v/v) (24:1) and centrifugation at 8000 rpm for 5 min at 4°C, the supernata was separated and transferred to a fresh tube. The warm CTAB/NaCl solution (10% (w/v) CTAB, 7 M NaCl) was added in 1/10 volume of the supernatant, immediately mixed with one volume of chloroform-octanol 24:1 (v/v). The mixture was centrifuged at 8000 rpm for 5 min at 4°C and supernatant was collected. One volume of CTAB precipitation buffer (1% (w/v) CTAB, 10 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0) was added to the supernatant. The samples were mixed well by inversion and centrifuged at 2000 rpm for 5 min at 4°C. The pellet washed with 70% (v/v) ethanol, dried at room temperature and resuspended in 250-400 µl of 10 mM Tris-HCl pH= 8.0 and after few hours was quantified on the agarose gel.

2.2.6.2 Extraction of total RNA from *C. plantagineum*

Total RNA was extracted from 50 mg of Craterostigma leaf tissues according to Valenzuela-Avendaňo et al. (2005). Leaf materials were ground in mortar and pestle using liquid nitrogen. 1.5 ml of extraction buffer was added to the material in Eppendorf tube and incubated at room temperature for 10 min. The suspension was centrifuged at 1000 g at room temperature for 10 min. The supernatant transferred to the new tube and mixed with 300 µl of the chloroform-isoamylalcohol (24-1) mixture. After centrifugation at 10000 g for 10 min at 4°C, clear aqueous phase transferred to the new tube, then 375 µl of isopropanol and 375 µl of a mixed solution (0.8M sodium citrate/1M sodium chloride) were added. It was mixed thoroughly and incubated at room temperature for 10 min. RNA was precipitated by centrifuging at 12000 g for 10 min, then washed with 1ml of 70% ethanol twice. The pellet was dried up at room temperature and dissolved with 100 µl of DEPC-treated water.

Extraction buffer: 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate pH 5.0, 38% (v/v) buffer-saturated phenol, 5% (v/v) glycerol **DEPC-treated** water**:** 0.2%(v/v) diethyl pyrocarbonate (DEPC) in distilled water, stirred vigorously for 1 hour and then autoclaved.

2.2.6.3 Extraction of genomic DNA from *A. thaliana*

DNA was extracted from about 100 mg of *Arabidopsis* leaf tissue in liquid nitrogen. 300 µl of 2x lysis buffer (4% sarcosyle; 1% (w/v) SDS; 40 mM EDTA, pH 8.0; 0.6 M NaCl; 0.1 M Tris-HCl, pH 8.0) were added to the ground material and homogenized. After centrifugation, 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the suspension, thoroughly mixed and centrifuged for 10 min at 13000 rpm at room temperature. The supernatant was transferred to a new tube and extracted once more with phenol/chloroform/isoamyl alcohol (25:24:1) when required. 0.7 volume of isopropanol and 0.1 volumes of 3 M sodium acetate (pH 5.2) was added to the supernatant and centrifuged for 20 min at 13000 rpm and 4°C. The DNA pellet was washed twice with 70% (v/v) ethanol, dried and dissolved in 25 µl of re-suspension buffer containing 10 mM Tris-HCl (pH 8.0) and 20-40 µg/ml RNase A. Extracted DNA was incubated at 37°C for 15-20 min to activate the RNase and degrade the RNAs in the samples. DNA quantity and quality were assessed using a nano-spectrophotometer (Shimadzu Biotech, Chiyoda-ku, Japan) or 1% (w/v) agarose gel, respectively.

2.2.6.4 Extraction of total RNA from *A. thaliana*

Total RNA was extracted from 100-200 mg of *A. thaliana* leaf tissues ground to a fine powder in liquid nitrogen. 500 µl of the extraction buffer (10 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 0.3 M LiCl; 6 M urea) were added to the leaf material. The suspension was centrifuged for 5 minutes at 13000 rpm and 4°C after adding one volume of phenol/ chloroform/ isoamyl alcohol (25:24:1). Then aqueous phase was extracted once more with one volume of phenol/ chloroform/ isoamyl alcohol (25:24:1). The supernatant was transferred to the new tube and RNAs were successively precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.7 volume of isopropanol and centrifuging for 20-30 minutes at 13000 rpm and 4°C. The RNA pellet was washed twice with 1 ml of 70% (v/v) ethanol and after each washing step centrifuged for 2 minutes at 4°C. Air-dried pellets were dissolved in 20-25 µl RNase free water and stored at -70°C.

2.2.7 Purification of extracted DNA:

To obtain pure DNA for PCR reactions, purification of manually extracted DNA is very important. DNA samples were brought to 100 µl with sterile water, supplemented with one volume of phenol/ chloroform/ isoamyl alcohol (25:24:1) and thoroughly mixed. The suspension was centrifuged for 10 minutes at 14000 rpm and room temperature followed by the transfer of the upper phase to the fresh tube. The DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 96% ethanol by centrifuging for 20 minutes at 14000 rpm and 4°C. The pellet was washed with 70% (v/v) ethanol and dissolved in 20 µl of 10 mM Tris-HCl (pH 8.0). The concentration of DNA was measseard using a nano-spectrophotometer

2.2.8 Agarose gel electrophoresis

Agarose gel electrophoresis was used for separating DNA fragments after digestion or PCR amplification. 1 g of agarose was dissolved in 100 ml 1 X Tris-acetate-EDTA (TAE) buffer and boiled in microwave to be well melted. The solution was poured in a gel tray and solidified for 1 hour. The solidified gel was laid in an electrophoresis chamber and run at 90 V in 1 X TAE buffer. Finally the agarose gel was stained with ethidiumbromide (0.5 mg/ml) solution, rinsed with distilled water and visualized by ultraviolet (UV) light.

2.2.9 Estimation of DNA and RNA

The quantity of DNA or RNA was measured spectrophotometrically at 260 nm, while the quality was assessed by both, electrophoresis in 1% agarose gel and spectrophotometrical measurement at 230, 260 and 280 nm. A value of OD_{260} = 1 is considered as 50 µg/µl for a DNA solution and 40 µg/µl for RNA solution. The ratios of OD_{260}/OD_{230} and OD_{260}/OD_{280} are important to have it pure DNA. The value of pure DNA (OD_{260}/OD_{280}) is around 1.8 – 2. It becomes smaller when contaminates with protein. The ratio of OD_{260}/OD_{230} in pure DNA is around 2.2 and decreases when the DNA is contaminated with phenolic compounds.

2.2.10 RNA blot analyses

RNA blot analyses were carried out with 20 μg total RNA per sample. 1 g of agarose was boiled in 62 ml of dH_2O and cooled down at room temperature to 60°C. The mixture of 20 ml of 10 X MEN buffer and 18 ml of 37% deionized formaldehyde were added to the melted agarose. It was immediately poured on the tray and allowed to solidify under the hood. One volume of RNA-blot loading buffer was mixed with RNA samples, heated at 70°C for 5 minutes and loaded into the gel. The electrophoresis was carried out at 100 mA till the loading dye moved at least 8 cm from the wells. The blotting was performed according to Bartels et al. (1990) using nylon membrane (Hybond ^{TM}-N, Amersham Biosciences; Buckinghamshire, UK) and transfer buffer (10 X SSC). RNA was bound to the membrane by exposing it to UV light for 2-3 min, afterwards; the membrane was baked at 80°C for 1 hour.

RNA running buffer: 100 ml of 10 X MEN buffer mixed with 820 ml sterile distilled water; 80 ml of 37% (v/v) deionized formaldehyde mixed to reach 1 liter.

RNA-blot loading buffer for 1 ml: 175 μl of 37% (v/v) deionized formaldehyde, 50 μl 10 X MEN buffer; 500 μl formamide; 255 μl of 100% glycerol and 20 μl of 10% (w/v) bromophenol blue were mixed.

2.2.10.1 Staining of membranes

To check the efficiency of the transfer, the membrane was stained with a methylene blue solution (0.04% (w/v) methylene blue in 0.5 M sodium acetate, pH=5.2) for 8 minutes and washed three times with dH_2O. The membrane was slightly moved until the blue-stained RNA bands were appeared. The photograph was taken and the membrane was used, either for pre-hybridization or kept in Whatman paper and stored until use. The amount of RNA > 20 ng can be detected and stained with methylene blue. The bluish color in the background can be removed from the membrane by washing with 0.1-1% SDS, which is present in the pre-hybridization solution as well.

2.2.10.2 Preparation of a^{32}P-dCTP hybridisation probe (Feinberg and Vogelstein, 1983)

Preparation of prob was according to Feinberg and Vogelstein (1983). To synthesize the probe, a cDNA or PCR fragment of the respective gene was used. The PCR product was purified using the NucleoSpin® Extract II kit. The radioactive labeling probe was performed using the Hexalabel^{TM}DNA labeling Kit (MBI Fermentas; St. Leon-Rot, Germany). 100 ng of DNA was mixed with 10 μl of 10 X hexanucleotides buffer and dH_2O to reach a final volume of 40 μl. The DNA probe was heated for 5 minutes at 95°C and immediately transferred onto the ice. The reaction mixture was mixed with 2 μl a^{32}P-dCTP, 3 μl of Mix C (dNTPs without dCTP) and 1 μl of Klenow fragment. The reaction was incubated at 37°C for 10 min, and then 4 μl dNTP–Mix was added to the mixture, incubated at 37°C for 5 minutes and finally mixed with 50 μl of 1 X TE (pH 8.0) to stop the reaction. The labeled probe was separated from non-incorporated dNTPs by Sephadex G-50 column pre-equilibrated with 1 X TE buffer. After measuring the counts of the fractions using Geiger counter as first peak was taken, the sample was denaturised at 95°C for 5 minutes stored on ice and used as radioactive probe for hybridization.

2.2.10.3 Hybridization procedures

The membrane was incubated with nucleic acids and northern pre-hybridization buffer for approximately 2-3 hours in a water bath at 42°C. The buffer was exchanged by the hybridization buffer prior to adding the denatured a^{32}P-dCTP probe. The hybridization was performed overnight at 42°C under rotated condition. Next day, hybridization buffer was removed and the membrane was washed twice with 0.1% (w/v) SDS and 2 X SSC for 20 min at 42°C and once for 20 min at 65°C to remove unspecific probes from the membrane. The radioactivity level was checked with a Geiger counter. When little radioactivity was left on the membrane, it was dried on filter paper with RNA side up and kept in a transparent plastic bag. The membrane was exposed to a signal on phosphor imager screens for a few days. The screens were analysed with the Typhoon 8610 imager system (Amersham Biotech, Freiburg, Germany).

Hybridization buffer: 50% (v/v) formamide, 10 mM PIPES (pH 6.8), 5 X SSC, 0.1% SDS (w/v), 1 X Denhardt's solution (100 X 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). **Washing Buffer:** 0.1% (w/v) SDS, 2 X SSC.

2.2.11 Quantitative estimation of extracted protein

The concentration of the extracted protein was determined according to Bradford (1976) using a Bio-RAD protein assay kit. 5-10 µl of extracted protein was mixed with 200 µl of Bradford dye and 800 µl of sterile water and incubated at room temperature for 5 minutes followed by an OD measurement at 595 nm. The amount of protein was calculated from a standard curve made for different concentrations of BSA (bovine serum albumin).

2.2.12 Cloning of DNA fragments

2.2.12.1 Polymerase chain reaction (PCR)

A standard PCR reaction in a total volume of 20 µl was prepared as followed:

16.4 µl	H_2O (sterile double distilled)
2 µl	10 X PCR-buffer containing $MgCl_2$
0.5 µl	Forward-primer (10 µM/µl)
0.5 µl	Reverse-primer (10 µM/µl)
0.4 µl	10 mM dNTPs
1.0 µl	Genomic DNA (50-100 ng/µl) or plasmid DNA (5 ng/µl) or bacterial colonies
0.2 µl	Taq-polymerase

Reactions were mixed briefly and run in a PCR thermocycler (Thermo block, Biometra, Göttingen, Germany). Depending on the level of gene expression, the number of PCR cycles was adjusted. The following PCR program was used for amplification of plasmid DNA:

94°C	3 min (denaturation)
94°C	30 sec (denaturation)
X °C (Annealing)*	30 sec (primer binding)
72°C	30 sec (elongation)

72	5 min for final extension
4°C	For keeping the samples stable

* Annealing temperature = $T_M \pm 4$ °C; T_M = melting temperature of the primers. In the case of primers with different T_M, the lower one was used for calculation of the annealing temperature.

2.2.12.2 Restriction endonuclease treatments

DNA digestion was performed by restriction endonucleases according to the following criteria: For digestion of 1 µg of DNA 5 U of restriction enzyme and 1/10 dilution of reaction buffer (10 X) of the final volume were used. For double digestion, suitable buffers were used to ensure that both restriction enzymes are active.

2.2.12.3 Dephosphorylation

Digested DNA fragments were dephosphorylated at the 5' end with shrimp alkaline phosphatase (SAP, Boehringer/Roche, Mannheim, Germany) in order to avoid self ligation of 5' and 3' compatible ends of plasmid DNA. The reaction was performed in a total volume of 10 µl containing 1 µl 10 X SAP buffer, 1 µl (1 unit) SAP, and sufficient amount of the plasmid vector. The mixture was made up to 10 µl with sterile distilled water and incubated at 37°C for 10-20 minutes. Afterwards, the SAP enzyme was inactivated by heating at 65 °C for 15 minutes.

2.2.12.4 Ligation

To make plasmid DNA construct, the desired insert-DNA was ligated with a linearized vector according to Maniatis et al. (1989) and the recommendation of Fermentas (http://www.fermentas.de/product_info.php?info=p580) in the following steps: The ligation reaction was made in 20 µl (final volume) which comprises of 1 µl ligase buffer (10 X), X µl digested and purified plasmid DNA vector (50 ng), 1 µl T4 DNA ligase (MBI-Fermentas; St. Leon-Rot, Germany), and y µl insert-DNA. The mixture was adjusted to 20 µl with sterile H_2O and incubated for 20 h at 16°C. The molar ratio of DNA insert and plasmid vector in the ligation reaction should be at least 3 to 1. The amount of vector and insert DNA was determined according to the following formula: X ng of insert = (3) x (bp insert) (50 ng linearized plasmid) ÷ (size of plasmid in bp).

2.2.13 Preparation of competent cells and bacterial transformation methods

2.2.13.1 Preparation of rubidium chloride competent cells for *E. coli*

Cells from *E. coli* DH10B strain was used to make competent cells. A single colony was grown in 3 ml LB liquid medium. Following the overnight incubation at 37°C, 1 ml of overnight pre-culture was inoculated into 50 ml of LB medium. The bacteria culture was grown at 220 rpm at 37°C till OD_{600} was between 0.35 and 0.45. After centrifugation the pellet was resuspended in 15 ml of cold TBFI and incubated on ice for 10 minutes followed by centrifugation for 10 minutes at 4000 rpm and 4°C. Supernatant was discarded and the pellet resuspended once more in 15 ml of cold TBFI followed by centrifugation at the same conditions as before. The suspension was incubated on ice for 5 minutes and centrifuged. The pellet was resuspended in 2 ml TBFII. Finally, the suspension was aliquoted in 50 µl, frozen in liquid nitrogen and stored at -80°C.

TBFI: KAc (Potassium acetate) 30 mM; RbCl 100 mM; $CaCl_2$ $2H_2O$ 10 mM; $MnCl_2$ $4H_2O$ 50 mM; Glycerol 15 (v/v) %; pH adjusted to 5.8 using 0.2 M acetic acid. **TBFII:** MOPS 10 mM; $CaCl_2$ $2H_2O$ 75 mM; RbCl 10 mM; Glycerol 15% (v/v); pH adjusted to 6.5 using potassium hydroxide (KOH).

2.2.13.2 Preparation of calcium chloride competent cells for *E. coli*

DH10B strain of *E. coli* cells was used to make competent cells. A single colony was inoculated in 3 ml LB medium and incubated overnight and few hours at 37°C until the OD_{600} reached 0.5. Afterwards, the culture was centrifuged for 5 minutes at 5000 rpm and 4°C. The pellet was resuspended in 1 ml of pre-chilled 0.1 M $CaCl_2$ and centrifuged. After resuspending the pellet in 0.9 ml pre-chilled 0.1 M $CaCl_2$ centrifugation was repeated once more. Subsequently, the pellet was resuspended in 1 ml of pre-chilled 0.1 M $CaCl_2$ supplemented with 15% (v/v) glycerol and frozen in liquid nitrogen in 100 µl aliquots.

2.2.13.3 Preparation of electrocompetent cells of *A. tumefaciens*

A single colony of *A. tumefaciens* containing the binary vector GV3101 was inoculated in 3 ml YEB-rifampicin medium and grown overnight at 250 rpm. Next day, the suspension was diluted in 50 ml of fresh YEB-rif medium and further cultured until the OD_{600} reached 0.5. Afterwards, the cell culture was centrifuged for 30 minutes at 5000 rpm. The supernatant was discarded and the pellet was resuspended in following solutions:

25 ml 1 mM Hepes pH 7.5

12.5 ml 1 mM Hepes pH 7.5

10 ml 10% (v/v) glycerol, 1 mM Hepes pH 7.5

5 ml 10% (v/v) glycerol, 1 mM Hepes pH 7.5

2 ml 10% (v/v) glycerol

1 ml 10% (v/v) glycerol

After adding of each solution the suspension was centrifuged for 10 minutes at 4°C and 5000 rpm. Finally after adding 1 ml 10% (v/v) glycerol, the competent cells were frozen in liquid nitrogen in 50 µl aliquots.

2.2.14 Transformation of bacteria

2.2.14.1 Transformation of *E. coli* cells by heat shock method

For transformation of *E. coli* cells 5-10 ng/µl of DNA or 2-5 µl of a ligation product was mixed with one aliquot (50 µl) of competent cells, incubated on ice for 20 min with tapping every 5 minutes and the incubation on ice was followed by heat shock in a water bath for 45 second at 42°C. Afterwards 650 µl of Soc medium (for 1 liter: 10 mM NaCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$; 2% (w/v) trypton; 0.5% (w/v) selected yeast extract) was added to the tube and incubated at 37°C (180 rpm) for 1 hour. Subsequently, 100-200 µl of cell suspension was spread on the plate containing appropriate antibiotics and the plate was incubated overnight at 37°C.

2.2.14.2 Transformation of *A. tumefaciens* cells by electroporation method

Before starting the transformation one aliquot of electro-competent cells was thawed on ice and 5-10 ng/µl DNA or 1 µl of ligation product was added to them. The mixture was mixed by slight tapping and loaded into pre-chilled electro-cuvette (Bio-Rad, Germany). A single pulse which brings the foreign DNA into the cells (GenePulser II, Bio-Rad) was applied. 1 ml YEB-medium was added into the cuvette and mixed thoroughly. Afterwards, the cells were transferred to a new Falcon tube grown for 1-2 hours at 28°C. 100-150 µl of the cell culture were spread on selective media and incubated at 28°C.

Table 2-2: Electroporation parameters of *A. tumefaciens*

Transformation parameters	*A. tumefaciens*
DNA	Only plasmid
Elecro-Cuvette	2 mm
Resistance	400 Ω
Power	2.5 kv
Capacity	25 µlF
incubation medium	YEB
incubation temperature	28°C

2.2.15 Plant transformation

2.2.15.1 *S*table transformation of *A. thaliana* by floral dip method

Transgenic *Arabidopsis* plants were produced by the floral dip method in wild-type plants (Col-0) according to Clough and Bent (1998). The *Agrobacterium* clone carring the transgene was cultured in 250 ml YEB media containing 50 µg/ml kanamycin and rifampicin at 28°C under shaking (250 rpm). The bacteria culture was grown until the OD_{600} reached 0.6-0.7. Then 0.05% (v/v) of Silwet L-77 (surfactant) were added to the cell suspension and mixed thoroughly. In the flowering *Arabidopsis* plants open flowers were removed, while young and non-open ones were inverted and immersed in the bacteria culture with gentle rotation for 20-25 seconds. The dipped plants were covered with a transparent plastic bag with some holes to allow for ventilation. Two

days after transformation, the plastic bags were removed and the plants were grown to produce the first generation of seeds.

2.2.16 Transient transformation

2.2.16.1 *Agrobacterium*-mediated transient transformation, FAST assay

The *Agrobacterium* co-cultivation based on the procedure described by Li et al. (2009) was optimized for 3 species of Linderniaceae family. The *A. tumefaciens* strain GV3101 (Koncz and Schell 1986) was transformed with the binary vector pBIN19 containing selected promoter::*GUS* constructs. Afterwards, leaves of *C. plantagineum, L. brevidens* or *L. subracemosa* were transiently transformed via *Agrobacterium*-mediated transient transformation.

A single colony was grown in 3 ml YEB (Yeast Extract and Beef) medium containing 5 g/l beef extract, 1 g/l yeast extract, 5 g/l peptone, 5 g/l sucrose, 0.5 g/l $MgCl_2$ with the appropriate antibiotic (50 μg/ml rifampicin and 50 μg/ml kanamycin) for 24-36 hours at 28°C. Then 20 ml YEB medium containing a selective antibiotics was inoculated with the *Agrobacterium* pre-culture to reach an OD_{600} = 0.3 and grown at 28°C; continuously shaking at 220 rpm until OD_{600} = 1.7-1.8. *Agrobacterium* cells were collected by centrifugation for 10 min at 6000 *g* and 4°C. The pellet was resuspended in 20 ml 10 mM $MgCl_2$ washed twice and resuspended in 2 ml 10 mM $MgCl_2$. *A. tumefaciens* cells were diluted to a density of OD_{600} = 0.7 for *L. brevidens* or *L. subracemosa* and OD_{600} = 1 for *C. plantagineum* with 1/4 MS medium supplemented with 1% (w/v) sucrose and 0.005% (v/v) Silwet L-77 for *L. brevidens*, *L. subracemosa,* or 0.0075% (v/v) Silwet L-77 for *C. plantagineum* to generate the co-cultivation medium. Co-cultivation was carried out in 24-well plates with three leaves per well. Leaves were handled gently with flat forceps to avoid damage and soaked with co-cultivation medium. Co-cultivation was performed in darkness for 60 hours for *C. plantagineum or L. brevidens* or 48 hours for *L. subracemosa* at 20°C. The transformed leaves were washed with 0.05% (v/v) sodium hypochlorite and rinsed with water before performing stress treatments.

2.2.16.2 Biolistic method

Leaves of *C. plantagineum* were transiently transformed with *Cp LEA-like 11-24 promoter::GUS* fusion constructs, provided by van den Dries et al. (2011), via particle bombardment. Transformed leaves were incubated in 150 mM NaCl solution and water as control for 48 hours. GFP signal was analysed 24 hours after bombardment by screening each leave under a confocal laser-scanning microscope (Nikon Eclipse TE2000-U/D-Eclipse C1, Nikon, Düsseldorf, Germany). GFP was excitated at a wavelength of 488 and detected at 515 nm. The software of EZ-C1version 3.20 (Nikon, Düsseldorf, Germany) was used for processing the images. Chlorophyll autofluorescence was detected at 570 nm after excitation at 543 nm.

2.2.16.2.1 Preparation of gold particles

Preparation of gold particles was performed according to Sanford et al. (1993). Before starting the preparation, three-month-old detached *in-vitro* leaves from *Craterostigma* arranged in the middle of a petri dish containing ½ MS medium. Briefly, 30 mg of gold particles which are 1.6 µM in diameter (Bio-Rad, Munich, Germany), were sterilized with 1 ml of 70% (v/v) ethanol via vigorous vortexing for 5 minutes and soaking for 15 minutes. Afterwards, the micro-particles were shortly spun down and the supernatant was discarded. Centrifugation was followed by triple washing with 1 ml of sterile water and subsequent vortexing for one minute, incubation for 1 minute to settle down the particles and short spinning down. After the third washing step, the gold particles were resuspended in 500 µl of sterile 50% (v/v) glycerol to a final concentration of 60 mg/ml, assuming no loss during the preparation. Finally, 100 µl aliquots of gold micro-particles were stored at 4°C.

2.2.16.2.2 Coating of gold micro-particles with DNA and bombardment

Before coating the gold particles with DNA, the tubes containing gold particles in 50% glycerol were vortexed on a platform shaker for 5 minutes to ensure an even suspension. Afterwards, 50 µl (3 mg) of gold particles were taken into a 1.5 ml sterile Eppendorf tube and used for two bombardments. In the next step, 30 µl of plasmid DNA containing 15 µl (1 µg/µl) *LEA-like 11-24* promoter::*GUS* and 15 µl (1 µg/µl) *35S CaMV::GFP* construct were added to the tube. Then,

20 µl of 100 mM freshly prepared spermidine and 2.5 M $CaCl_2$ were added to the tube and mixed with continuous shaking. The suspension was briefly centrifuged and the supernatant was discarded. Afterwards, 140 µl of 70% (v/v) ethanol was added to the suspension for washing the particles, and the suspension was spun down followed by rewashing with 140 µl 100% (HPLC grade) ethanol . After removing the supernatant, 30 µl of 100% ethanol was added to the particles and the mixture was gently mixed by tapping, briefly spun down followed by placing of 15 µl coated gold particles on the macrocarier to use for bombardment. Helium-driven microprojectile gene gun (Biolistic® Particle Delivery System-1000/He Device, Bio-Rad, Munich, Germany) was used for the bombardment. Prior bombarding, equipment was sterilized with 70% (v/v) ethanol and stopping disc as well as macrocarrier were placed in the macrocarrier holder. The plate containing leaves in the middle position was placed on position 6 below the stopping screen, and then vacuum was applied to the bombardment chamber using helium pressure until the rupture disk burst. Helium pressure of 1350 pounds per square inch (9.3 Mega Pascal (MPa)) under a vacuum of 27 mm of mercury (3.6 MPa) was applied to the *C. plantagineum* leaves. Under these conditions the macrocarrier was burst and held by the stopping screen while DNA-coated gold particles were transferred into the leaf tissues. Bombarded leaves were treated either with 150 mM NaCl or water.

2.2.17 Vitality test

Fluorescein-diacetate (FDA) is a non-fluorescent dye hydrolyzed by different enzymes such as esterases used in the vitality test. The reaction results in the production of fluorescein, which can be visualized within cells by fluorescence microscope (Schnurer and Rosswall, 1982). To perform the vitality test *Craterostigma* leaves which were transiently transformed with "*Cp LEA-like 11-24::GUS + 35S::CpbZIP1*'' construct via *Agrobacterium* co-cultivation, the leaves were firstly sectioned using razor blades and afterwards incubated in a 500-fold dilution of 0.5% (w/v) FDA solution in acetone. After 5 minutes incubation, the staining solution was washed off with water to reduce background staining. The tissues were visualized by fluorescent microscopy.

2.2.18 Screening methods

2.2.18.1 Screening of bacteria colonies

Bacteria colonies were picked from the plate and arranged in a new plate with different numbers. The colonies were either directly used for amplification of DNA insert *via* colony PCR or cultured in LB medium with appropriate antibiotics for plasmid DNA miniprep. The extracted plasmid DNA was digested with specific restriction enzymes to prove the presence of respective DNA insertion. All obtained results were confirmed by sequencing of plasmid DNA constructs.

2.2.18.2 Screening of transgenic plants

After transformation, the first set of seeds was collected from completely dried plants and sown on MS-agar plates containing 50 µg/ml kanamycin. 10-15 day after sowing transgenic seedlings (T1), kanamycin resistant seedlings could be distinguished from non resistant ones. Transgenic seedlings were appeared with green cotyledon, whereas non-transgenic ones showed yellow and dead cotyledonous. The green seedlings were transfered to soil and, later on, screened for the transgene using PCR and specific primers. The seeds of these plants were collected and germinated to produce T2 seedlings.

2.2.19 RT-PCR analyses

Reverse transcription analyses were performed with 2 µg of total RNA. RNAs were extracted from either from *C. plantagineum* leaves or *Arabidopsis* transgenic lines. To remove the DNA contaminations of extracted RNA, the samples were subjected to DNase I treatment. Briefly the reaction mixture containing 10 U DNase I, 1 µl 10 X DNase buffer (20mM Tris-HCl , pH 8.0), 50 mM KCl 2 mM $MgCl_2$ was incubated for 30 minutes at 37°C followed by adding 1 µl. 25 mM EDTA and incubation for 10 minutes at 65°C to stop the reaction. After this step, RNA can be stored at -80°C or used for the synthesis of complementary DNA (cDNA). Single strand cDNA was synthesized using 10 µl RNA pre-treated with DNase according to the protocol provided by the manufacturer (Fermentas, Burlington, USA).

2.2.20 Site-directed mutagenesis

Site-directed mutations were generated with the Quik Change® II Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). This method allows for site-specific mutations in a double-stranded plasmid, without requirement of any specialized vector or unique restriction site.

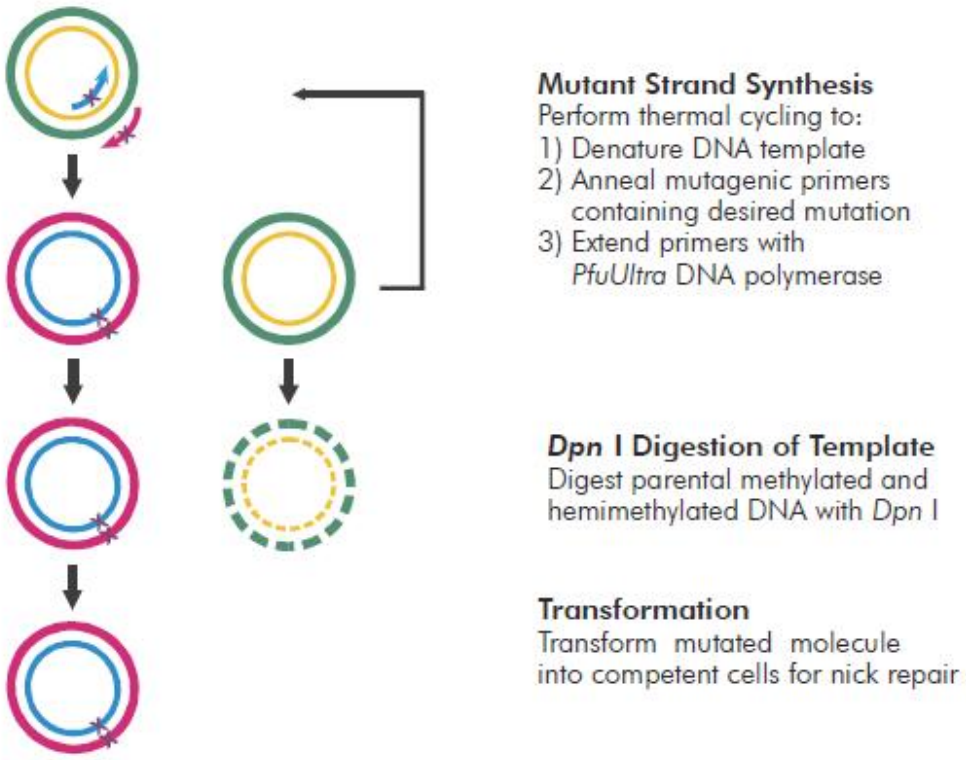


Fig. 2-1: Overview of the QuikChange® site-directed mutagenesis method (QuikChange® II Site-Directed Mutagenesis protocol, Stratagene, Heidelberg, Germany).

The method is based on the amplification of a plasmid in a PCR reaction with specific primers and subsequent digestion of the methylated parental plasmids with the restriction enzyme DpnI. The principle of this method is shown in Fig. 2-1. All steps were performed according to the manufacturer's instruction.

2.2.20.1 Designing of primer and introducing of mutations

To generate a single mutation, 'Quick Change Site-Directed Mutagenesis' program (http://www.stratagene.com/qcprimerdesign) was used. The designed primers should contain the same mutation and anneal to the same sequence on the opposite strands of the plasmid. They

should also contain 40% GC, be 25-45 bp in length and possess the melting temperature (Tm) higher than 78°C. The melting temperature is calculated with the following formula:

$$T_m = 81.5 + 0.41\ (\%GC) - 675/N$$

Where 'N' is the primer length in bases. It doesn't include the bases which are being inserted or deleted.

2.2.20.2 PCR reaction and digestion of parental DNA

Preparation according to the recipe presented as follows:

X µl (5-50 ng)	dsDNA template
0.5 µl (125 ng)	Oligonucleotide primer 1 (10 µM/µl)
0.5 µl (125 ng)	Oligonucleotide primer 2 (10 µM/µl)
0.4 µl	dNTP mix (10 mM)
16.4 µl	ddH_2O to a final volume of 50 µl
0.2 µl	PfuTurbo DNA polymerase (2.5 U/µl)

Specific mutations were introduced by performing a PCR reaction with mutagenized primers and program shown below (Table 2-3).

Table (2-3): PCR program in site direct mutagenesis

Segment	Cycle	Temperature	Time
1	1	95°C	30 seconds
		95°C	30 seconds
2	*12-18	55°C	60 seconds
		68°C	1 min per 1 kbp of plasmid length

* For single amino acid ex-change 16 cycle, for point mutations (our study) 12 cycles and for multiple amino acids deletion or insertion 18 cycles are required.

The PCR amplification resulted in a formation of a plasmid with the desired mutations. From this plasmid, the parental non-mutated and methylated template was removed by adding 1 µl of the *Dpn I* restriction enzyme (10 U/µl) followed by the incubation for 1 hour 37°C.

2.2.21 Relative water content

For the analyses of relative water content *C. plantagineum* leaves were taken from the pots and treated either with salt or kept at room temperature for 0, 3, 6 and 9 hours. Fresh weight (FW) of the samples was measured. For each sample fresh weight (FW), turgor weight (TW), after 24 hours incubation in tap water, dry weight (DW) after 24 hours incubation at 80°C were determined. Relative water content (RWC) of plants was calculated in percentage (%) using the following formula:

$$\%RWC = (FW - DW)/ (TW - DW) \times 100$$

In *Arabidopsis* overexpressing *CpbZIP1* (35S::bZIP1) lines relative water content of soil and leaves was measured in different time points.

2.2.22 Plant stress treatments

Stress treatments were performed in *Agrobacterium* mediated transient transformed plants. After co-cultivation the leaves were soaked in 100 μM ABA (100 mM stock solution in absolute ethanol) or 0.8 M mannitol for 48h in the growth room and immediately frozen in liquid nitrogen. The samples were subsequently used for GUS quantification or gene expression analyses.

To analyze the effect of stress treatments on expression of genes in *C. plantagineum*, the mature plants grown in soil were removed from the pots and kept in water supplemented with different concentration of NaCl (0, 50, 150, 300 mM) or 100 μM ABA. The plants were treated at different time points 0, 3, 6, 9 and 24 h. In the case of dehydration treatment the plants were removed from the pots and kept at room temperature. For each treatment three plants were used. The leaves and roots of these plants were separated, immediately frozen in liquid nitrogen and used for further analysis.

2.2.23 Biological and biochemical methods

2.2.23.1 Proline determination

The proline content was determined according to Bates et al (1973). Briefly, 80-100 mg of plant material was ground in liquid nitrogen with metal beads and subsequently homogenized in 2 ml

of 3% (w/v) sulphosalicylic acid. The mixture was centrifuged at 5000 rpm for 10 minutes and afterwards 1 ml of the supernatant or standard L-proline solution (1, 5, 10, 25 and 50 μM) was transferred into a Falcon tube containing 1 ml ninhydrin acid and 1 ml of glacial acetic acid. The mixture was incubated in a water bath at 100°C for one hour followed by cooling down to room temperature and proline extraction with 2 ml toluene. After centrifugation for 10 minutes at 5000 rpm and 4°C, upper organic phase was used for spectrophotometrical quantification of proline content at 520 nm against toluene which used as blank. Obtained OD_{520} values were subsequently used for the calculation of proline concentration in the plant extract using a standard curve drawn for various concentrations of standard L-proline solution. Calculated concentration was used for the estimation of free proline content in the extract according to the following formula:

Free proline content (μmol g^{-1}FW) = Estimated concentration x volume of extract in L/FW.

2.2.23.2 Determination of chlorophyll content

Total chlorophyll was extracted according to the method of Arnon (1949). Briefly, 20-60 mg of leaf material ground in liquid nitrogen was extracted with 2 ml 80% (v/v) acetone. The suspension was incubated for 30 minutes in the darkness at room temperature and subsequently centrifuged for 5 minutes at 12000 rpm and room temperature. The chlorophyll content was estimated from the absorption of the extract at 663 and 645 nm according to the following formula:

$$C= 0.002 \text{ x } (20.2 \text{ x } OD645 + 8.02 \text{ x } OD663)/g \text{ FW},$$

where C expresses the total chlorophyll content (chlorophyll A + chlorophyll B) in mg/g FW.

2.2.23.3 Lipid peroxidation assay (MDA assay)

A lipid peroxidation assay based on the reaction of lipid peroxides with thiobarbituric acid (TBA) was performed according to Hodges et al. (1999) and Kotchoni et al. (2006). The amount of malondialdehyde (MDA), a final product of lipid peroxidation process, can be quantified spectrophotometrically and used as stress indicator. In short, 20-60 mg of plant material was ground with metal beads in Eppendorf tubes, mixed by vortexing with 1 ml of pre-chilled 0.1%

(w/v) trichlotoacetic acid (TCA solution) and incubated at room temperature for 5 min. Afterwards, the mixture was centrifuged for 5 minutes at 13000 rpm and 4ºC followed by the transfer of one aliquot (0.5-0.6 ml) to a new 15 ml Falcon tube. The aliquot was subsequently mixed with 1 volume of the reagent solution II (RSII: RSI+0.65% TBA) and incubated for 25 minutes at 95ºC in a water bath. The reaction was stopped by cooling down on ice. After centrifugation for 5 minutes at 13000 rpm and 4ºC, the absorbance of supernatant was measured at 440 nm (absorbance of sugars), 532 nm (the maximum absorbance of chromagen being the product of MDA and TBA reaction) and finally at 600 nm corresponding to the turbidity. 0.1% (w/v) TCA solution was used as blank. The amount of MDA was calculated based on the following formula:

MDA equivalents (nmol/ml) = [(A-B)/157000] x 106, where

A= [(Abs 532RSII – Abs 600RSII)] and

B= [(Abs 440RSII – Abs 600RSII) x 0.0571]

MDA equivalents (nmol/g fresh weight) = MDA equivalents (nmol/ml) x total volume of the extracts (ml)/g FW or number of seedlings.

Reagent solution I (RSI): 0.01% (w/v) Butylated hydroxytoluene (BHT) and 20% (w/v) TCA.

2.2.23.4 GUS expression in plant leaves or seedlings

The *E.coli* b-*glucuronidase* (*GUS*) gene has been developed as a reporter gene system for the transformation of plants. The GUS enzyme hydrolyses wide range of ß-glucuronides. It catalyzes cleavage of colourless substrate X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D glucuronic acid). After cleavage and oxidation of indole derivatives, dimerization occurs and produces an insoluble blue dye the so called dichoro-dibromo-indigo (CIBr-indigo).

2.2.23.5 Histochemical GUS assay via tissue staining

Here, histochemical GUS assays were performed for 5-10 leaves of *C. plantagineum* after co-cultivation and stress treatment as well as for transgenic *A. thaliana* seedlings at different developmental stages. The leaves or seedlings were stained in GUS staining buffer overnight at

37ºC and de-stained in 80% (v/v) ethanol solution. Afterwards, the tissues were stored in 10% (v/v) glycerol and subsequently analysed with a dissecting binocular microscope. For the fluorometric GUS assays, 10-15 leaves (in three repeats, in each repeat 3-4 leave were used) were assayed for GUS activity according to Michel et al. (1994) using a fluorometer (Bio-Rad, München, Germany).

GUS staining solution: 0.5 mg/ml X-Gluc dissolved in DMF, dimethylformamide,(100 µl DMF per 10 mg X-Gluc); 0.1% (v/v) Triton X-100; 50 mM NaH_2PO_4 buffer, pH 7.2; 8 mM β-mercapto-ethanol freshly added.

2.2.23.6 Fluorometric GUS assay via X-Gluc substrate

Fluorometric assay of the GUS activity was performed according to Jefferson et al. (1987). Fluorogenic substrate 4-methylumbelliferyl-glucuronid (4-MUG) is cleaved by GUS enzyme to the fluorescent product 4-methylumbelliferone (4-MU) which can be detected spectro-photometrically. To perform the GUS assay, 50-100 mg of plant tissues was ground with metal beads in Eppendorf tubes and subsequently homogenized with 100-200 µl extraction buffer (50 mM sodium phosphate, pH= 7, 0.1% (v/v) TritonX-100, 10 mM EDTA, 0.1% (w/v) sodium-lauryl sarcosyle). Afterwards, the homogenate was centrifuged for 10 minutes at 14000 rpm and 4°C. The supernatant was used for the quantification of GUS activity. To perform the assay, 15 µl of the extract was mixed with one volume of 1 mM 4-MUG and the reaction mixture was incubated at 37°C for 15 minutes. A reaction mixture containing one volume of extraction buffer and one volume of 4-MUG was prepared as a control. After 15 and 30 min incubation at 37°C, 5 µl from each sample was removed, diluted in 2 ml of stop buffer (0.2 M sodium carbonate: Na_2CO_3, pH=9.5) and used for the measurement of fluorescent intensity (FI) with a flurometer (Bio-rad). The filter with excitation at 365 nm and emission at 455 nm was used. The GUS activity was calculated from the standard curve (FI versus pmol 4-MU) prepared for Na_2CO_3 (pH 9.5) containing different concentrations (5, 10, 25, 50 and 100 nM) of 4-MU to analyze the GUS activity. The specific GUS activity for each sample was calculated on the protein level (measured with Bio-Rad assay; see 2.2.11) and expressed as 4-MU pmol/min/mg protein based on the following formula:

GUS activity of the extract (pmol 4-MU/min/mg protein) = (corrected y/x) x [reaction volume (ml)/volume per test (ml)] x [1 / sample volume (ml)] x [1/extract concentration (mg protein/ml)].

3. Results

3.1 Optimization of *Agrobacterium*-mediated transient transformation in *C. plantagineum*, *L. brevidens* and *L. subracemosa*

The method of *Agrobacterium*-mediated transformation has been optimized for the wide range of plant species (Grant et al. 2004, Sharma et al. 2009). In this study the *Agrobacterium*-mediated transient transformation method described by Li et al. (2009) was adapted and optimized to transform leaves of 3 to 4 months-old *in vitro* cultured plants of the three closely related *Linderniaceae* species namely, *C. plantagineum L. brevidens and L. subracemosa*. The aim was to achieve a high transformation efficiency combined with a minimum of necrosis.

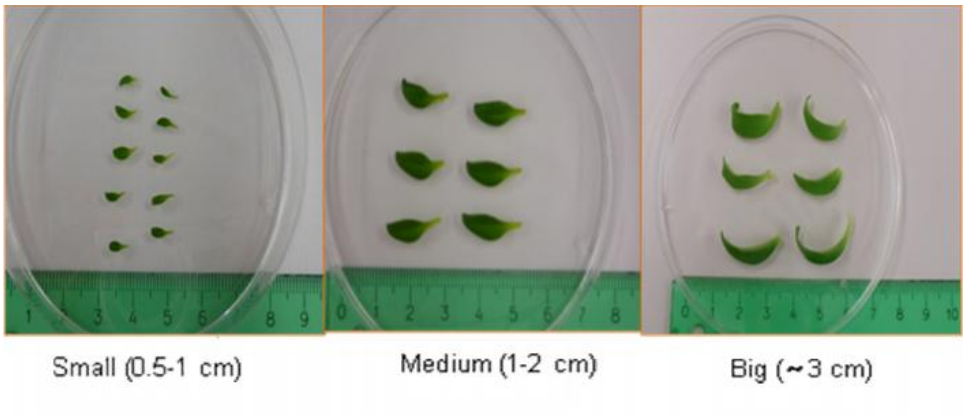
3.1.1 Parameters optimized in *Agrobacterium*-mediated transient transformation of three Linderniaceae species

The *CaMV35S::GUS* construct was used to determine the optimal conditions of transient transformation. Four parameters which have a key role in achieving high transformation efficiency were taken into account: leaf size, surfactant (Silwet L-77) concentration, bacteria density and duration of co-cultivation. As a control leaves were incubated in co-cultivation media containing wild type *A. tumefaciens* cells. In this control no GUS activity was observed whereas GUS activity was detected in the leaves transformed with *CaMV35S::GUS* construct.

3.1.1.1 Leaf size

To choose the best size of leaves for *Agrobacterium*-mediated transient transformation, different sizes of plant leaves (0.5-1 cm, 1-2 cm, approximately 3 cm), at the age of 3-4 months-old *C. plantagineum*, *L. brevidens* and *L. subracemosa* were tested. Quantitative GUS assays showed highest activity in 1-2 cm long leaves. The efficiency was reduced in bigger and smaller leaves (Fig. 3-1). For further experiments leaves of 1-2 cm were used.

a)



b)

Fig. 3-1: Leaves of *C. plantagineum* used for optimization of *Agrobacterium*-mediated transient transformation. a) Different size of leaves b) Histochemical GUS staining of *C. plantagineum* leaves in two different sizes (1-2 cm and approximately 3 cm) transiently transformed with a 35S::GUS fusion construct.

3.1.1.2 Silwet concentration

Different concentrations of Silwet L-77 (0.0%, 0.005%, 0.0075% and 0.01% (v/v)) in the co-cultivation medium were tested for Linderniaceae species and quantitative GUS assays were performed. To analyze the efficiency of transformation using co-cultivation medium containing different concentration of Silwet L-77, quantitative GUS assays were performed (Fig. 3-2). The highest GUS activity of *C. plantagineum* was observed in 0.0075% (v/v) Silwet L-77 concentration. In contrast, *in L. brevidens* and *L. subracemosa* the optimal GUS activity was detected using 0.005% (v/v) Silwet in the co-cultivation medium. It is shown that Silwet L-77 concentration had the strongest effect on transformation efficiency in *L. subracemosa*. Thus the GUS activity in the plants cultivated in medium containing 0.005% (v/v) Silwet L-77, was 3.1 fold higher than the control plants (without Silwet L-77 treatment) (Fig. 3-2). Nevertheless

higher concentrations of Silwet L-77 affected the viability of leaf cells and led to necrosis of leaf tissues.

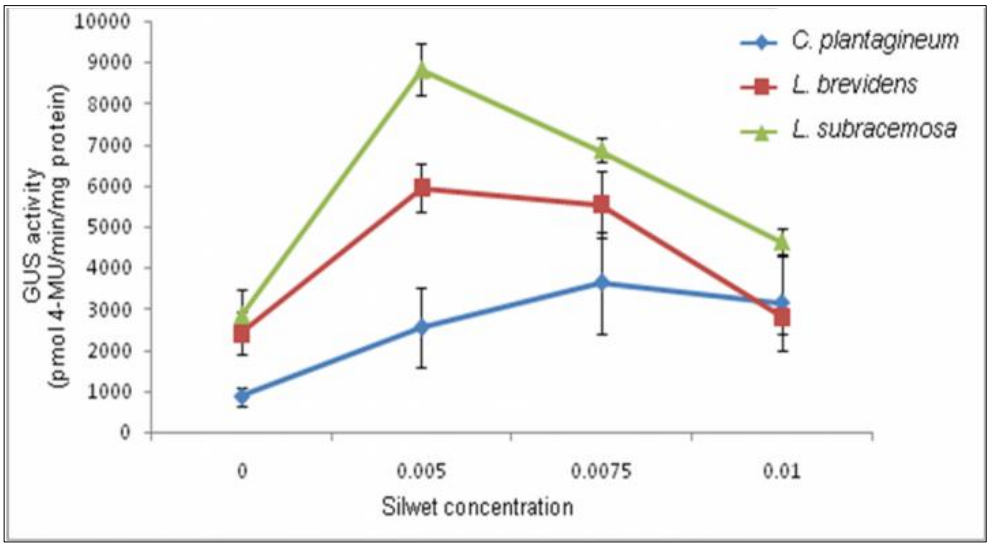


Fig. 3-2: Effect of Silwet L-77 concentration on expression of the GUS reporter gene in transient transformed *C. plantagineum* (Cp*), L. brevidens* (Lb), *L. subracemosa* (Ls) via *Agrobacterium*-mediated transformation. Transient expression efficiency was measured by expressing the GUS activity in the leaves co-cultivated for 36 h in ½ MS medium containing various concentration of Silwet L-77. Bacteria density and duration of co-cultivation was followed the FAST methos as described for *Arabidopsis* seedlings. The expression was quantified as pmol 4-MU/min/mg protein. Values represent the mean ± SE of three biological replicates.

3.1.1.3 Bacteria density

When the optimal Silwet L-77 concentration in co-incubation medium was determined, different bacterial densities (OD_{600}= 0.5, 1, 1.5, 2, 3, 4, 5) were tested. The optimal densities were obtained at λ=600 nm as 1-2 for *C. plantagineum* and 1 for *L. brevidens and L. subracemosa* (Fig. 3-3). Higher bacteria densities generally increased the GUS activity, but resulted in severe necrosis of leaf tissues. Only *C. plantagineum* plants which have thicker leaves than *L. brevidens* and *L. subracemosa* tolerated higher concentrations of bacteria (up to OD_{600}=3-4) and increased the GUS activity. Nevertheless, at these concentrations necrosis in the leaves was observed. Since the standard error in OD_{600}=1 is high enough to cover the other OD_{600}=1.5-2, the OD_{600}=1 was used for further analyses.

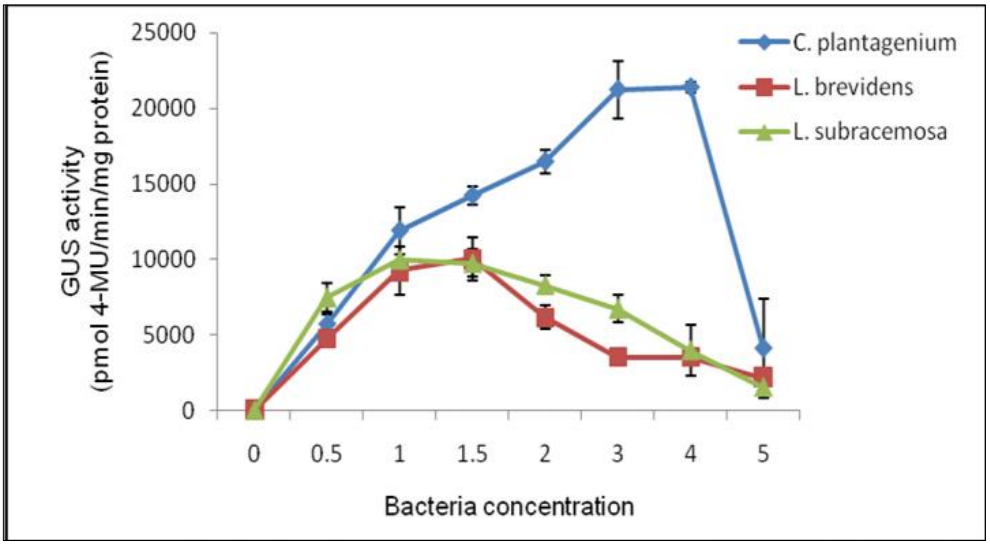


Fig. 3-3: Effect of *Agrobacterium* density on expression of GUS reporter gene in transient transformed *C. plantagineum* (Cp), *L. brevidens* (Lb), *L. subracemosa* (Ls). Transient expression efficiency was measured by determining the GUS activity in the leaves co-cultivated for 36 h in ½ MS medium containing various bacteria density and optimized Silwet L-77 concentration (0.005% (v/v) for *L. brevidens* and *L. subracemosa* and 0.0075% (v/v) for *C. plantagineum*). The expression was quantified as pmol 4-MU/min/mg protein. Values represent the mean ± SE of three biological replicates.

3.1.1.4 Duration of co-cultivation

Finally under the optimal Silwet L-77 concentration and bacteria density, various times of incubation (0, 24, 36, 48, 60, 72 and 96 h) were tested (Fig. 3-4).

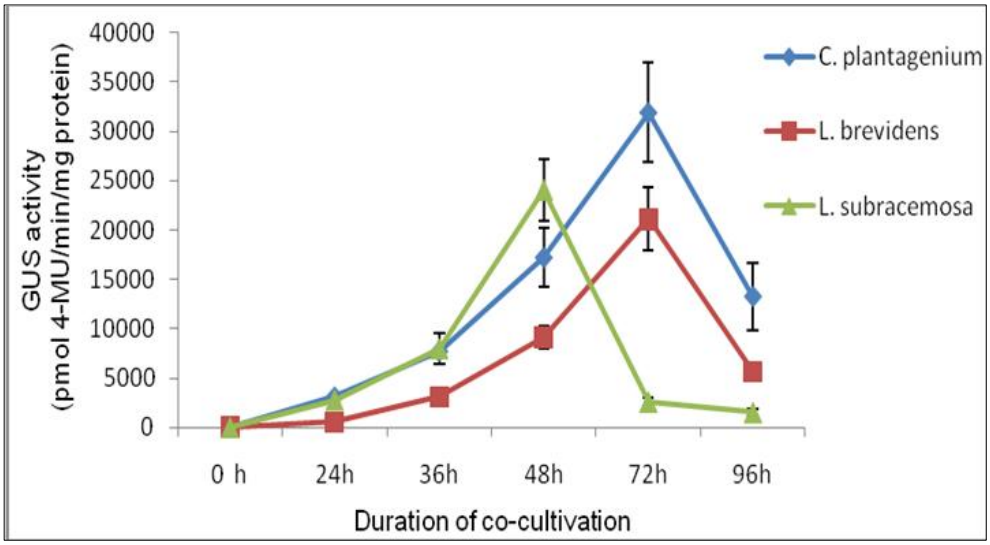


Fig. 3-4: Effect of duration of co-cultivation on expression of GUS reporter gene in transient transformed *C. plantagineum* (Cp), *L. brevidens* (Lb), *L. subracemosa* (Ls). Transient expression efficiency was measured by determining the GUS activity in the leaves co-cultivated for various times in ½ MS medium containing optimized Silwet L-77 concentration and bacteria density. The expression was quantified as pmol 4-MU/min/mg protein. Values represent the mean ± SE of three biological replicates.

The optimal GUS expression was observed in *C. plantagineum* after 60-72 hours, after 54-60 hours in *L. brevidens* and after 48 h in *L. subracemosa*.. Table 3-1 summarizes the optimal condition for each of three species.

Table 3-1: Optimal transformation conditions for the three tested Linderniaceae species

	C. plantagineum	*L. brevidens*	*L. subracemosa*
Silwet L-77 concentration	0.0075% (v/v)	0.005% (v/v)	0.005% (v/v)
Bacteria density	OD_{600}=1	OD_{600}=0.7	OD_{600}=0.7
Duration of co-cultivation	60 h	60 h	48 h

3.1.2 Preparation and cloning of the *LEA-like 11-24::GUS* construct from *C. plantagineum*, *L. brevidens*, *L. subracemosa* in pBIN19 binary vector

Three different GUS fusion constructs containing the *LEA-like 11-24* promoter (307 bp for *C. plantagineum*, 321 bp for *L. brevidens* and 488 bp for *L. subracemosa*) were used for cloning in pBIN19 binary vector. The *LEA-like 11-24::GUS* constructs were digested in *HindIII* and *BglII* sites of pBT10::GUS vector (Sprenger-Haussels and Weisshaar 2000) and cloned in the pBIN19 binary vector between *HindIII* and *BamH1* restriction sites. The *Cp LEA-like 11-24* promoter fragment harbours two conserved ABREs, namely ACGT box 1 (A1) and ACGT box 2 (A2) as well as one non conserved ABRE-ACGT box 3 (A3) (Fig. 3-5). Additionally one ACCGAC motif termed DRE motif (D) is located in *Cp LEA-like 11-24* promoter fragment. The *Lb LEA-like 11-24* promoter fragment contains two conserved ABREs (A1, A2) and one non conserved ABRE (A4), while the *Ls LEA-like 11-24* promoter fragment contained two conserved ABREs (A1, A2) and two non conserved ABREs (A5, A6) in a more distal part of the promoter fragment (van den Dries et al. 2011)

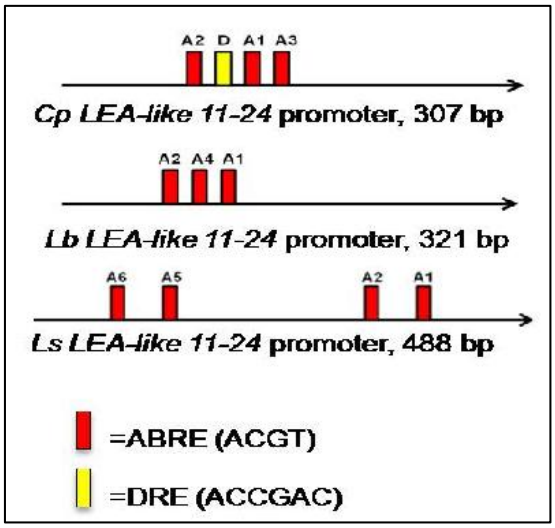


Fig. 3-5: *LEA-like 11-24* promoter fragments from *C. plantagineum*, *L. brevidens* and *L. subracemosa* used for transient transformation assays. The ACGT motif determines the ABRE *cis*-acting element (A), ACCGAC motif determines DRE *Cis*-acting element (D). Two conserved ABREs are indicated with A1 and A2 and non-conserved ABREs are indicated with A3, A4 and A5.

3.1.3 Reliability of the optimized method and transcript analysis

After optimization of the *Agrobacterium*-mediated transient transformation method in three tested species, the reliability and efficiency of this method was tested using *LEA-like 11-24* promoter::*GUS* construct of *C. plantagineum, L. brevidens and L. subracemosa*. *Agrobacterium*-mediated transient transformation was performed successfully and the efficiency of 100% was obtained in all three species as all leaves were stained positively in GUS assay. In this assay, ABA and mannitol treatment after *Agrobacterium*-mediated transient transformation induced *Cp LEA-like 11-24* promoter activity stronger than water control. These results were comparable with those obtained for biolistic method (van den Dries et al. 2011). This indicates that transient *Agrobacterium* transformation is a reliable method to study promoter activities. Thus, promoter activities can be analysed in the three tested species by two independent methods, namely *Agrobacterium* co-cultivation and particle bombardment.

To test whether the *Agrobacterium*-mediated transient transformation induces host defences in leaves of tested plants (Pruss et al. 2008; Rico et al. 2010) the accumulation of the stress-inducible transcripts *Cp LEA-like 11-24* (Velasco et al. 1998) and *Cp LEA 6-19* (Michel et al. 1993) were examined after different periods of co-cultivation (24, 36, 48 and 60 h) in *C.*

plantagineum leaf tissues (Fig. 3-5). The accumulation of these transcripts was examined also in desiccated wild-type leaves as positive control and in non-treated wild-type leaves as negative control. No transcript accumulation was observed in the transformed leaves, indicating that the tested genes are not induced by *Agrobacterium*-mediated transient transformation. These results show that the *Agrobacterium* co-cultivation does not induce host-defence reactions.

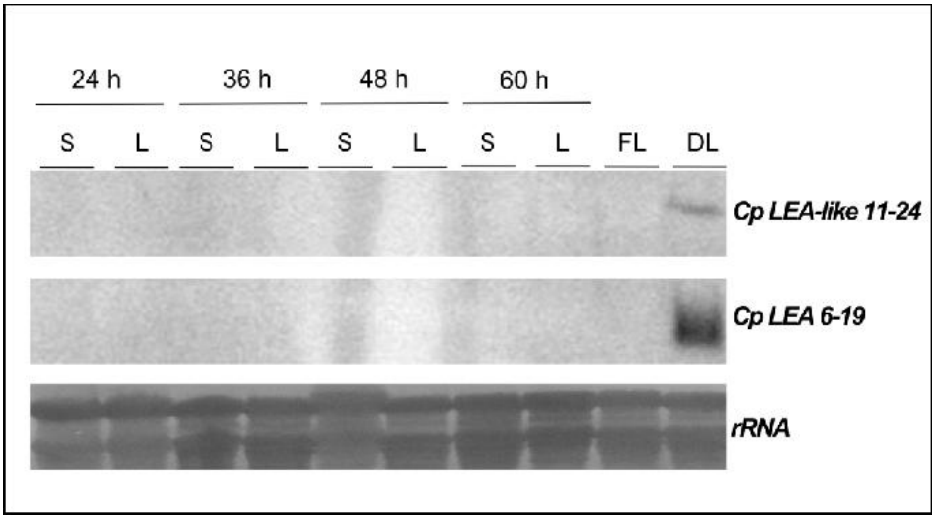


Fig. 3-5: RNA blot analyses of *Cp LEA-like 11-24* and *Cp LEA 6-19* transcript expression in leaves of *C. plantagineum* after *Agrobacterium*-mediated transient transformation. Leaves were co-incubated with *Agrobacterium* and RNAs were extracted 24, 36, 48 and 60 h after inoculation. Small leaves (S = 1-2 cm), and large leaves (L = 3) were used in the experiment. The filters were hybridized with ^{32}P -labeled probes for *Cp LEA-like 11-24* and *Cp LEA 6-19*. FL and DL indicate fresh and dehydrated leaf tissues. Methylene blue stain shows ribosomal RNA (rRNA) bands to indicate equal loading of RNA.

3.1.4 Activity of the *LEA-like 11-24* promoter fragments in leaves via *Agrobacterium*-mediated transient transformation in the homologous genetic background

The optimized *Agrobacterium* co-cultivation method was used to transiently transform leaves with the *LEA-like 11-24* promoter*::GUS* constructs. The transformed leaves were treated with 100 μM ABA, 0.8 M mannitol or water as control for 48 hours and afterwards screened for GUS activity. To compare the induction of promoters in different species the GUS activities of the water treated samples set to 100 %. The GUS activities measured after stress treatment were calculated based on water control to monitor the promoter induction. The *LEA-like 11-24* promoter fragment of *C. plantagineum*, *L. brevidens* and *L. subracemosa* were expressed

differentially in homologous leaves. The highest activity was obtained for *Cp LEA-like 11-24* promoter fragment in comparison to *Lb* and *Ls LEA-like 11-24* promoter fragments (Fig. 3-6). Osmotic stress (mannitol) and ABA treatment induced the activity of *Cp, Lb* and *Ls LEA-like 11-24* promoter fragments (Fig. 3-7). These results are in accordance with those obtained by van den Dries (2011) using a biolistic method. In this assay, the activity of *Cp LEA-like 11-24* promoter fragment was 1.3-fold higher in response to exogenous ABA, 2.7-fold higher than that of *Lb LEA-like 11-24* promoter fragment in response to mannitol treatment respectively. The *Ls LEA-like 11-24* promoter fragment (488 bp) showed very low activity compared to *Cp LEA-like 11-24* promoter fragment (Fig.3-6). It was 2.2 fold lower than that of the *Cp LEA-like 11-24* promoter fragment in response to ABA and 3.3 fold in response to mannitol treatments.

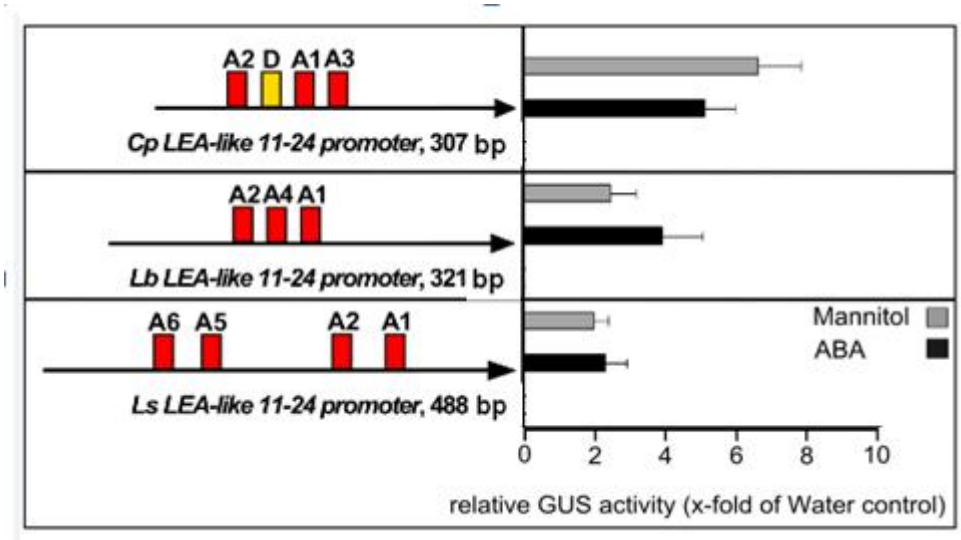


Fig. 3-6: Promoter activity of *C. plantagineum* (Cp*), L. brevidens* (Lb), *L. subracemosa* and (Ls) LEA-like 11-24 promoter fragments in after transient transformation in a homologous genetic background. Putative ABREs within the LEA-like 11-24 promoter fragments are shown in the red boxes (A1-A6) and the DRE motif is shown in a yellow box (D). The two conserved ABREs are indicated with A1 and A2. Promoter activities were measured after treatment with 100 µM ABA, 0.8 M mannitol or water for 48 hours. Promoter activity is expressed as pmol 4-MU/min/mg protein and normalized with regards to the water control which was set to 100%. Values represent the mean ± SE of three independent experiments.

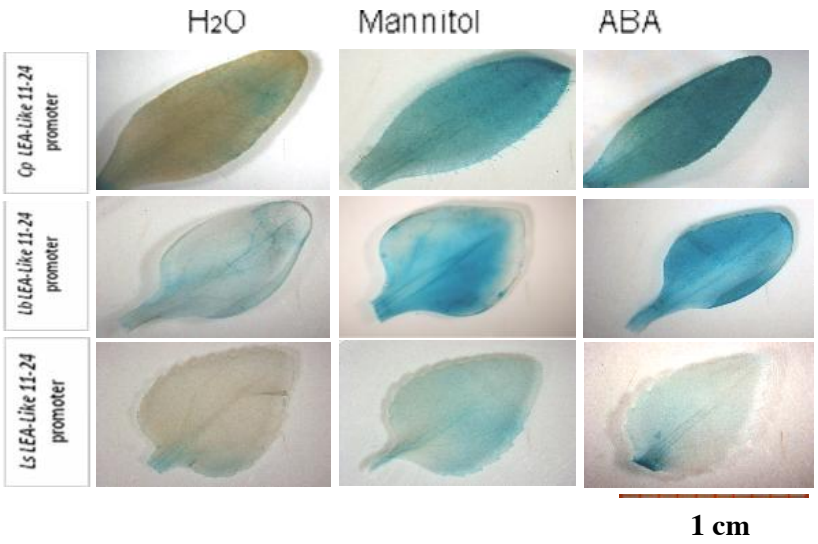


Fig. 3-7: Activity of the *LEA-like 11-24* promoter in homologous leaf tissues of transiently transformed *C. plantagineum* (Cp), *L. brevidens* (Lb) *and L. subracemosa* (Ls) in response to abiotic stress. The leaves were treated either with mannitol, ABA or water after transient transformation and subsequently stained for GUS activities (Jefferson et al 1987) at 37°C in darkness for 4 h in *C. plantagineum*, 16 h in *L. brevidens and L. subracemosa*.

3.1.5 Activities of the *LEA-like 11-24* promoter fragments in a heterologous genetic background

To examine whether essential *trans*-regulatory environment required for promoter induction in dehydration and osmotic stress are present in the three species, cross comparison of promoter fragments in three species was performed. The *Cp LEA-like 11-24* promoter fragment was analyzed in *L. brevidens* and *L. subracemosa* leaves in response to ABA and mannitol. *Lb LEA-like 11-24* promoter was analyzed in *C. plantagineum* and *L. subracemosa* leaves. The *Ls LEA-like 11-24* promoter fragment was evaluated in *C. plantagineum* and *L. brevidens* leaves.

3.1.5.1 Expression of the *Cp LEA-like 11-24* promoter fragment

The induction of promoter activity in *L. brevidens* and *L. subracemosa* in response to expression of the *Cp LEA-like 11-24* promoter fragment indicates that the trans-acting environments required for dehydration and osmotic stress-induced promoter activities are present in both species (Fig. 3-8). Although, a decrease in mannitol responsiveness of 24% and ABA responsiveness of 40% was observed in *L. brevidens* in comparison to that of *C. plantagineum*. Similarly, in *L. subracemosa*, a decreased activity was detected in response to ABA (28%).

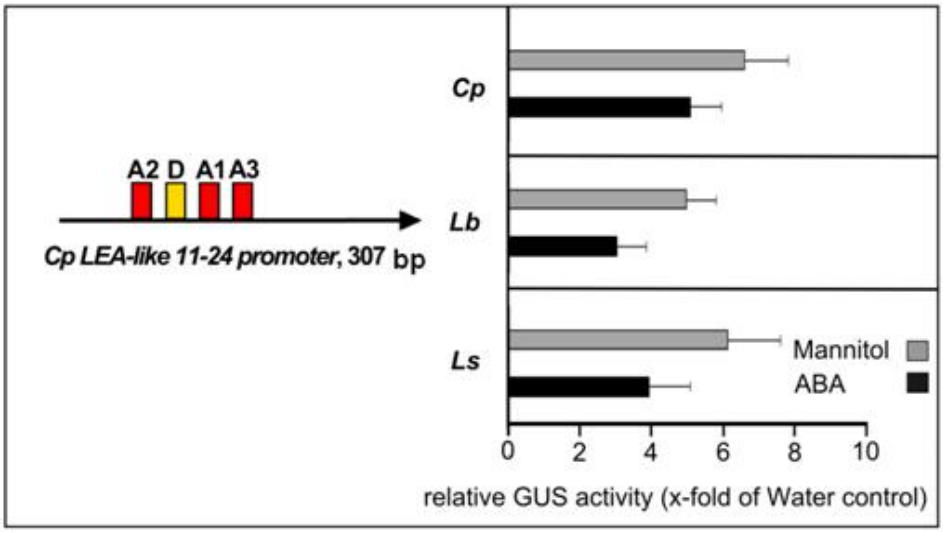


Fig. 3-8: Activity of *Craterostigma plantagineum* (Cp) *LEA-like 11-24* promoter fragment in *C. plantagineum, Lindernia brevidens* (Lb) and *Lindernia subracemosa* (Ls) leaves in a *Agrobacterium*-mediated transiently expression assay. Putative ABREs (A1-A3) within the Cp LEA-like 11-24 promoter fragments are shown as red boxes and the DRE motif (D) is shown as a yellow box. The two conserved ABREs among three species are indicated with A1 and A2. Promoter activities were measured as pmol 4-MU/min/mg protein after treatment with 100 µM ABA, 0.8 M mannitol or water for 48 hours and normalized to water control as 100%. Bars represent mean values of three independent experiment for each treatment (n = 3). Standard errors are displayed as error bars.

3.1.5.2 Expression of *Lb LEA-like 11-24* promoter fragment

The *Lb LEA-like 11-24* promoter fragment harbours two conserved ABREs (ABRE1, ABRE2) and one non-conserved ABRE (ABRE4) *cis*-elements (van den Dries et al. 2011). ABRE1 and ABRE2 motifs regulate promoter activity in ABA and mannitol treated homologous leaves (van den Dries 2011). The Lb *LEA-like 11-24* promoter fragment was expressed in *C. plantagineum* as well as in *L. subracemosa*. The trans-acting environment in *C. plantagineum* and *L. subracemosa* leaves was able to activate ABA and mannitol responsiveness of *Lb LEA-like 11-24* promoter fragment (Fig. 3-9). Higher promoter activity was observed in response to ABA than mannitol treatment in both species. However, decreased ABA-induction of 45% and mannitol-induction of 38% in *C. plantagineum* were detected in comparison to that of *L. brevidens*. In *L. subracemosa,* ABA induction of *Lb LEA-like 11-24* promoter fragment was also reduced (34%), but mannitol-induction was comparable to homologous leaves.

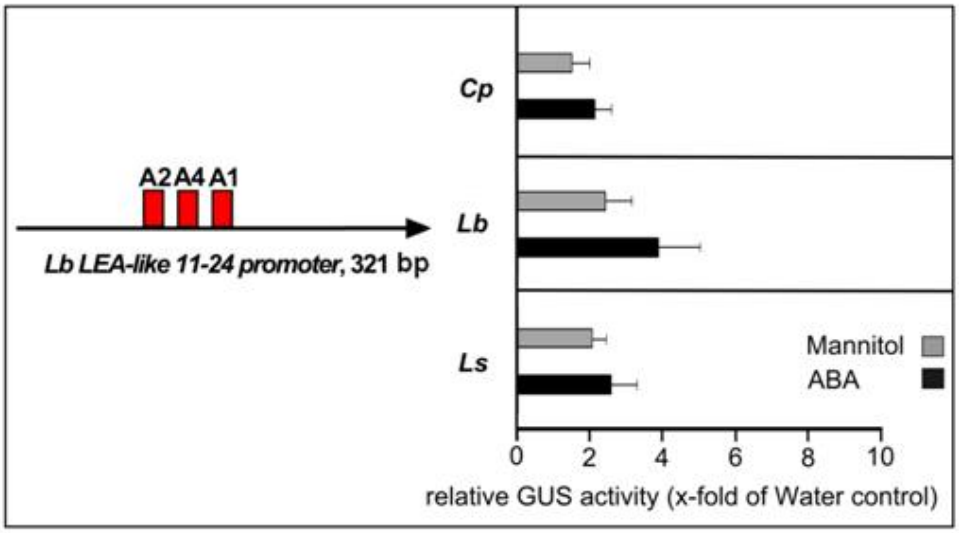


Fig. 3-9: Activity of *Lindernia brevidens* (Lb) *LEA-like 11-24*, promoter fragment in transiently transformed *Craterostigma plantagineum* (Cp), *Lindernia brevidens* (Lb) and *Lindernia subracemosa* (Ls) leaves. Putative ABREs (A1-A4) within the *Cp LEA-like 11-24* promoter fragments are shown in red. The two conserved ABREs are indicated with A1 and A2. Promoter activities were measured as pmol 4-MU/min/mg protein after treatment with 100 µM ABA, 0.8 M mannitol or water for 48 hours and normalized with water control as 100%. Bars represent mean values of three independent experiments for each treatment (n = 3). Standard errors are displayed as error bars.

3.1.5.3 Expression of *Ls LEA-like 11-24* promoter fragment

The 488 bp fragment of *Ls LEA-like 11-24* promoter which harbours two conserved (A1, A2) and two non-conserved ABREs (A5, A6) elements (van den Dries et al. 2011), was introduced into *C. plantagineum* and *L. brevidens* leaves. ABA induction in *C. plantagineum* and *L. brevidens* leaves was similar to that observed in *L. subracemosa* (Fig. 3-10). The mannitol responsiveness was shown to reduce the activity in *C. plantagineum* leaves. It is similar in *L. brevidens* leaves. The reduction of the mannitol response in *C. plantagineum* leaves was confirmed by particle bombardment experiments (Braun 2011).

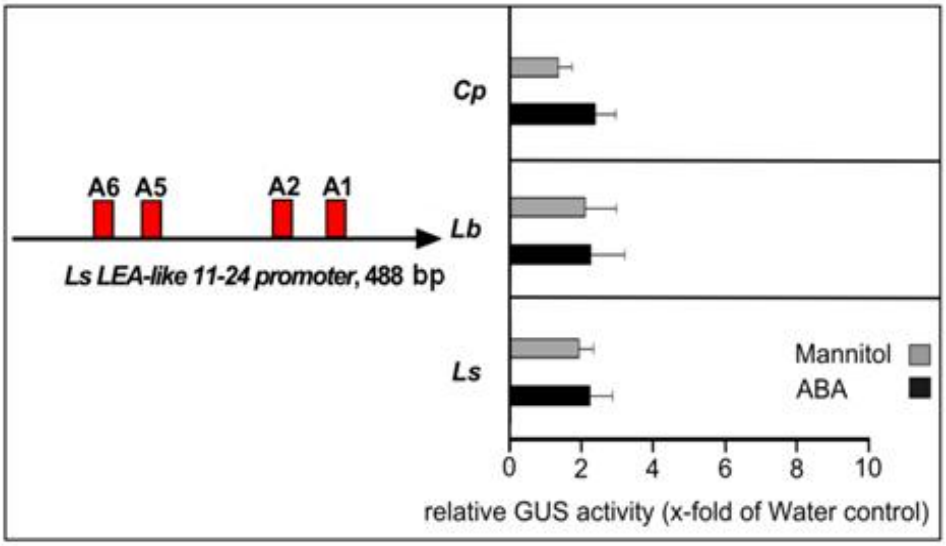


Fig. 3-10: Activity of *Lindernia subracemosa* (Ls) *LEA-like 11-24* promoter fragment in transiently transformed *Craterostigma plantagineum* (Cp), *Lindernia brevidens* (Lb) and *Lindernia subracemosa* (Ls) leaves. Putative ABREs (A1-A6) within the *Cp LEA-like 11-24* promoter fragments are shown as red. The two conserved ABREs are indicated with A1 and A2. Promoter activities were measured as pmol 4-MU/min/mg protein after treatment with 100 µM ABA, 0.8 M mannitol or water for 48 hours and normalized with water control as 100%. Bars represent mean values of three independent experiments for each treatment (n = 3). Standard errors are displayed as error bars.

3.2 Trans-activation of *CpbZIP1* transcription factor in *Cp LEA-like 11-24-GUS* promoter

Using yeast-one-hybrid assay CpbZIP1 transcription factor shown to bind to the *Cp LEA-like 11-24* core promoter (van den Dries 2010). The CpbZIP1 transcription factor was also found to bind to the *CpC2* promoter (stress inducible promoter from *C. plantagineum*) (Ditzer et al. 2006). To understand the effect of *CpbZIP1* transcription factor on the activity of *Cp LEA-like 11-24* promoter, trans-activation studies were performed via *Agrobacterium*-mediated transient transformation.

3.2.1 Generation of *"Cp LEA-like 11-24::GUS"* and *"35S::CpbZIP1+Cp LEA-like 11-24::GUS''* constructs

To perform the transient transformation of *C. plantagineum* leaves, two constructs were made namely *''Cp LEA-like 11-24::GUS''* and *''35S::CpbZIP1+Cp LEA-like 11-24::GUS''* (Fig. 3-12). The minimal stress inducible promoter fragment of *Cp LEA-like* 11-24 (307 bp) was used in

both constructs. This core promoter fragment has been shown to be the minimal stress-inducible promoter fragment and possessing similar induction pattern by ABA and osmotic stress to the full length promoter (Velasco et al. 1998; van den Dries et al. 2011). The β-glucuronidase (*GUS*) reporter gene driven by the *Cp LEA-like 11-24* promoter fragment (*Cp LEA-like 11-24 promoter::GUS::nos_terminator)* was isolated from the pBT10-GUS vector (van den Dries et al. 2011) by digesting with *HindIII* and *BglII* restriction enzymes and sub-cloned in *HindIII* and *BamHI* site of pBIN19 vector. A single colony of this clone was used to transform *A. tumefaciens*.

The cassette *35S::CpbZIP1* (1400 bp) was isolated from PA17 (pBT10-GUS vector) construct (Ditzer 2006) using *HindIII* restriction enzyme. The isolation was preceded by generation of point mutation via QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) to disrupt one internal *HindIII* digestion site. The primers MU-Hind-bzip F/R were used for the mutagenesis. The *35S::CpbZIP1* cassette was removed by using the *HindIII* restriction enzyme (Fig. 3-11) and subsequently sub-cloned either in front of the *Cp LEA-like 11-24::GUS* cassette in *HindIII* restriction site of pBIN19 vector or in pJET1.2 vector.

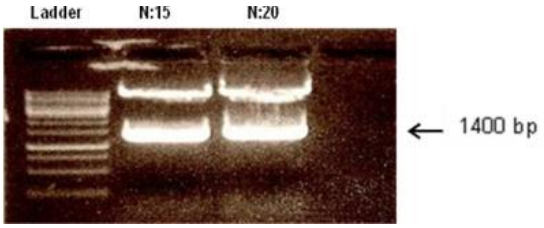


Fig. 3-11: Digestion of mutated PA17 construct with *HindIII* restriction enzyme: After digestion, the second band (1400 bp) was purified from the gel and cloned into pBIN19 vector in front of *Cp LEA-like 11-24::GUS* cassette.

Kanamycin resistant transformants were screened using a combination of primers specific for the inserted gene. After sequencing analysis a clone was selected having *Cp LEA-like 11-24::GUS* and *35S::CpbZIP1* in the same direction as of pBIN19 vector (Fig. 3-12). The posisitive clone was used to transform *Agrobacterium tumefaciens* competent cells. However, the clone containing *35S::CpbZIP1* in opposite direction of *Cp LEA-like 11-24::GUS* was also obtained and remained in glycerol stock at -80°C for furher use.

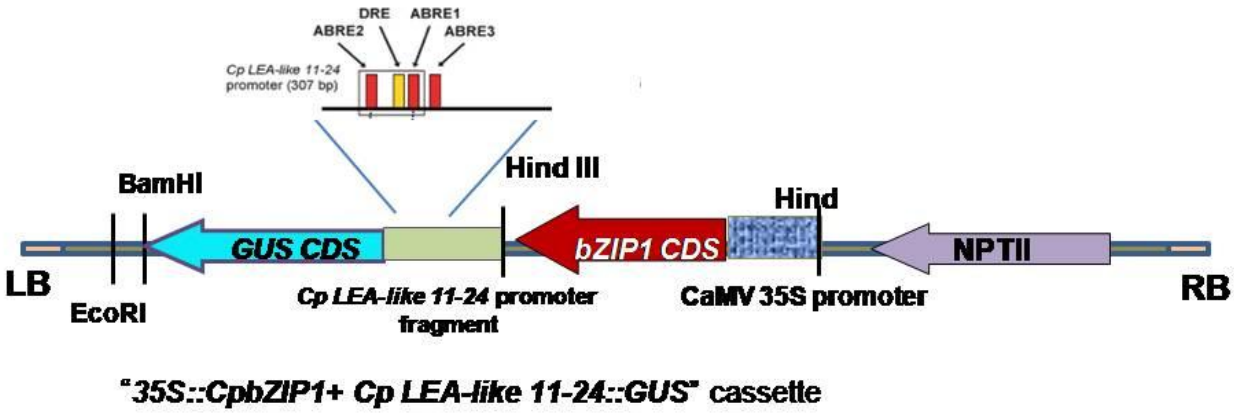


Fig. 3-12: Schematic diagram of the T-DNA region carrying ''*35S::CpbZIP1+Cp LEA-like 11-24::GUS''* cassette. It contains the *Cp LEA-like 11-24::GUS* coding sequence with *35S prom::CpbZIP1*coding sequence. Three ABRE and One DRE motifs is located in *Cp LEA-like 11-24* promoter fragment. The gene coding for the kanamycin resistance (NPTII) is located in the right border. **LB**: Left border; **RB**: Right border.

3.2.2 Analysis of C. plantagineum leaves transiently transformed with ''*Cp LEA-like 11-24::GUS*'' and ''*35S::CpbZIP1+Cp LEA-like 11-24::GUS*'' constructs

Agrobacterium-mediated transient transformation of two constructs, namely ''*Cp LEA-like 11-24::GUS''* and ''*35S::CpbZIP1+Cp LEA-like 11-24::GUS'',* was performed according to the optimized method in *C. plantagineum*, *L. brevidens* and *L. subracemosa* leaves (in this study, Table 3-1). The transformed leaves were treated for 48 hours with 0.8 M mannitol, 100 µM ABA for osmotic and dehydration stress. Leaves incubated in tap water served as a control. GUS activity in these leaves was assessed both qualitatively (histochemical staining) and quantitatively (fluorometric assay).

3.2.2.1 Histochemical and fluorometric detection of GUS activity

Histochemical (Fig. 3-13b) and fluorometric (Fig. 3-13a) detection of GUS activity in the leaves of *C. plantagineum,* transformed with ''*35S::CpbZIP1+Cp LEA-like 11-24::GUS''* cassette indicated much weaker GUS expression in comparison to the leaves transformed with ''*Cp LEA-like 11-24::GUS''* construct (Fig. 3-13a). In addition, low GUS activity was detected in the leaves of *L. brevidens* and *L. subracemosa* transiently transformed with ''*Cp LEA-like 11-24::GUS +35S::CpbZIP1''* cassette (Fig. 3-14).

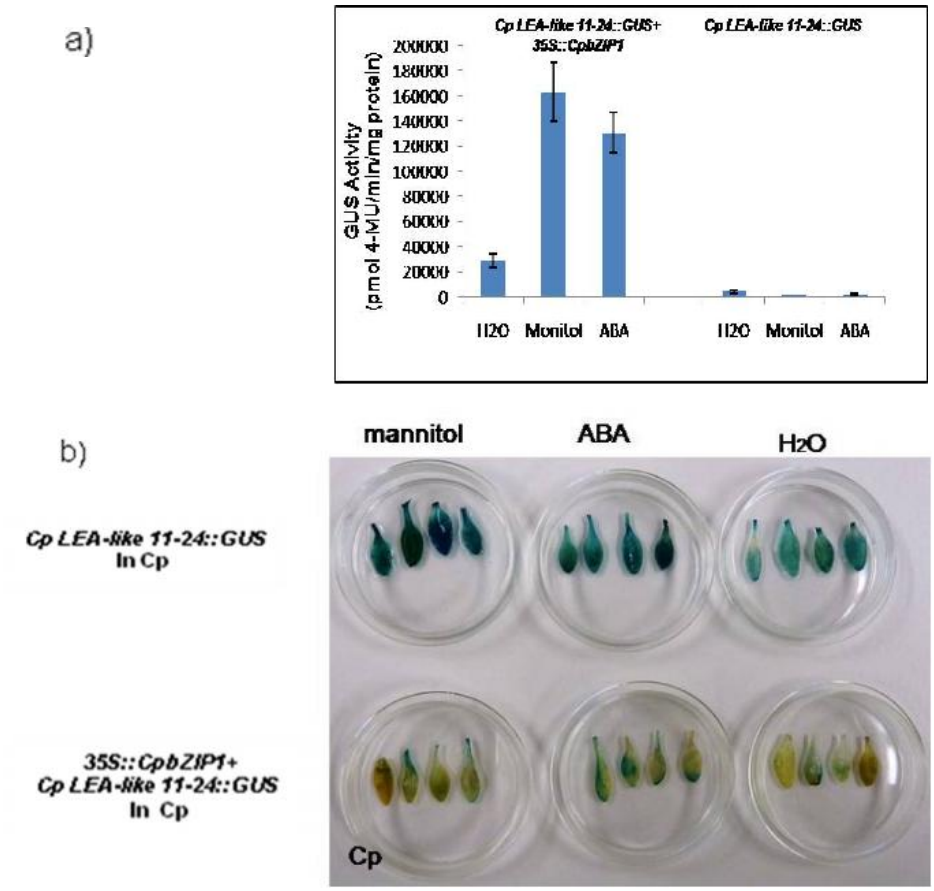


Fig. 3-13: GUS activity in the transiently transformed *C. plantagineum* leaves (Cp). a) Fluorometric and b) Histochemichal GUS activity of transiently transformed leaves of *C. plantagineum* with *''Cp LEA-like 11-24::GUS''* and *''35S::CpbZIP1+Cp LEA-like 11-24::GUS''* cassette after subjection to the 48 hours-long treatment with 0.8 M mannitol, 100 µM ABA or H_2O (control).

Fig. 3-14: Histochemichal detection of GUS activity in leaves of *L. brevidens* (Lb) *and L. subracemosa* (Ls) transiently transformed with *''Cp LEA-like 11-24::GUS''* and *''Cp LEA-like 11-24::GUS+ 35S::CpbZIP1''* cassette after subjection to the 0.8 M mannitol, 100 µM ABA or H_2O treatment for 48 hours.

Similarly, the promoter activity was not increased with the construct containing the opposite direction of 35S::CpbZIP1 in''*35S::CpbZIP1+Cp LEA-like 11-24::GUS''* cassette . To check

whether *CpbZIP1* gene product down-regulated the *Cp LEA-like 11-24* promoter or damaged the cells, viability test and GUS gene expression analyses were performed.

3.2.2.2 Viability test using Fluorescein diacetate (FDA)

To understand whether transformed cells showing low GUS activity are alive, viability tests using fluorescein diacetate was performed (Schnurer and Rosswall, 1982). In both un-transformed and transformed leaves, the fluorescence signal is observed thus indicating the viability of the cells (Fig. 3-15). Therefore, cell death is excluded as a reason of lower GUS activity in the leaves of *C. plantagineum*, *L. brevidens* and *L. subracemosa* transiently transformed with *35S::CpbZIP1+Cp LEA-like 11-24::GUS*.

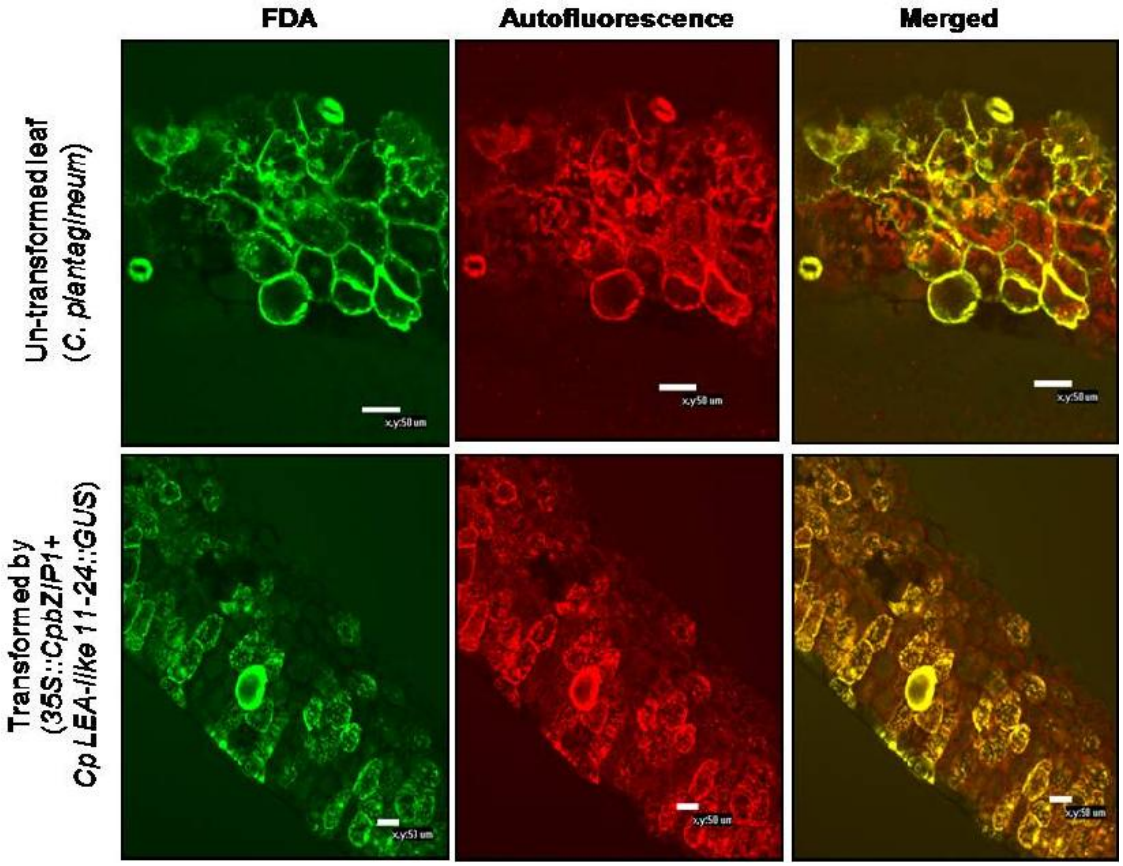


Fig. 3-15: FDA test of *C. plantagineum* leaves transiently transformed with *35S::CpbZIP1+Cp LEA-like 11-24::GUS* construct. Leaf section of transiently transformed leaves was soaked in FDA solution (see Materials and methods). Un-transformed WT leaves were used as control. Living cells show a yellow fluorescence in the merged picture.

3.2.2.3 Transcript expression analyses of *GUS* gene

To prove the lower activity of *Cp LEA-like 11-24* promoter trans-activated by CpbZIP1 protein compared to non-transactivated *LEA-like 11-24* promoter in response to mannitol and ABA, the expression level of the *GUS* gene was evaluated in all analysed samples (Fig. 3-16). The analyses showed down-regulation of the *GUS* gene in the construct containing 35S::*CpZIP1* suggesting that *CpbZIP1* might be a negative regulator of *Cp LEA-like 11-24* promoter.

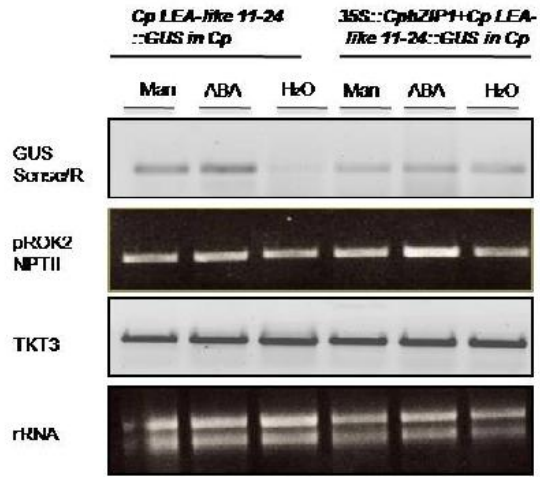


Fig. 3-16: GUS gene expression from the leaves of *C. plantagineum* transiently transformed with ''*Cp LEA-like 11-24::GUS''* and *''35S::CpbZIP1+Cp LEA-like 11-24::GUS''* construct after exposure to 0.8 M mannitol (Man) 100 µM ABA or H_2O (control) for 48 h. Expression pattern of transcripts encoding the neomycin phosphotransferase (NPTII), transketolase3 (TKT3) genes are constitutively expressed in *C. plantagineum* and thus served to monitor an equal use of RNA in the RT-PCR. Ribosomal RNA (rRNA) bands indicate the equal loading of RNA. TKT3 was amplified in 28 cycles, while NPTII and GUS were amplified in 30 cycles.

3.2.3 Trans-activation study of *Cp LEA-like 11-24* promoter by CpbZIP1 transcription factor

3.2.3.1 Generation of *Arabidopsis* transgenic plants containing either ''*Cp LEA-like 11-24::GUS*'' or ,''*35S::bZIP+Cp LEA-like 11-24 GUS*''cassette

To study the physiological consequences and verify the results of GUS activity measurements obtained for the *C. plantagineum* leaves transiently transformed with *35S::CpbZIP1+Cp LEA-like 11-24::GUS* and *Cp LEA-like 11-24::GUS, Arabidopsis thaliana* Col-0 plants were stably transformed with both constructs. In addition, since *Cp LEA-like 11-24* (307 bp) promoter fragment is the minimal stress inducible promoter fragment

(van den Dries et al. 2011), a full length promoter fragment (1.5 kbp) (Velasco et al. 1998) was used to generate transgenic plants. For the studies of the function of CpbZIP1 protein, generation of transgenic lines containing *35S::CpbZIP1* gene were also performed.

3.2.3.2 Screening of the transgenic lines

The first generation of seeds (T1) were collected after transformation, surface sterilized and are sown on MS plates supplemented with kanamycin (50 mg/ml). After 2 weeks of growth, transgenic seeds which were able to produce green leaves in the presence of kanamycin were selected as positive seedlings. Positive transgenic seedlings (transgenic lines) were transferred to soil and screened for the transgene using combination of specific primers. The positive transgenic plants were grown for the next generation (T2). Transgenic *Arabidopsis* plants carring the *Cp LEA-like 11-24::GUS,* minimal promoter (307 bp) or ''*35S::CpbZIP1+Cp LEA-like 11-24 GUS''* minimal promoter (307 bp) constructs, were respectively named G-Lines and GZ-lines (Table 3-2). The transgenic *Arabidopsis* plants harbouring the *Cp LEA-like 11-24::GUS,* full promoter (1.5 kbp), and ''*35S::CpbZIP1+ Cp LEA-like 11-24::GUS''*, full promoter cassette, were respectively called L-Lines (Velasco et al. 1998) and LZ-lines. The transgenic lines containing the *35S::CpbZIP1* were named S-lines.

Table 3-2: List of generated *Arabidopsis* transgenic lines:

Studied lines	Generated in:	Description
G-Lines	this study	*Cp LEA-like 11-24::GUS*, minimal promoter (307 bp)
GZ-Lines	this study	*35S::CpbZIP1+Cp LEA-like 11-24 GUS*, minimal promoter (307 bp)
L-Lines	Velasco (1998)	*Cp LEA-like 11-24::GUS*, full promoter (1.5 kbp)
LZ-Lines	this study	*35S::CpbZIP1+ Cp LEA-like 11-24::GUS*, full promoter (1.5 kbp)
S-Lines	this study	*35S::CpbZIP1*

As *Cp LEA-like 11-24::GUS* full promoter lines (Velasco et al. 1998) were already available in the laboratory, LZ-lines were generated by transformation of *Cp LEA-like 11-24::GUS* full promoter (line *16-11*) with the *35S::CpbZIP1* construct. The screening of transgenic seedling of LZ lines was difficult, because of the presence of the gene providing kanamycin resistance in the

T-DNA region of both constructs. However presence of *CpbZIP1* in the transgenic lines gave a phenotype which can be recognized from seedling stage (Fig. 3-17 b). Independent LZ transgenic seedlings were first selected in seedling (Fig. 3-17 b) phenotyping (transgenic seedlings showed different phenotype than the wild type seedlings) and next screened by DNA-based PCR amplification using combination of primers specific for *CpbZIP1* gene and CaMV35S promoter such as *CpbZIP1* F/R or *pROK-35S/CPbZIP1* primers (Fig. 3-17b and 3-18).

Fig. 3-17: Different phenotypes observed by the transgenic *Arabidopsis* plants harboring *35S::CpbZIP1+Cp LEA-like 11-24 GUS*,either minimal (307 bp) or full promoter (1.5 kbp) of CpLEA-like 11-24. Pictures were taken from transgenic seedling and 6 week-old plants harboring *35S::CpbZIP1+Cp LEA-like 11-24 GUS,* minimal promoter. of *Arabidopsis* seeds were germinated either in soil or on MS-medium. 6 week-old *Arabidopsis* plants in a) soil and b) seedlings on MS-medium displayed different phenotypes. 1. Normal like wildtype seedlings 2. Abnormal seedlings or plant.

Four transgenic lines were confirmed in T1 generation, but the expression of *CpbZIP1* gene in LZ44 line was not detected by RT-PCR whereas genomic DNA amplification showed the presence of the gene encoding for *CpbZIP1* (Fig. 3-18b). Silencing might explain this result as reported for transgenic S- lines too (in this study, see Fig. 3-30). The transgenic plants in GZ, LZ, S lines revealed phenotype, in the T1 and T2 generation with respect to the shoot and root growth, flowering time, flower structure, siliques size and seed yield.

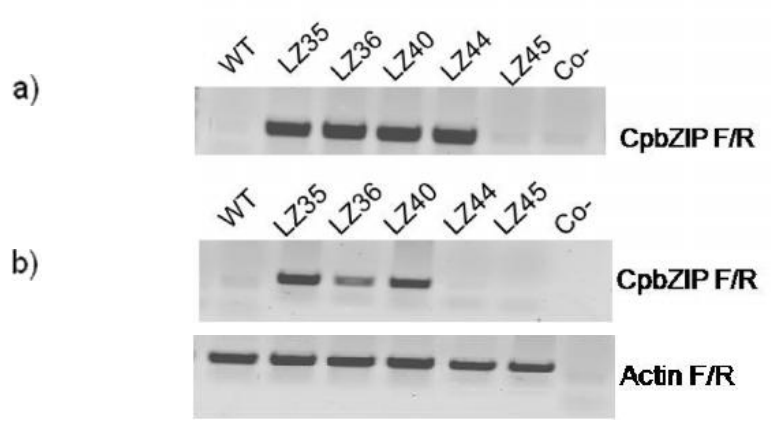


Fig. 3-18: Screening of transgenic a*rabidopsis* plants (LZ lines) containing *35S::CpbZIP1+Cp LEA-like 11-24::GUS* full promoter. a) Genomic DNA amplification using different combination of primers such as *CpbZIP1* F/R specific for *CpbZIP1* gene. b) Transcript level of *CpbZIP1* gene.

The expression of *CpbZIP1* was checked in independent transgenic lines in the T2 generation (Fig 3-19b). It was found that the plants which contain high expression of *CpbZIP1*, showes severe abnormalty and dwarfism. This is true for all transgenic lines which containing *CpbZIP1* in the genome. Further experiments were done using transgenic lines with moderate expression of *CpbZIP1* gene, Fig. (3-19 a, b).

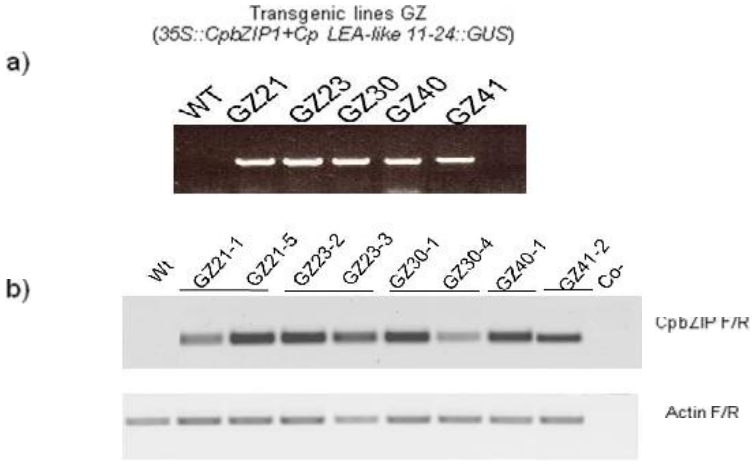


Fig. 3-19: Screening of transgenic lines. a) Genomic DNA amplification of *CpbZIP1* in wild type and T1 generation of independent transgenic GZ lines b) Expression analyses of *CpbZIP1*gene in T2 generation using RT PCR.

3.2.4 Analysis of *Cp LEA-like 11-24* promoter activity in the presence or absence of *CpbZIP1* in *Arabidopsis*

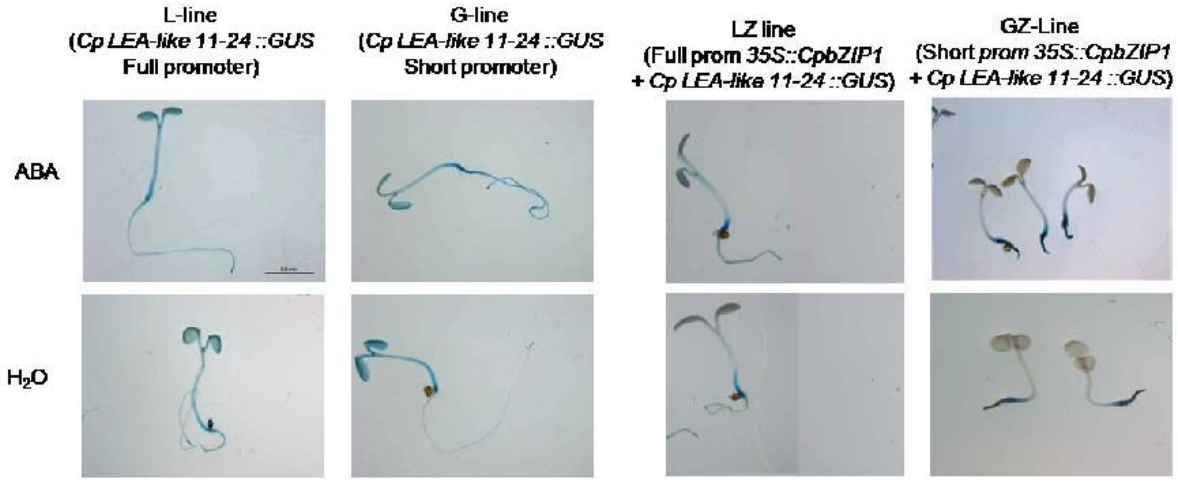
In plants, ABRE binding factors which belong to bZIP transcription factor families are the important regulator of ABRE-containing genes such as stress inducible genes (Hurst HC 1994; Yamaguchi-Shinozaki et al. 2005). The interaction of CpbZIP1 transcription factor with *Cp LEA-like 11-24* promoter fragment was confirmed using yeas-one-hybrid system (van den Dries et al. 2011). Therefore, it is assume that this transcription factor may play an important role in *C. plantagineum* during dehydration.

To analyze the effect of presence of *CpbZIP1* in trans-activation of the *Cp LEA-like 11-24* promoter, GUS histochemical and fluorometric assays were performed in independent transgenic lines (G-lines, GZ-lines, L-lines and LZ-lines) subjected to 18 hours of ABA treatment or water (control). Analysis of promoter activity was carried out in transgenic lines at different developmental stages of 7, 14 and 21 day-old seedlings. As different GUS expression was observed in the leaf and root (visualized by GUS staining), the GUS activity of transgenic lines in the leaf and root were separately analyzed.

3.2.4.1 GUS activity in 7 day-old seedlings

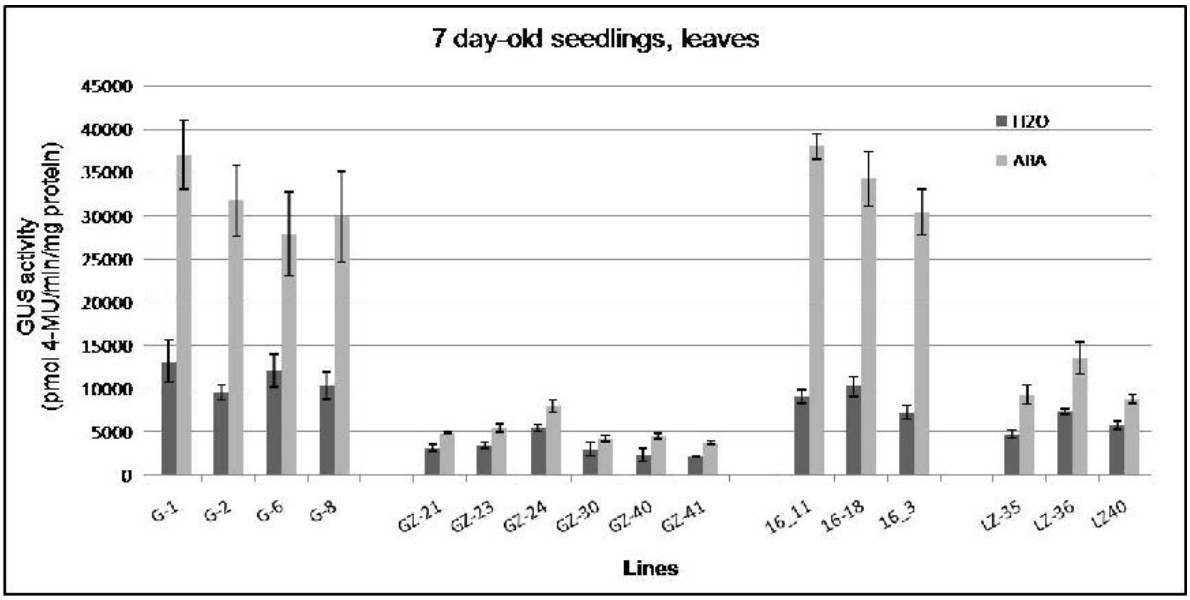
7 day-old seedlings of transgenic lines (G-line, GZ-lines, L-lines, LZ-lines) were treated for 18 hours with either ABA or H_2O as control. In this assay GZ-lines, which contain the short fragment of *Cp LEA-like 11-24* promoter were compared with G-lines with the same size of promoter and LZ lines which contain the full promoter of *Cp LEA-like 11-24,* were compared with L lines. The Short promoter fragment in G-lines consists of 3 ABRE (ABA response element) namely ABRE I, ABRE II, ABRE III and one DRE motif (dehydration response element) (Fig. 3-4). In 7 day-old seedlings, fluorometric and histochemical analyses of GUS activity revealed lower GUS expression in the leaves of GZ-lines than in L-lines treated with ABA. These results were confirmed using three independent lines of LZ transgenic plants (Fig. 3-20).

In 7 day-old seedlings of G-lines and L-lines which, only contain respectively short or full promoter of *Cp LEA-like 11-24*, the GUS reporter gene was active only in the young tissue of cotyledon leaves and root tips. However, in G-lines, *GUS* gene was constitutively expressed in the whole root tissue, whereas in L-lines only in the root tips (Fig. 3-20 a, b). Thus, comparison of G-lines and L-lines treated with ABA demonstrated that GUS expression in the roots of G-lines is stronger than those of the L-lines in both ABA treated and not-treated seedlings. The observation suggests that there are *cis*-elements upstream of the promoter fragment in the G-lines, which control the tissue-specific expression of the promoter.



ig. 3-20: 7 day-old seedlings of independent transgenic lines were treated for 18 h with 100 μM ABA or H_2O (control) and histochemically stained for GUS activitiy (Jefferson et al. 1987). GUS expression in G-lines (*Cp LEA-like 11-24::GUS* short promoter) and GZ-lines (*Cp LEA-like 11-24::GUS+35SCpbZIP1* short promoter), L-lines (*Cp LEA-like 11-24::GUS* full promoter) and LZ lines (*Cp LEA-like 11-24::GUS+35SCpbZIP1* full promoter).

a)



b)

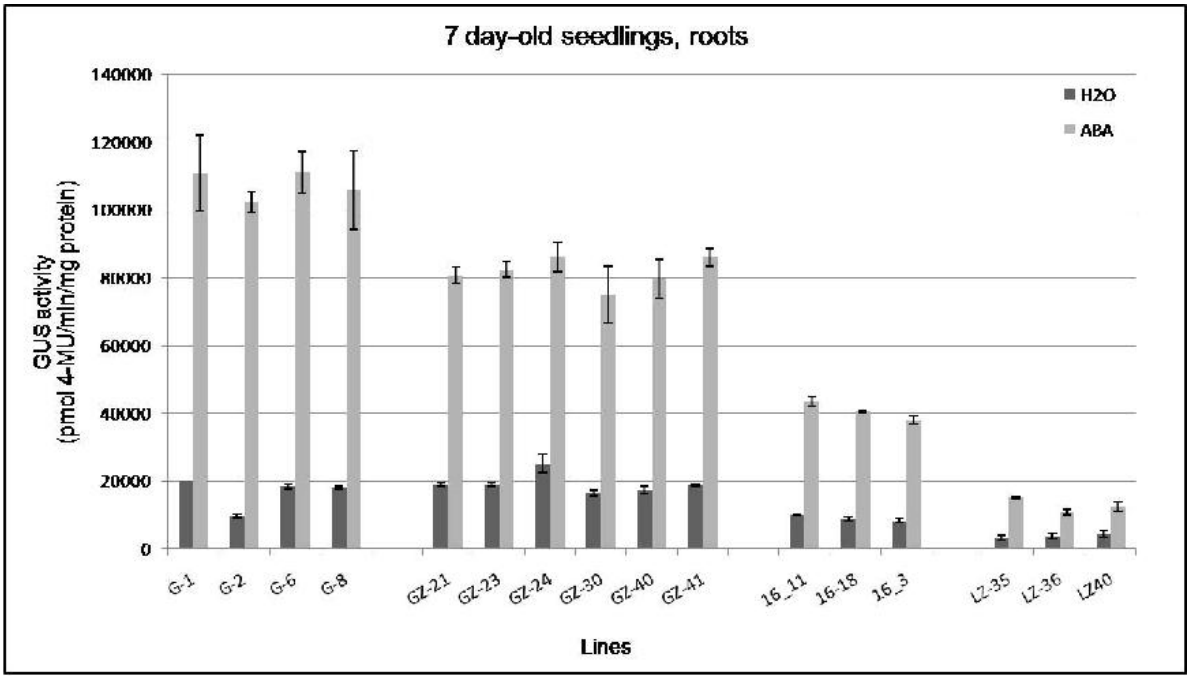
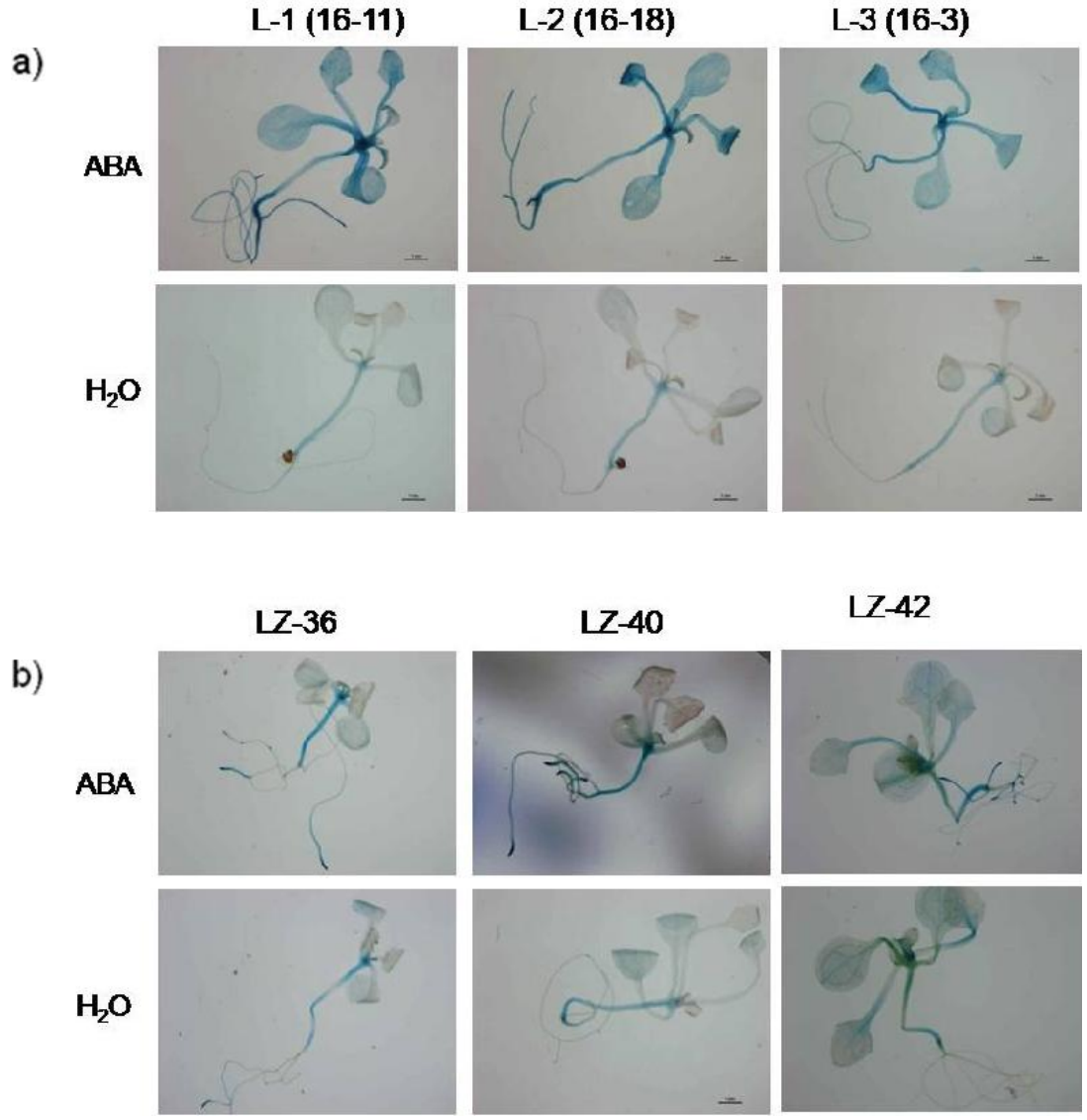


Fig. 3-20: 7 day-old seedlings of independent transgenic lines were treated for 18 h with 100 μM ABA or H_2O (control) and GUS activity (Jefferson et al. 1987) was measured. a) GUS expression in G-lines (*Cp LEA-like 11-24::GUS* short promoter) and GZ-lines (*Cp LEA-like 11-24::GUS+35SCpbZIP1* short promoter), L-lines (*Cp LEA-like 11-24::GUS* long promoter) and LZ lines (*Cp LEA-like 11-24::GUS+35SCpbZIP1* long promoter). c and d) Quantitative GUS assays of G-lines, GZ-lines, L-lines and LZ-lines in response to ABA or H_2O treatment (H_2O as control) in leaves and roots respectively. Values represent the mean ± SE from three biological replicates.

3.2.4.2 GUS activity in 14 day old seedlings

In 14 day-old seedlings of transgenic lines containing *35S::CpbZIP1*, GUS expression followed the same pattern as in 7 day-old seedlings. However in general the promoter activity was decreased in 14 day-old seedlings compared to 7 day-old seedlings (Fig. 3-20 and 3-22). Moreover, GUS activity in the leaves and roots of transgenic lines containing *CpbZIP1* was lower than in the transgenic lines without *CpbZIP1* coding sequence (Fig. 3-21, 3-22).

The comparison of GUS expression in G-lines and L-lines revealed that ABA-response in the roots of G-lines is higher than those of the L-lines, whereas in the leaves, the GUS gene was expressed slightly stronger in L-lines than in those of the G-line (Fig. 3-21, 3-22).



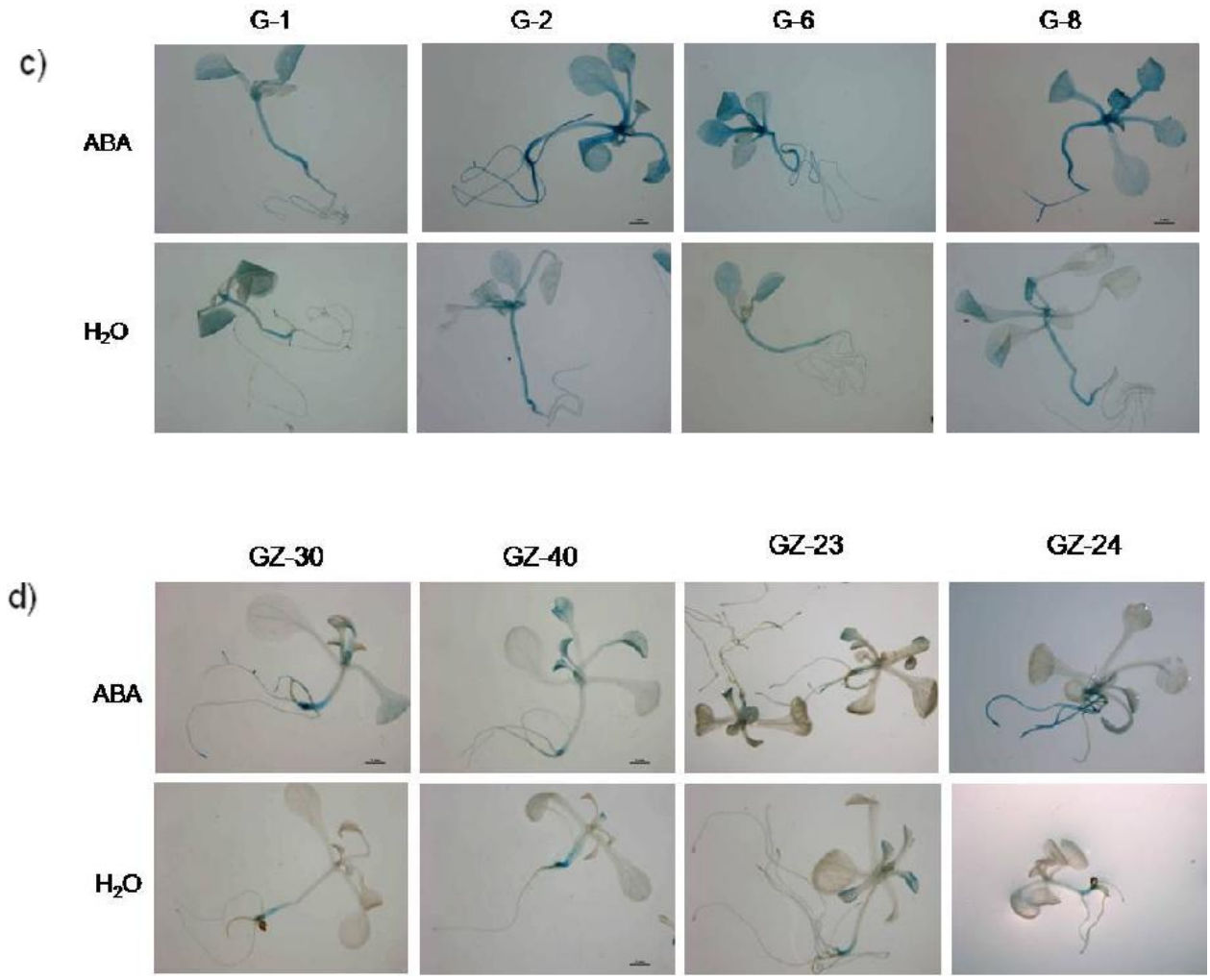
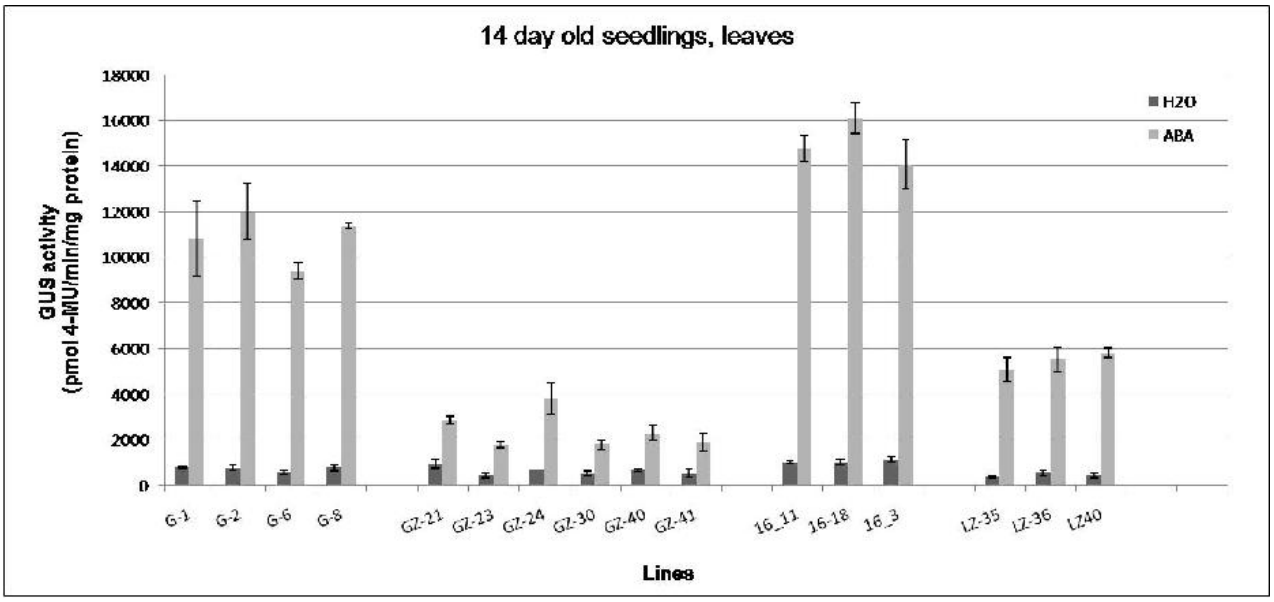


Fig. 3-21: Histochemical GUS staining of 14 day-old *Arabidopsis* transgenic lines treated with ABA or H_2O (as control). a) *Arabidopsis* transgenic L-lines containing the full promoter of *Cp LEA-like 11-24::GUS*, (lines: L-1 (16-11), L-2 (16-18), L-3 (16-3). b) *Arabidopsis* transgenic LZ-lines containing the full promoter of *Cp LEA-like 11-24::GUS+35S::CpbZIP1* (lines LZ-36, LZ-40, LZ-42). c) *Arabidopsis* transgenic G-lines containing short promoter of *Cp LEA-like 11-24::GUS* (lines*:* G-1, G-2, G-6, G-8). d) *Arabidopsis* transgenic GZ-lines, containing short promoter of *Cp LEA-like 11-24::GUS+35S::CpbZIP1* (lines: GZ-30, GZ-40, GZ-23, GZ-24).

a)



b)

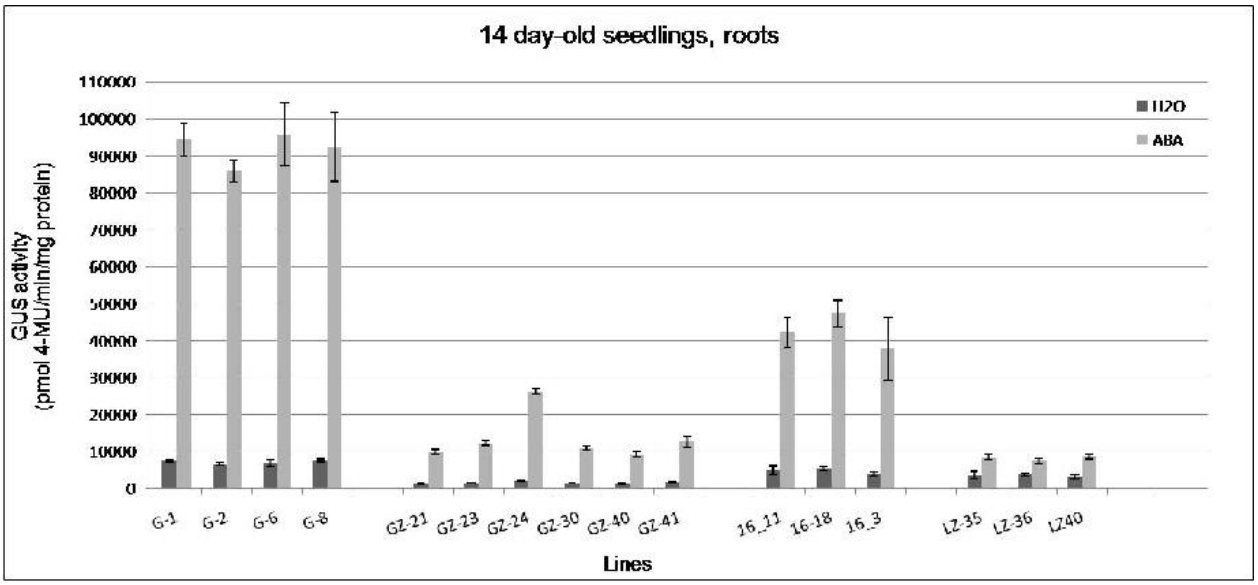


Fig.3-22: Quantitative GUS activity of 14 day-old seedlings of G-lines (*Cp LEA-like 11-24::GUS* short promoter) GZ-lines (*Cp LEA-like 11-24::GUS+35SCpbZIP1*short promoter), L-lines (*Cp LEA-like 11-24::GUS* long promoter) and, LZ-lines (*Cp LEA-like 11-24::GUS+35SCpbZIP1* long promoter) in response to ABA and H_2O treatment (H_2O as control) . a) in leaves or b) in roots. Values represent the mean ± SE from three biological replicates.

3.2.4.3 GUS activity in 21 day old seedlings

In 21 day-old seedlings, quantitative GUS activity in the leaves although did not showed any significant induction in all transgenic lines tested under ABA treatment (Fig. 3-23a). The GUS activity in the roots of *Cp LEA-like 11-24* plants was induced under ABA treatment in all lines. The promoter activity in the roots of G-lines is much higher than those of other lines.

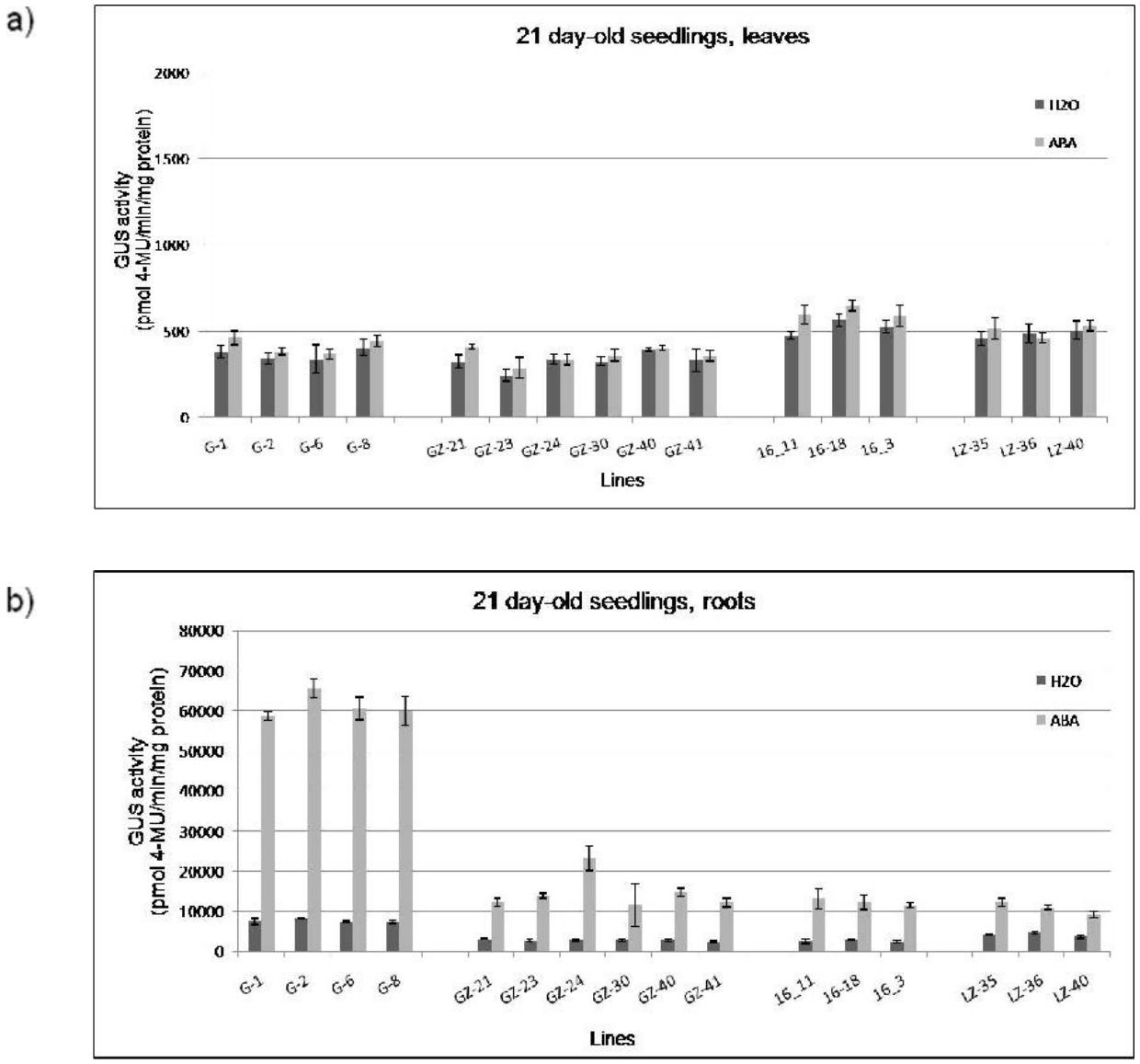


Fig. 3-23: Quantitative GUS activity of 21 day-old seedlings of G-lines (*Cp LEA-like 11-24::GUS* short promoter) GZ-lines (*Cp LEA-like 11-24::GUS+35SCpbZIP1*short promoter), L-lines (*Cp LEA-like 11-24::GUS* long promoter) and, LZ-lines (*Cp LEA-like 11-24::GUS+35SCpbZIP1* long promoter) in response to ABA and H_2O treatment (H_2O as control), in leaves a) or in roots b). Values represent the mean ± SE from three biological replicates.

3.3 Transcript expression analyses of *CpbZIP1* and *Cp LEA-like 11-24* coding gene

3.3.1 Expression of the *CpbZIP1* gene in leaves and roots of *C. plantagineum* exposed to various abiotic stress conditions

Transcript level of *CpbZIP1* gene was analysed in *C. plantagineum* leaf and root tissues subjected to different abiotic stresses. Leaves and roots were separately analysed for gene expression. Since the expression of *CpbZIP1* in *C. plantagineum* leaves is weak (Ditzer 2003), 2 μg of RNA was initially taken for cDNA preparation and without diluting the cDNA 1 μl was used for PCR with 30 cycles of amplification. Experimental conditions for all RT-PCR reactions were identical. To analyze the gene induction, transcript abundance of treated samples were compared with the expression level of water treated plants. To monitor the effectiveness of the stress treatments and to show that the stress stimuli were strong enough to induce the stress inducible genes, the expression of the *Cp LEA-like 11-24* gene was analysed in parallel for all samples. The accumulation of *Cp LEA-like 11-24* transcript upon stress is known (Bartels et al. 1990; Hundertmark and Hincha 2008). The expression of transketolase 3 (*tkt3*) was used as internal control. Constitutively expression of *tkt3* in untreated, dried or rehydrated leaves of *C. plantagineum* has been reported (Bernacchia et al. 1995).

3.3.1.1 Gene expression under dehydration condition at different time points

Transcript expression analysis was carried out in dehydrated and non-stressed samples at different time points in leaves and roots (Fig. 3-24a). The plants were removed from the pots and kept on a paper towel to dehydrated for various time periods. Gene expression in leaf and root tissues were analysed in untreated samples (control) and after 3, 6, 9 and 24 hours of dehydration as well as in completely desiccated plant (DS). Total RNA was extracted from the leaves and roots of respective plants and the expression level of *CpbZIP1* gene was investigated (Fig. 3-24a). Transcript analysis demonstrated that the *CpbZIP1* gene was transcribed in untreated leaves and roots as well as in the dehydrated samples. However, there was a slight

upregulation of the expressionafter dehydration in leaves. The expression of *Cp LEA-like 11-24* gene was up-regulated after 3 and 9 hours of dehydration in the both leaves and roots.

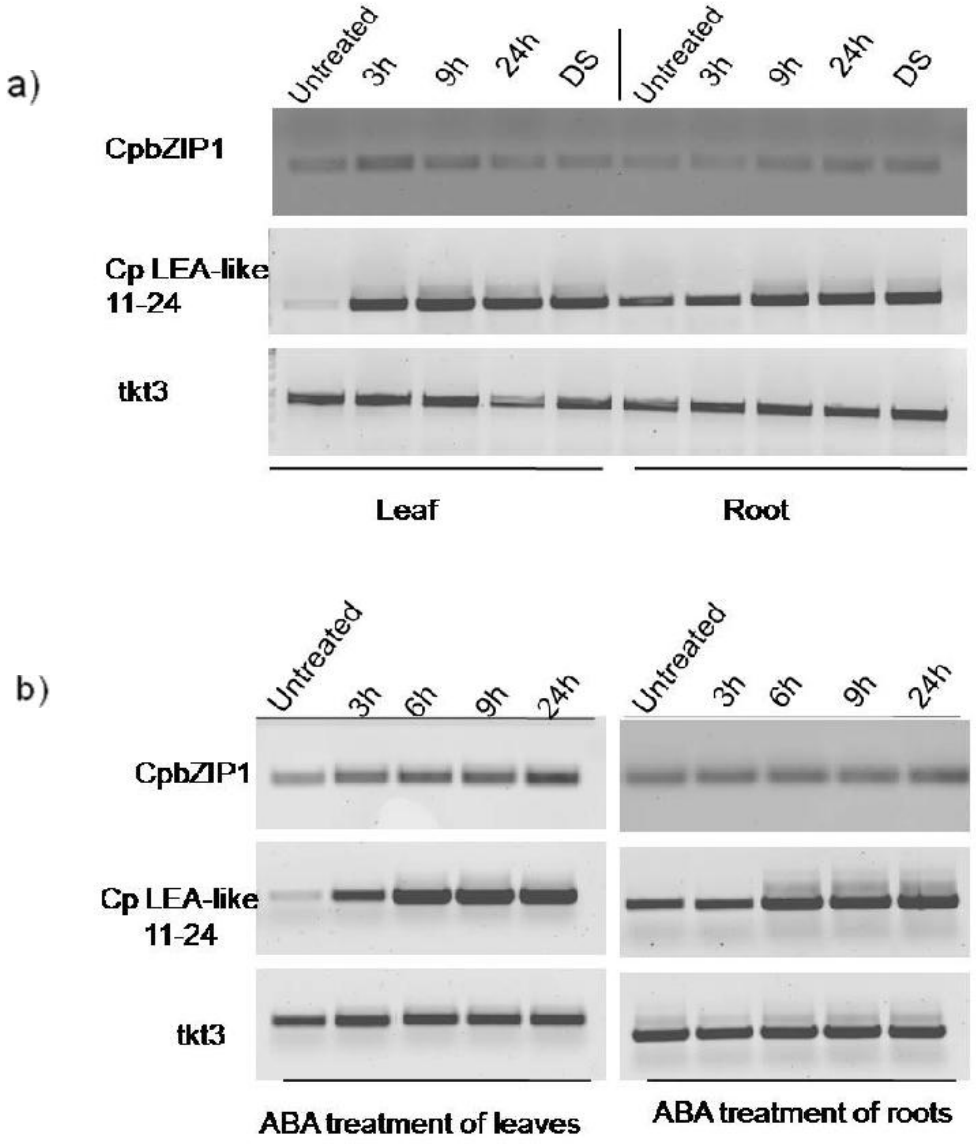


Fig. 3-24: Analysis of *CpbZIP1* gene expression in leaves and roots of *C. plantagineum* subjected to either different dehydration periods or ABA treatment for different time periods. a). Expression of *CpbZIP1* in response to dehydration stress. Plants were kept under drought conditions for 0, 3, 9 and 24 h. DS indicates desiccated leaves. b) Expression of *CpbZIP1* in response to ABA treatment after 3, 6, 9 and 24 h. 'Untreated' fresh leaves and roots were used as control. *Cp LEA-like 11-24* gene expression was used as control for a stress-inducible gene. tkt3 is constitutively expressed in *C. plantagineum* and served to monitor equal loading of RNA in the RT-PCR (28 cycles). The *CpbZIP1*gene was amplified in 30 cycles.

3.3.1.2 Gene expression under ABA treatment at different time points

To study the effect of ABA treatment on the expression of the *CpbZIP1* gene, *C. plantagineum* plants were treated with 100 μM ABA for 3, 6, 9 or 24 hours. Untreated fresh leaves and roots were used as control. Total RNA was separately extracted from the leaves and roots and the expression of *CpbZIP1* gene was evaluated using RT-PCR (Fig. 3-24b). Transcript analysis demonstrated that *CpbZIP1* was constitutively expressed in untreated leaves and roots. Transcript level of the *CpbZIP1* slightly increased in the leaves after 24 hours ABA treatment. In contrast, no change in the expression of *CpbZIP1* was observed in the roots. Differentially *Cp LEA-like 11-24* gene was expressed already after a short treatment with ABA (3 h). Afterwards, expression of this gene progressively increased until 24 hours of treatment. Likewise, the expression of the *Cp LEA-like 11-24* gene in the roots was induced after 6 hours of ABA treatment.

3.3.1.3 Gene expression profiling of *C. plantagineum* leaves and roots subjected to elevated sodium chloride concentration

Effect of salt treatment on the expression of the *CpbZIP1* was analysed in the leaves and roots of *C. plantagineum*. The plants were subjected to salt stress by treating them with different concentrations of NaCl (50, 150, 300 mM) for 3, 6 and 9 hours. untreated fresh leaves and roots were used as controls (Fig. 3-25, 3-26). Transcript expression analyses demonstrated up-regulation of the *CpbZIP1* gene under salt treatment. The expression was induced in the roots treated for 9 hours with 50 mM salt, whereas it peaked after only 3 hours of treatment with 300 mM NaCl. This suggests that the *CpbZIP1* gene is a salt stress responsive gene. The *Cp LEA-like 11-24* gene expression showed that the transcript level was induced by salt treatment as well.

3.3.1.3.1 Relative water content of *C. plantagineum* exposed to dehydration and salt treatment

Relative water content of *C. plantagineum* leaves was determined under dehydrations conditions and under salt treatment at different time points (0, 3, 6, 9 and 24 h). The percentage of relative

water content in fresh leaves was set as 100%. During the first 3 hours of dehydration the leaves lost approximately 55% of water, whereas plants treated with 50 mM salt lost 20% of water content (Fig. 3-25). In contrast to 50 mM salt, the plants in 300 mM salt treatment lost 40% of water content which is comparable with dehydration conditions.

a)

b)

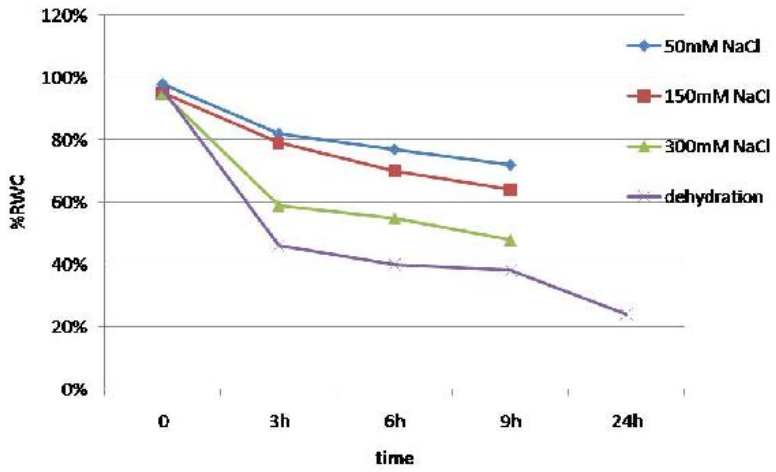


Fig. 3-25: Salt stress treatment in *C. plantagineum*. a) Untreated and salt-treated *C. plantagineum* plants for 3 h in 300 mM NaCl. b) Relative water content of *C. plantagineum* leaves dehydrated and treated in different concentrations of salt.

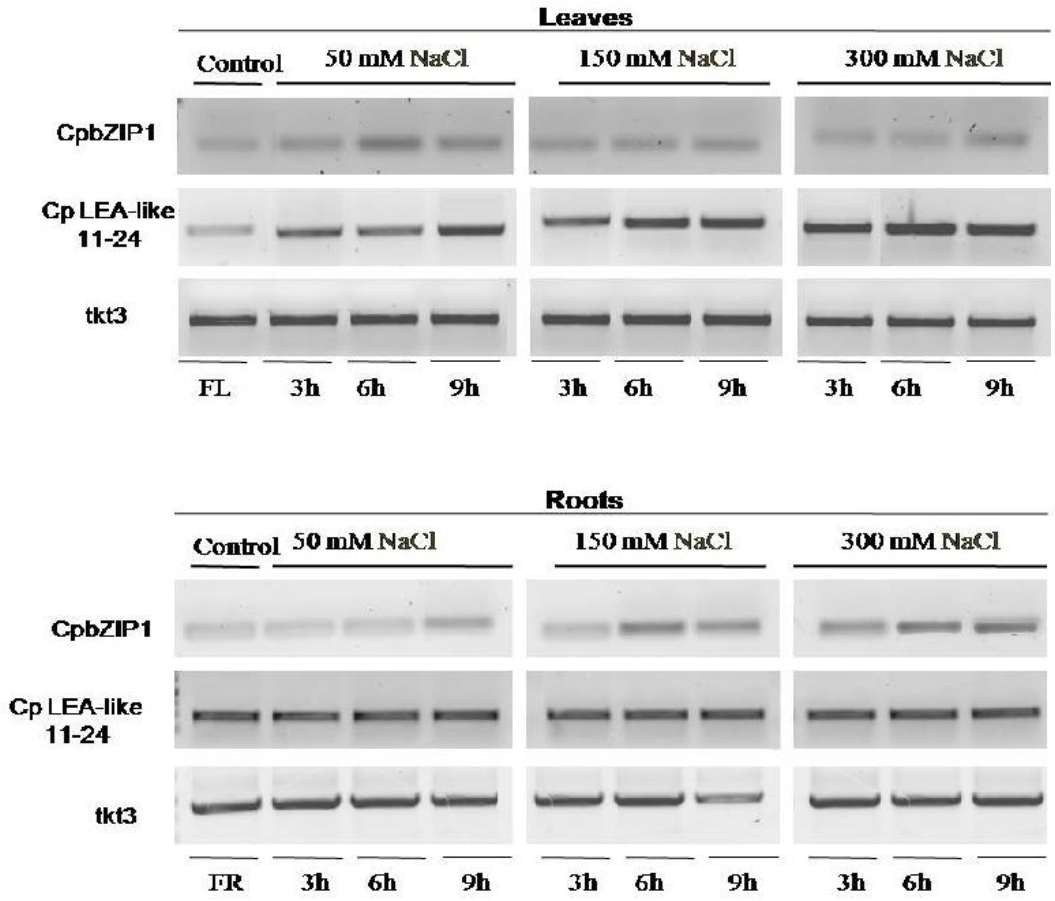


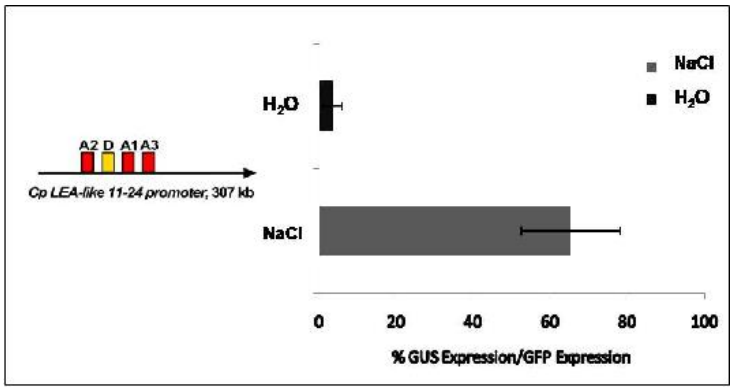
Fig. 3-26: Expression analyses of *CpbZIP1* transcript in the leaves and roots of *C. plantagineum* in response to different concentration of NaCl. a) Expression of *CpbZIP1* in leaves b) Expression of *CpbZIP1* in roots. Plants were treated with in different concentrations of sodium chloride (0, 50, 150 and 300 mM) for 3, 6 and 9 hours. Fresh leaves and roots were used as control for gene expression. *Cp LEA-like 11-24* gene expression was used as control for a stress-inducible gene (28 cycles). Tkt3 is constitutively expressed in *C. plantagineum* and served to monitor equal use of RNA in the RT-PCR (28 cycles). The *CpbZIP1*gene was amplified for 30 cycles.

3.3.1.3.2 Activity of the *Cp LEA-like 11-24* promoter fragment in response to sodium chloride via particle bombardment

The expression of *LEA-like 11-24* promoter fragment of *C. plantagineum* under salt stress condition (150 mM sodium chloride) was also analysed in transient transformation method. The *Cp LEA-like 11-24::GUS* fusion construct (van den Dries et al. 2011) was introduced in to the leaf cells of *C. plantagineum* via particle bombardment. The construct 35S CaMV::*GFP* was used as an internal control and co-bombarded with the *Cp LEA-like 11-24::GUS* construct. The bombarded leaves were treated for 48 hours with 150 mM NaCl or water as control. For analysing the GFP signal and GUS spots, the previous procedure was followed (van den Dries et al. 2011). GFP signal was analysed 24 hours and GUS spots were counted 48 hours after

bombardment (Fig. 3-27). Normalization of GUS spots to GFP signals was performed by dividing the number of counted GUS spot to the number of GFP spots. The relative expression (% GUS/GFP) confirmed the induction of *Cp LEA-like 11-24* promoter in response to NaCl treatment (Fig. 3-27). However, the detected spots were very small due to plasmolysises of cells in the sodium chloride solution.

a)



b)

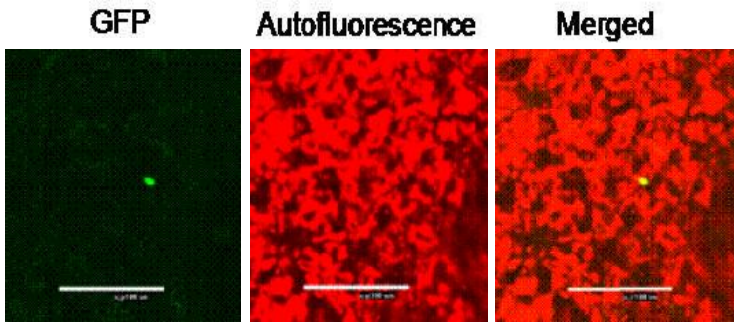


Fig. 3-27: a) Analyses of promoter activity of *LEA- like 11-24* gene in response to 150 mM sodium chloride treatment via particle bombardment. b) Expression of *LEA-like 11-24* gene expression after sodium chloride treatment (150 mM). The green signal corresponds to GFP, red is autofluorescence of chloroplasts and the third picture is the merged signal of autofluorescence and the GFP signal.

3.3.2 Time dependent of the *CpbZIP1* and *Cp LEA-like 11-24* gene in *C. plantagineum* leaves

It has been reported that some of the stress inducible genes follows circadian rhythms (Bieniawska et al. 2008; Legnaioli et al. 2009; Wilkins et al. 2010). To analyze the expression kinetics of *CpbZIP1* and *Cp LEA-like 11-24* gene at the transcript level, detached plants were taken from the growth room every four hours. Immediately leaves, roots were separated and frozen in -80ºC. For each time point three plants were taken at different time point including 11 am, 3 pm, 7 pm, 11 pm which is corresponds to light period, 3 (3 am) and 7 hours (7 am) after

darkn period (Fig. 3-28). Transcript expression results showed that the expression of *CpbZIP1* was up-regulated after 7 hours darkness (at 7 am), whereas during the day (in growth room) the gene was expressed constitutively. Three hours of dark treatment (at 3 am) were not sufficient to induce the *CpbZIP1* gene expression. *Cp LEA-like 11-24* gene was weakly expressed during the day in growth room. While the expression completely was abolished at 11 pm and 3 am which corresponds to the light and dark conditions respectively. Upregulation of this gene was observed at 7 am in the morning, when the light was still off. Apart from that, to evaluate the expression of *CpbZIP1* and *Cp LEA-like 11-24* gene under low energy condition in darkness *C. plantagineum* plants were kept in prolonged darkness for 24 and 72 hours. Expression of *CpbZIP1* increased after 72 hours dark treatment while it was changed slightly after 24 hours of dark treatment (Fig. 3-28). The upregulation of *CpbZIP1* expression under prolonged dark condition indicate the regulation of *CpbZIP1* under low energy condition. Prolonged darkness up to 72 hours led to upregulation of *Cp LEA-like 11-24* expression show the function of *Cp LEA-like 11-24* in circadian clock.

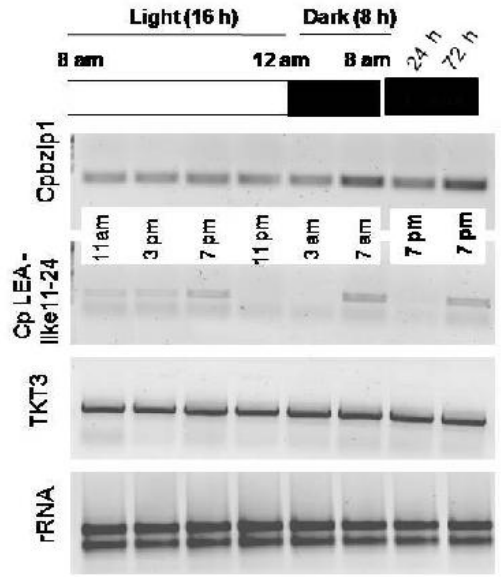


Fig. 3-28: Expression of *CpbZIP1* and *Cp LEA-like 11-24* during one day light/dark cycle in the growth room. a) Detached leaves and roots of *C. plantagineum* were harvested every 4 hours during one day light/dark cycle. The leaves were harvested at 11 am, 3 pm, 7 pm and 11 pm, when the light in growth room was on. Remaining plants were harvested at 3 am, 7 am when the light was off. As control for *CPbZIP1* gene expression in the darkness, harvested leaves after 24 and 72 h under darkness were used. tkt3 is constitutively expressed in *C. plantagineum* and served to monitor equal use of RNA in the RT-PCR (*tkt3* was amplified in 28 cycles). *Cp LEA-like 11-24* gene was amplified in 28 cycles and *CpbZIP1* gene for 35 cycles. rRNA bands indicate equal loading of RNA.

3.3.3 Sequence similarity of CpbZIP1 protein with other plant species

The bZIP proteins are characterized by basic region and leucine zipper domain in *Arabidopsis*. The CpbZIP1 protein from *C. plantagineum* consists of 139 amino acids with a molecular weight of 16 kDa (Ditzer et al. 2006). The CpbZIP1 protein sequence was aligned the bZIP protein sequence of different species to get the percentage similarity. According to the transcriptome data of *C. plantagineum*, *Vitis vinifera, Populus tricocarpa, Solanum lycopersicum* are shown high homology (Dinakar and Bartels 2013). The sequence of the bZIP proteins were aligned along with AtbZIP53 from *A. thaliana, Zea mays, Oryza sativa* (Fig. 3-29). *In addition,* the CpbZIP2 protein sequence was added as close homologue of CpbZIP1. The CpbZIP1 protein showed the highest similarity (94%) with CpbZIP2 from *C. plantagineum*, followed by 55% and 51 % identity with *Populus tricocarpa* and *Arabidopsis thaliana*. In contrast, a bit lower amino acid identity was obtained for *Orysa sativa* (48%), *Zea mayze* (46%) and *Vitis vinifera* (45%). Among the analysed species only *AtbZIP53* from *A. thaliana* which shows 47% amino acid identity with *CpbZIP1,* has been demonstrated to have specific function in seeds (Alonso et al. 2009).

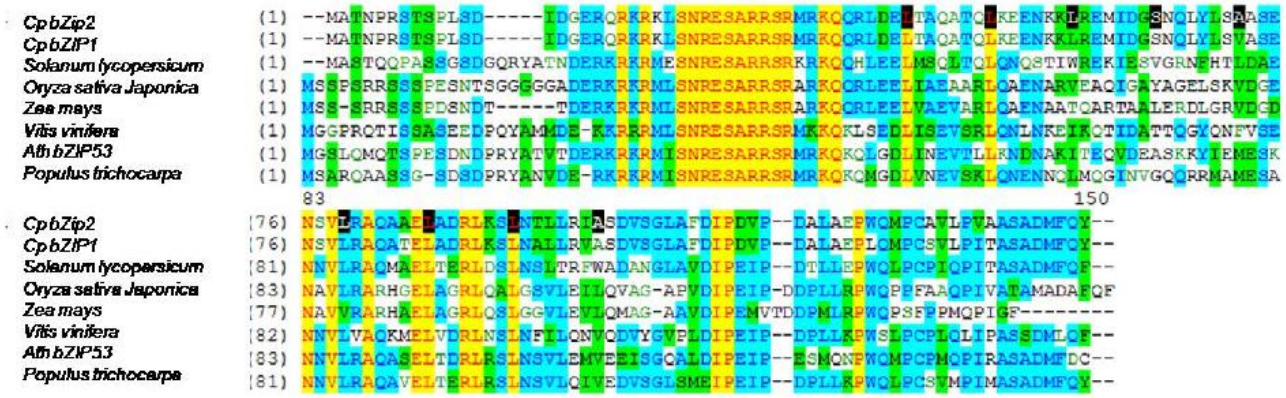


Fig. 3-29: Alignment of amino acid sequences of different bZIP proteins. Sequences of CpbZIP1 protein were compared between a CpbZIP2 from *C. plantagineum* (accession Nr: AAZ72654) as close relative of CpbZIP1 protein, *Solanum lycopersicum* (accession Nr: NP_001234339), *Oryza sativa* (accession Nr. NP_001054544), *Zea mays* (accession Nr: NP_001105684), *Vitis vinifera* (accession Nr: XP_002282195), *Arabidopsis thaliana bZIP53* (accession Nr: NP_191801) and *Poulus trichocarpa* (accession Nr: XP_002301511).

3.4 Generation and molecular characterisation of transgenic plants ectopically expressing the *CpbZIP1* gene, (35S::Cp*bZIP1*, S-lines)

To further characterise and understand the role of CpbZIP1 protein in response to abiotic stresses, plant overexpressing *CpbZIP1* were generated through stable transformation (For the isolation of the *''35S::CpbZIP1''* cassette and cloning procedure see in part 3.2.1).

3.4.1 Screening of the transgenic plants overexpressing the *CpbZIP1* gene and correlation of *CpbZIP1* expression with the level of dwarfism

After 14 day kanamycin-resistant seedlings were transferred from MS-plates supplemented with kanamycin into the soil. Afterwards, the transgenic seedlings were screened with the combination of specific primers flanking the *CpbZIP1* coding sequence and CaMV35S promoter such as RTCpbZIP1 F/R, 35S-pRok-F/RTCpbZIP-R in T1 and T2 generation. Totally, more than 10 transgenic lines were screened in T1 generation. Transcript level of *CpbZIP1* gene was analysed in 5 lines namely S3, S10, S25, S28, S31, S36 (Fig. 3-32). Different phenotype was found between transgenic lines which can be attributed to the overexpression of *CpbZIP1*. Similar phenotype also was found in GZ and LZ lines harbouring (*35S::CpbZIP1+Cp LEA-like 11-24::GUS*). None of these phenotypic traits was observed in the *Cp LEA-like 11-24::GUS* lines either short or full promoter. The gene was found to be silenced in one of the independent lines (S10) in T2 generation. In these lines *CpbZIP*1 expression was abolished at the transcript level whereas it was present in the genomic PCR amplification. Therefore, the S10 line was removed from the list of study. Also S12 line was removed from the list, because the seeds were not viable and only few seedlings were grown. Finally, three independent lines (S28, S31, S36) were chosen for further analyses.

In these analyses the expression level of transgene was investigated in T1 and T2 generation of independent lines. 4 week-old plants cultivated in growth chamber were removed from the pots and the leaves were immediately frozen in liquid nitrogen. Two different phenotypes of each line were chosen to analyze the transcript expression of *CpbZIP1* gene (Fig. 3-31). Higher expression of CpbZIP1 was often found in plants showing more severe phenotype.

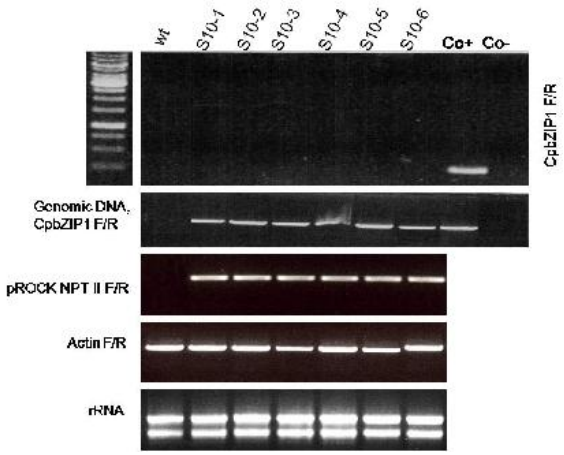


Fig. 3-30:. Silencing of the CpbZIP1 gene in T2 generation of overexpressing S10 line. No transcript expression of *CpbZIP1* gene was observed in the 6 members of S10 line. Genomic DNA amplification proved the presence of *CpbZIP1*. Expression of neophosphotransferase II gene proved the insertion of transgene in transgenic plants. Actin gene expression shows equal use of RNA in the RT-PCR. rRNA bands indicate equal loading of RNA.

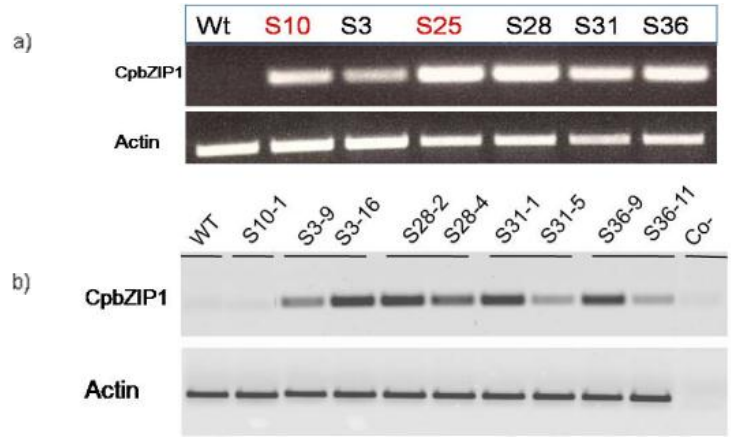


Fig. 3-31: Expression analyses of *CpbZIP1*gene in a) T1 generation and b) T2 generation of transgenic lines overexpressing *CpbZIP1* (S-lines). Actin expression determines the equal use of RNA in the RT-PCR.

3.4.2 Phenotypic analyses of S-lines plants

Phenotypic analyses of transgenic *Arabidopsis* plants ectopically expressing *CpbZIP1* gene were peformed. 4 week-old Wild-type and transgenic *Arabidopsis* lines were transferred to the long-day conditions for flowering. The transgenic lines showed dwarf phenotype with short roots and stems, abnormal flowers with small siliques and delayed flowering time. Moerover, in these lines floral buds appeared open with shorter sepal and petal in early stage of flowering when flowers are supposed to be closed (Fig. 3-32c).

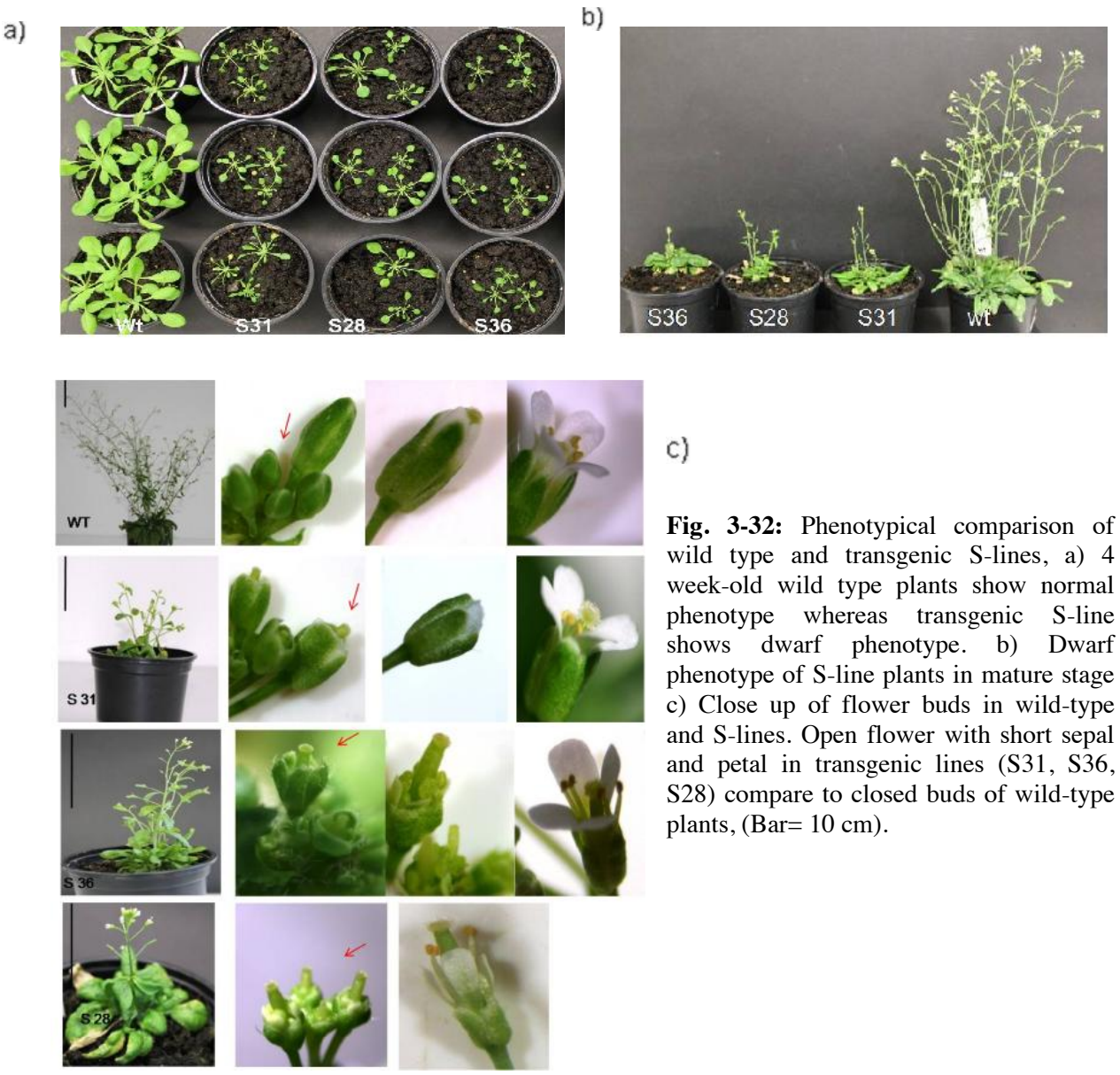


Fig. 3-32: Phenotypical comparison of wild type and transgenic S-lines, a) 4 week-old wild type plants show normal phenotype whereas transgenic S-line shows dwarf phenotype. b) Dwarf phenotype of S-line plants in mature stage c) Close up of flower buds in wild-type and S-lines. Open flower with short sepal and petal in transgenic lines (S31, S36, S28) compare to closed buds of wild-type plants, (Bar= 10 cm).

3.4.3 *CpbZIP1* overexpressing (S lines) plants under salt stress

The CpbZIP1 protein is a member of S-class bZIP transcription factor. All members of this class have been demonstrated to function very specifically in the regulation of stress responsive drives from abiotic stresses or changing energy balances (Weltmeier et al. 2004, Dietrich et al. 2011). Therefore, the stress responsiveness of *CpbZIP1* was analysed in transgenic lines ectopically expressing the bZIP1 protein. (also transcript of the gene showed slight upregulation in *C. plantagineum*). Responsiveness of transgenic plants overexpressing *CpbZIP1* (S-lines) to the salt stress was tested at the seedling stage. Transgenic S-lines were grown on MS-kanamycin plates for 7 days, while wild-type seeds were cultured in MS-medium without kanamycin.

Afterwards wild-type and kanamycin-resistant seedlings were transferred on MS-medium supplemented with different concentration of NaCl (0, 100 and 150 mM) and subsequently grown for 10 days in the respected medium (Fig. 3-33). As 150 mM NaCl was an effective concentration in inhibiting the seedling growth of wild type plants, the number of seedlings survived in 150 mM NaCl was counted (Table 3-3). Higher percentage of survived seedlings was obtained in transgenic lines (89-98%) compared with wild type (56%). Moreover, Mmeasurement of proline content indicated higher accumulation of proline in response to 150 mM NaCl in transgenic lines than in wild-type plants (Fig. 3-34). Plants accumulate proline as compatible solute to cope with the stress conditions. Also plants membrane lipids oxidizes under stress conditions leading to producing malondialdehyde (MDA) which being a toxic compound for plant, MDA content was measured (Fig. 3-39). MDA level increased in wild-type plants more than the transgenic S-lines when seedlings were grown on MS medium supplemented with 150 mM NaCl.

Table 3-3: Total number, survived and Percentage of survived seedlings under salt stress.

Salt stress	WT	S28	S31	S36
Total Number of seedlings	75	75	75	75
Number of survived seedlings	57	67	60	74
Percentage of survived seedlings (%)	76	89	80	98

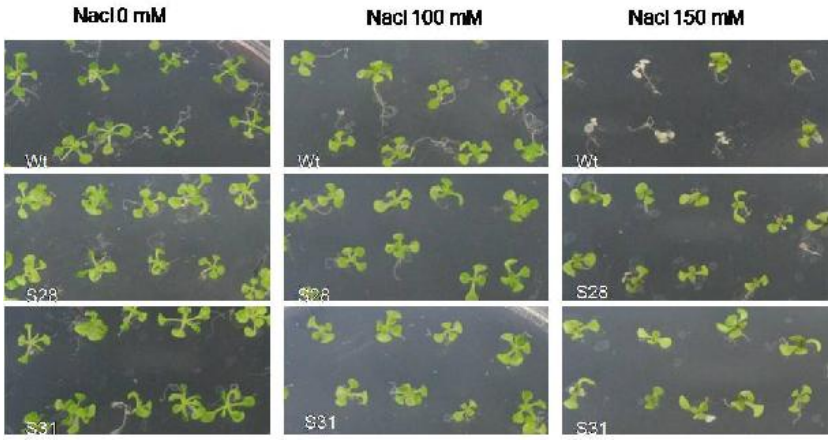


Fig. 3-33: Photographs of WT and transgenic S-line seedlings grown on MS plates. The photos were taken after 10 day from transferring them to the MS supplemented with 0, 100 and 150 mM NaCl.

a) b)

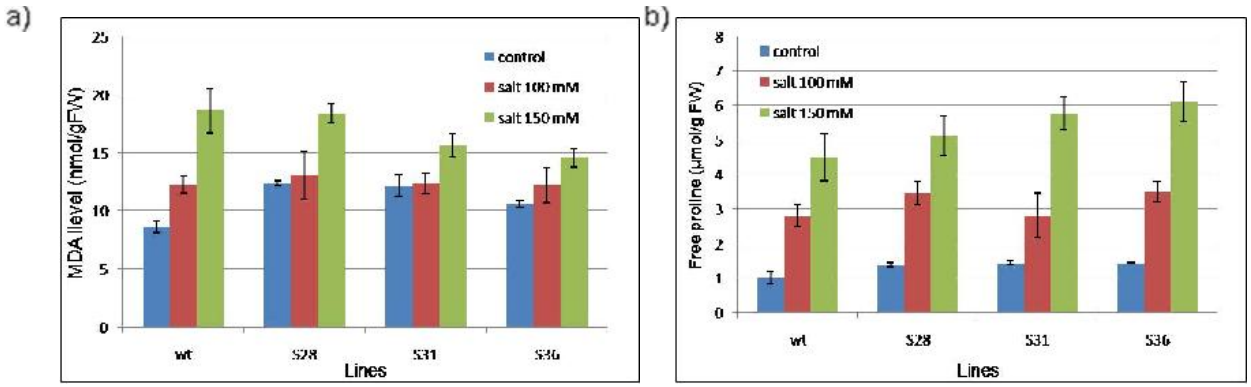


Fig. 3-34: Seedlings of wild-type and transgenic S-lines treated with 100 and 150 mM NaCl. a) MDA level and b) Free proline content. Values represent the mean ± SE from three biological replicates.

3.4.4 *CpbZIP1* overexpressing (S lines) plants under drought stress

To check the response of transgenic lines to drought conditions, five week-old wild-type, S28 and S31 overexpressing lines were exposed to drought stress by stopping irrigation (Fig. 3-35). In general transgenic plants were smaller than the wild-type plants (Fig. 3-35a). Therefore, different physiological stages of plants could make the comparison of wild-type plants and transgenic lines difficult. The water content of the soil was measured in the plants grown in a an equal amount of soil as at the beginning of the experiment

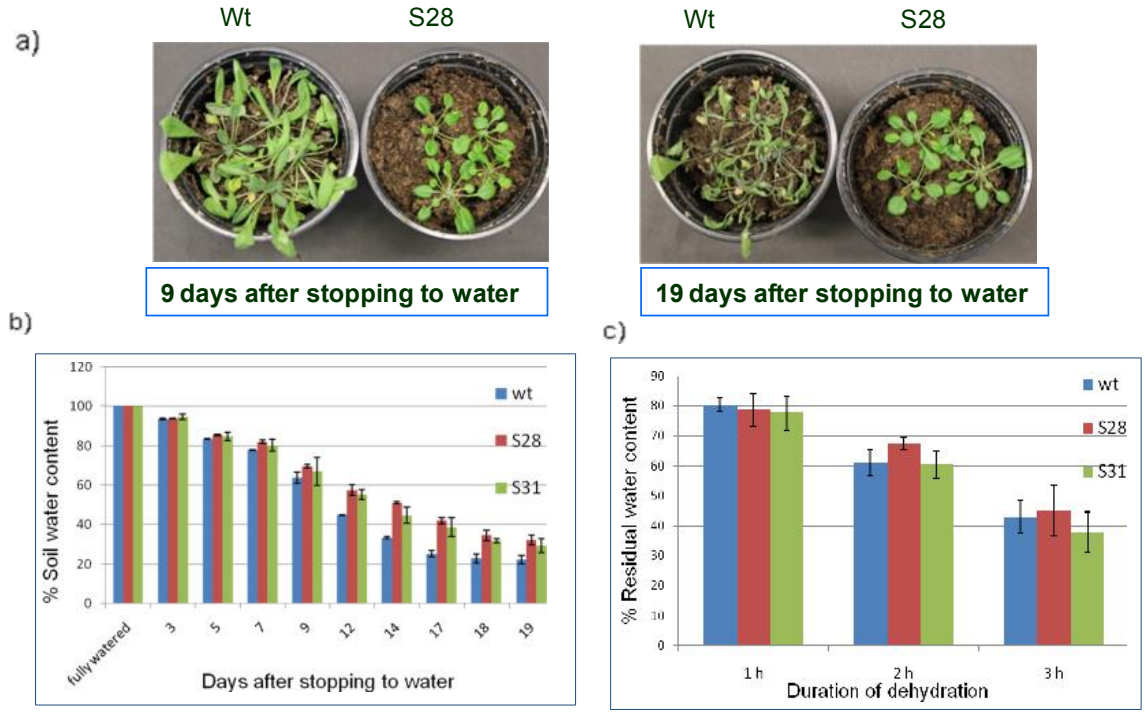


Fig. 3-35: a) Photos of transgenic and wild-type plants subjected to drought, b) Relative water content of the soil in the pots with wild-type or transgenic plant after stress application. c) Percentage of water loss in wild type and transgenic plants, 5 days after stopping to water.

The pots were weighted every 2-3 days for 18 days. After this time, the transgenic plants look healthier than the wild-type. However, high percentage of soil relative water content was obtained in transgenic lines than in wild-type plants (Fig. 3-35b).

In addition, %water loss of leaves taken from wild type and transgenic plants were calculated after 1, 2 and 3 hours (Fig. 3-35c). Wild type and transgenic plants showed similar percentage of water loss in different time point. Since the biomass of the transgenic plants is less, it is not surprising that the transgenic plants take less water than wild type plants. Logically the water content in soil where transgenic plants are grown will be higher.

3.4.5 Photosynthesis rate in non-stressed plants overexpressing *CpbZIP1* gene

Since transgenic plants overexpressing *CpbZIP1* showed a dwarf phenotype, photosynthetic parameters were measured in untreated wild-type and overexpressing *CpbZIP1* plants to check whether transgenic plants are healthy (Fig. 3-36). The leaves of 4-5 week-old wild-type and transgenic plants from three pots were used for this purpose. The graph shown on CO_2 assimilation demonstrates that the transgenic plants at different light intensities have higher photosynthetic rates than wild-type plants. However, no significant differences was observed among transgenic and wild-type planats, suggesting higher rates of CO_2 assimilation in these plants (Fig. 3-36a). The differences between wild type and transgenic plants in yield of PSII are marginal as shown in Fig. 3-36b.

The photosynthetic electron transport rate is higher in transgenic lines than in wild-type plants (Fig. 3-36c). However, it shows variation among the transgenic plants. The increase in non-photochemical quenching at all light intensities in one transgenic plant (S28) suggests that this transgenic plant is better photoprotected than the others (Fig. 3-36d). These parameters suggest that transgenic plants are healthy. Moreover, the enhanced photosynthetic performance, electron transport rate and better photo protection mechanisms show better performance of these transgenic plants than wild-type plants.

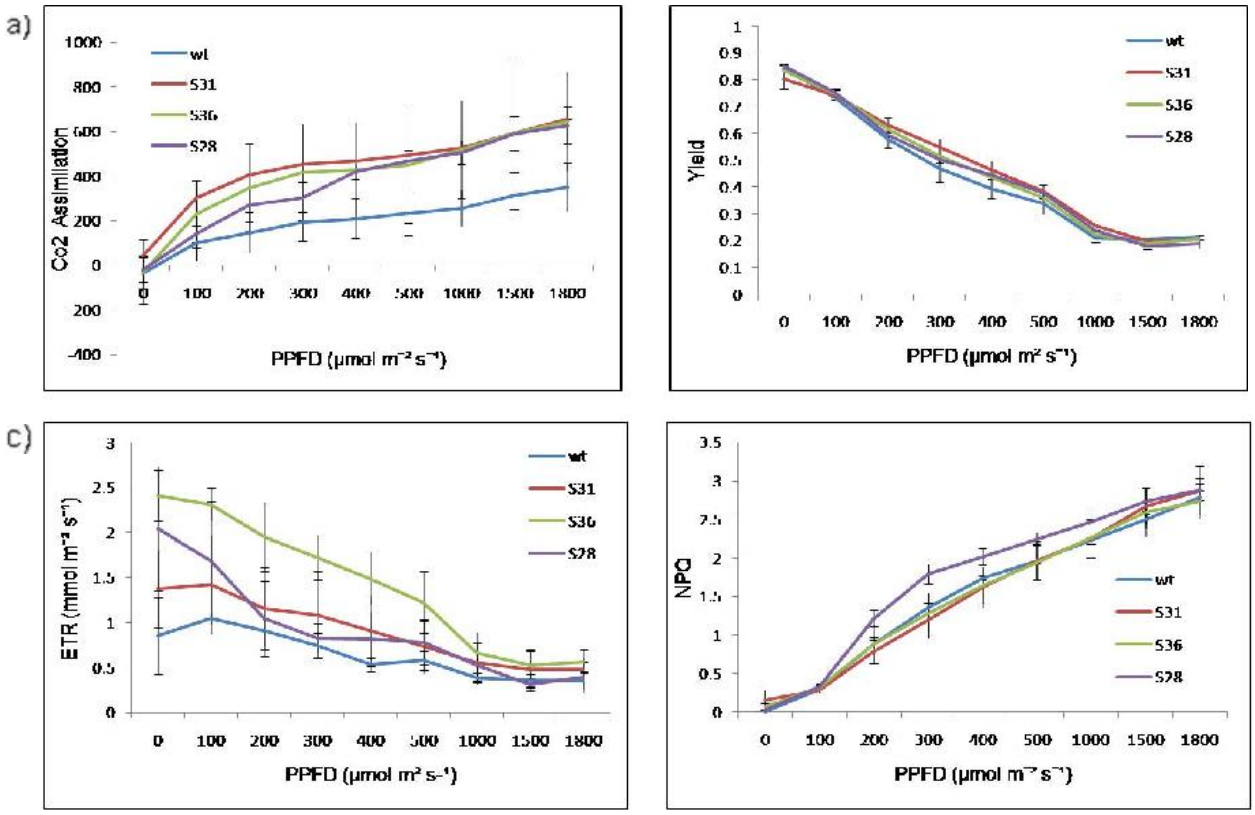


Fig. 3-36: Photosynthesis measurements in wild-type and transgenic plants overexpressing *CpbZIP1* under non-stressed conditions in various light intensity. a) CO2 assimilation rate, b) The yield of photosystem II, c) Electron transport capacity, d) Non photochemical quenching. All photosynthetic parameters were measured in 4-5 week-old transgenic (S lines) and wild-type plants. The measurements are mean ± SE. from three biological replicates.

3.4.6 *CpbZIP1* overexpressing (S-lines) plants under dark stress

In previous sections (part 3.3.2 Fig. 3-28), the kinetic expression of gene encoding *CpbZIP1* showed demonstrated the upregulation of gene during dark period and after 7 hours darkness, while it expressed constitutively during the day. The upregulation of *CpbZIP1* during dark conditions may have a specific function. To analyses the function of *CpbZIP1* in the dark, 4-5 week-old *Arabidopsis* plants overexpressing *CpbZIP1* were cultivated in dark condition for 4 and 8 days (Fig. 3-37a). Control plants were cultivated in similar condition along with light. The proline level was higher in untreated transgenic plants than that of wild type. However, differentially than in wild-type, proline content in transgenic lines sharply decreased after 4 days of dark treatment. After 8 days of darkness, the proline content was increased in wild type as well as transgenic lines. The proline level almost reached the level of control conditions in wild

type. This suggests that plants possess particular regulatory mechanism to compensate the carbon limitation in severe starvation. Expression of *ProDH* after 4 days of darkness showed upregulation of this gene in both, dark- treated or non-treated plants (Fig. 3-37c).

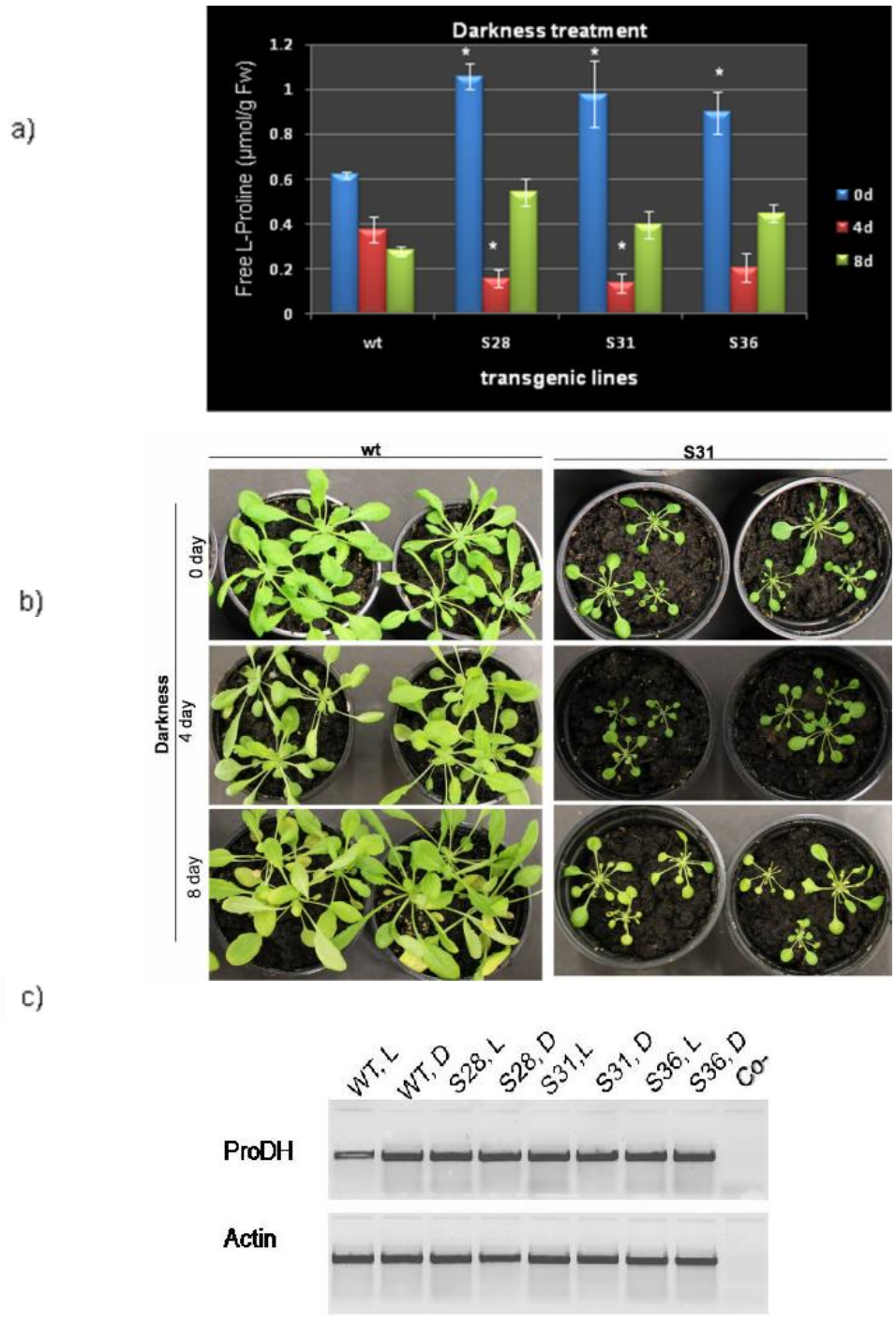


Fig. 3-37: 5-week-old wild type and transgenic plants overexpressing *CpbZIP1* were subjected to dark treatment. a) Proline content of wild-type and transgenic plants under prolonged darkness, control plant kept in short day room. Standard errors are displayed as error bars. *Asterisks* indicate statistical significance (* $P < 0.05$) in comparison with the corresponding wt plants. b) Photos of wild-type and transgenic lines after 0, 4 and 8 days of dark treatment. c) Expression of *ProDH* in wild-type and transgenic lines under light (L) or dark (D) conditions.

3.4.7 Effect of nitrogen source on the growth rate of transgenic plants overexpressing *CpbZIP1* gene

To support the results obtained from transgenic plants overexpressing *CpbZIP1*, one independent transgenic plant (GZ21) belongs to the GZ line harbouring *'35S::CpbZIP1+Cp LEA-like 11-24::GUS'* (short promoter) was used in all physiological experiments.

3.4.7.1 Effect of proline in rescuing the growth of transgenic plants

Proline is the major component of cell wall proteins, therefore the presence of this amino acid is crucial for plant growth (Verslues and Sharma 2010). ProDH plays a crucial role in proline degradation in *Arabidopsis* plants after releasing the stress conditions. Since, dwarf phenotype were observed in transgenic plants overexpressing *CpbZIP1* (S lines), in addition, the *ProDH* gene was up regulated in these plants, the effect of proline in rescuing the growth rate of transgenic plants was analysed. The seedlings were grown on MS+kan (kanamycin) and after 7-days transferred to the MS+N (nitrogen source) as described by Murashige and Skoog (1962) or MS+N+5 mM proline.

The seedlings were subsequently grown for two weeks on MS+N+5 mM. However proline could not rescue the growth of transgenic plants (Fig. 3-38). Measurement of chlorophyll contents resulted in significantly lower chlorophyll contents in the transgenic seedlings grown on MS+N+5 mM proline compared with those grown on MS+N.

It suggests that addition of proline to the MS medium has a toxic effect in transgenic plants. Toxicity of proline accumulation in plant cells is known from different plants (Mani et al. 2002, Nanjo et al. 2003; Verbruggen and Hermans 2008). It has been reported that toxicity of proline in these plants is derived from pyrroline-5-carboxylate (P5C) accumulation, which increases reactive oxygen species (ROS) production and causes stress for plants (Hellmann et al. 2000; Deuschle et al. 2004; Verbruggen and Hermans 2008).

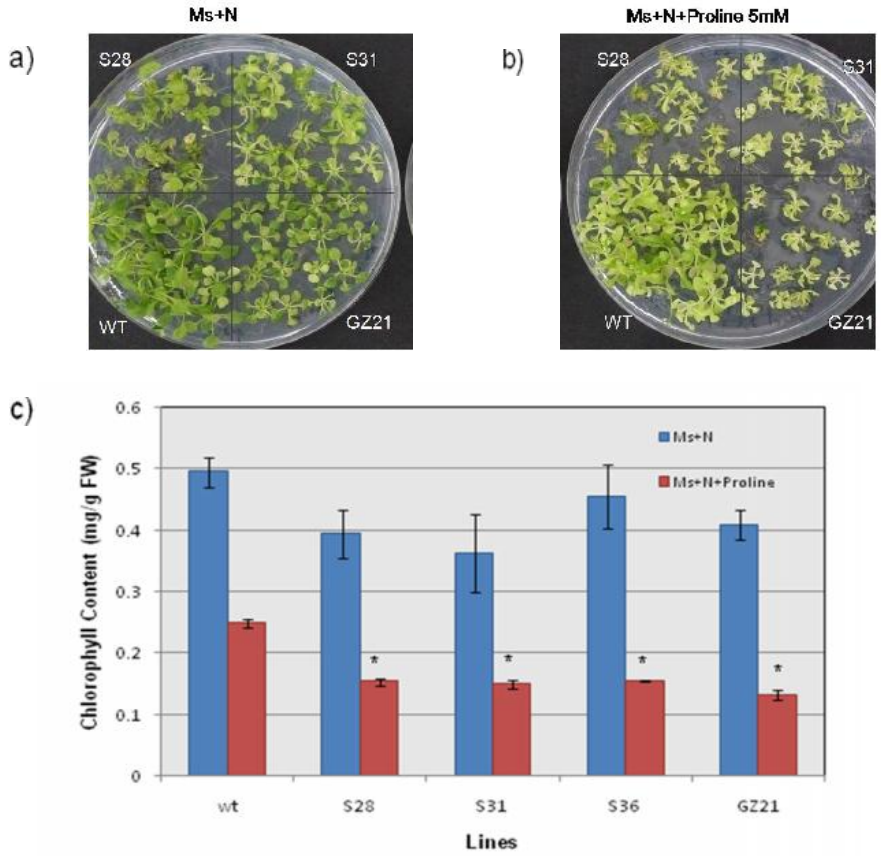


Fig. 3-38: Effect of proline on the growth rate of transgenic lines: a) MS+N (Murashige and Skoog 1962). b) MS+N+5 mM. c) Chlorophyll content of treated and non-treated transgenic lines. Data are means± SE from three replicates. The significance of differences between treatments was determined by one-way ANOVA (p<0.05).

3.4.7.2 Utilization of proline as a nitrogen source in transgenic plants overexpressing the *CpbZIP1* gene

To understand whether transgenic plants can utilize the proline as nitrogen source, wild type and transgenic lines were grown in MS-medium with and without nitrogen source. Nitrogen sources in MS-medium (NH_4NO_3 and KNO_3) were substituted with 20 mM KCl, (Murashige and Skoog 1962) and supplemented with 15 mM sucrose. Transgenic lines were first grown in MS+Kan for one week and then transferred to the respected plates for two weeks. The comparison of plants grown on MS-medium without nitrogen source and with those grown on the similar medium supplemented with proline indicated that transgenic plants are able to use proline as a nitrogen source. It demonstrates no defect in the nitrogen utilization pathway (Fig. 3-39 a,b).

The comparison of chlorophyll content of transgenic lines with that of the wild-type plants showed that transgenic lines could grow in MS-N medium better than wild-type plants. In this medium transgenic seedlings retained higher chlorophyll levels than wild type (Fig 3-39 d,e,f,g).

In addition, transgenic seedling in MS medium with out nitrogen source started bolting earlier than wild type.This suggests the use of endogenous proline as nitrogen source for growth. The transcript accumulation of *ProDH* gene was also up regulated in transgenic plants (Fig. 3-39 c). Therefore, it is assumed that transgenic lines may contribute in remobilizing the amino acids in nitrogen deficiency conditions.

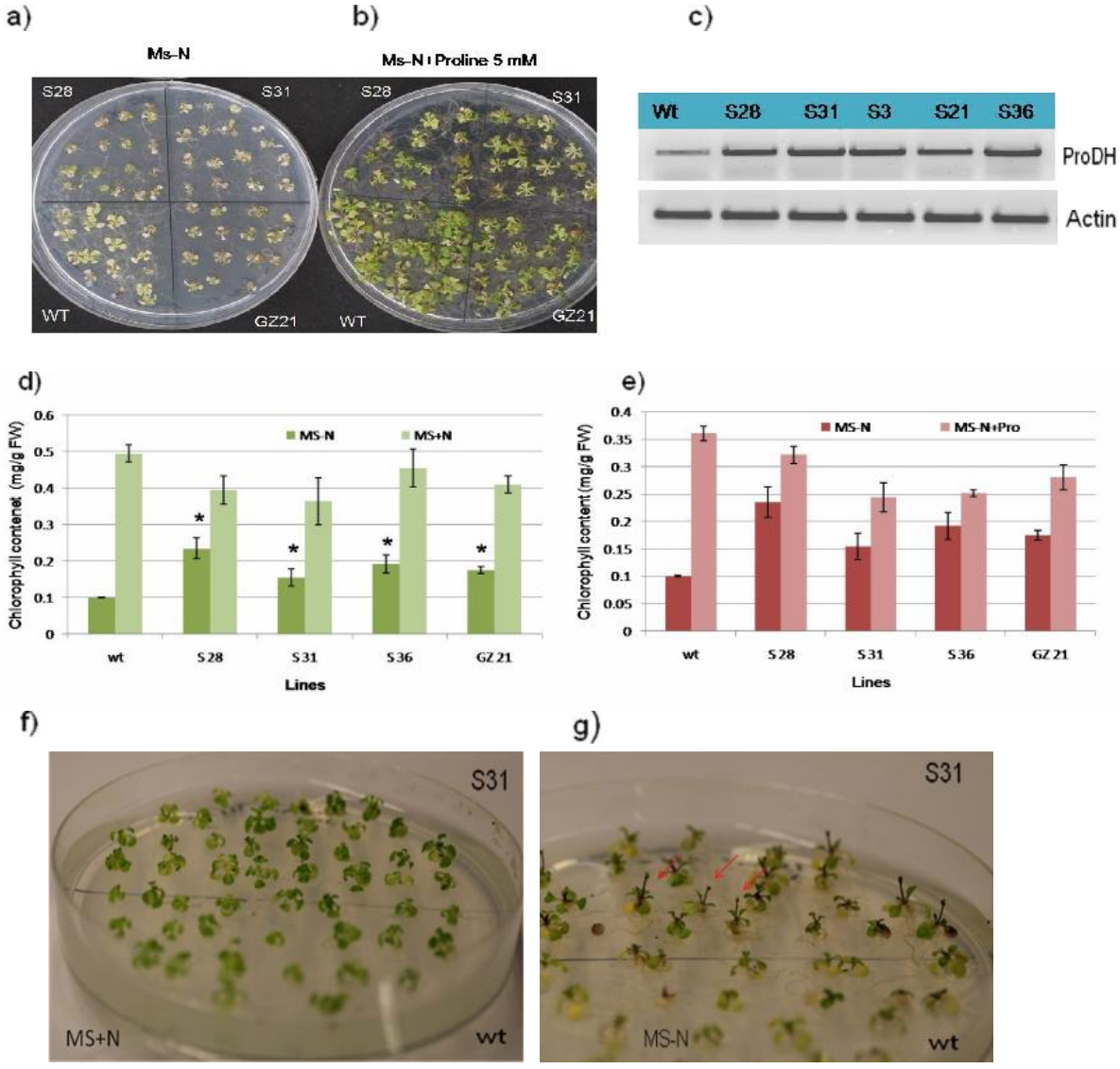


Fig. 3-39: Use of proline as nitrogen source in wild-type and transgenic plants: a) 7 day-old seedlings were grown in MS-N (MS-medium without nitrogen source) b) seedlings were grown in MS-N+5 mM proline at a concentration of 5 mM. c) Transcript expression of ProDH in untreated transgenic lines and wt. d) Chlorophyll content of wt and transgenic lines in MS-N and MS+N medium. e) Chlorophyll content of wt and transgenic lines grown on MS-N plate supplemented with 5mM proline. f and g) 7 day-old wt and transgenic S31seedlings were grown on MS+N or MS-N plate for 2 weeks. Transgenic seedlings grew better than wild type and start bolting. Red signs show bolted seedlings. Data are means ± SE from three replicates. The significance of differences between treatments was determined by one-way ANOVA (p<0.05).

3.4.8 Proline content in transgenic plants overexpressing *CpbZIP1*

As proline dehydrogenase (*ProDH*) was upregulated in the transgenic plants overexpressing the *CpbZIP1* gene, proline accumulation was measured in different organs of these lines and subsequently compared to wild type. The proline level was measured in vegetative organs of 2, 3 and 4 week-old plants and reproductive organs as flowers and siliques. Since, the proline level can vary during the day or night, the plant material was harvested always in the afternoon. In both 2 week-old seedlings, 3 and 4 week-old plants, the proline was accumulated at higher level in leaves of transgenic plants than that of wild type. In addition proline level was higher in the flower, young and mature siliques of transgenic plants, suggesting the up-regulation of the genes responsible for proline biosynthesis in transgenic plants.

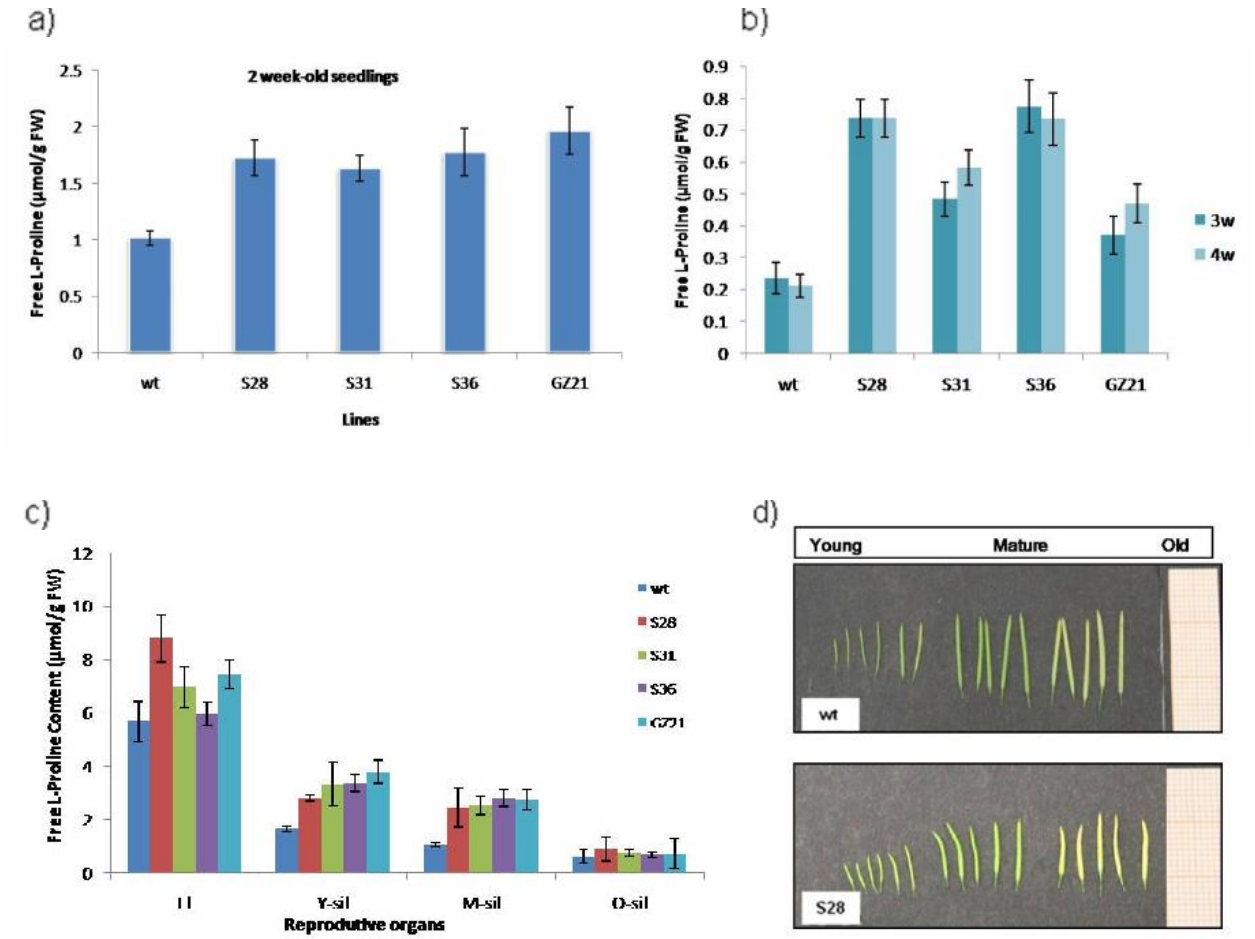


Fig. 3-40: Proline accumulation in wild-type and transgenic lines overexpressing *CpbZIP1*. Proline was measured in the leaves of a) 2 week-old seedlings, b) 3 week and 4 week-old plants c) proline level was measured in flowers and siliques. Y-sil=young siliques, M-sil=mature siliques, O-sil=old siliques; d) three different sizes of siliques were used for proline measurements (Yung, Mature, Old).

3.4.9 Effect of proline on root elongation of transgenic plants overexpressing *CpbZIP1*

To evaluate the effect of external supply of proline on root elongation in transgenic plants, different concentrations of proline (5, 10 and 20 mM) were tested for 10 days in 7 day-old seedlings. Seedlings grown in 20 mM of proline were dead while 10 mM did not show any alteration in phenotype of wild type and transgenic seedlings. Thus, 10 mM proline was added to the media. In Arabidopsis the concentration of proline higher than 10 mM has been reported (Mani et al. 2002). The experiment was performed in two levels, with sugar and without sugar for better mimic the environment of soil grown plants (Sharma et al. 2011). After one week of growing in the respected media, root elongation was analysed (Fig. 3-41). The results demonstrated that addition of proline to the medium supplemented with/without sucrose did not have any effect on the root elongation of transgenic lines and did not rescue the root defect.

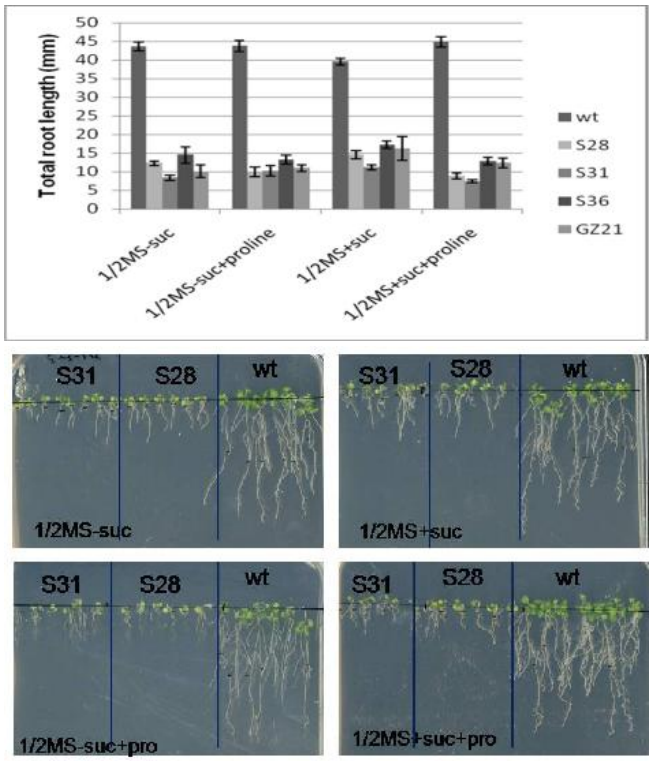


Fig. 3-41: Effect of proline on root elongation in transgenic lines. 7 days-old seedlings were transferred to the MS±sucrose with/without 10mM proline. a) Root length was measured after 10 days in different media. b) Photo of root elongation in different media. The photos were taken after 10 days of growth.

4. Discussion

Desiccation tolerance is a very complex trait. Numerous genes involved in desiccation tolerance have been characterized in *C. plantagineum* (Michel et al. 1994; Velasco et al. 1994; Ditzer et al. 2006; van den Dries et al. 2011). Promoter regions play an important role in transcriptional expression of genes regulated by desiccation tolerance (van den Dries et al. 2011). Since promoters are the main regulatory elements for enhancing the transcriptional efficiency, our focus was at the study on transient transformation methods to understand promoter function in response to different abiotic stresses. The first objective of this study was optimization of the *Agrobacterium*-mediated transient transformation method to analyze the promoter activity in three closely related species of Linderniaceae family (*C. plantagineum*, *L. brevidens* and *L. subracemosa*). The optimized method was used for cross comparison of the *LEA-like 11-24* promoter fragments of these species. The trans-activation of the *Cp LEA-like 11-24* promoter by the CpbZIP1 transcription factor was examined in homologous and heterologous systems. Also, the stress responsiveness of the gene coding CpbZIP1 was studied under different abiotic stress conditions. Finally, for functional analyses transgenic *Arabidopsis* plants overexpressing CpbZIP1 were generated.

4.1 Optimization of a new transient transformation method was essential for studying the promoter function in *C. plantagineum*

Transient transformation is a suitable method for analysing promoter activity in different plants species (Basu et al., 2003; Endo et al. 2008). Transient transformation methods are a rapid choice to analyze the promoter function in compare to stable transformation. On the other hand, stable transformation allows the insertion of a gene in random genomic position. As a result, it may have an effect on the expression level of gene based on chromosomal insertion position.(Yang et al. 2000).

Different transient expression studies including biolistic bombardment (Christou 1995; van den Dries et al. 2011; Liu et al. 2014), protoplast transfection (Michel et al. 1994; Yoo et al. 2007; Ohkama-Ohtsu et al. 2008) and *Agrobacterium*-mediated transient transformation methods (Yang et al. 2000; Li et al. 2009; Xu et al. 2014) have been applied in different studies. These transient transformation methods practically faced some difficulties in *C. plantagineum*. For

instance, protoplast isolation in *C. plantagineum* leaves is difficult because of the presence of thick leaves and the fragile nature of protoplasts. Both methods of protoplast isolation and particle bombardment are not suitable for quantitative measurements of promoter activities. Therefore, development of a new transient expression method was proposed (van den Dries 2010). In this study *Agrobacterium tumefaciens*-based large-scale transient transformation method was optimized in three closely related species to analyze and compare the *LEA like-11-24* promoter activities in homologous and heterologous backgrounds in response to ABA and osmotic stress. The method was optimized according to the FAST procedure (Fast Agro-mediated Seedling Transformation) which has been developed for *A. thaliana* seedlings (Lie et al. 2009).

4.1.1 *Agrobacterium*-mediated transient transformation has been successfully optimized in two desiccation tolerant and one desiccation sensitive Linderniaceae members

Comparative analysis using physiological, biochemical and molecular approaches, between desiccation tolerant *C. plantagineum, L. brevidens* and desiccation sensitive species, *L. subracemosa* is very important for understanding desiccation tolerance mechanisms (van den Dries et al. 2011; Dinakar et al. 2012). To analyze the comparative promoter functions in these three species, the important parameters in enhancing the transformation efficiency such as leaf size, Silwet L-77 concentration, bacterial density and duration of co-cultivation was optimized for three species. Silwet L-77 plays a similar role to the vacuum infiltration and facilitates the delivery of *A. tumefaciens* cells (Desfeux et al. 2000; Li et al. 2009). Silwet L-77 in co-cultivation medium reduces plant surface tension with less phytotoxicity (Whalen et al. 1991). Therefore, the dissolved Silwet L-77 in aqueous solution spreads over the leaf and penetrates in open stomata (Whalen et al. 1991). In *L. subracemosa*, GUS activity increased with 0.005% (v/v) Silwet L-77 and declined sharply with 0.0075% (v/v), whereas 0.0075% (v/v) Silwet L-77 concentration enhanced GUS activity in *C. plantagineum* and was slightly reduced with 0.01 % Silwet concentration, indicating essential requirement for optimizing the method in different species. The transformation with optimal Silwet concentration enhanced GUS activity 4.2, 2.4

and 3.1 fold in comparison to control conditions (Silwet concentration = 0%) in *C. plantagineum*, *L. brevidens*, *L. subracemosa* respectively. The results for the optimal Silwet concentration are in agreement with other *Agrobacterium* mediated-transformation studies in wheat, soybean or *Arabidopsis* that showed the positive effect of Silwet L-77 on transformation efficiency (Clough and Bent 1998; Wu et al. 2003; Li et al. 2009). In addition, optimization of the bacteria concentration in the co-cultivation medium improved the transformation efficiency which is consistent with the reports of legumes (Kapila et al. 1997), cottonwood (Han et al. 2000), tobacco (Krugel et al. 2002) and *Arabidopsis* (Clough and Bent 1998; Li et al. 2009). In the case of *C. plantagineum* a concentration of bacteria equal to $OD_{600=}$ 2 increased the GUS activity, but caused necrosis in leaves. Higher bacteria density $OD_{600} > 2$ damaged the leaf tissues and decreased the GUS activity demonstrating toxicity of high bacteria density in plant cells (Voinnet et al. 2003).

We were able to optimize the duration of co-cultivation using the optimal Silwet L-77 and bacteria concentration in the co-cultivation medium. The highest transformation efficiency in *L. subracemosa* was observed after 48 hours co-cultivation, whereas in *C. plantagineum* and *L. brevidens* 60 hours co-cultivation caused highest transformation efficiency. Duration of 72 hours co-cultivation was lethal for *L. subracemosa* while 96 hours for *C. plantagineum* and *L. brevidens*. That corresponds with previous data where the duration of *Agrobacterium* co-cultivation varies from 2-7 days (Han et al. 2000; Somleva et al. 2002; Li et al. 2009; Xu et al. 2014) whereas a period of 2-3 days is commonly use. Finally, after optimization we observed a 12.5, 3.5 and 2.7 fold increase of GUS activity in *C. plantagineum, L. brevidens* and *L. subracemosa* leaves in comparison to transformation before optimization, when only the FAST method was followed.

4.1.2 Promoter activities can be analyzed in the three species of Linderniaceae by *Agrobacterium* co-cultivation method

After optimization of co-cultivation method in three species using control construct (35S::GUS), small inducible promoter fragments of the *LEA-like 11-24* from three species (promoter::*GUS*) were introduced into homologous leaves via co-cultivation. When the optimized method was employed, a similar activity was observed for the *LEA-like 11-24* promoter fragments compared

to both stable transformation (Velasco et al. 1998) and particle bombardment (van den Dries et al. 2011) in response to ABA and mannitol. The host defense response to *Agrobacterium* co-cultivation has been suspected to have an influence on activity of promoters (Pruss et al. 2008; Rico et al. 2010). But the expression pattern of *Cp LEA-like 11-24* in response to mannitol and ABA in homologous leaves is comparable with that of particle bombardment (van den Dries et al. 2011). Higher GUS expression of *Cp LEA-like 11-24* promoter in response to mannitol than ABA in *C. plantagineum* leaves (Fig. 3-6) is in agreement with particle bombardment observations (van den Dries et al. 2011). High promoter activity of *Cp LEA-like 11-24* in co-cultivation confirmed the fundamental role of the DRE motif (dehydration responsive element) (Fig. 3-5) in *Cp LEA-like 11-24* promoter fragment in stress responsive conditions (van den Dries et al. 2011). The DRE motif, which is presents only in the *Cp LEA-like 11-24* promoter fragment (van den Dries et al. 2011), mediates the stronger expression of the *C. plantagineum* promoter than *Lb* and *Ls LEA-like 11-24* promoter fragments.

While both transient transformation methods (co-cultivation and particle bombardment) have their own benefit, remarkable advantages of co-cultivation can overcome that of particle bombardment method (Ko and Korban 2004; Lopez et al. 2004; Takata and Eriksson 2012; Xu et al. 2014). These advantages are including large scale transformation of plant tissue, high transformation efficiency, low cost procedure, capability in quantitative measurement of promoter activity and possibly transcript expression analyses. These results demonstrate that the optimized protocol is an efficient and suitable method for analysing the promoter functions in transiently transformed leaves of *C. plantagineum*, *L. brevidens* and *L. subracemosa*.

4.1.3 Trans-regulatory factors responsible for *Cp LEA-like 11-24* promoter are present in drought tolerant and sensitive species

The comparison of *Cp LEA-like 11-24* promoter activity in two other heterologous backgrounds such as *L. brevidens and L. subracemosa* revealed the induction of *Cp LEA-like 11-24* promoter fragment in two other species. Similarly, the *Lb* and *Ls LEA-like 11-24* promoter fragments showed activity in *C. plantagineum* and two *Lindernia* species, suggesting the presence of trans-regulatory factors essential for the induction of *LEA–like 11-24* promoter fragments in two

desiccation tolerant and a desiccation sensitive species. The *Cp LEA-like 11-24* promoter which contains two conserved ABA-responsive elements (ABRE *cis*-elements), similar to that of *L. brevidens* and *L. subracemosa,* and additional DRE motif revealed high promoter activity in homologous species and two heterologous background, confirming the fundamental role of promoter type and architecture in the expression level of the *Cp LEA-like 11-24* promoter fragment (van den Dries et al. 2011). Functional promoter analyses of *LEA-like 11-24* from three related species of Linderniaceae via particle bombardment revealed that the level of GUS expression is related to the promoter type, number, position and distribution of functional *cis*-acting elements (Braun 2011, van den Dries et al. 2011). However, we cannot exclude the *trans*-environment effect, specific for each species in the activity of promoters (Fig. 3-8). For example the *Cp LEA-like 11-24* promoter showed highest activity in homologous background, while the expression was slightly decreased in *L. brevidens* and *L. subracemosa* background. Protein sequence similarity of the *LEA-like 11-24* between *C. plantagineum* and *L. brevidens* is 46% while, it is 51% between *C. plantagineum* and *L. subracemosa* proteins (van den Dries 2010). These differences may relate to species-specific proteins as *C. plantagineum* belongs to *Craterostigma* genus, whereas *L. brevidens and L. subracemosa* belongs to *Lindernia* genus (Rahmanzadeh et al. 2005).

However, little is known about the *trans*-acting factors participating in transcriptional regulation of the *LEA-like 11-24* promoters. Recently, Braun (2011) showed that when a DRE motif from the *Cp LEA-like 11-24* promoter fragment was inserted in the same position in *Lb* and *Ls LEA-like 11-24* promoter fragments promoter activities were significantly enhanced in response to ABA and osmotic stress. Similarly, when the DRE motif was removed from the Cp LEA-like 11-24 promoter, the GUS activity was comparable with that of L. brevidens and L. subracemosa. This demonstrates trans-regulatory elements which is binding to the DRE element is present in three species and promoter sequence and architecture is more important in the activity of promoters. Altogether these findings demonstrate the presence of essential trans-regulatory factors responsible for *Cp LEA-like 11-24* promoter in three species and

4.2 GUS activity was not increased in trans-activation of *Cp LEA-like 11-24* promoter by CpbZIP1 protein

The interaction of *CpbZIP1* transcription factor with *Cp LEA-like 11-24* promoter fragment has been reported in *C. plantagineum* using yeast-one hybrid assay (van den Dries et al. 2011). It is assumed that CpbZIP1 binds to the *Cp LEA-like 11-24* promoter in *C. plantagineum* during desiccation. Trans-activation was performed to evaluate the effect of over-expression of the CpbZIP1 transcription factor in activating the *Cp LEA-like 11-24* promoter. GUS activity was analyzed in stably transformed *Arabidopsis* plants with a short (307 bp) or a long promoter fragments (1.5 kbp) of *Cp LEA-like 11-24*. GUS expression did not increase in response to ABA in transgenic *Arabidopsis* lines containing ''*35S::CpbZIP1+Cp LEA-like 11-24 GUS*'' compared to ''*Cp LEA-like 11-24 GUS''* in leaves. This is in agreement with the results of trans-activation assay via *Agrobacterium*-mediated transient transformation in *C. plantagineum*, *L. brevidens* and *L. subracemosa* (in this research Fig. 3-13; 3-14). In previous studies, the CaMV *35S::CpbZIP1* construct was co-bombarded with *Cp LEA-like 11-24*::*GUS* and CaMV 35S::*GFP* vectors into *C. plantagineum* leaves via particle bombardment (van den Dries 2010). Even though *trans*-activation experiments were performed with different concentration ratios, the level of *Cp LEA-like 11-24* promoter activity co-expressed with *CpbZIP1* was not enhanced in comparison with that of the *Cp LEA-like 11-24* promoter alone in response to ABA or mannitol (van den Dries 2010). Similar results were obtained in trans-activation of another stress inducible promoter the *CpC2* by CpbZIP1 using particle bombardment in *C. plantagineum* (Ditzer et al. 2006). The interaction of CpbZIP1 protein with the *CpC2* promoter has also been reported. These authors proposed that 1) transient expression of the *CpbZIP1* was not sufficient to enhance the activity of the *Cp LEA-like 11-24* promoter fragment, 2) the *CpbZIP1* required post translational modification to be active and likely activation of CpbZIP1 protein depends on other proteins to induce the promoter. Though, based on our observations, other reasons might explain the low GUS activity in the concerned transgenic lines. It must be noticed that, the transgenic *Arabidopsis* plants overexpressing *CpbZIP1* (35S::CpbZIP1) have shown similar phenotype with transgenic plants containing the ''*35S::CpbZIP1+Cp LEA-like 11-24::GUS*'' cassette, in addition both direction of *CpbZIP1* in the cassette showed similar results in

Agrobacterium-mediated transient transformation assay. Therefore, we should exclude the effect of construct structure in obtained low GUS activity in transformed plants.

1) Contribution of heterologous background for *CpbZIP1* mediates lower promoter activity:

The first reason could be that the partner proteins are not available in *A. thaliana* as heterologous background for CpbZIP1 protein to form dimmers. The CpbZIP1 protein sequence shows homology with the S1-class of bZIP proteins from *Arabidopsis* (Ditzer et al. 2006; van den Dries 2009). Members of this class of bZIP proteins preferentially heterodimerize with members of the C-class in tobacco (Strathmann et al. 2001), parsely (Rügner et al. 2001) and *Arabidopsis* (Ehlert et al. 2006, Hanson et al. 2008; Dietrich et al. 2011) in response to different abiotic stresses. But the homodimerization capacity is very weak, for instance, homodimerization of AtbZIP63 (C-class) or AtbZIP1 (S1-class) in a yeast-two-hybrid assay produces GUS activity of 1.0 and 0.38 units respectively compared to 22.1 units in heterodimerization of these two bZIPs (Ehlert et al. 2006). In *C. plantagineum* the trans-activation experiment in homologous leaves of *Craterostigma* also was performed via *Agrobacterium*-mediated transient transformation, and the results were identical with these in stable transgenic lines. Therefore, the formation of CpbZIP1 dimers in homologous or heterologous background may be less relevant to explain our observations. The CpbZIP1 protein in homologous background also was not able to increase GUS activity.

2) Repressor functions of CpbZIP1 protein:

As bZIP transcription factors in the S1-class do not contain any activation domain, they might exert their function through either heterodimerization with other bZIPs or constitutive binding to DNA, thus preventing other transcription factors to bind to the *cis*-elements in the target gene promoters (Jakoby et al. 2002). The bZIP proteins regulate the expression of the target genes through dimerization specificity. The dimerization specificity of bZIP proteins in *Arabidopsis* has been predicted (Deppmann et al. 2004). Heterodimerization of AtbZIP53 (S1-class) and AtbZIP10 (C-class) may mediate activation of transcription independently from the DNA-binding properties. This demonstrates a specific mechanism in regulation, activation and function of transcription factors (Weltmeier et al. 2006). Specific heterodimerization of bZIP53 was shown to change the function and activity in *Arabidopsis* (Weltmeier et al. 2006; Ehlert et

al. 2006). For example AtbZIP53 and all S1-class bZIP proteins are able to bind the ACTCAT motif in the proline dehydrogenase gene (PrODH) promoter in combination with C-class bZIP and differentially regulate the gene (Weltmeier et al. 2006). Therefore, heterodimerization of transcription factors allow numerous combinations of protein complexes which can have different and sometimes opposite function. (Naar et al. 2001). Over-expression of AtbZIP11 down regulates a few transcription factors such as AtbZIP10, AtbZIP25 and AtbZIP1 (Hanssen 2009). Analysis of the downregulated genes demonstrated that the mechanism of repression differs from that of activation. It has been reported that AtbZIP11 represses the genes via indirect binding to the conserved motif in the promoter region (Hanssen 2009). But most transcription factors can repress the genes by binding a conserved site with specific heterodimer formation (Gaston and Jayaraman 2003).

3) Inhibition of DNA-protein interaction by non-equimolar ratio of protein:

Negative DNA-protein interaction might be due to non-equimolar ratio of the protein (Kang et al. 2010). Inhibition of *LEA-like 11-24* promoter activity by constitutively expressed CpbZIP1 protein could be due to heterodimer formation by non-equimolar ratio of partner proteins. For instance, heterodimer formation of AtbZIP63 (C-class) and AtbZIP1 (S1-class) have a negative effect on binding to the ACGT motif in a concentration dependent manner, thus reducing the DNA-protein dimerization (Kang et al. 2010).

In *Arabidopsis* different heterodimer transcription factors regulate a single gene in different ways. For example, ProDH can be regulated with different heterodimer complexex of all bZIPs in S1-class (AtbZIP1, AtbZIP2, AtbZIP11, AtbZIP44, AtbZIP53) (Satoh et al. 2004; Weltmeier et al., 2006). AtbZIP11 has been demonstrated to form dimers with other proteins based on the availability of proteins and activates the ProDH gene in ACTCAT motif in response to hypoosmolarity, starvation and sucrose level differentially. However, homodimer formation poorly activates the ProDH (Weltmeier et al. 2006).

4) Absence of uORF in the 5' region of *CpbZIP1:*

The *CpbZIP1* belongs to S1-class bZIPs (Ditzer et al. 2006), which is characterized by a conserved upstream open reading frame (uORF) (Martinez-Garcia et al. 1998; Jakoby et al. 2002; Strathmann et al. 2001; Wiese et al. 2004; Dietrich et al. 2011). The protein deduced from

this region is known to regulate post-translational modification of proteins (Morris and Geballe, 2000). It has been shown to have SIRT activity (sucrose-induced repression of translation) which represses the bZIP protein translation in high sugar conditions (Morris and Geballe, 2000; Wiese et al. 2004; Dietrich et al. 2011). SIRT mechanism as post transcriptional repression has been reported in all members of S1-class (Weltmeier et al. 2009) which controls the sugar level in the cells. The *AtbZIP11* controls nitrogen and carbon level through SIRT mechanism (Gutierrez et al., 2007).

In a previous study, the *CpbZIP1* gene was isolated from *C. plantagineum* (Ditzer et al. 2006). The binding of this transcription factor to the small stress inducible promoter fragment, the *CpC2*, was confirmed in yeast-one-hybrid assay (Ditzer et al. 2006; van den Dries 2010). But the effort to isolate the upstream region of the *CpbZIP1* coding sequence failed. Therefore, it is not clear whether this *CpbZIP1* gene also contains a long 5'-leader sequence containing small upstream open reading frames (uORF) (Ditzer et al. 2006). On the other hand, the second isolated gene (*CpbZIP2*) which shows 94% identity with the CpbZIP1 protein contains uORF sequence. If the *CpbZIP1* coding sequence actually contains the uORF in *C. plantagineum,* the transgenic *Arabidopsis* lines generated in this work thus lack the uORF in the coding sequence of *CpbZIP1* gene. Therefore, the function is affected. Most likely, during photosynthesis when sugar levels go up in the cells; all S1-class bZIPs of *Arabidopsis* are repressed through SIRT mechanism. Only CpbZIP1 proteins will likely form homodimer or heterodimerize with the bZIP proteins from other groups. No increase of the promoter activity will observed if such homodimers or heterodimers bind to the promoter of *Cp LEA-like 11-24-GUS*.

4.3 *CpbZIP1* transcript expression under abiotic stress conditions

To understand the biological function of CpbZIP1 transcription factor, the expression of *CpbZIP1* gene was evaluated in *C. plantagineum* leaves and roots in response to ABA, dehydration and salt treatments (sodium chloride) during different time points or different concentrations. Expression analyses of the gene encoding *CpbZIP1* in *C. plantagineum* demonstrated that the gene is slightly induced in response to dehydration and ABA in leaves, while constitutive expression is obtained in roots (Fig. 3-24). In contrast, expression of *CpbZIP1*

in response to salt treatment increased in the roots, whereas it remained unchanged in leaves (Fig. 3-26). In previous studies, only three stages of *C. plantagineum* plant (untreated, dehydrated and rehydrated leaves and roots) were analysed without considering different time points (Ditzer et al. 2006; van den Dries 2010). Consequently, constitutive expression of *CpbZIP1* under stressed (drought, ABA) and non-stressed conditions was observed, which may not determine the expression of the *CpbZIP1* gene accurately. The results obtained in this study using different time points or concentrations for all stress treatments led to accurate estimation of *CpbZIP1* expression in response to abiotic stresses and demonstrated stress induciblity of the *CpbZIP1* gene.

4.3.1 *CpbZIP1* gene from *C. plantagineum* is slightly induced under drought and ABA treatments in leaves

RT-PCR analyses demonstrated slightly induction of *CpbZIP1* gene by stress treatments, drought and ABA, in leaves. Similarly, the moderate transcriptional responses of S1-class AtbZIPs from *Arabidopsis* in stress conditions have also been reported (Kaminaka et al. 2006; Weltmeier et al. 2009). In *A. thaliana AtbZIP1* has been shown to be slightly induced in response to ABA (Zimmermann et al. 2004) .Induction in *CpbZIP1* gene under stress conditions shows the stress responsibility of transgene.

4.3.2 The *CpbZIP1* gene from *C. plantagineum* is induced by salt stress in roots

The expression analyses of the *CpbZIP1* gene revealed accumulation of its transcript in response to different concentrations of salt in roots, while transcript levels remained unchanged in leaves. Weltmeier et al. (2009) using *in silico* analyses showed the expression profile of all members of S1-class of *AtbZIPs* in *Arabidopsis* under different abiotic stresses. Among them only *AtbZIP1* and *AtbZIP53* transcripts were inducible by salt treatment in roots. In contrast, expression of *AtbZIP1* was repressed by salt in leaves, whereas that of *AtbZIP53* remained unchanged. *AtbZIP11* transcript was strongly induced only in leaves under salt stress. Therefore, transcript expression of *CpbZIP1* in response to salt treatments is in agreement with the expression of

AtbZIP53 in leaves and roots. *AtbZIP53* is the only gene in the S-class of *Arabidopsis*, which the expression is abundantly induced during seed development (Alonso et al. 2009).

4.3.3 Distinct expression pattern of *CpbZIP1* in response to different abiotic stresses

The observation that the expression of *CpbZIP1* is induced only in roots after salt treatments but not under dehydration or ABA treatments indicates the differential regulation of *CpbZIP1* gene in response to dehydration/ABA and salt treatments in roots. In addition, the expression of *CpbZIP1* in leaves slightly induced under dehydration and ABA. Complex regulation of *AtbZIP* transcription factors in the leaves and roots is reported by Kilian et al. (2007) using a transcriptome data set. The authors showed that exposure to stress treatments usually leads to the induction of *AtbZIPs* only in one organ of the plants. It has been shown that five members of S1-class *AtbZIPs* (*AtbZIP1, AtbZIP2, AtbZIP11, AtbZIP44* and *AtbZIP53*) are differentially expressed in the leaves and roots of *Arabidopsis* in response to different stresses (Zimmermann et al. 2004; Alonso et al. 2009; Weltmeier et al. 2009; Kang et al. 2010; Dietrich et al. 2011; Ma et al. 2011). For instance, expression of *AtbZIP53* can be observed in mid and late maturation stage of seeds and under the particular stress conditions such as salt treatment in roots (Weltmeier et al. 2009). In contrast, *AtbZIP1* is induced by cold and salt stresses in roots, while the transcript level decreased in leaves under the same treatments (Weltmeier et al. 2009). Therefore, these observations suggest a specific and complex expression of *CpbZIP1* gene in leaves and roots under different abiotic stresses.

4.4 Molecular and functional analyses of the CpbZIP1 protein in transgenic plants overexpressing *CpbZIP1*

Transcription factor analysis involves studying of the gene expression, function and regulation (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki 2003; Sakuma et al. 2006). One way to identify the biological function of the genes is by generating ‘’gain of function’’ or ‘’loss of function’’ mutant plants. This can be done by analysing knock-out plants or transgenic lines

that overexpress the gene of interest. Most of the resurrection plants are polyploid and have large genomes, which makes it difficult to knock out a particular gene (Rodriguez et al. 2010; Dinakar et al. 2013). *C. plantagineum* is a polyploid species for which generation of loss of function mutant was not possible by using currently available methods (Furini et al 1994; Dinakar et al. 2012). Therefore, *Arabidopsis* transgenic lines overexpressing *CpbZIP1* coding sequence were generated to study selective functions of CpbZIP1 protein in *Arabidopsis*.

4.4.1 Dwarf phenotype in transgenic plants

Arabidopsis transgenic plants overexpressing *CpbZIP1* (S-lines) from *C. plantagineum* were analyzed based on morphological characteristics. Dwarf phenotypes with abnormal flower, small siliques and delayed bolting were observed in transgenic lines overexpressing *CpbZIP1* (Fig. 3-31). Strong dwarf phenotypes showed higher expression of the *CpbZIP1* gene, whereas lower expression of the *CpbZIP1* was correlated with a slight alteration in the phenotype. Overexpression of bZIPs from *Arabidopsis* such as *AtbZIP11* and *AtbZIP2* also generated dwarf phenotypes (Dietrich et al. 2011). Moreover, Alonso et al. (2009) showed that over-expression of *AtbZIP53* in *Arabidopsis* resulted plants slowly growth phenotype and dwarfism. As *AtbZIP53* is highly expressed during seed development, dwarf phenotype*s* in transgenic plants overexpressing *AtbZIP53* were correlated with missexpression of maturation genes (*MAT*) in vegetative tissues. These authors showed that transcripts of maturation genes such as albumin (*2S2*), cruciferin (*CRU3*) were accumulated.

Similarly, *CabZIP1* from pepper (*Capsicum annuum*) which is also stress responsive transcription factor leads to the alteration in phenotype when overexpressed in *A. thaliana*. Altered phenotype is probably due to defects in the ability to transmit hormones signaling and is presumed to play a negative regulatory role in development of hormone signaling (Karlowski et al. 2003; Kuhlmann et al. 2003).

Observation of dwarf phenotypes in overexpressing *CpbZIP1* plant could be due to one of those reasons mentioned above. Further analyses are needed to confirm the function of the CpbZIP1 protein in growth and development.

4.4.2 Moderate stress tolerance of transgenic plants

Stress responsiveness of transgenic lines overexpressing *CpbZIP1* (S-lines) was investigated by exposure of the seedlings to different concentrations of salt (Fig. 3-33, 34). Stress tolerance of these plants is explained via higher accumulation of proline (Kreps et al. 2002) and reduction of malondialdehyde (MDA) as a marker of free-radical-catalyzed lipid peroxidation (Weber et al. 2004). According to our results, accumulation of proline in untreated transgenic seedlings is higher than in the wild-type plants (Fig. 3-34). The proline content was increased and MDA level decreased in the seedlings grown on 150 mM salt. However, proline content and MDA level were not significantly different from wild-type plants in the seedling grown on 150 mM salt suggesting moderate salt stress tolerances of transgenic lines.

In addition, stress responsiveness of of transgenic plants in dehydration condition showed moderately, better performace of transgenic plants. However, relative water content of soil and % water loos in leaves of wild type and transgenic plants demonstrated that wild-type plants which have more biomass than transgenic plants loos water faster than transgenic plants.

Looking at the picture (Fig. 3-35a), although both have similar water loos, transgenic plants seems to sustain dehydration stress. This could be due to remobilizing nutrient from the old leaves.

Altogether, dehydration tolerance of transgenic plants can not be confirmed based on different size and consequently different physiological stages.

4.4.3 The involvement of CpbZIP1 protein in energy homeostasis/starvation

Time dependent expression of the *CpbZIP1* gene demonstrated constitutively expression of gene during light. However, the induction of *CpbZIP1* gene was observed during the dark period (Fig. 3-28). This induction was increased under prolonged darkness (72 hours). Consistent with the results that obtained in this study, transcripts of the *AtbZIP1* and *AtbZIP53* from *Arabidopsis* are up-regulated in dark condition (Kang et al. 2010; Dietrich et al. 2011). Diurnal changes in life cycle mediate environmental fluctuations, which bring temporary energy deprivation (Baena-González and Sheen 2008). Plant metabolism has to be adjusted to these fluctuations (Weltmeier et al. 2009). For instance, to cope with the diurnal changes in carbon availability, plants retain some photosynthates such as starch or other storage carbohydrates that can be remobilized

during the night to compensate the low energy conditions in the cells (Usadel et al. 2008). These resources are depleted within 2 to 4 hours of extended night, consequently severe carbohydrate starvation occur, which is not a favour for plant. To cope with the carbohydrate depletion during night, expression of many genes related to remobilization of carbon/nitrogen is induced; thereby amino acids as source of nitrogen and carbon are recycled to compensate the starvation condition (Usadel et al. 2008; Dietrich et al. 2011). In the cell asparagine is used to store and transport nitrogen in stress condition (Lam et al. 1994). Several members of S1-class of *AtbZIP* from *Arabidopsis* have been reported to be involved in starvation response and amino acid metabolism (Gutiérrez et al. 2008; Dietrich et al. 2011). *AtbZIP1* and *AtbZIP53* has been reported to be involved in dark-induced starvation (Dietrich et al. 2011). The authors showed that dark-induced expression of *CpbZIP1* depends on the sugar depletion and not the light absence.

Based on the high sequence homology between *AtbZIP1* and *AtbZIP53* from *Arabidopsis* (Dietrich et al. 2011) and *CpbZIP1* from *C. plantagineum*, similarly the *CpbZIP1* may function in energy homeostasis in *C. plantagineum*. Controlling energy homeostasis is a critical task for plant surviving in response to biotic and abiotic stresses (Dietrich et al. 2011). Similar starvation conditions may occur at the beginning of dehydration period due to shut down or decrease of metabolic pathways which is related to photosynthesis. Consequently, sugar depletion and finally starvation occur in the plants. Therefore, to cope with low energy condition due to decrease of photosynthesis activity in the cell, CpbZIP1 transcriptionally and posttranslationally being active to bind the target genes for remobilizing the amino acids. Since CpbZIP1 expresses in control condition, the transcript is available in nucleus and mediates faster reponse to minor altereation in sugar level.

Similar function has been reported for dark-induced transcription factors, *AtbZIP1* and *AtbZIP53*. Homologs of *Arabidopsis* S1 *AtbZIPs* are present in other plant species which are transcriptionally induced under abiotic stresses such as drought, cold, salt and wounding (Kusano et al. 1995; Ito et al. 1999; Stankovic et al. 2000; Shimizu et al. 2005). *AtbZIP1* and *AtbTIP53* has been found to be as a crucial transcription factors in low energy conditions that is transcriptionally and posttranscriptionally regulated (Dietrich et al. 2011).

Further support for the hypothesis of involvement of CpbZIP1 in homeostasis/starvation comes from overexpressing *CpbZIP1* lines. When transgenic *Arabidopsis* plants overexpressing *CpbZIP1* were cultivated under dark regime for four days, high expression of *ProDH1* was found in both untreated and dark-treated transgenic plants (Fig. 3-37c; 3-39c) in compared to wild type. In addition, lower proline accumulation was observed in transgenic plants which were cultivated in dark regime for four days compared to wild type (Fig. 3-37a), confirming degradation of proline by high expression of ProDH enzyme. ProDH is known as the first enzyme in the proline catabolism pathway. This enzyme upregulates in hypoomolarity (when the sugar is depleted) and high proline accumulated conditions (Kiyosue et al. 1996; Satoh et al. 2004; Weltmeier et al. 2006). It catabolises the conversion of proline to P5C (pyrroline-5-carboxylate) in mitochondria, consequently proline is degraded and glutamate is produced (Verbruggen and Hermans 2008; Verslues and Sharma 2010).

These observations are in agreement with *AtbZIP1* and *AtbZIP53* function in darkness. Transgenic lines overexpressing *AtbZIP1* and *AtbZIP53* revealed that these proteins are crucial transcription factor involved in regulating proline level (Dietrich et al. 2011). In addition, direct binding of these proteins to the promoter of proline dehydrogenase (ProDH) has been shown (Weltmeier et al. 2009; Dietrich et al. 2011). A 6-bp sequence, ACTCAT, in the promoter region of the ProDH gene is known as *cis*-element. This sequence is similar to the ATGA(C/G)TCAT motif that is recognized by bZIP proteins. The authors proposed that AtbZIP53 and AtbZIP1 proteins regulate the control of energy homeostasis in the cell through a carbon/nitrogen accessibility network and clock day/night rhythms.

4.4.4 Seedlings overexpressing *CpbZIP1* utilize remobilized nitrogen to continue the growth under hypoosmotic conditions

Growth rate and chlorophyll content of transgenic and wild-type seedlings were compared on MS-medium supplemented with 15 mM sucrose (0.015 M) with or without nitrogen source (proline) (Fig. 3-39d,f and g). The results demonstrated that transgenic lines overexpressing *CpbZIP1* retained more chlorophyll (Fig. 3-39d,g) in the medium without nitrogen source.

In *Arabidopsis*, upregulation of the *ProDH* gene in proline accumulation or hypoosmolarity-response has been reported (Kiyosue et al. 1996; Nakashima et al. 1998). Hypoosmotic conditions can be obtained by the cultivation of *Arabidopsis* seedlings in MS-medium supplemented with sugar (such as mannitol or sucrose) less than 0.1 M (Satoh et al. 2004) whereas, 0.9 M sugar is used for control conditions.

The observations in this study suggest that during hypoosmolarity condition, where carbon/nitrogen sources are limited, transgenic seedlings overexpressing *CpbZIP1* may recycle amino acids to support carbon and nitrogen demands. This hypothesis id in agreement with the hypothesis of Albert et al. (2012) and Dietrich et al. (2011). Also, upregulation of the gene encoding *ProDH* in transgenic plants overexpressing *CpbZIP1* (fig. 3-39c) supports this hypothesis. The *ProDH* which is involved in amino acid catabolism (Verslues and Sharma 2010), has been reported as direct *in vivo* target of the AtbZIP53 transcription factor (Weltmeier et al. 2006).

Altogether, these results reveal that *CpbZIP1* involvement in remobilizing the amino acids in hypoosmolarity condition. As explained above, similar hypoosmolarity may occur in dehydration condition and mediate the activation of CpbZIP1 in binding to the target genes such as ProDH and asparagine synthetase to remobilize the nitrogen from prolin and produce aspagine respectively.

4.4.5 More proline accumulated in overexpressing *CpbZIP1* lines in control condition

Higher accumulation of proline was observed in the leaves, florets and siliques of transgenic plants overexpressing *CpbZIP1* than in the same organs of wild-type plants (Fig. 3-40). Although, plant age, leaf age and position of leaves influence the proline content (Chiang and Dandekar 1995; Sharma et al. 2011), proline level that was measured in transgenic lines in all experiments under non-stressed conditions was higher than in wild-type plants. In *Arabidopsis* high proline content in seeds and flowers, especially in pollen grains under non-stressed conditions is already known (Verbruggen 1993; Verbruggen and Hermans 2008). High accumulation of proline has been reported during flowering stage due to the requirement of

carbon and nitrogen (Verbruggen 1993; Verslues and Sharma 2010). Transgenic lines overexpressing *AtbZIP53* in *Arabidopsis* has been shown to accumulate less proline in leaves due to upregulation of the *ProDH* gene, which is responsible for degradation of proline in mitochondria (Dietrich et al. 2011). In contrast, transgenic lines overexpressing *CpbZIP1* accumulated more proline in leaves compared to wild type. However, upregulation of *ProDH* in these transgenic lines also was observed. Upregulation and downregulation of *ProDH* was shown to be independent from ABA or proline accumulation (Sharma and Verslues 2012).
In addition, these lines on the medium without nitrogen source initiated flowering and started bolting earlier than wild-type plants (Fig. 3-39g). The positive correlation of high accumulation of proline and early flowering has been reported in *Arabidopsis* plants overexpressing pyrroline-5-carboxylate synthetase (*P5CS*) (Kavi Kishor et al. 1995; Mattioli et al. 2007). Therefore, the results obtained for CpbZIP1 suggests that this protein may functionally related to some transcription factor regulators that enhance proline accumulation in leaves and reproductive organs of *Arabidopsis*.

Apart from the contribution of *CpbZIP1* in abiotic stress, The *CpbZIP1* is found to be involved in another pathway that is discussed below:

4.4.6 Kinetic expression analyses of *CpbZIP1* and *Cp LEA-like11-24*

4.4.6.1 Expression of *Cp LEA-like11-24* and *CpbZIP1* gene depends on the time of the day

Diurnal fluctuation was observed in expression of *Cp LEA-like11-24* and *CpbZIP1* from *C. plantagineum* under control conditions. This rhythmic expression of gene is correlated with the circadian clock (McClung 2006). The circadian clock is an endogenous timer that plays a crucial role in biological activities through diurnal variation for adaptation of organisms (Hanano et al. 2008; Marcolino-Gomes et al. 2014). The correlation between plant responses to abiotic stresses (such as drought, cold and heat) and circadian clock has been reported in many studies (Bieniawska et al. 2008; Legnaioli et al. 2009; Wilkins et al. 2010). For instance, Dsp22 is a chloroplastic desiccation inducible protein from *C. plantagineum* that shows expression fluctuation under dark treatment (Alamillo and Bartels 1996). Dsp22 is a homologue of the gene encoding early light-inducible protein (*ELIP*) which is regulated by the circadian clock (Grimm

et al. 1989). The involvement of the circadian clock in regulation of *Dsp22* has been porposed by Alamillo and Bartels (1996).

Another clock-regulated gene is the *AtbZIP1* from *Arabidopsis*. The involvement of the *CCA1* (circadian clock-associated 1) gene in regulation of the *AtbZIP1* has been proposed (Yakir et al. 2007; Dietrich et al. 2011). It has been shown that the CCA1 protein binds to the promoter of the *AtbZIP1* gene (Gutierrez et al. 2008). However the exact molecular base of this interaction is not known yet. Similarly, Hanano (2008) using *in silico* analyses showed that members of three transcription factor families, MYB, bHLH, bZIP show a circadian clock waveform of transcript abundance.

According the obtained results in this study, oscillation in accumulation of *Cp LEA-like 11-24* and *CpbZIP1* transcripts during day and night suggests that the circadian clock may also regulate these genes. The circadian rhythm of *CpbZIP1* and *Cp LEA-like 11-24* genes might be proved by kinetic expression analyses of the circadian related genes. According to the central clock of *Arabidopsis thaliana,* the homologue of *CCA1* (circadian clock-associated *1)*, *LHY* (late elongated hypocotyls) and *TOC1* (timing of expression 1) genes (Li et al. 2011) in *C. plantagineum* could be examined for gene expression analyses.

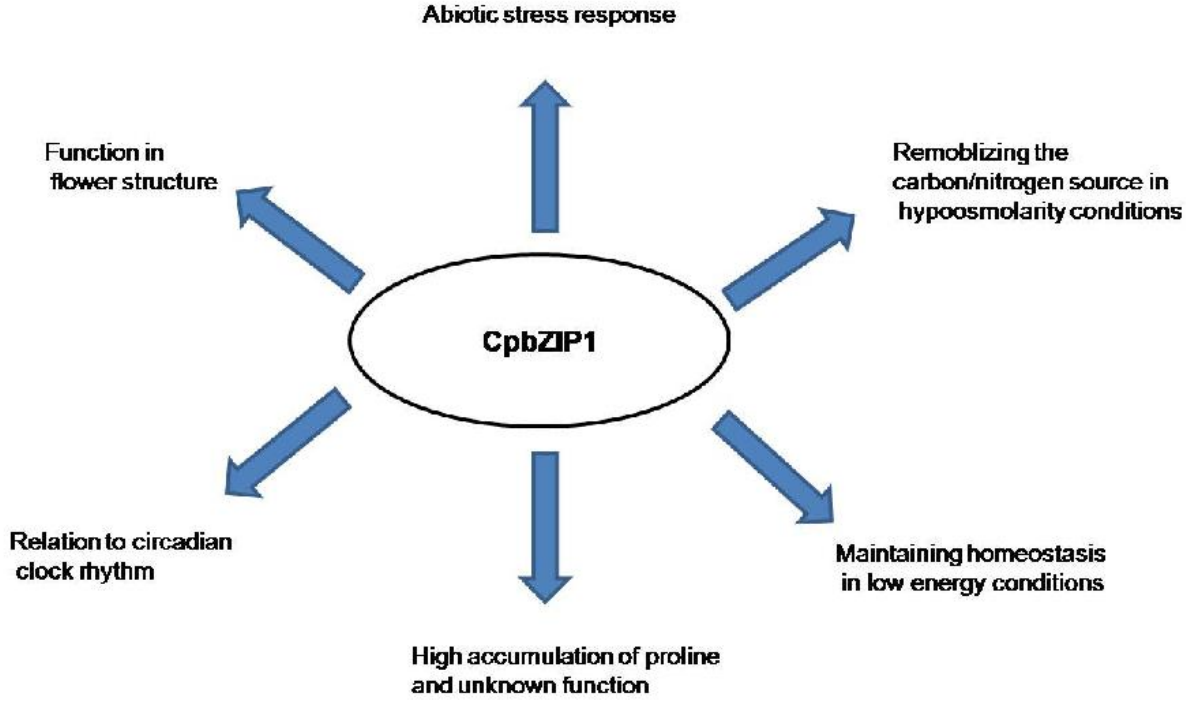


Fig. 4-1: Putative functions of CpbZIP1 transcription factor from *C. plantagineum*.

Conclusions

In this study, an efficient transient transformation method was standardized to study promoter functions in three species, *C. plantagineum*, *L. brevidens* and *L. subracemosa*. The ABA and mannitol-induction pattern of the *LEA-like 11-24* promoter fragments from three species were in agreement with that of particle bombardment. Using the optimized method, quantitative measurement of promoter activities will be possible in these three species. Cross comparison of *LEA-like 11-24* promoters between three species revealed that the trans-regulatory factors responsible for *LEA-like 11-24* promoter fragments are present in drought tolerant and sensitive species.

Trans-activation activity did not show an increase in the activity of the *Cp LEA-like 11-24* promoter by the CpbZIP1 transcription factor. Many reasons could be responsible for this, such as repressor function of CpbZIP1 protein in *Cp LEA-like 11-24* promoter or likely, absence of an uORF in the upstream region of the CpbZIP1 coding sequence. This region functions in the repression of bZIP proteins of S1-class in *Arabidopsis* in response to sucrose level. The results obtained in this study shows that CpbZIP1 is inducible by abiotic stress in a very distinct manner.

Owing to the fact that current methods do not allow to generate stable transgenic *C. plantagineum* plants, the functional characterization of *C. plantagineum* genes can currently be done in a heterologous system. Gain of function mutants was thus generated in this study in *Arabidopsis* to study the function of CpbZIP1 transcription factor. The function of the CpbZIP1 is completely unknown in *C. plantagineum* so far. This protein has been shown to bind to the stress inducible *Cp LEA-like 11-24* promoter (van den Dries 2010). The gene encoding the *Cp LEA-like 11-24* protein is known to be induced during desiccation in *C. plantagineum*. Therefore, understanding the function of CpbZIP1 protein can help to follow the signaling pathways of desiccation tolerance in resurrection plants. The results obtained in this study, demonstrated the contribution of CpbZIP1 in remobilization of carbon/nitrogen or maintaining their homeostasis under low energy conditions as obtained for the bZIP homologue in *Arabidopsis* (AtbZIP53). More research needs to be done inorder to find its partner proteins in *C. plantagineum*.

It is conceivable that during desiccation, photosynthesis decreases followed by the reduction of photoassimilates in the plant. Carbon and nitrogen must be remobilized and used for the synthesis of particular sugars (serving as compatible osmolytes) during desiccation as demonstrated in C. *plantagineum*. The transcription factors involved in starvation response play an important role in this process and *CpbZIP1* appears to be one of these factors in *C. plantagineum*.

Apart from stress responsiveness of *CpbZIP1*, the involvement of this protein in circadian clock regulation was also proposed. However, further studies are required to prove this hypothesis. Overexpression of *CpbZIP1* in *Arabidopsis* opens new doors of research for the bZIP protein function from *C. plantagineum*. The multiple function of the *CpbZIP1* in proline level, flower structure, circadian rhythms should be studied extensively. The Fig. (4-1) shows putative function of CpbZIP1 transcription factor from *C. plantagineum*.

5. Summary

The resurrection plant *Craterostigma plantagineum*, a member of Linderniaceae family, is distributed in areas with variable water availability in Southern Africa. Two closely related species, *L. brevidens* which is endemic to montan rain forest of Africa and *L. subracemosa* which is found in central and Southern Africa, are used for comparative analyses for unravelling the molecular mechanisms of desiccation tolerance. While *C. plantagineum* and *L. brevidens* are desiccation tolerant, *L. subracemosa* represents a desiccation sensitive species. Desiccation tolerance is a very complex trait. Many genes that are involved in desiccation tolerance and the proteins that are encoded by these genes have been characterized in *C. plantagineum*. The *Cp LEA-like 11-24* is one of these stress inducible genes in *C. plantagineum* that has been extensively studied both at the transcriptional and posttranscriptional level. Studies have been performed on the promoter regulation and transcription factor binding along with comparative promoter analyses between two desiccation tolerant and a desiccation sensitive species. Since promoter regions are the main regulatory elements for enhancing the transcriptional efficiency, developing an efficient transient transformation system was required to analyze promoter functions in response to different abiotic stresses. In this study, an efficient *Agrobacterium*-mediated transient transformation method was developed in three closely related Linderniaceae species. Different parameters that are important for enhancing the transformation efficiency were considered. These parameters include leaf size, Silwet L-77 concentration, bacterial density and duration of co-cultivation. After optimization, the activities of the minimal stress inducible promoter fragments of the *LEA-like 11-24* from three species were examined under ABA and osmotic stress using optimized *Agrobacterium* co-cultivation method.

The transformation efficiency was found to be 100% in all three species. In addition, the induction pattern of each promoter fragment under ABA and osmotic stress was comparable with that of stably transformed plants or particle bombardment, suggesting this as the most suitable method for quantitatively analyzing promoter activity in the three species. The optimized method was used for cross comparison of the *LEA-like 11-24* promoter fragments among two desiccation tolerant and a sensitive species to understand the effect of trans-regulatory factors in activating the *LEA-like 11-24* promoter fragments. The results demonstrated that both desiccation tolerant and sensitive species contain essential *trans-*

regulatory factors for the activity of *LEA-like 11-24* promoter fragments. Using a yeast-one-hybrid system, it was previously shown the *Cp LEA-like 11-24* promoter fragment interacts with CpbZIP1 protein. The *CpbZIP1* belongs to the S1-class of AtbZIP transcription factors from *Arabidopsis* and has been shown to have 47% protein sequence identity with AtbZIP53. Transcript expression analysis of *CpbZIP1* in response to ABA, dehydration and salt stress demonstrated the stress responsiveness of this gene. S1-class members of *AtbZIP* from *Arabidopsis* have complex expression patterns in different stress responses. The *CpbZIP1* from *C. plantagineum* also showed similar complex expression pattern in response to stress. The function of CpbZIP1 transcription is completely unknown in *C. plantagineum*. In a functional study of the CpbZIP1 transcription factor, transgenic *Arabidopsis* plants overexpressing *CpbZIP1* exhibited dwarf phenotype, abnormality in flower structure and small siliques suggesting that CpbZIP1 interferes with the developmental processes. Physiological and molecular data from transgenic plants overexpressing *CpbZIP1* revealed the contribution of *CpbZIP1* in remobilization of carbon/nitrogen or maintaining homeostasis of energy, as obtained for the *bZIP* homologue in *Arabidopsis* (*AtbZIP53*). Time dependent expression of *CpbZIP1* revealed that apart from the function of *CpbZIP1* in abiotic stress, most likely *CpbZIP1* is regulated by circadian clock rhythm. The results from this study suggests that *CpbZIP1* is stress inducible and involved in multiple functions like growth, flower development, accumulation of proline, remobilizing carbon/nitrogen and maintaining homeostasis of energy in low energy conditions.

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ABBREVIATIONS

4–MUG	4 –Methylumbelliferyl glucuronide
ABA	Abscisic acid
ABRE	ABA responsive element
ABF	ABRE-binding factor
Amp	Ampicillin
bp	Nucleotide base pair
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
CaMV	Cauliflower mosaic virus
CE	Coupling element
cDNA	Complementary DNA
Cp	*Craterostigma plantagineum*
CRT	C-repeat
CTAB	Cetyl trimethyl ammonium bromide
dATP	Desoxy-adenosin-triphosphate
dCTP	Desoxy-cytidin-triphosphate
dGTP	Desoxy-guanosin-triphosphate
DMF	N,N-Dimethylformamid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DRE	Dehydration responsive element
DREB	DRE-binding protein
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetate
Fw	Fresh weight
g	gram
GUS	β–glucuronidase
TW	Turgor weight
DW	Dry weight
GFP	Green Fluorescent Protein
GUS	*E. Coli* β-glucuronidase gene (*uidA*)
h	Hour
HEPES	Hydroxyethyl)-1-piperazinethansulfonic acid
Kan	Kanamycin sulfate
Kb	Kilobase
LB	Luria and Bertani medium
LEA	Late Embryogenesis Abundant
Ls	*Lindernia subracemosa*
Lb	*Lindernia brevidens*
M	Molar, mole(s) per liter
mA	Milliamperes
MCS	Multiple cloning site
MDA	Malondialdehyde
M	Meter
min	Minute

ml	Milliliter
mRNA	messenger RNA
MS	Murashige and Skoog (1962)
OD	Optical density
Oligo	Oligodeoxythymidylic acid
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
rev	Reverse
Rif	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate buffer
ssDNA	Single-stranded DNA
TA	Annealing temperature
TAE	Tris-Acetate-EDTA
Taq	*Thermophilus aquaticus*
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TE	Tris (10mM)-EDTA (1 mM)
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	Melting temperature
Tris	Tris-(hydroxymethyl)-aminomethane
Triton X-100	Poly(ethylenglycolether)n-octylphenol
TW	Turger weight
U	Unit
UV	Ultraviolet
V	Volts
v/v	Volume/volume
w/v	Weight/volume
WC	Water content
X-Gal	5-bromo-4-chloro-3-indolyl-b-Dgalactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide