Molecular analysis of the plastid-targeted gene and protein (CpPTP-1) associated with desiccation tolerance in the resurrection plant *Craterostigma plantagineum*

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To my parents, my wife, and my sweet daughter

I. ABBREVIATIONS

3-AT	3-Amino-1,2,4-triazole
ABA	Abscisic acid
ABRE	ABA responsive element
Amp	Ampicillin
BLAST	Basic local alignment search tool
bp	Nucleotide base pair
CaMV	Cauliflower mosaic virus
CBF	C-repeat binding factor
cDNA	Complementary DNA
CE	Coupling element
Cp	*Craterostigma plantagineum*
CRT	C-repeat
DNA	Deoxyribonucleic acid
DRE	Dehydration responsive element
DREB	Dehydration-responsive element binding
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium bromide
fwd	Forward
g	Gram
GFP	Green Fluorescent Protein
GUS	E. coli β-glucuronidase gene (uidA)
h	Hour
His	Histidine
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin sulfate
kb	Kilobase
kDa	Kilodalton
LB	Luria and Bertani medium
LEA	Late embryogenesis abundant
Leu	Leucine
Ls	*Lindernia subracemosa*
m	Meter

M	Molar, mole(s) per liter
min	Minute
ml	Milliliter
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog
MYC	myelocytoma
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Rev	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Synthetic defined media
SDS	Sodium dodecyl sulfate
sec	Second
TAE	Tris-acetate-EDTA
Taq	Thermophilus aquaticus
TE	Tris-EDTA
Trp	Tryptophan
TF	Transcription factor
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
WT	Wild type
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
Y2H	Yeast two hybrid
YEB	Yeast extract broth
YEPD/YPD	Yeast extract peptone dextrose
YPAD	Adenine supplemented YPD

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SUMMARY

Water scarcity causes drought stress and enormous losses in agricultural production, and consequently this leads to a worldwide food crisis. *Craterostigma plantagineum* is one of the best studied resurrection plants that is able to adapt to water scarcity and survive under severe drought stress conditions (desiccation). This plant has been widely studied to understand the molecular basis of desiccation tolerance. The study of the genes and their corresponding proteins related to desiccation tolerance may help to improve tolerance of sensitive plants, particularly crops.

In the present study, an ABA and desiccation-induced gene from *C. plantagineum* called *CpPTP-1* which belongs to a plastid-targeted protein (PTP) family was investigated. This study was carried out to have a better understanding of the role of the CpPTP-1 protein in acquisition of desiccation tolerance in *C. plantagineum*.

Promoter regions and corresponding regulatory *cis*-acting elements of the *CpPTP-1* gene were studied to understand and identify the motifs involved in the regulation of gene expression at the transcriptional level as the most important step in the activation and the suppression of gene expression. It is observed that a combination of *cis*-acting elements including coupling element 3 (CE3), MYC and dehydration-responsive element (DRE) regulate the full activity of *CpPTP-1* during abiotic stress. The MYC motif which is a binding site for NAC transcription factors showed a great effect on the regulation of expression of the *CpPTP-1* gene in *C. plantagineum*. The promoter of the *CpPTP-1* gene exhibited high activity in stably transformed *A. thaliana* plants in both stress conditions and non-stress conditions.

The CpPTP-1 unlike any other plant protein uniquely contains a bacterial receptor domain called Methyl-accepting chemotaxis-like conserved domain (MCP) which mediates chemotaxis to different environmental and intracellular signals in bacteria and archaea.

In this work, a protein-protein interaction (PPI) study was performed to understand the function of the CpPTP-1 protein during desiccation. A yeast two-hybrid screening approach revealed that the CpPTP-1 protein interacts with a chloroplastic lipocalin (CpCHL) protein in yeast, suggesting the same interaction in *C. plantagineum*. The interaction between those proteins is mediated by two coiled-coil domains of the CpPTP-1 protein. The transcript of the *CpCHL* is highly up-regulated upon desiccation. The CpCHL protein is localized in the chloroplasts of *C. plantagineum.* On the basis of the CpCHL cellular function, the findings of this study suggest

that the CpPTP-1 and CpCHL proteins form a functional complex to protect the photosynthesis apparatus in chloroplasts during desiccation by preventing lipid peroxidation of thylakoid membrane lipids of chloroplasts.

1. INTRODUCTION

1.1 Water scarcity is a major factor limiting plant production

Recently, global climate change has affected food security and reduced food availability worldwide. The climate change leaves no way to escape from a food crisis in the near future. Water crisis has become a global issue for human survival all over the world. Zhang and Bartels (2018) distinguished water stress using three definitions including drought, dehydration, and desiccation to describe the water status of plants. Drought stress is related to agriculture and occurs in a slow process while water uptake out of the soil is exceeded by transpiration. The term dehydration is used when a steady loss of water occurs in the whole plant or detached organs. Desiccation is described as extreme dehydration in plants which almost leads to the same water content as it exists in the air (Zhang and Bartels 2018).

Various environmental disasters especially drought stress due to the limited access to fresh water and consequently the accumulation of salt due to irrigation have seriously threatened agricultural production in the world. Most of the major crops face abiotic stress at a certain time of their life cycle. Drought and dehydration could inhibit normal physiological activities and damage cellular structures of plants which lead to a substantial reduction in crop production (Deng et al. 2002). Dehydration could cause different forms of damage such as denaturation, oxidative and mechanical stress to cellular components such as macromolecules (DNA and proteins), cell walls and membranes (Giarola and Bartels 2015). Drought stress could reduce carbon dioxide entry and activity of photosynthetic enzymes, and increase oxidative stress which leads to an inhibition of photosynthesis as a central response of both desiccation-tolerant and desiccation-sensitive plants (Challabathula et al. 2018).

1.2 Desiccation tolerance of resurrection plants

Bartels and Salamini (2001) described desiccation as the most severe status of water deficit which leads to a substantial loss in protoplasmic water of the plant cell. A group of flowering plants termed ‘‘resurrection plants’’ have evolved desiccation tolerance (DT) and are able to survive under extreme water stress conditions as desiccation-tolerant angiosperm plants (Bartels 2005). Most angiosperm plants die when the cellular water content falls below 60 % relative water content but resurrection plants revive from an air-dried state when water becomes

available (Giarola and Bartels 2015). The desiccation tolerance in resurrection plants is specified to seeds and vegetative tissues (Gaff 1971). Desiccation-tolerant plants apply various strategies such as expression of specific genes and corresponding proteins, accumulation of protective sugars, cell structure changes, anti-oxidative reactions and production of the plant hormone abscisic acid (ABA) to gain desiccation tolerance (Zhang and Bartels 2018). The phytohormone ABA plays an essential role in acquisition of dehydration tolerance by leading a complex regulatory network, participating in seed germination and development, closure of stomata, and signal transduction which regulates the activation of transcription factors and, consequently induction of expression of stress-related genes (Cutler et al. 2010; Kim et al. 2010; Hirt and Shinozaki 2004). Expression patterns of stress-related genes upon dehydration stress play a key role in protective mechanisms and many dehydration-induced or constitutively expressed genes are involved in acquisition of a complex trait like desiccation tolerance in resurrection plants (Giarola et al. 2017). The late embryogenesis abundant proteins (LEA) are examples of proteins which are associated with the desiccation tolerance in *C. plantagineum* and their encoding genes are inducible under desiccation (Bartels 2005). The LEA proteins exist in different organisms such as plants, fungi, bacteria and nematodes and induce during osmotic stress (Garay-Arroyo et al. 2000; Browne et al. 2002).

1.3 The Linderniaceae family contains resurrection plants as models for desiccation tolerance study

To understand the molecular basis of desiccation tolerance (DT), three members of the Linderniaceae family including *Craterostigma plantagineum* and *Lindernia brevidens* as desiccation-tolerant species, and *Lindernia subracemosa* as a desiccation-sensitive species have been widely studied (Giarola et al. 2017). Although, the desiccation-sensitive species shows DT in seeds, the desiccation-tolerant species exhibit DT in seeds and vegetative tissues, suggesting that the DT of the vegetative tissues is acquired from seeds by developing additional mechanisms for the whole plant (Illing et al. 2005; Farrant and Moore 2011). The study of tolerant and sensitive species provides valuable information about similarities and differences of specific mechanisms which are involved in acquisition of desiccation tolerance.

C. plantagineum is originated from south Africa and known as a dicotyledonous angiosperm with a highly complex octoploid genome (x=7; n=4x=28; 2n=8x=56) (Bartels et al. 1990; Giarola et al. 2017). Most of the higher plants including crops cannot survive when the relative water content is less than 60 % but *C. plantagineum* survives under even 1% of relative water

content and resumes normal growth and development within several hours after rehydration (Fig. 1) (Giarola and Bartels 2015; Bartels et al. 1990).

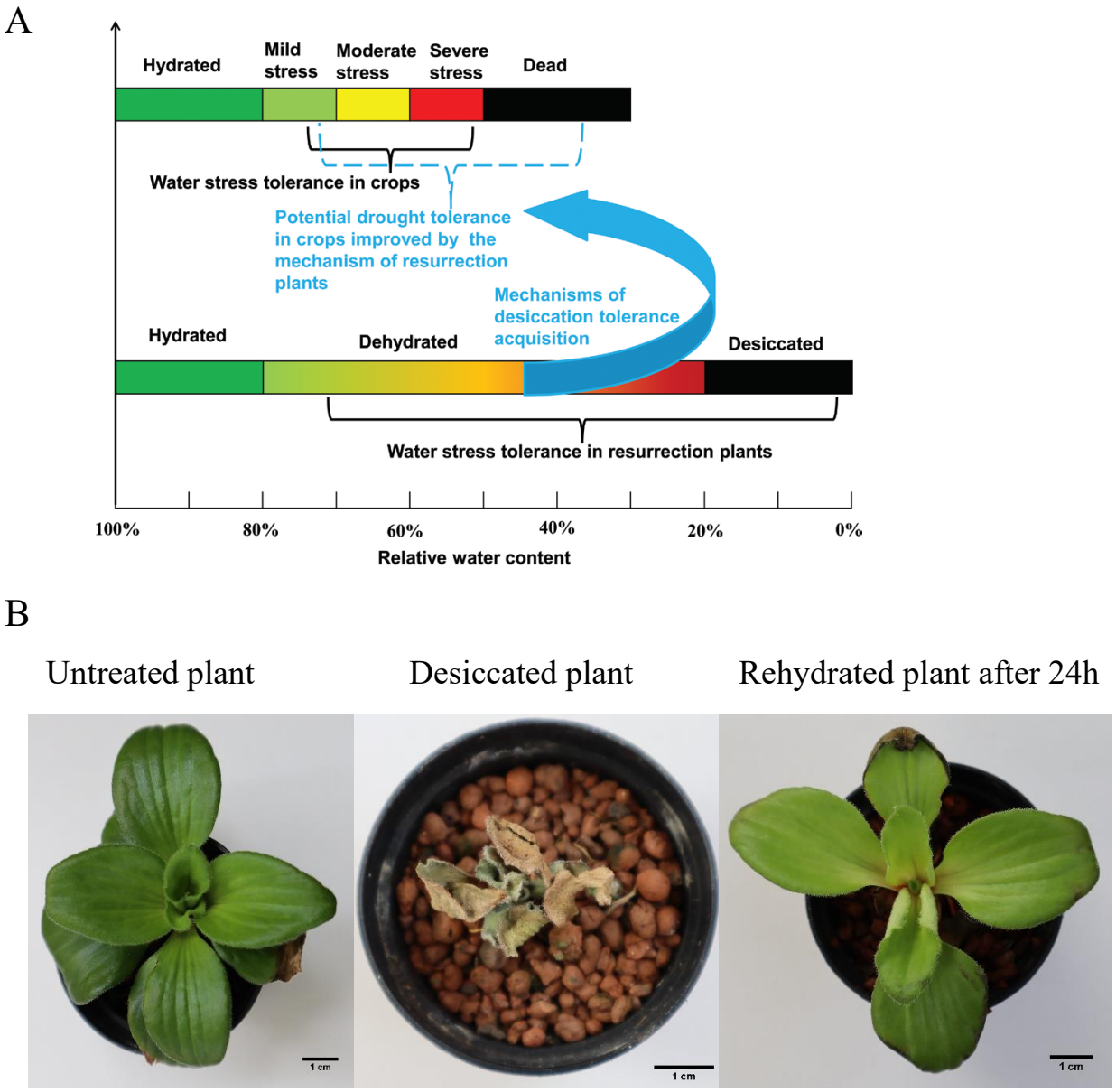


Fig. 1 (A) A comparative scheme of crops and resurrection plants tolerance in different relative water content and resurrection plants' potential to improve the water stress tolerance in crops (Zhang and Bartels 2018). (B) *C. plantagineum* before dehydration, desiccated plant and after 24 h of rehydration.

1.4 The effect of desiccation on the photosynthetic machinery

Resurrection plants are divided into two groups poikilochlorophyllous plants which lose chlorophyll and thylakoid membranes during dehydration, and homoiochlorophyllous plants like *C. plantagineum* which maintain chlorophyll and photosynthetic structures of chloroplasts during dehydration (Tuba et al. 1998).

C. plantagineum tolerates harsh desiccation by shrinking and folding leaves during dehydration and reversing them to normal conditions after rehydration (Giarola et al. 2016). Farrant (2000) proposed leaf folding as a mechanism to reduce the generation of reactive oxygen species (ROS) which leads to retain chlorophyll in *Craterostigma wilmsii* and *Myrothamnus flabellifolius.* A reduction in photosynthesises activity occurs in both desiccation tolerant and sensitive plants during dehydration but the desiccation tolerant plants engage different mechanisms like antioxidant enzymes/metabolites to protect the photosynthetic machinery which leads to conservation of the structural integrity of leaves to finally recover the photosynthetic rates after rehydration. In contrast, desiccation-sensitive plants face an irreversible damage of the photosynthetic machinery (Challabathula et al. 2018).

Closure of stomata is a primary response to the limitation of cellular water availability during dehydration which causes a reduction in carbon dioxide (CO_2) diffusion to chloroplasts in mesophyll cells, leading to a decrease of photosynthetic efficiency (Smirnoff 1993). Photoreduction of oxygen in chloroplasts elevates formation of ROS such as singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\cdot$) which could cause oxidative damage to cellular components such as DNA, RNA, proteins and lipids, and chloroplast structures by inactivating enzymes and raising lipid peroxidation of the membranes (Smirnoff 1993; Mittler 2002).

Plants employ an efficient scavenging system to minimize the damages of ROS formation through enzymatic and non-enzymatic antioxidants. The superoxide dismutase enzyme (SOD) plays a primary role at the front-line of antioxidant defence mechanisms to scavenge and convert superoxide (O_2^-) to oxygen and H_2O_2, whereas catalase (CAT) and ascorbate peroxidase (APX) scavenge H_2O_2 (C Bowler et al. 1992; Willekens et al. 1997; Noctor and Foyer 1998). Other enzymatic antioxidants including monodehydroascorbate reductase (MDAR), glutathione peroxidase (GPX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) also play a role as part of the scavenging system of the cell (Sherwin and Farrant 1998). Non-enzymatic antioxidants form another group of scavengers including ascorbic acid (AA),

reduced glutathione (GSH), α-tocopherol, carotenoids, flavonoids and proline (Mittler 2002; Das and Roychoudhury 2014).

The thylakoid membranes of chloroplasts are the main site of photosynthesis which generates chemical energy and oxygen from sunlight in green plants and algae. The thylakoid membrane comprises membrane multiprotein complexes of photosynthesis including the light-harvesting complexes I and II (LHCI and LHCII), photosystems I and II (PSI and PSII), the cytochrome b6/f complex (Cyt b6f), and ATP synthase (Daum et al. 2010). Oxidative stress forms singlet oxygen ($^1O_2$) as an excited form of molecular oxygen and hydroxyl radical (HO·) which cause irreparable photo-oxidative damage by photo-inhibiting PSI and PSII proteins which leads to the loss of photosynthesis and growth rate of plants (Takagi et al. 2016; Pospisil et al. 2004). Radical ROS such as singlet oxygen and hydroxyl radical cause peroxidation of thylakoid membrane lipids which leads to severe damage of PSI and PSII which are embedded in the lipid bilayers of the thylakoid membrane (Chan et al. 2012). Hydroxyl radical and singlet oxygen initiate lipid peroxidation by forming lipid hydroperoxides (LOOH) as primary products of lipid peroxidation (oxylipins) which is decomposed to lipid hydroxides (LOH), reactive carbonyl species (RCS), and electronically excited species as secondary products of lipid peroxidation during oxidative stress (Pospíšil 2016).

Cells employ β-carotene, tocopherol and plastoquinone as scavengers of singlet oxygen ($^1O_2$) to protect PSI and PSII (Cazzaniga et al. 2012; Dogra and Kim 2020). ROS and particularly singlet oxygen play an important role in signaling from chloroplast to nucleus to regulate the expression of nuclear genes as a response to stress (Beck 2005; Laloi et al. 2007). Oxylipins as products of lipid peroxidation contain α,β-unsaturated carbonyl groups which make them electrophilic and known as lipid-derived reactive electrophile species (RES) such as 12-oxo-phytodienoic acid (OPDA), phytoprostanes, aldehydes, and ketones which regulate the expression of genes to promote stress acclimation, detoxification, defence, and cell death as stress responses (Dogra and Kim 2020).

A protein called ATP-dependent zinc metalloprotease (FtsH) plays an important role in PSII repair in grana margins (GM) (Kato and Sakamoto 2009). Wang et al. (2016) reported a chloroplast protein EXECUTER1 (EX1) which is affected by singlet oxygen, suggesting participation of the EX1 in singlet oxygen signaling. An association between EX1 and FtsH in grana margins was found, suggesting that the EX1 may be involved in PSII repair (Wang et al. 2016).

Liu and Last (2015) reported a proline-rich protein named Maintenance of PSII under High

light 1 (MPH1) which is involved in preservation of PSII under photooxidative damage as photoinhibitory stress. Two knock-out *Arabidopsis thaliana* lines of the *MPH1* gene showed severe damages to PSII reaction proteins compared to wild-type *A. thaliana* under photooxidative stress. The knock-out lines of the *MPH1* exhibited a reduction in PSII quantum yield and electron transport rate under excess light, suggesting that the MPH1 protein plays an important role in PSII photoprotection in *A. thaliana* (Liu and Last 2015).

Lipocalin proteins are a group of ancient proteins which are widely distributed in a wide range of plant, bacterial and animal species; they are involved in binding and transporting hydrophobic ligands such as fatty acids, steroids, odourants, retinoids and pheromones (Flower 1996; Boca et al. 2014). Lipocalin proteins are responsive to physiological conditions and in humans and animals are considered as biochemical markers of cancer, coronary diseases, inflammatory problems, liver and kidney diseases, lipid disorder and neurodegenerative (Xu and Venge 2000). In contrast, lipocalin proteins are not well studied in plant species. Lipocalin proteins in plant species are divided into two classes including chloroplastic lipocalins (CHLs) and temperature-induced lipocalins (TILs) (Charron et al. 2005). The temperature-induced lipocalin (TIL) is a plasma membrane localized protein and chloroplastic lipocalin (CHL) is a chloroplast-localized protein from *A. thaliana* (Abo-Ogiala et al. 2014; Levesque-Tremblay et al. 2009). Both AtTIL and AtCHL proteins are involved in the protection of lipids against oxidative damage, particularly AtCHL plays an important role in the protection of thylakoid membrane lipids by protecting non-enzymatic antioxidant such as tocopherol and zeaxanthin to consequently prevent ROS damage to the thylakoids of chloroplasts (Levesque-Tremblay et al. 2009; Boca et al. 2014).

The *C. plantagineum plastid-targeted protein* (*CpPTP*) gene family has been described as an abscisic acid (ABA) and dehydration-inducible gene family encoding chloroplast-localized proteins which probably protect chloroplast structures during desiccation via interacting with chloroplast DNA using its two coiled-coil domains (Phillips et al. 2002). Crick (1953) described that two or more α-helices twist around each other and form a supercoil which is called a coiled-coil structure. Coiled-coil structures with particular packing properties are present in almost 10% of all proteins and mediate biological interactions of proteins among other essential biological functions such as multimerization and stabilization of proteins, providing structural rigidity, transporting of molecules and conformational changes (Watkins et al. 2015; Lupas and Bassler 2017; Ludwiczak et al. 2019). The role of the CpPTP protein has not been well investigated during desiccation and the protective role of the CpPTP protein in chloroplasts and

photosynthesis apparatus is poorly understood.
There are only a few other identified dehydration responsive proteins in *C. plantagineum* which are involved in the protection of photosynthetic machinery in chloroplasts such as early light-inducible protein (ELIP), desiccation stress protein 21 and 43 (dsp 21 and dsp 43) (Bartels et al. 1992; Schneider et al. 1993).

1.5 Transcriptional regulation of genes during dehydration

Acclimation of plants to environmental changes is acquired in a set of responses at the molecular, physiological and morphological levels. These responses at the molecular level start from transcriptional regulation of gene expression and continue by post-transcriptional, and post-translational modifications to production of proteins which lead to physiological and morphological changes of cells and plant organs. These changes could be either positive by gaining tolerance against damages in stress-tolerant plants or negative by irreversible damages in stress-sensitive plants. Transcriptional regulation of gene expression is mainly controlled by DNA sequences within the promoter upstream of the coding sequence of a gene and corresponding *cis*-acting elements which are binding sites for transcription factors (TF) which largely initiate and regulate transcription of a gene (Zou et al. 2011; Hernandez-Garcia and Finer 2014).
Promoters are classified into four groups including constitutive, spatiotemporal and inducible native promoters, as well as synthetic promoters. The constitutive promoters can be highly active and provide constant level of gene expression in all tissues and developmental stages but the spatiotemporal promoters drive gene expression in certain tissues and developmental stages (tissue- or stage-specific expression). The inducible promoters are another group of promoters which control regulation of gene expression under certain environmental conditions and are responsive to external and internal hormonal stimuli. Synthetic promoters are designed to provide a unique combination of features of native promoters (Hernandez-Garcia and Finer 2014).

Yamaguchi-Shinozaki and Shinozaki (2006) categorized stress responsive genes into two groups including functional and regulatory genes. The functional genes encode reactive oxygen species detoxifying enzymes, molecular chaperones, late embryogenesis abundant (LEA) proteins, and osmoprotectants such as sugars and proline biogenesis enzymes and the regulatory genes encode proteins which play crucial roles in signal transduction cascades and regulation of gene expression such as transcription factors, proteins for ABA and lipid signalling, protein

kinases (Yamaguchi-Shinozaki and Shinozaki 2006). The transcriptional regulation of genes is mediated by ABA-dependent and ABA-independent signal transduction cascades (Yamaguchi-Shinozaki and Shinozaki 2006).

The transcription factors are regulatory proteins which mainly regulate the expression of genes by binding to *cis*-acting elements of promoter sequences. The transcription factors are categorized based on their recognition DNA binding domains and interaction mechanism which they are involved in to bind to the *cis*-regulatory elements of the genes (Nakashima et al. 2009). The abscisic acid-responsive element binding protein (ABRE) as a specific ABA-dependent transcription factor regulates expression of ABA-inducible genes by binding to the ABRE *cis*-acting elements (ABRE; CACGTGGC) of the promoter sequences (Guiltinan et al. 1990; Nakashima et al. 2009). More than one copy of the ABRE motifs is required to regulate expression of ABA-inducible genes, whereas a single copy is not sufficient (Skriver et al. 1991). Although the ABRE motif requires another copy of an ABRE *cis*-acting element, other motifs as coupling *cis*-acting elements such as coupling element 1 and 3 (CE1 and CE3), and dehydration-responsive element/C-repeat (DRE/CRT) as an ABA-independent pathway *cis*-acting element are able to act as coupling motif of the ABRE *cis*-acting elements in an ABA-dependent pathway of gene expression (Shen et al. 1996; Narusaka et al. 2003).

The expression of dehydration-inducible genes is also regulated by an ABA-independent pathway through binding of the dehydration-responsive element binding protein (DREB) to the DRE *cis*-acting element (TACCGACAT). DREB transcription factors belong to the AP2 (APETALA2)/ ERF (ethylene-responsive element-binding factor) protein family consisting of 145 members in *A. thaliana* as AP2/ERF transcription factors (Sakuma et al. 2002). In *A. thaliana*, three *DREB1/CBF* (*DRE Binding protein 1/C-repeat Binding Factor*) homologous genes encoding DREB1A/CBF3, DREB1B/CBF1 and DREB1C/CBF2 transcription factors, and two *DREB2* homologous genes encoding DREB2A and DREB2B transcription factors participate in ABA-independent regulation of stress-inducible genes at the transcriptional level (Nakashima et al. 2009). The genes coding for DREB1A, DREB1B and DREB1C transcription factors are induced by low temperature and consequently these TFs regulate the expression of cold-inducible genes by binding to their DRE/CRT motif of the promoter sequences (Nakashima et al. 2009). Gilmour et al. (2000) reported that over-expressing *A. thaliana* lines of *DREB1* elevated cold tolerance by accumulating proline and various sugars. On the contrary, the gene coding for DREB2A is induced by salt stress and involved in regulation of gene

expression under salt stress and DREB2B is involved in regulation of gene expression under drought stress (Nakashima et al. 2009).

NAM, ATAF, and CUC (NAC) constitute another group of transcription factors having more than 100 members in *A. thaliana* and rice, the ones which are involved in ABA-independent regulation of drought- or other stress-responsive gene expression are termed stress-responsive NAC (SNAC) (Hu et al. 2008; Jensen et al. 2010). Tran et al. (2004) reported that three SNAC transcription factors including ANAC019, ANAC055, and ANAC072/RD26 regulate gene expression by binding to MYC *cis*-acting elements (CATGTG) of promoter sequences of *ERD1* (*early response to dehydration1*) in *A. thaliana*. MYC and MYB proteins are involved in the ABA-dependent regulatory pathways by binding to MYC and MYB *cis*-acting elements (Abe et al. 1997). Therefore, the MYC *cis*-acting element is involved in both ABA-independent and ABA-dependent regulatory pathways in the expression of stress-responsive genes.

It is suggested that changes in *cis*-regulatory elements of genes cause large physiological and morphological diversities in plants and animals, and these changes may occur by genomic stress of adaptation to severe environmental challenges which leads to diverse gene expression (Doebley and Lukens 1998; Prud'homme et al. 2007; Giarola and Bartels 2015). Functional studies of stress-related genes in *C. plantagineum* suggest that *cis*-regulatory elements may cause variation in the expression patterns of stress-related genes in desiccation tolerant and non-desiccation-tolerant plants. The study of regulatory *cis*-acting elements may suggest if changes in regulatory regions lead to evolution of desiccation tolerance in *C. plantagineum* (Giarola and Bartels 2015). The study of regulatory *cis*-acting elements of promoter sequences and interacting transcription factors with the contributing *cis*-acting elements provides a better understanding of mechanisms involved in the regulation and the initiation of transcription of target genes.

1.6 Objectives of the study

The importance of the chloroplast as an organelle where photosynthesis takes place has made the CpPTP an interesting chloroplastic protein family which may be involved in acquisition of desiccation tolerance in *C. plantagineum.* This study was carried out to elucidate the role of the *CpPTP-1* gene from the transcriptional level to its corresponding protein during desiccation to gain a broad understanding of the protective role of the CpPTP-1 protein in the photosynthesis apparatus. The present work was divided in two objectives:

1. The first objective was to isolate 5'-upstream regions from the desiccation responsive genes *CpPTP-1/2/3/4* to perform a comparative functional promoter analysis in *C. plantagineum* and *A. thaliana* by constructing a series of deletion and site-directed mutagenesis constructs containing different *cis*-acting regulatory elements. The comparative mutant analysis is an essential step toward understanding of regulatory mechanism of the *CpPTP-1* transcription during desiccation stress. This analysis was done to provide insight in the mechanisms of transcriptional regulation during desiccation.

2. The second objective was to investigate the role of the CpPTP-1 protein in protecting the photosynthesis apparatus in chloroplasts by performing a yeast two-hybrid screening as an *in vivo* approach to identify interacting proteins. Identification of interacting proteins of the CpPTP-1 protein and their functions may provide a better understanding of the role of the CpPTP-1 protein in the protection of the photosynthesis machinery in chloroplast during desiccation in *C. plantagineum*.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant materials

2.1.1.1 *Craterostigma plantagineum*

Craterostigma plantagineum was used as the main plant in this study. This plant was initially collected in South Africa (Volk and Leippert 1971).

2.1.1.2 *Arabidopsis thaliana*

Arabidopsis thaliana ecotype Col-0 was used for this study (Rédei 1992).

2.1.2 Microorganisms

2.1.2.1 *Escherichia coli* DH10B

E.coli DH10B was used as a host strain for cloning (Lorow and Jessee 1990).

2.1.2.2 *Escherichia coli* BL21 (DE3)

E.coli BL21 (DE3) (Pharmacia, Freiburg, Germany), RIPL (Agilent Technologies, USA) and C43 (Sigma-Aldrich, USA) were used for over-expression of proteins.

2.1.2.3 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens (Koncz and Schell 1986) was used for stable transformation of wild-type *A. thaliana* (ecotype Col-0).

2.1.2.4 *Saccharomyces cerevisiae*

The model yeast, *Saccharomyces cerevisiae* Y190 (Durfee et al. 1993) was used for yeast two-hybrid screening.

All the microorganisms are stored in 500 µl 100 % glycerol at -80 °C.

2.1.3 Vectors

2.1.3.1 pJET1.2/blunt (Thermo Fisher Scientific, USA)

The linearized cloning vector was used to clone PCR products according to the manufacturer's instructions. Resistance to ampicillin and a deadly gene which is deactivated by ligation of an external DNA insert into the multiple cloning sites makes it a suitable cloning vector for a broad range of molecular biology studies.

2.1.3.2 pBT10-GUS

The pBT10-GUS harbours a reporter gene β-glucuronidase (*GUS/uidA*) and an ampicillin resistance gene (β-lactamase) (Sprenger-Haussels and Weisshaar 2000). This vector has been widely used for gene promoter studies with GUS as a reporter gene.

2.1.3.3 pGJ280

pGJ280 carries a dual *CaMV35S* promoter and a Green Fluorescent Protein (GFP) coding sequence. This vector confers ampicillin resistance through a *bla* gene as well.

2.1.3.4 pBIN19

pBIN19 is classified as a binary vector which is widely used for transformation of the *Agrobacterium tumefaciens* (Bevan 1984; Frisch et al. 1995). This vector is capable of conferring kanamycin resistance through its *NPTII* gene which encodes a neomycin phosphotransferase enzyme.

2.1.3.5 pET28a (Novagen, Germany)

This vector is widely used for over-expression of proteins. The pET28a contains N/C-terminal Histidine-Tag sequences (6 × His) and confers kanamycin resistance.

2.1.3.6 pAS2-1 (Stratagene, USA)

pAS2-1 plasmid is used for a yeast two-hybrid screening approach to construct a protein bait. This vector can be transformed into *E.coli* and *S. cerevisiae* and confers ampicillin resistance

through the *bla* gene. The *TRP1* nutritional gene allows transformed *E.coli* and *S. cerevisiae* to survive and grow on minus tryptophan media. The pAS2-1 contains a GAL4-BD coding sequence.

2.1.3.7 HybriZAP™ lambda vector (Stratagene, USA)

This vector was used to prepare a primary plasmid library for the yeast two-hybrid screening.

2.1.3.8 pAD-GAL4 (Stratagene, USA)

This vector is used to prepare a secondary plasmid library or a specific target protein as prey protein or proteins for the yeast two-hybrid screening. The pAD-GAL4 shares the same features as the pAS2-1 plasmid but harbours a *LEU2* nutritional gene which allows transformed *E.coli* and *S. cerevisiae* to survive and grow on minus leucine media.

2.1.3.9 pDONR201 (Thermo Fisher Scientific, USA)

The pDONR201 is used for BP clonase reaction in a gateway cloning system. This is a gateway donor vector which confers kanamycin resistance.

2.1.3.10 pQlinkHD (Thermo Fisher Scientific, USA)

The pQlinkHD is an expression vector with the N-terminal His-tag (7 × His) for LR clonase reaction in a Gateway cloning system. This vector is used for over-expression of a protein and confers ampicillin resistance to the host bacterial cells.

All the vectors are stored as plasmids at -20 °C or as glycerol stock as bacteria strains at -80 °C.

2.1.4 Primers

All the primers were designed using online tools such as Primer3web and reverse-complement websites. Primers were synthesized by Eurofins Genomics (Ebersberg, Germany) and are listed in Table 1. The primers were dissolved in TE buffer to a final concentration of 100 µM and stored at -20°C.

Table 1 list of primers

Name	Sequence (5' to 3')	Restriction site
Plasmid specific primers and adapters		
pJET1.2_For	CGACTCACTATAGGGAGAGCGGC	
pJET1.2_Rev	AAGAACATCGATTTTCCATGGCAG	
5'-pBT10-GUS-fwd-F	AATACGCAAACCGCCTCT	
pBT10-GUS -F	GAAGAGCGCCCAATACGCAAAC	
pBT10-GUS-Start-R	GGTTGGGGTTTCTACAGGACG	
pBIN19-M13-F	TGTAAAACGACGGCCAGT	
pBIN19-M13-R	CAGGAAACAGCTATGACC	
T7-Promoter	TAATACGACTCACTATAGGG	
T7-Terminator	GCTAGTTATTGCTCAGCGG	
pGJ280-Rev	TGTGCCCATTAACATCACCA	
attB1-adapter	GGGGACAAGTTTGTACAAAAAAGCAGGCT	
attB2-adapter	GGGGACCACTTTGTACAAGAAAGCTGGGT	
pDONR201-seq-LA	TCGCGTTAACGCTAGCATGGATCTC	
pDONR201-seq-LB	GTAACATCAGAGATTTTGAGACAC	
pQlinkHD-pQE276	GGCAACCGAGCGTTCTGAAC	
pQlinkHD-pQE65	TGAGCGGATAACAATTTCACACAG	
pAS2-1-Fwd	TCATCGGAAGAGAGTAG	
pAS2-1-Rev	CTGAGAAAGCAACCTGAC	
pAD-Fwd	CACTACAGGGATGTTTAATACCAC	
pAD-Rev	TGCACGATGCACAGTTGAAG	
Expression analysis of *CpPTP-1* and *CpCHL* genes		
CpPTP-1-RT-F	TAGGTGTTCTTCCCTCCCTATG	
CpPTP-1-RT-R	CTTGATCTGCTGCTTCCTGA	
Cp-CpCHL-RT-F	GCTTCGTTGGCATCGCTATT	
Cp-CpCHL-RT-R	AGGTTGCCTGGACAGAATCC	
Sequencing of the genomic sequence of the *CpPTP-1*, *CpPTP-2*, *CpPTP-3* and *CpPTP-4*		
CpPTP-1-F	ACAGCAGAGTGAAGAGTTCTATTTAGC	
CpPTP-1-R	TCGATACTCAAATAAAATAAAAAACTAGGAGATAC	
CpPTP-2-F	TTGAAGTATACTGTCTAAATCTTGAC	
CpPTP-2-R	CTTATAATGTCCATCAATACTCAAAC	

CpPTP-3-F	GAGTTCTAGTTAGCATCCG	
CpPTP-3-R	GTCCATCAATACTCAAATAAAATAAAAAC	
CpPTP-4-F	TGGCATTCATAAAAGCCATAAATCG	
CpPTP-4-R	TCCAATCACTGCTCGCATACAATC	
Genome walker primers and adaptors		
CpPTP-1-R-primary PCR	GAGGTTAATAAAGATGGCTTCATAGG	
CpPTP-1-R-secondary PCR	TGGATGCTAAATAGAACTCTTCACTCTGC	
CpPTP-2-R-primary PCR	GATAAAAGCACAACTGTAGTGAAAGAGTAAATC	
CpPTP-2-R-secondary PCR-1	GGAAAAAAAATACACTTTCTTGATG	
CpPTP-2-R-secondary PCR-2	AGCCATGGAAAAAAAATACACTTTCTTGATG	
CpPTP-3-R-primary PCR	GAGGTTAATAAAGATGGCTTCATAGG	
CpPTP-3-R-secondary PCR	GATTTAAAGGCTTTGTACGGATGCTAAC	
CpPTP-4-R-primary PCR	ATAGTGAAAGAATAAATGACAACTGAAAACCA	
CpPTP-4-R-secondary PCR-1	CGATTTATGGCTTTTATGAATGCC	
CpPTP-4-R-secondary PCR-2	CTTCAACGATTTATGGCTTTTATGAATGCC	
Adaptor primer 1	GTAATACGACTCACTATAGGGC	
Adaptor Primer 2 (Nested)	ACTATAGGGCACGCGTGGT	
Primers for promoter study		
CpPTP-1- EcoRI-F	AAAAAGATGAATTCGCGTA	*EcoRI*
CpPTP-1-R	GAGGTTAATAAAGATGGCTTCATAGG	*NcoI* in upstream
pBT10-GUS-NOS-HindIII-R	AGCAGAAGCTTCTAATTCCCGATC	*HindIII*
CpPTP-1-sd-C1- HindIII-F	GCACAAGCTTGACAAATCAAAC	*HindIII*
CpPTP-1-sd-C2- HindIII-F	CACGAAAGCTTGAGACAAAATG	*HindIII*
Site-directed mutagenesis primers		
CpPTP-1-SDM-MYC-F	GATGACTGCTTATGTTAGAGAACATATGCTCTTCGTTTGCT	Single mutation
CpPTP-1-SDM-MYC-R	AGCAAACGAAGAGCATATGTTCTCTAACATAAGCAGTCATC	Single mutation
CpPTP-1-SDM-DRE-F	CACAAGTTGACAAATCAAACACAGATAAGGGACACAGCTATATTT	Single mutation
CpPTP-1-SDM-DRE-R	AAATATAGCTGTGTCCCTTATCTGTGTTTGATTTGTCAACTTGTG	Single mutation
Primers for protein study		
CpPTP-1-BamHI-F	GGATCCATGGCTAGTTCTCTTATTATTAGG	*BamHI*
CpPTP-1-St-SacI-R	ATCCCGAGCTCTAAAATATAGGTTGATCG	*SacI*
CpPTP-1-NcoI-F	TTTCCATGGCTAGTTCTCTTATTATTAGG	*NcoI*
CpPTP-1-no-St-SacI-R	GAGCTCGAGGCTTTTGGAGTATCACTTGCATTTTCAA	*SacI*
Gateway cloning primers		
CpPTP1-F-attB1	AAAAAGCAGGCTTAATGGCTAGTTCTCTTATT	
CpPTP1-R -attB2	AGAAAGCTGGGTATTAGGCTTTTGGAGTATCA	

CpPTP1-F2-attB1	AAAAAGCAGGCTTAACATTCAAAGAGAAAGAG	
CpPTP1-F3-attB1	AAAAAGCAGGCTTACTGGGGCGATTAGGGTTT	
Yeast two-hybrid screening primers		
CpPTP-1-C1-Y2H-NcoI-F	CCATGGCATTCAAAGAGAAAGAGGGAAGA	*NcoI*
CpPTP-1-C2-Y2H-NcoI-F	CCATGGTGGGGCGATTAGGGTTTTTATTA	*NcoI*
CpPTP-1-Y2H-BamHI-R	GGATCCAAGCTCTAAAATATAGGTTGAT	*BamHI*
Cp-CpCHL-sp-EcoRI-F	GAATTCATGTCGAAGAAAGACATGG	*EcoRI*
Cp-CpCHL-XhoI-R	CTCGAGCTTCCCAAAGAGGGATTTG	*XhoI*
Primers for subcellular localization of the CpCHL		
Cp-CpCHL-sp-EcoRI-F	GAATTCATGTCGAAGAAAGACATGG	*EcoRI*
Cp-CpCHL-NcoI-R	CCATGGACTTCCCAAAGAGGGATTTGA	*NcoI*
Cp-CpCHL-sp-NcoI-R	CCATGGTACCATCAGAGTTGAGGTT	*NcoI*

2.1.5 Chemicals

Chemicals, enzymes and kits were bought from the following companies

Agilent technologies, (USA); Becton Dicknson and Company (Sparks, USA); BIOMOL (Hamburg, Germany); Clontech (Saint-Germain-en-Laye, France); Duchefa Biochemie bv (Haarlem, Netherland); Fermentas (St. Leon-Rot, Germany); Macherey-Nagel (Düren, Germany); Merck (Darmstadt, Germany); Novagen (Germany); PEQLAB (Erlangen, Germany); Roth (Karlsruhe, Germany); Sigma-Aldrich (Steinheim, Germany); Spiess-Urania; Stratagene (USA) ;Chemicals (Hamburg, Germany); Th. Geyer (Renningen, Germany); Thermo Fisher Scientific (USA).

2.1.6 Media

LB-medium

20 g/L LB powder for liquid media. 35 g/L LB-Agar powder for solid media.

MS-medium

4.6 g/L MS-salt mixture; 20 g/L sucrose; 1 ml vitamin solution (see below); pH 5.8; 8 g//L bacto-agar for plate.

YEB-medium

5 g/L sucrose, 5 g/L of meat extract, 5 g/L peptone, 1 g/L yeast extract; 2 mM MgSO4, pH to 7.0 and 15 g bacto-agar for solid medium.

YPAD medium

20 g/L of difco peptone, 10 g/L of yeast extract, pH to 5.8, 40 mg of adenine sulfate, Autoclave. Add glucose to 2% (v/v) (40 ml of autoclaved 50% stock solution). 15–20 g/L of agar for solid medium.

SD medium

6.7 g/L of difco yeast nitrogen base without amino acids, 182.2 g/L of D-sorbitol, 20 g bacto-agar for solid medium, pH 5.8 and autoclave. Add 40 ml sterile 50% glucose and 100 ml of the appropriate sterile 10× Dropout Solution after autoclaving.

2.1.7 Kits

CloneJETTM PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany); Genome Walker Universal Kit (Clontech, France); RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon–Rot, DE); NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, DE); NucleoBond Xtra Midi Kit (Macherey-Nagel, Germany); QuikChange II Site-Directed Mutagenesis Kit (Agilent technologies, USA); Gateway cloning system kit (Thermo Fisher Scientific, USA); Thermo Scientific Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA).

2.1.8 Media supplements

Vitamin solution for plant growth medium: 2 mg/ml glycine; 0.5 mg/ml niacin (nicotine acid); 0.5 mg/ml pyridoxine-HCl; 0.1 mg/ml thiamine-HCl. Use 1:1,000 dilution of the autoclaved solution; store at 4 °C

Vitamin solution as 10× Dropout Solution (1L) for Yeast two-hybrid: 200 mg adenine hemisulfate salt; 200 mg arginine HCl; 200 mg histidine HCl monohydrate; 300 mg isoleucine; 300 mg lysine HCl; 1,000 mg leucine; 200 mg methionine; 500 mg phenylalanine; 2,000 mg threonine; 200 mg tryptophan; 300 mg tyrosine; 200 mg uracil; 1500 mg valine. Autoclave and store at 4 ºC.

Ampicillin (stock solution): 100 mg/ml in dH2O; filter sterilize. 1:1000 dilution (working solution).

Kanamycin (stock solution): 50 mg/ml in dH2O; filter sterilize. 1:1000 dilution (working solution).

Rifampicin (stock solution): 50 mg/ml in methanol; add ~5 drops 10 N NaOH per milliliter to facilitate dissolving. Alternatively dissolve in DMSO.1:500 dilution (working solution).

3-Amino-1, 2, 4-triazole (3-AT): 30 mM of 3-AT for SD selection media.

2.1.9 Equipment and machines

- Agarose gel electrophoresis chamber, EasyCast, Owl-Scientific, Portsmouth, USA.
- SDS-PAGE Minigel system, Biometra, Göttingen, Germany.
- Blotting chambers for proteins, XCell IITM Blot Module, Invitrogen, Carlsbad, USA and Criterion Blotter, Bio-rad, Munich, Germany.
- Power supply, Electrophoresis power supply, Gibco BRL, Carlsbad, Canada.
- PCR cycler, T3 Thermocycler, Biometra, Göttingen, Germany and Bio-rad, USA.
- Centrifuges 5415D, 5417R, 5810R, Eppendorf, Hamburg, Germany.
- pH-meter, SCHOTT GLAS, Mainz, Germany.
- Spectrophotometer SmartSpec 3000, Bio-rad, Hercules, Canada.
- Nanodrop BioSpec-Nano, Shimadzu Biotech, Chiyoda-ku, Japan.
- Electroporation system GenepulserII Electroporator, Bio-Rad, Hercules, USA.
- ScannerTyphoon 9200, Amersham, Piscataway, USA and Image scanner, Amersham, Buckinghamshire, UK.
- Ultrasonic Processor, UP200S, Hielscher, Teltow, Germany.
- Rotator neoLab–Rotator 2–1175, neoLab, Heidelberg, Germany.
- Particle Gun Biolistic®, Bio-Rad, Hercules, USA.
- Azure Imager c300, Azure Biosystems, USA.

- Binocular microscope SMZ-800, Nikon, Düsseldorf, Germany.
- Confocal Laser Scanning Microscope ZE2000 with Laser D-eclipse C1, Nikon, Düsseldorf, Germany.
- UV-light table PeQlab, Vilber, Eberhardzell, Germany.
- Leica DMi8 confocal laser scanning microscope, Germany.

2.1.10 Online sources and software

Thomson Reuters EndNote X9.2

Microsoft office 2019

SnapGene 2.3.2

Vector NTI 11.5.1

ApE 2.0.49

GATC Viewer 0.0.0.0

GraphPad Prism 8.3.0

MEGA4

ImageJ

Mmdb (Molecular Modeling Database)

PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/)

National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/)

ExPASy Bioinformatics Resource Portal‖ (https://www.expasy.org/tools/)

EMBL (https://www.ebi.ac.uk/services)

Primer3web (http://primer3.ut.ee/)

Conserved domains tool (NCBI, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)

QuikChange Primer Design (https://www.agilent.com/store/primerDesignProgram.jsp)

ChloroP (http://www.cbs.dtu.dk/services/ChloroP/)

Kyte-Doolittle scale (https://web.expasy.org/protscale/)

Science launcher (http://www.sciencelauncher.com/oligocalc.html)

Primer3 (http://frodo.wi.mit.edu/primer3/)

https://www.bioinformatics.org/sms2/color_align_cons.html

MARCOIL online software

Inkscape

2.2 Methods

2.2.1 Plant cultivation

C. plantagineum seeds were collected form the botanic garden at the University of Bonn and were placed and grown on MS agar plates after sterilization. Young *C. plantagineum* seedlings were propagated on MS agar at 22°C in a day/night cycle of 16/8 h and repropagated every eight weeks. *C. plantagineum* plants were also cultivated in LamstedtTon® granulate (Leni, Bergneustadt, Germany) at 18°C in a day/night cycle of 13/11 h (Bartels et al. 1990). *Arabidopsis thaliana* plants were grown on soil while a light intensity of 80 µE/m2/s in a day/night cycle of 8 h of light at 22°C and 16 h of darkness at 20°C. The four-leaf seedlings were recultivated in new pots. Six week-old plants were transferred to a long-day growth chamber with exposure of 16 h of light at 22°C and 8 h of darkness at 20°C for seed production. Propagation of seeds was done on MS agar plates.

2.2.2 Gene expression changes during desiccation and salt stress

Three biological replicates of *C. plantagineum* seedlings were treated with 150 mM and 250 mM salt (NaCl) for 24 h and fully desiccated seedlings were collected from our plant bank. The total RNA was isolated from treated and untreated seedlings as control according to Valenzuela-Avendaño et al. (2005). Concentration of RNA samples were measured using nanodrop BioSpec. The cDNA of each sample was synthesized from mRNA based on manufacturer's instructions (Fermentas).

2.2.3 Identification of introns

The introns of the *CpPTP-1*, *CpPTP-2*, *CpPTP-3* and *CpPTP-4* genes were identified. For this, genomic DNA was isolated from leaves of *C. plantagineum* as described by Murray and Thompson (1980). The genes of interest were amplified with specific primers (Table 1) from genomic DNA and fused into the pJET1.2/blunt sequencing plasmid according to the manufacturer's instructions (Fermentas). The recombinant plasmids were then transformed into *E.coli* DH10B cells and transformed bacterial cells were grown on LB-agar plates containing ampicillin as selectable marker and incubated overnight at 37°C. Analysis of recombinant colonies was done using PCR with pJET1.2 forward and reverse sequencing primers. The PCR products were visualized using EtBr-stained agarose gel electrophoresis in 1x TAE buffer. A single positive bacterial colony from each transformation was inoculated in 10 ml LB medium containing ampicillin antibiotic and incubated overnight at 37 °C with shaking. The positive plasmids were isolated using the alkaline lysis method (Birnboim and Doly 1979). The remaining RNA was digested using RNase A (100 μg/ml) for 2 h at 37°C. Digestion of the fused inserts from recombinant plasmids was done using appropriate restriction enzymes and then analyzed on an agarose gel. Finally, the positive plasmids were sequenced.

2.2.4 Promoter studies

2.2.4.1 Genome walker

The Genome walker approach was used to isolate and to identify the promoter sequence of *CpPTP-1*, *CpPTP-2*, *CpPTP-3* and *CpPTP-4* according to the manufacturer's instructions (Clontech). To perform the Genome walker approach, four different DNA libraries of *C. plantagineum* were constructed using *DraI, EcoRV PvuII* and *StuI* restriction sites and ligation of Genome Walker adaptor fragments and primary PCR amplification with primers AP1 and GSP1 were done and finally a secondary PCR amplification was performed with primers AP2 and GSP2. The fragment which was obtained by the secondary PCR of the Genome walker approach was cloned into the pJET1.2/blunt sequencing plasmid and sent for DNA sequencing.

2.2.4.2 Identification of *cis*-acting regulatory elements

Crucial *cis*-acting regulatory elements were identified in promoter sequences using the plant *cis*-acting regulatory DNA elements database PlantPAN 2.0 (Chow et al. 2016) (Fig. 2 A).

2.2.4.3 *β–glucuronidase (GUS)* reporter system

To analyze the promoter activity, the *β-glucuronidase (GUS)* reporter gene approach was performed as described by Jefferson et al. (1987). *CpPTP-1* promoter::*GUS* reporter plasmid was constructed. For this purpose, the 637 bp long promoter fragment of the *CpPTP-1* genomic clone was fused to the *β-glucuronidase* (*GUS*) cDNA in the plasmid pBT10gus. The desired promoter fragment was amplified from the genomic DNA introducing an *EcoR I* and an *Nco I* restriction site. Digestion of the PCR products and pBT10gus with appropriate restriction enzymes was done for 3 to 4 h at 37°C. The digested pBT10gus was treated with 2 µl of Fast Alkaline Phosphatase enzyme (AP) for the last 1 h of the digestion to prevent recirculation of the pBT10gus plasmid. The restricted PCR products and pBT10gus were loaded onto the agarose gel and purified from the gel using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). The restricted PCR products were cloned into the *EcoR I/Nco I* sites of the pBT10gus plasmid. The recombinant vector was transformed into *E.coli* DH10B cells. Analysis of recombinant colonies was done using PCR with pBT10gus forward and reverse primers (Table 1). The positive and recombinant plasmid was isolated as described by Birnboim and Doly (1979). Digestion of the recombinant plasmid was done using *Ecor I* and *Nco I* restriction enzymes. The digested products were visualized using an agarose gel. The positive plasmids were confirmed by DNA sequencing.

2.2.4.4 Stable transformation of *Arabidopsis thaliana* plants

Stably transformed *A. thaliana* lines of the *CpPTP-1* promoter::*GUS* construct were generated through *Agrobacterium*-mediated transformation.

2.2.4.4.1 Preparation of transformed *Agrobacterium tumefaciens*

The pBIN19 vector which is classified as a binary vector was used for transformation of *Agrobacterium tumefaciens*. The appropriate full-length promoter::*GUS* fragments in the plasmid pBT10gus were restricted with *BamH I* and *Hind III* and then ligated into the pBIN19

vector. The recombinant *CpPTP-1* promoter::*GUS*::pBIN19 vector was confirmed by sequencing. Transformation of the *CpPTP-1* promoter::*GUS*::pBIN19 into *Agrobacterium tumefaciens* was carried out through transformation via electroporation as described by Tung and Chow (1995).

2.2.4.4.2 *Agrobacterium tumefaciens*-mediated transformation

The floral dip procedure was used for *Agrobacterium*-mediated transformation of wild-type *Arabidopsis thaliana* (Clough and Bent 1998). Ten independent transgenic lines of the *CpPTP-1* promoter::*GUS* were generated. The transgenic plants were treated with dehydration stress, 200 mM NaCl or 100 µm ABA to evaluate the promoter activity. The leaves of the first-generation plants were treated with 200 mM NaCl and dehydrated for 4, 8, 24 and 48 h. The second generation of transgenic plants was also analyzed. For this, 4 week-old seedlings were dehydrated for 24 h. Dehydration of fully grown plants was performed for 8 days (flowers and siliques were examined). ABA (100 µm) treatment of 4 week-old seedlings was done. The activity of the *GUS* reporter gene in treated transgenic leaves, seedlings and seeds was analyzed using histochemical GUS staining assay (Jefferson et al. 1987).

2.2.4.5 Promoter deletion analysis

A promoter deletion analysis was performed to evaluate *cis*-acting elements of the *CpPTP-1* promoter. Two different combinations of identified *cis*-acting elements were made to make two different deletion constructs to identify the *cis*-acting element which is responsible for promoter activity within *CpPTP-1* (Fig. 2 B,C). The deletion constructs 1 and 2 were prepared by ligating 256 bp and 447 bp long promoter fragments of the *CpPTP-1* into pBT10gus, respectively. The construct 1 only contains the DRE *cis*-acting element and construct 2 contains MYC and DRE *cis*-acting elements. For this purpose, the desired promoter fragments were amplified from the genomic DNA introducing *Hind III* and *Nco I* restriction sites. Digestion of the PCR products and pBT10gus with appropriate restriction enzymes was done for 3 to 4 h at 37°C. The restricted PCR products of the deletion constructs 1 and 2 were cloned into the *Hind III/Nco I* site of the pBT10gus plasmid and were processed and confirmed by DNA sequencing as described in 2.2.3. The *CpPTP-1* promoter deletion construct 1 and 2 containing the chosen *cis*-acting element::*GUS* fusion plasmids, and *CpPTP-1* full-length promoter::*GUS* as control were

introduced into *C. plantagineum* and *A. thaliana* leaves via particle bombardments as described by Van den Dries et al. (2011).

2.2.4.5.1 Site-directed mutagenesis

To evaluate the effect of single nucleotide mutations of the MYC and core sequence of the DRE *cis*-acting elements, point mutations were made in the *CpPTP-1* promoter using the QuikChange II site-directed mutagenesis (SDM) kit from Stratagene (Heidelberg, Germany) according to the manufacturer's instructions (Fig. 2D,E). Specific mutagenic primers introduced a point mutation within the MYC (CAT**G**TG) *cis*-acting element that led to generate CAT**A**TG as deleted MYC, and within the core sequence of the DRE (AC**C**GA) *cis*-acting element that led to generate AC**A**GA as deleted DRE. The pBT10gus plasmid containing the *CpPTP-1* full-length promoter was used as template for a PCR reaction to generate the pBT10gus plasmid containing the *CpPTP-1* full-length promoter with desired point mutations. The *DpnI* enzyme is able to digest isolated plasmids from *E.coli* which is methylated (Stratagene). The parental or non-mutated pBT10gus plasmids which were used as templates were digested with 1 µl *DpnI* enzyme overnight at 37°C. The mutated plasmids were transformed into the *E.coli* DH10B cells and confirmed by colony PCR and DNA sequencing as described in 2.2.3.

2.2.4.5.2 Particle bombardment transformation

All constructs of pBT10gus including the deletion construct 1 and 2, and site-directed mutagenesis MYC and DRE constructs were transiently transformed into *C. plantagineum* and *A. thaliana* leaves via particle bombardments as described by Van den Dries et al. (2011). For this purpose, gold particles were used as microcarriers for the bombardment. Because of the high variability of particle bombardments, each *CpPTP-1* promoter::*GUS* construct was co-bombarded with the pGJ280 plasmid. The plasmid pGJ280 contains a cauliflower mosaic virus *(CaMV) 35S* promoter fused to the green fluorescent protein (GFP) gene. To measure the efficiency of each particle bombardment, the GFP signal was used as an internal standard. GFP signals were visualized and scanned using a confocal laser scanning microscope (Nikon) 24 h after particle bombardment. Bombarded leaves were dehydrated or treated with ABA (1 mM) for 4 h. The bombarded leaves under normal or non-stress conditions were used as control. The activity of the *GUS* reporter gene was analyzed 48 h after the particle bombardment. Six leaves

of *C. plantagineum* and *A. thaliana* were used for dehydration, ABA treatment and non-stress conditions as biological replicates.

2.2.4.6 Determination of GUS activity in transformed plant samples

Stably and transiently transformed *C. plantagineum* and *A. thaliana* plant samples were histochemically stained with 5-bromo-4- chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) to evaluate the activity of the promoter through *GUS* reporter gene activity (Jefferson et al. 1987). The GUS enzyme is able to convert the X-Gluc substrate and subsequently produce an insoluble blue precipitate.

2.2.4.7 Relative promoter activity

After bombardment of *C. plantagineum* and *A. thaliana* leaves the relative promoter activities was calculated by the following formula based on the number of the GFP signals (expressing cells) and GUS (blue) spots.

$$Relative\ promoter\ activity\ [\%] = \frac{\text{Number of GUS spots}}{\text{Number of GFP spots}}\ x\ 100$$

Scheme of the *CpPTP-1* promoter constructs

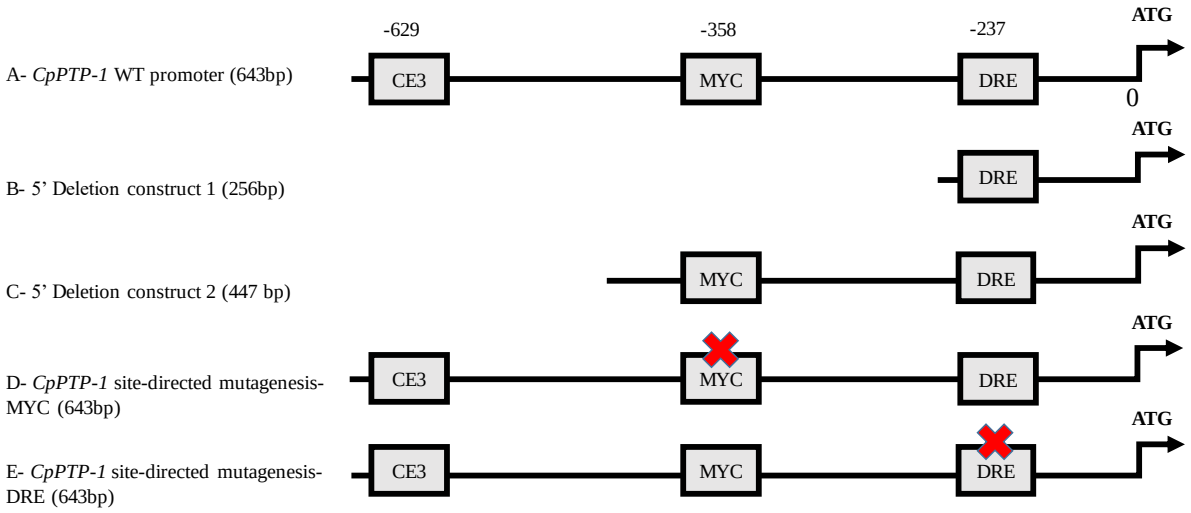


Fig. 2 CE3, coupling element 3 is sufficient for ABA induced gene expression. MYC recognition sequence which is a specific binding site for NAC proteins. DRE, a core sequence for binding of dehydration responsive transcription factors.

2.2.5 Protein studies

To evaluate PTP-1 protein expression, the *CpPTP-1* gene was cloned in the *E.coli* expression vectors pET28a+ or PQlinkhd (Gateway expression system) to overexpress the corresponding protein.
Total RNA was isolated from *C. plantagineum* leaves according to Valenzuela-Avendaño et al. (2005) and cDNAs were synthesized based on manufacturer's instructions (Fermentas). The cDNA was used as template to amplify the *CpPTP-1* coding sequence.

To overexpress the CpPTP-1 protein five different expression constructs were prepared through restriction-ligation cloning (two constructs) and gateway cloning (three constructs) systems.

- pET28a- CpPTP-1 (0 to +603; 201 amino acids) with fused N-terminal His-tag (6 × His) (705 nt; 234 aa resulting protein).
- pET28a- CpPTP-1 (0 to +600; 200 amino acids) with fused C-terminal His-tag (6 × His) (657 nt; 218 aa resulting protein).
- PQlinkHD-CpPTP-1 plasmid (0 to +603; 201 amino acids) with fused N-terminal His-tag (7 × His) (666 nt; 222 aa resulting protein).
- PQlinkHD-CpPTP-1 (without signal peptide) plasmid (+112 to +603; 164 amino acids) with fused N-terminal His-tag (7 × His) (558 nt; 185 aa resulting protein).
- PQlinkHD-CpPTP-1 (only functional domain) plasmid (+301 to +603; 100 amino acids) with fused N-terminal His-tag (7 × His) (366 nt; 122 aa resulting protein).

2.2.5.1 Restriction-ligation cloning approach

Two constructs of the *CpPTP-1* coding sequence were designed. The first one had 6×His-tags at the N-terminus (N-His) and the other one had 6×His-tags at the C-terminus (C-His). The desired *CpPTP-1* fragments were amplified with cloning primers which introduced a *BamHI* and a *SacI* (N-His), and an *NcoI* and a *SacI* (C-His) restriction site (Table 1). The purified PCR products were cloned in the pJET1.2 vector and then sequenced as described in 2.2.3. The empty vector pET28a+ and pJET1.2-PTP-1 were restricted using the appropriate restriction enzymes *BamHI* and *SacI* (N-His) for 3 h, and *NcoI* and *SacI* (C-His) overnight at 37°C.

The amplified coding sequence of the *CpPTP-1* (0 to +603; 201 amino acids) was subcloned into the pET28a (+) expression vector via *BamHI/SacI* sites yielding a fusion protein of 234 aa

(705 nt) with an N-terminal His-tag (6 × His). To construct a CpPTP-1 protein fused with the C-terminal His-tag (6 × His) (657 nt; 218 aa) the stop codon (TAA) of *CpPTP-1* was removed and the coding sequence of *CpPTP-1* (0 to +600; 200 amino acids) was subcloned into the pET28a (+) expression vector via *NcoI/SacI* sites.

The recombinant pET28a- CpPTP-1 constructs were verified by DNA sequencing and pET28a-CpPTP-1 (N-His) was introduced into the *E. coli* strains BL21 (DE3), RIPL and C43. The expression of the recombinant protein was tested by induction at 15°C, 20°C, 25 °C, 30°C and 37°C by adding IPTG (isopropyl-β-D-thiogalactopyranoside) at different concentrations from 0.1 mM to 1mM from 5 h to 24 h.

2.2.5.2 The Gateway cloning system

The Gateway expression system was tested to overexpress the CpPTP-1 protein. Two steps gateway PCR experiments were carried out. Step 1 was done through amplification of template cDNA using template *CpPTP-1* specific primers containing 12 nucleotides of the attB sites. Step 2 was performed through amplification of the product produced in Step 1 using universal attB adapter primers. The final PCR product was purified from the agarose gel and then used for the BP and LR clonase reaction. For the BP clonase reaction, the amplified coding sequence of the *CpPTP-1* (0 to +603; 201 amino acids), from step 2 was cloned into the PDONR201 donor vector. The PDONR201-CpPTP-1 was transformed into the *E. coli* strain DH10B cells. For the LR clonase reaction, the recombinant PDONR201-CpPTP-1 was used to fuse the *CpPTP-1* coding sequence into the PQlinkHD expression vector with the N-terminal His-tag (7 × His).

The signal peptide of the *CpPTP-1* (111 nt; 37 amino acids) was removed to eliminate the effect of the signal peptide on the expression of the corresponding protein. The gateway expression system was used to overexpress the CpPTP-1 protein (+112 to +603; 164 amino acids).

The functional domain of the *CpPTP-1* (+301 to +603; 100 amino acids) was also constructed into Gateway expression system as described before.

The recombinant PQlinkHD-CpPTP-1 plasmids were verified by sequencing and introduced into the *E. coli* strains BL21 (DE3), RIPL and C43. The expression of the recombinant protein was tested by induction at 15°C, 20°C; 25 °C, 30°C and 37°C by adding IPTG at different concentrations from 0.1 mM to 1 mM for 5 h.

2.2.5.3 Identification of IPTG-induced recombinant proteins

Total proteins form IPTG-induced bacterial cells were extracted following the Laemmli (1970) protocol. Purification of inclusion bodies from *E. coli* was performed as well (Wingfield et al. 2001). The quality and identity of the proteins were validated by Coomassie blue staining of SDS-PAGE gels and by Western blot analysis using His-tag antibody according to Laemmli (1970); Towbin et al. (1979). The Coomassie stained gel and blotting membrane were visualized by Azure Imager c300 (Azure Biosystems).

2.2.6 Yeast two-hybrid screening

The yeast two-hybrid screening approach was used to identify the interacting proteins interacting with the CpPTP-1 protein.

2.2.6.1 Yeast two-hybrid library as prey

The HybriZAP™ lambda vector was used to prepare a primary plasmid library following the manufacturer's instructions (Stratagene). For this, 5 mg poly (A)+ RNA from 2 h dried *C. plantagineum* leaves was transcribed into cDNA and directionally cloned into the HybriZAP™ lambda vector. The HybriZAP™ lambda primary library was subsequently converted to a pAD-GAL4 as a secondary plasmid library (Frank et al. 1998). This library was used as prey or target for protein-protein interaction in the present study.

2.2.6.2 Preparation of bait constructs

Two different constructs of the *CpPTP-1* were designed with appropriate restriction sites and cloned into the plasmid pAS2 as bait.

The first construct presents the CpPTP-1 protein without signal peptide (+112 to +603; 164 amino acids) and the second one presents only the functional domain of the CpPTP-1 protein (+301 to +603; 100 amino acids). The desired *CpPTP-1* fragments were amplified from cDNA samples from *C. plantagineum* leaves with two sets of cloning primers which introduced an *NcoI* and a *BamHI* restriction site (Table 1). The purified PCR products were cloned in the pJET1.2 vector and then sequenced as described in 2.2.3. The empty vector pAS2 and pJET1.2-PTP-1 were restricted using the appropriate restriction enzymes *NcoI* and *BamHI* overnight at 37°C. The restricted *CpPTP-1* fragments were subcloned into the pAS2 vector via *NcoI/BamHI*

sites. The recombinant pAS2-CpPTP-1 plasmids were transformed into *E.coli* DH10B cells and then isolated according to Birnboim and Doly (1979). The positive pAS2-CpPTP-1 plasmids were confirmed by DNA sequencing.

2.2.6.3 Yeast transformation

Yeast transformation was performed using the LiAc method (Gietz and Schiestl 2007). The yeast Y190 strain was transformed by pAD-GAL4-2.1-cDNA libraries as prey and pAS2-CpPTP-1 as bait. The transformation mixture was transferred on SD agar plates without Leu and Trp as a selective medium 1 and incubated for 5 days at 30°C to confirm the transformation of the yeast cells.

2.2.6.4 Evaluation of protein-protein interaction

The colonies grown on selective plates (selective medium 1) were investigated for a specific interaction through reporter genes *HIS3* and *lacZ*. Selective medium 2 without Leu, Trp and His including 30 mM 3-AT was used for the evaluation of expression of the *HIS3* gene. 3-AT was used to increase the pressure of selection. To evaluate the expression of *lacZ* gene a filter lift assay was performed following the manufacturer's instructions (Stratagene). The positive colonies form selective medium 2 were recultured on fresh selective medium 2 every week. Fourteen individual colonies from selective medium 2 were analyzed by DNA sequencing.

2.2.6.5 Specific interaction of CpPTP-1 and CpCHL

To confirm specific interactions between the CpPTP-1 and CpCHL proteins, a targeted yeast two-hybrid approach was carried out.

To prepare a prey construct for the specific interaction, the desired *CpCHL* fragment (0 to + 552; 184 aa) was amplified from cDNA samples from *C. plantagineum* leaves with cloning primers (Cp-CpCHL-sp-EcoRI-F and Cp-CpCHL-XhoI-R) which introduced an *EcoRI* and a *XhoI* restriction site (Table 1). The purified PCR products were cloned in the pJET1.2 vector and then sequenced as described in 2.2.3. The empty vector pAD-GAL4 and pJET1.2-CpCHL were restricted using the appropriate restriction enzymes *EcoRI* and *XhoI* overnight incubation at 37°C. The restricted *CpCHL* fragment was subcloned into the pAD-GAL4 vector via *EcoRI/XhoI* sites. The recombinant pAD-GAL4- CpCHL plasmid was transformed into *E.coli*

DH10B cells and then isolated according to Birnboim and Doly (1979). The positive pAD-GAL4- CpCHL plasmids were confirmed by DNA sequencing.

2.2.6.5.1 Yeast transformation

Yeast transformation was performed using the LiAc method (Gietz and Schiestl 2007). The yeast Y190 strain was transformed with the pAD-GAL4- CpCHL plasmid as prey and pAS2-CpPTP-1 as bait. The transformation mixture was transferred on an SD agar plate without Leu and Trp as a selective medium 1 and incubated for 5 days at 30°C to confirm the transformation of the yeast cells.

2.2.6.5.2 Evaluation of specific interaction between CpPTP-1 and CpCHL

The colonies grown on selective plates (selective medium 1) were investigated for a specific interaction through reporter genes *HIS3* and *lacZ*. Selective medium 2 without Leu, Trp and His including 30 mM 3-AT was used to evaluate the expression of the *HIS3* gene.

2.2.7 Subcellular localization of the CpCHL

To perform subcellular localization of the CpCHL protein in *C. plantagineum* two localization constructs were prepared. The pGJ280 vector was used for the protein localization.

2.2.7.1 pGJ280-CpCHL localization constructs

- The first construct only contains the CpCHL protein signal peptide (0 to +150; 50aa) which was fused to the GFP sequence (714 nt; 238 aa) of the pGJ280 plasmid. The CpCHL protein signal peptide was supposed to act as a signal peptide and to translocate the GFP inside the chloroplast of *C. plantagineum*.
- The second construct contains the entire *CpCHL* gene including the signal peptide and the functional domain of the CpCHL protein (0 to +552; 184 aa). The *CpCHL* coding sequence was fused to the GFP sequence yielding a fusion protein of 423 aa (1269 nt) with a C-terminal GFP reporter protein.

2.2.7.2 Preparation of pGJ280-CpCHL constructs

The desired *CpCHL* fragments were amplified from cDNA samples from *C. plantagineum* leaves with cloning primers (Cp-CpCHL-sp-EcoRI-F, Cp-CpCHL-NcoI-R and Cp-CpCHL-sp-NcoI-R) which introduced an *EcoRI* and an *NcoI* restriction site (Table 1). The purified PCR products were cloned in the pJET1.2 vector and then sequenced as described in 2.2.3. The empty vector pGJ280 and pJET1.2-CpCHL were restricted using the appropriate restriction enzymes *EcoRI* and *NcoI* for 4 h at 37°C. The restricted *CpCHL* fragments were subcloned into the pGJ280 vector via *EcoRI/NcoI* sites. The recombinant pGJ280-CpCHL plasmids were transformed into *E.coli* DH10B cells and then isolated according to Birnboim and Doly (1979). The positive pGJ280-CpCHL plasmids were confirmed by DNA sequencing. Two constructs of pGJ280-CpCHL were transiently transformed into *C. plantagineum* leaves via particle bombardments as described by Van den Dries et al. (2011). GFP signals were visualized and scanned using a confocal laser scanning microscope (Leica DMi8) 12 h to 16 h after particle bombardment.

3. RESULTS

3.1 Phylogenetic analysis of the CpPTP protein

The PTP protein family has been found in members of the Linderniaceae family such as *C. plantagineum, Lindernia brevidens* and *Lindernia subracemosa.* Two homologues of the CpPTP protein family have been found in each *L. brevidens* and *L. subracemosa* from our lab's unpublished genome database. The alignment of the eight PTP proteins shows conserved amino acids (Fig. 3 A). The conserved amino acids are from the same amino acid categories with the same features. The phylogenetic relationship of PTP proteins in *C. plantagineum, L. brevidens* and *L. subracemosa* was analyzed based on the similarities of the amino acids using the Neighbor-Joining method of MEGA 4.0 software (Fig. 3 C). The phylogenetic tree shows that CpPTP-1 and CpPTP-3 are more related and have a more recent common ancestor. The CpPTP-4 is more related to CpPTP-1 and CpPTP-3 than the CpPTP-2. The Lbr_002697-RA protein from *L. brevidens* is more related and sharing a more recent common ancestor with the *C. plantagineum* CpPTP proteins than the other proteins in *L. brevidens* and *L. subracemosa.* The results show that Lbr_023434-RA from *L. brevidens* and Lsu_022940-RA from *L. subracemosa* are more closely related to each other and have more recent common ancestors than the other proteins in these two species. The phylogenetic analysis shows that homologues proteins from *L. brevidens* and *L. subracemosa* are diverged from the PTP protein family from *C. plantagineum* during evolution.

3.2 Functional domains of the CpPTP protein

The coiled-coil domains of the CpPTP-1 protein were predicted using MARCOIL online software (Zimmermann et al. 2018). The results show that there are two coiled-coil domains from 106 aa to 139 aa (length = 34) and from 147 aa to 194 aa (length = 48) at the C-terminal domain of the CpPTP proteins (Fig. 3 A). The online NCBI conserved domain search allows us to predict a methyl-accepting chemotaxis-like domain (chemotaxis sensory transducer) at the C-terminal domain of the CpPTP proteins (Fig. 3 B). The methyl-accepting chemotaxis proteins (MCPs) are classified as bacterial and archaeal receptors that mediate chemotaxis to different environmental and intracellular signals (Derr et al. 2006; Salah Ud-Din and Roujeinikova 2017).

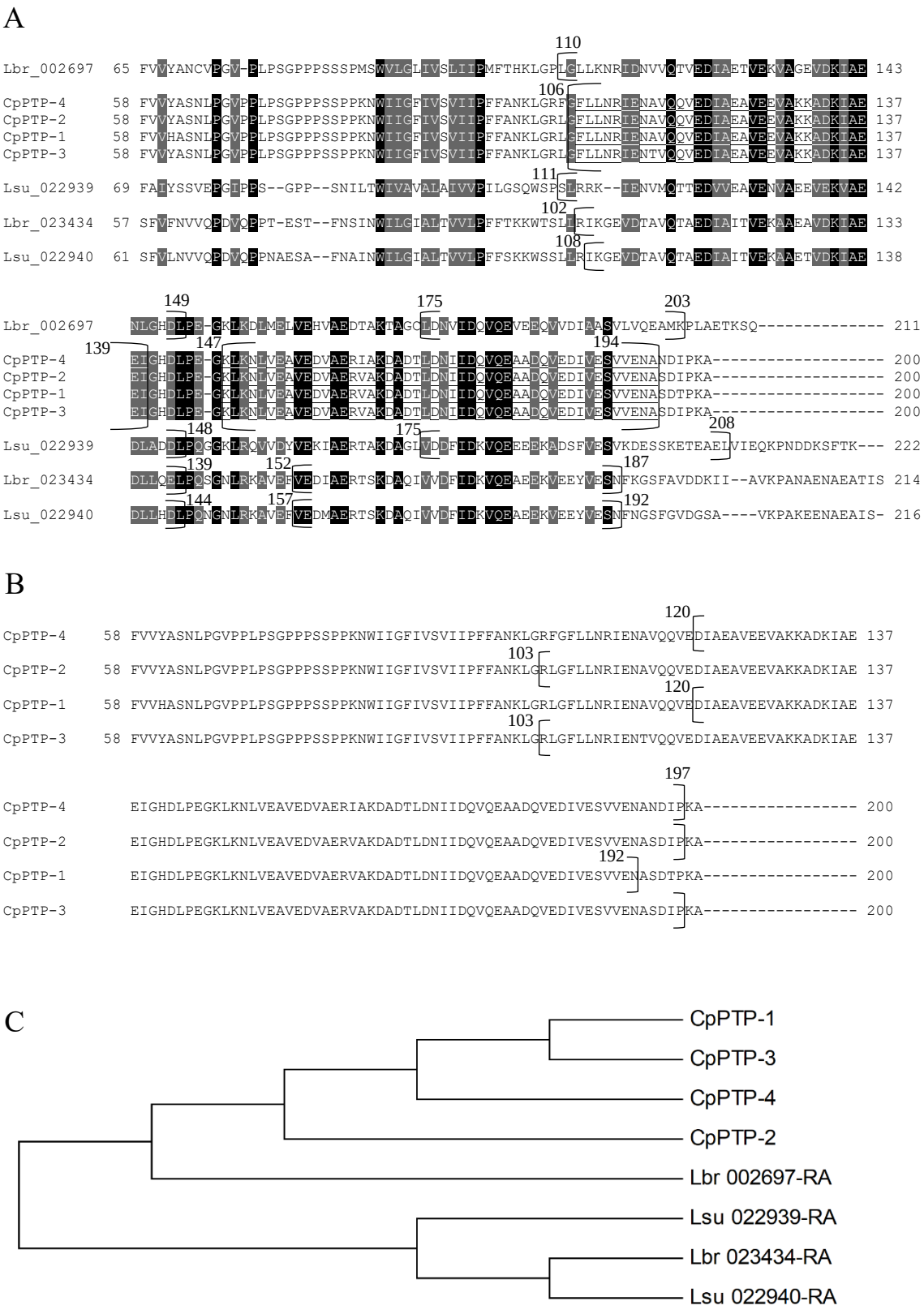


Fig. 3 (A) Conserved amino acids of the PTP protein family in *C. plantagineum*, *L. brevidens* and *L. subracemosa*. The sequences which are placed between two brackets are coiled-coil domains of the PTP protein family which were recognized using the MARCOIL online software. (B) The methyl-accepting chemotaxis-like domain of CpPTPs is placed between two brackets. (C) Phylogenetic analysis of the PTP proteins homologues in *C. plantagineum*, *L. brevidens* and *L. subracemosa*. The Neighbor-Joining (NJ) tree was constructed with MEGA 4.0 software based on the alignments. The position of each amino acid is placed next to the bracket.

3.3 Gene expression changes during desiccation and salt stress

The expression analysis of *CpPTP-1* on the transcript level was performed under 150 mM and 250 mM NaCl treatment, desiccation, and non-stress conditions in *C. plantagineum* (Fig. 4). The *elongation factor 1a (EF1a)* was used as a reference gene to relatively normalize the expression data. The result showed the highest transcript expression of *CpPTP-1* under desiccation conditions. A significant reduction was observed in the expression of *CpPTP-1* under 150 mM NaCl treatment. Although the data showed that the expression of the *CpPTP-1* gene is relatively high under non-stress conditions but the expression increased gradually after 250 mM NaCl treatment and desiccation (Fig. 4B,C).

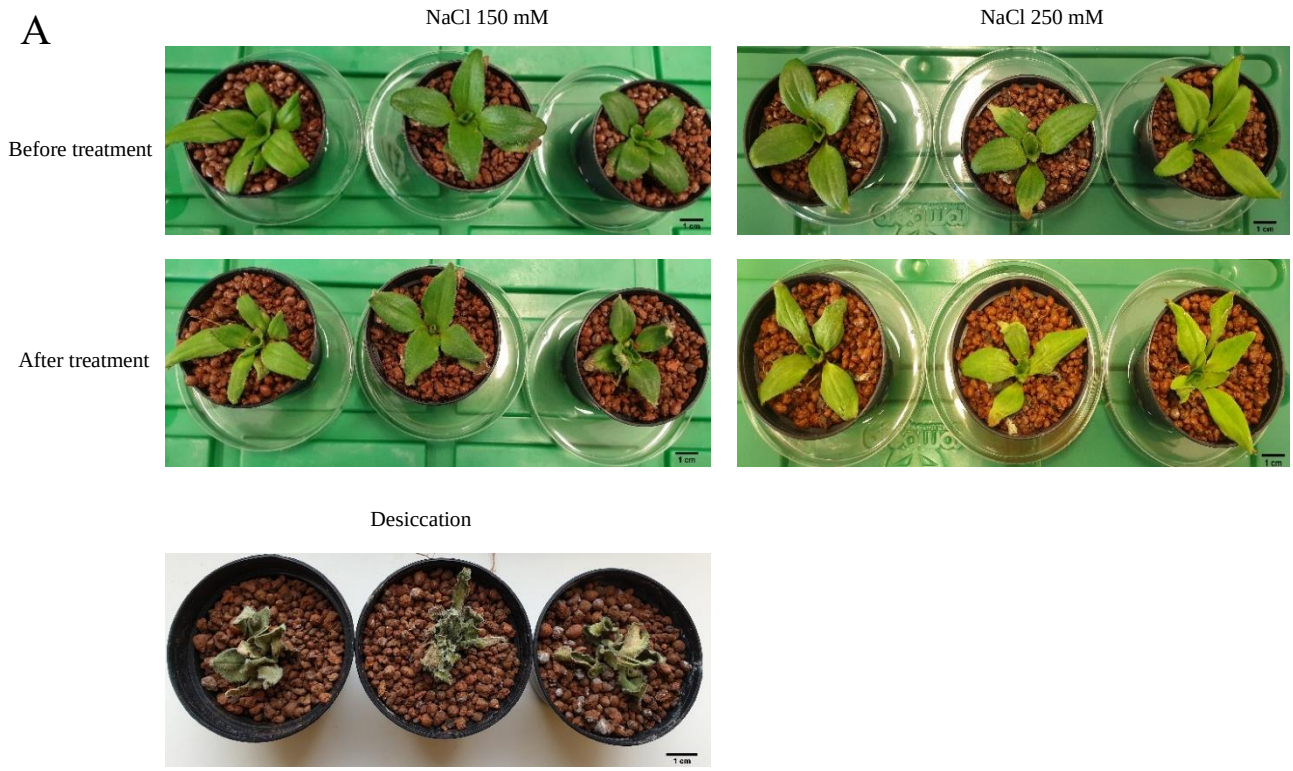


Fig. 4 Gene expression study of the *CpPTP-1* under NaCl 150 mM and 250 mM treatment, non-stress and desiccated *C. plantagineum* plants. *EF1a* gene was used as a reference gene. (A) Three biological replicates of *C. plantagineum* plants under different conditions. (B) RT-PCR of the *CpPTP-1*, and *EF1a* as a reference gene under different conditions. (C) Relative expression of the *CpPTP-1* under different conditions. The graphs were drawn and data were statistically analyzed using GraphPad Prism 8. One-way analysis of variance (ANOVA) was applied to the gene expression data. Signs of * and ** represent significance at 5% (*P<0.05) and 1% (**P<0.01) probability levels, respectively.

3.4 Introns of the *CpPTP* genes family

To identify the intron sequences of *CpPTP-1*, *CpPTP-2*, *CpPTP-3* and *CpPTP-4*, the DNA samples of *C. plantagineum* leaves were sequenced as described in part 2.2.3. The result showed that there are three long introns within the *CpPTP-1*, *CpPTP-2* and *CpPTP-4* genes and there was not any intron inside of the *CpPTP-3* gene (Fig. 5). The intron regions could be used for primary amplification of the Genome walker approach to isolate gene promoters.

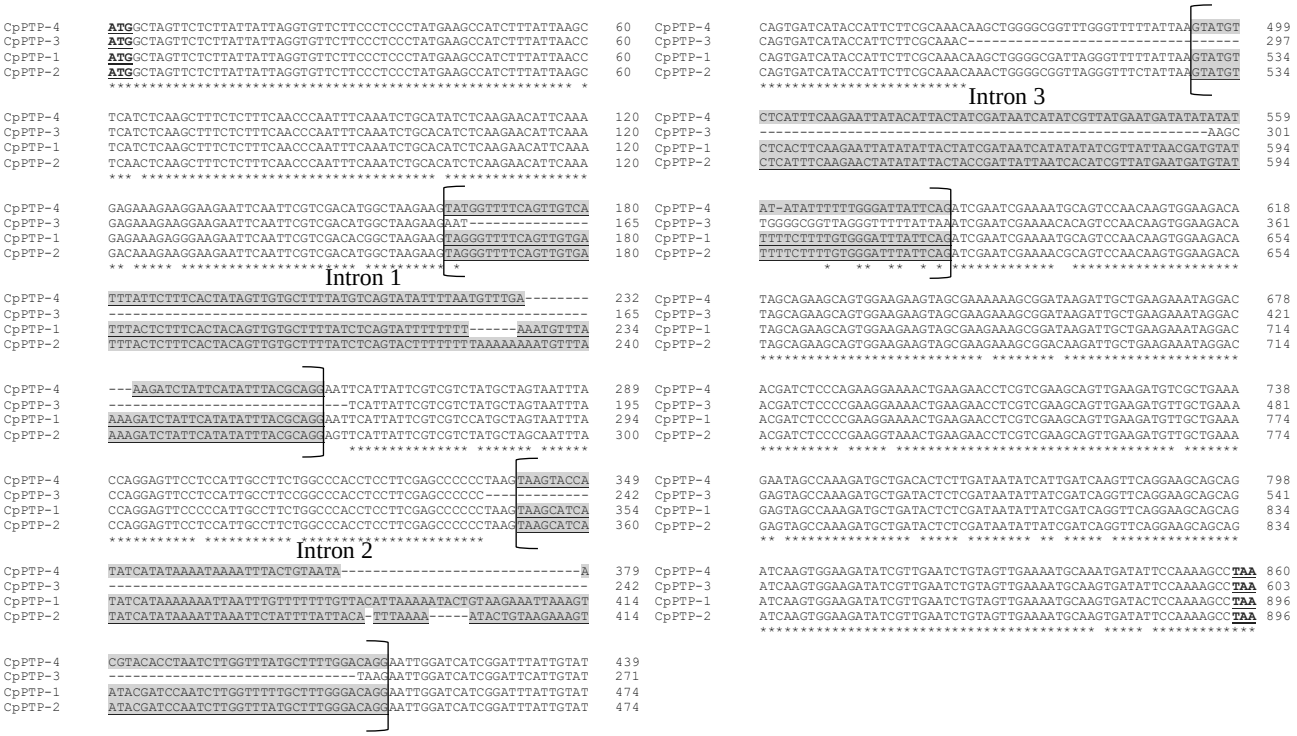


Fig. 5 Genomic sequences of *CpPTP-1*, *CpPTP-2*, *CpPTP-3* and *CpPTP-4*. The underlined and highlighted sequences which are placed between two brackets represent the intron sequences of the *CpPTPs*.

3.5 Promoter studies

3.5.1 Promoter isolation

To isolate and identify the promoter sequences of *CpPTP-1*, *CpPTP-2*, *CpPTP-3* and *CpPTP-4*, the Genome walker specific primers for each of these genes were designed based on single nucleotides which are different among these genes to set specific primers. The Genome walker results showed that the promoters of *CpPTP-1*, *CpPTP-2* and *CpPTP-4* are identical. However, for the *CpPTP-3*, there were several bands in secondary PCR of the Genome walker instead of only one band which may represent the promoter of the target gene and the promoter of the *CpPTP-3* was not amplified. This experiment was repeated for a second time to compare the validity of the data. The results were the same as the first experimental series. The length of the isolated *CpPTP-1* promoter is 643 bp which contains putative *cis*-acting regulatory elements. The most important *cis*-acting regulatory elements which might be involved in abiotic stress responses are indicated in the promoter sequence (Fig. 6) and the *cis*-acting elements are listed in the Table 2.

AAAAAGATATTCGCGTATTAATATTACAACATTCAATTTTATTTATTTAGTTATGCAAATAGTACTAAATTGTAACACAAA
AGTAAAGTCCGTAACTACTCTAGATCTTGACTAGTTGGTTGCATCCGCCAATAGCAAAGGTGATCTTTAATTAACTTCTGA
ATAGGAATAAAATAATAAATGAATCCTTAATATCACGAAAGGAGACAAAATGATATAGCACACATTATTGTACTTGCAAC
TTAAGATGTGGACCAGGTAAAGATGACTGCTTATGTTAGAGAACATGTGCTCTTCGTTTGCTTTGTTTTTACACTTTTTTAA
CATTTGTTTCGTTTTTCACATTCTACCTAACACGCGGAAGTAGATTAAAATTATTGAAAAAGCACAAGTTGACAAATCAAA
CACCGATAAGGGACACAGCTATATTTGCCTAGTTGAAGATTACAATATTTGAATTTTCATTGCAACAAGTCTTTGTTCTATA
AAATAATTGGGACCTTTCATCTAAAGTACGAAGCATCTTCCCtAAAACACCAAACAGCAGAGTGAAGAGTTCTATTTAGCA
TCCATACAAAGCCTTTAAATCTTTGAAGTATACTGTCTAAATCTTGATATCAAGAAAGTGTAATTTTTTTCCATG

CE3 MYC DRE

Fig. 6 *CpPTP-1* promoter sequence representing CE3, MYC and DRE in different colors.

Table 2 list of putative *Cis*-acting element

Cis-acting element	Sequence	Short description
CE3	GCGT	Coupling element 3 (CE3) is sufficient for ABA induced gene expression (Shen et al. 1996).
MYC	CATGTG	MYC motif is necessary for expression of erd1 (early responsive to dehydration) in *A. thaliana* during dehydration and is a specific binding site for NAC protein (Tran et al. 2004).
DRE	ACCGA	Dehydration responsive element (DRE) is responsive to dehydration and salt stress (Yamaguchi-Shinozaki and Shinozaki 1994).

3.5.2 Evaluation of *CpPTP-1* promoter activity in *A. thaliana*

To evaluate the *CpPTP-1* promoter activity in *A. thaliana* as a heterologous system the stable transformation was performed as described in part 2.2.4.4. To this, six T0 plants were transformed and 10 T1 seeds as independent transgenic lines were screened and selected on the MS agar plate containing Kan antibiotic. Therefore, ten independent *A. thaliana* transgenic lines were generated for two generations.

3.5.2.1 GUS activity in the first generation of the transformed *A. thaliana* lines

The intensity of blue color as a production of GUS activity in different samples was used to define the promoter activity. Histochemical GUS staining showed that there was high activity of the *CpPTP-1* promoter in the first generation (T1) of transgenic *A. thaliana* leaves after 4, 8, 24 and 48 h of dehydration, as well as in untreated leaves as control. The results revealed that in most of lines the activity of the *CpPTP-1* promoter in untreated samples was higher than in dehydrated samples. There was a significant difference in GUS activity in transgenic lines 1, 2, 3, 4, 5, 6, 9 and 10 in non-stress conditions compared to dehydration conditions (Fig. 7). This indicates that transcription factors of *A. thaliana* are able to regulate the activity of the *CpPTP-1* promoter and this promoter could play a regulatory role in *A. thaliana* as a heterologous system in non-stress conditions.

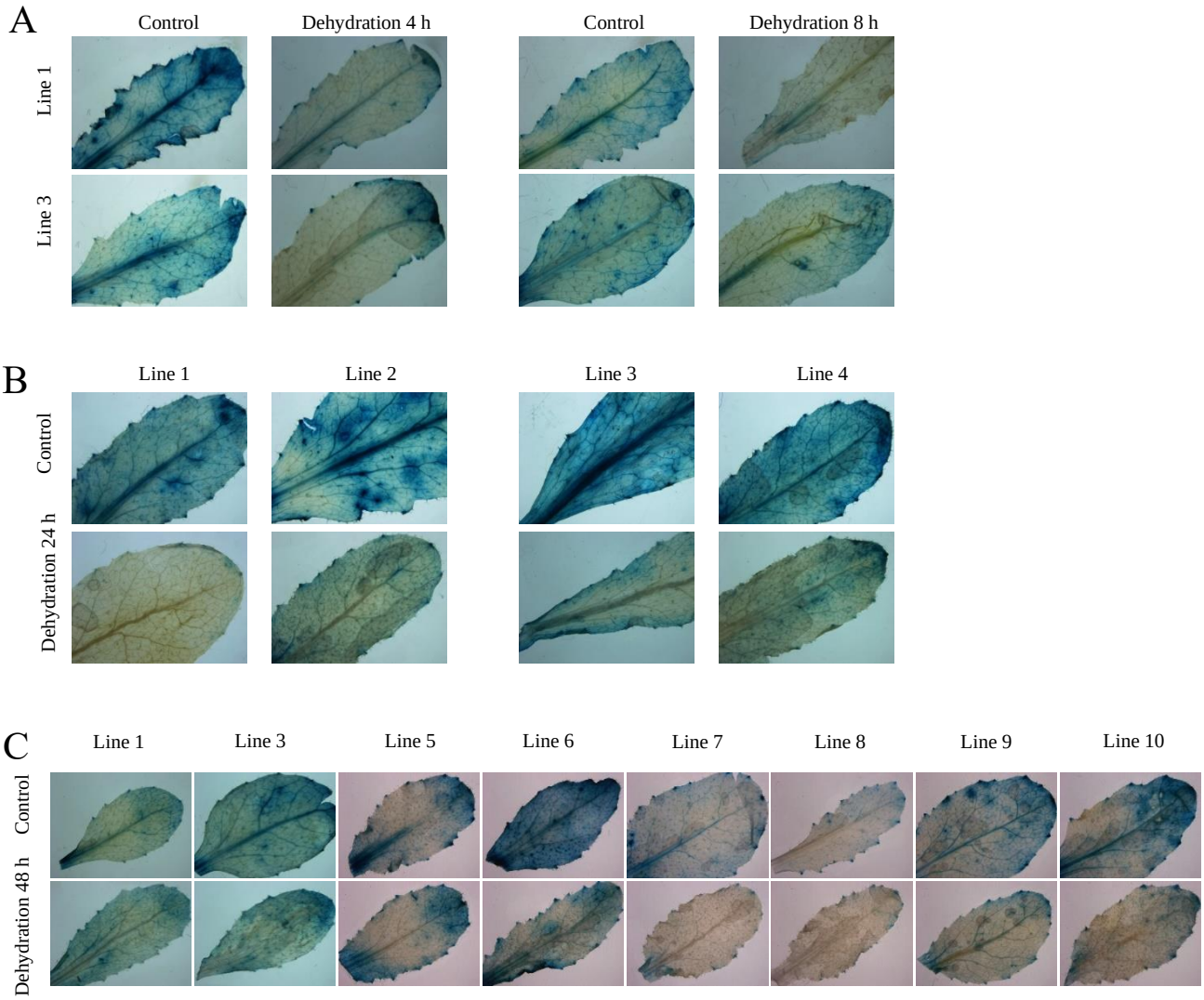


Fig. 7 Evaluation of *CpPTP-1* promoter activity through GUS-staining of the first generation (T1) of *A.thaliana* lines for different time periods. (A) Dehydration of the lines 1 and 3 for duration of 4 h and 8 h. (B) Dehydration of the lines 1,2, 3 and 4 for duration of 24 h. (C) Dehydration of the lines 1,3,5,6,7,8,9 and 10 for duration of 48 h.

3.5.2.2 GUS activity in the second generation of transgenic lines of *A. thaliana*

Second generation (T2) of transgenic *A. thaliana* lines of the *CpPTP-1* promoter were also examined. The 24 hours dehydration of the 4 week-old seedlings exhibited a high promoter activity in both treated and untreated seedlings (Fig. 8 A). There were significant and high promoter activity in the root system of most of the lines in both dehydration and non-stress conditions. Fully grown flowering plants were treated by stopping irrigation for 8 days to evaluate the promoter activity in dehydrated flowers and siliques, as well as the same lines were kept watered for 8 days as control samples. High promoter activity was observed in flowers and siliques of both treated and untreated plants lines 1, 6, 9 and 10. Transgenic line 2 showed high promoter activity after dehydration in the flowers compared to non-stress conditions. There was no promoter activity in flowers and siliques of lines 3, 7 and 8. Promoter activity was detected in flowers of line 4 after dehydration and non-stress conditions (Fig. 8 B).

The seeds of the ten T2 lines were scratched and the GUS activity was detected as described in 2.2.4.6. High promoter activity was observed in all seeds of the T2 lines as a result of *CpPTP-1* promoter activation during seed development (Fig. 9 A). The leaves of all T2 lines were subjected to 200 mM NaCl for 24 hours. The *CpPTP-1* promoter under salt treatment showed the same GUS activity as non-stress conditions (Fig. 9 B). The 4 week-old T2 lines were subjected to 100 µM ABA for 2 h. High promoter activity was observed in 4 week-old seedlings after ABA treatment and non-stress conditions (Fig. 9 C).

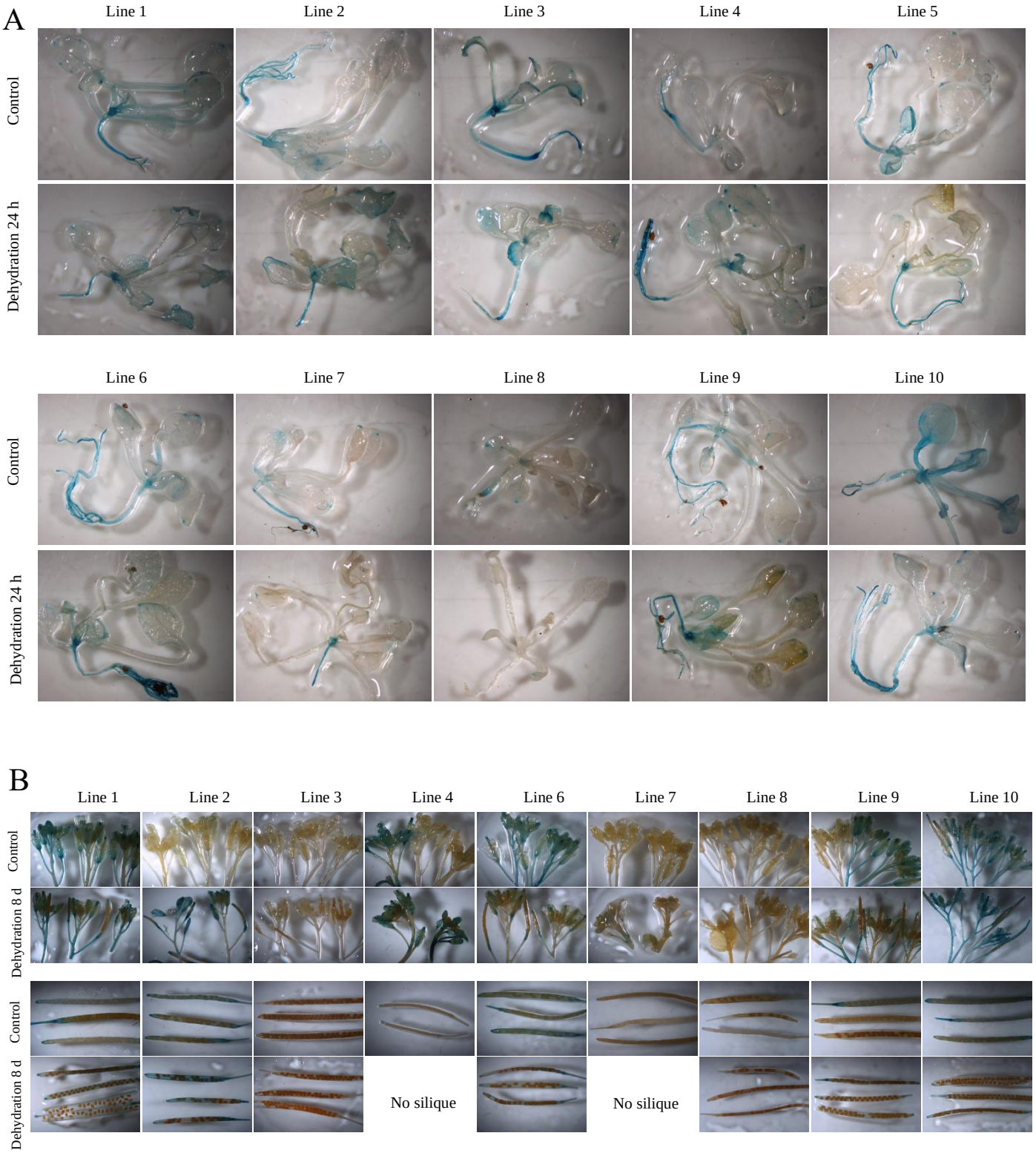


Fig. 8 Evaluation of *CpPTP-1* promoter activity through GUS-staining assay of second generation (T2) of transgenic *A.thaliana* lines at different growth and development stages. (A) Dehydration of the all 4 week-old transgenic lines for duration of 24 h. (B) Dehydration through withholding watering of all the flowering lines except line 5 for duration of 8 days to evaluate *CpPTP-1* promoter activity in flowers and siliques. Note, line 5 did not produce any flowers and lines 4 and 7 did not produce any siliques at the 8th day of dehydration treatment.

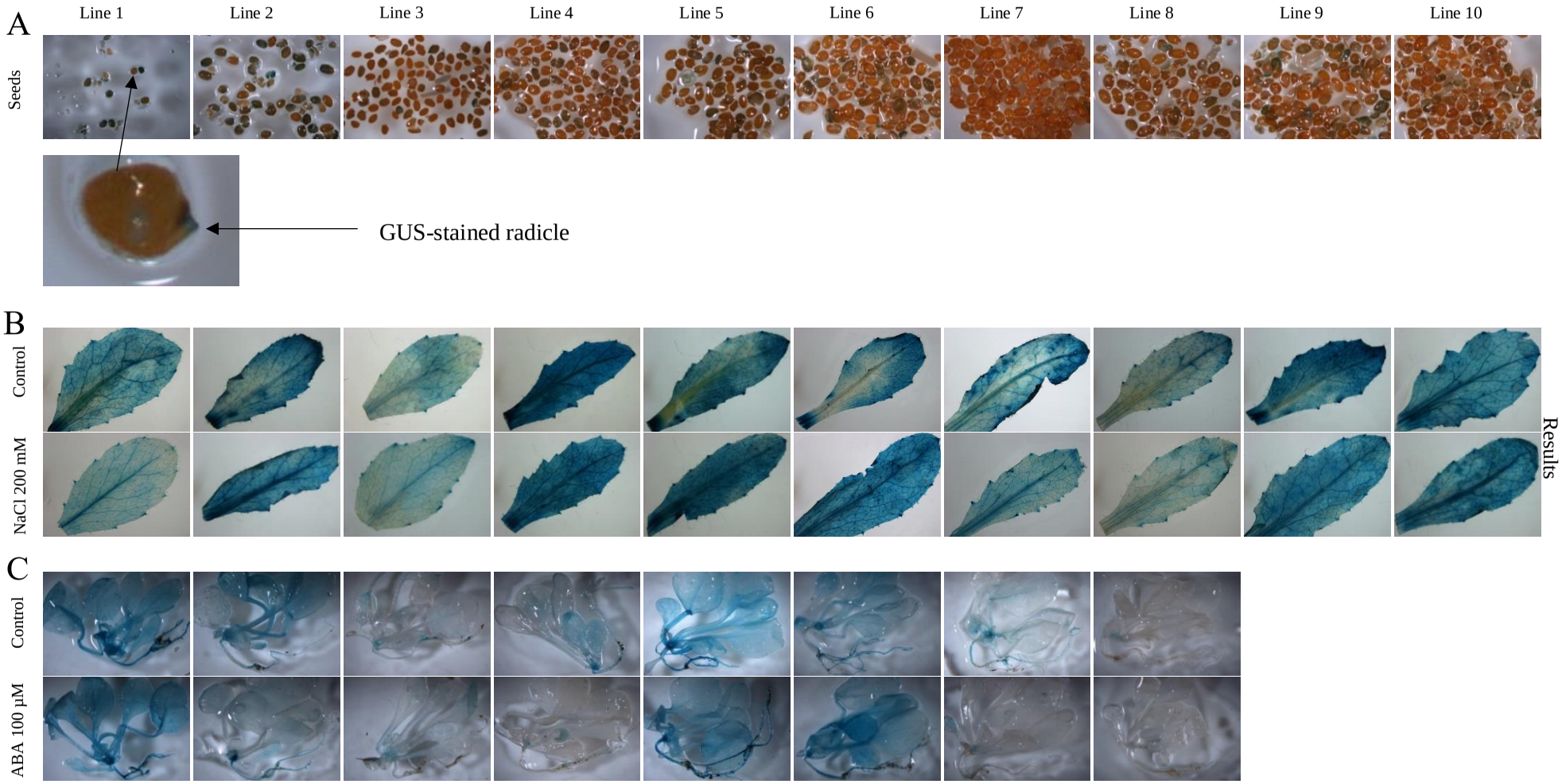


Fig. 9 Evaluation of *CpPTP-1* promoter activity through GUS-staining assay of second generation (T2) of *A.thaliana* lines at different growth and development stages. (A) All the ten lines seeds were scratched and assayed for the promoter activity. (B) Nacl 200 mM treatment of all the ten transgenic lines leaves for 24 hours. (C) 100 µM ABA treatment of the 4 week-old lines for 2 h.

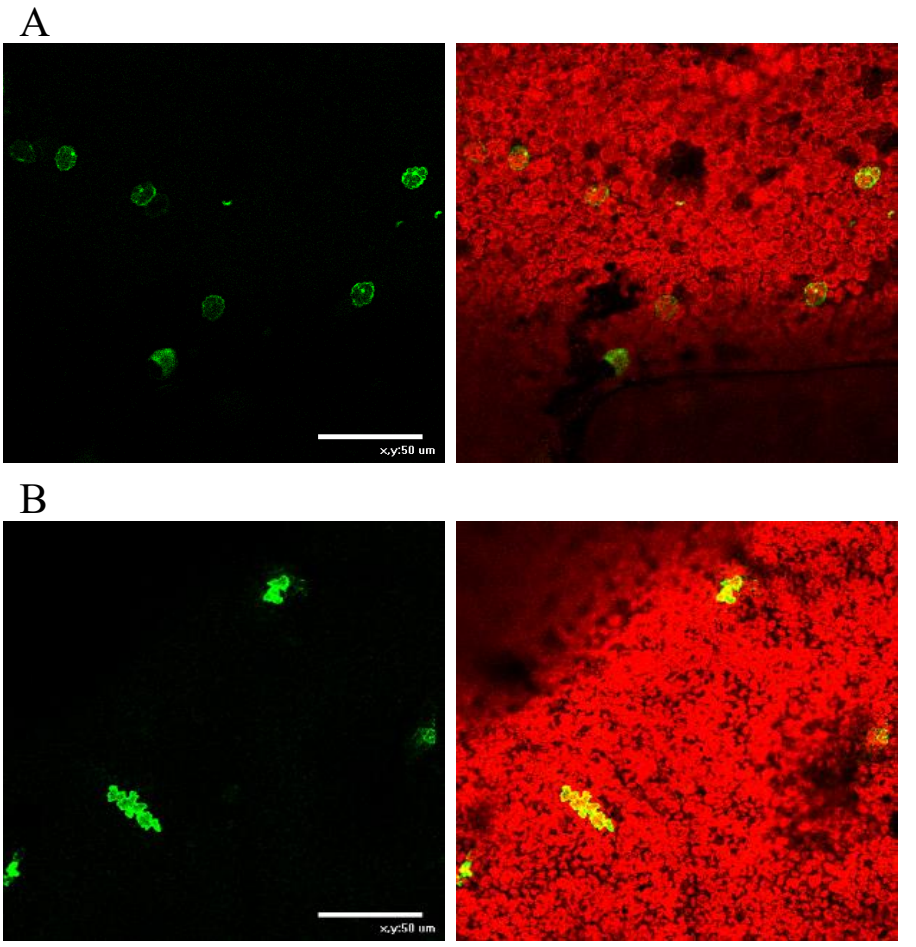
3.5.3 Transient transformation of the *CpPTP-1* promoter in *A. thaliana* and *C. plantagineum*

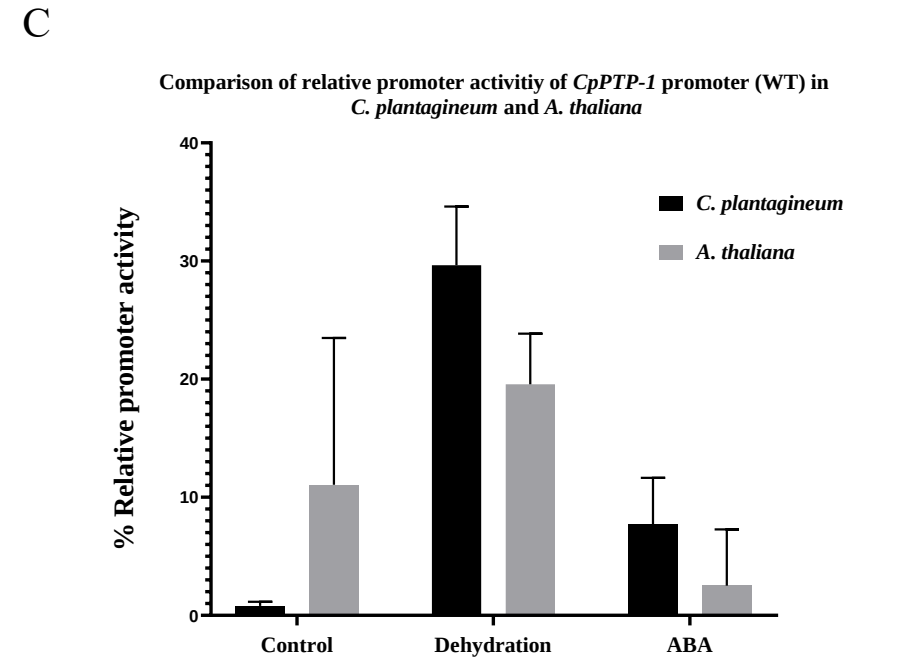
The *CpPTP-1* promoter activity was analyzed in two systems: in a homologous system *C. plantagineum* and in a heterologous system *A. thaliana*.

To further evaluate the *CpPTP-1* promoter activity, different *CpPTP-1* promoter fragments with the corresponding *cis*-acting elements::*GUS* fusion constructs including wild-type promoter, deletion construct 1 and 2, site-directed mutagenesis constructs MYC and DRE were designed and constructed (Fig. 2). All the *CpPTP-1* promoter::*GUS* fusion constructs were co-bombarded with the pGJ280 plasmid.

3.5.3.1 Evaluation of the *CpPTP-1* wild-type (WT) promoter

The 637 bp long promoter fragment of the *CpPTP-1* as a wild-type (WT) promoter was fused to the plasmid pBT10gus. The resulting pBT10gus-CpPTP-1 plasmid was transiently transformed into *C. plantagineum* and *A. thaliana* leaves via particle bombardments as described in 2.2.4.5.3. On the basis of the results obtained from transient transformation, GFP activity was detected as an internal standard in transformed leaves to measure the efficiency of each particle bombardment (Fig. 10A,B). The results showed higher promoter activity after dehydration than non-stress conditions in *C. plantagineum* and *A. thaliana* (Fig. 10 C). Although, the *CpPTP-1* promoter activity increased in *C. plantagineum* after ABA treatment compared to non-stress conditions, the *CpPTP-1* promoter activity decreased after ABA treatment in comparison to non-stress conditions in *A. thaliana*. The results showed that the dehydration has a greater effect on the promoter activity than ABA treatment and subsequently increased the activity of promoter in both *C. plantagineum* and *A. thaliana*. Higher promoter activity was observed in *A. thaliana* in comparison to *C. plantagineum* in non-stress conditions (Fig. 10 C).





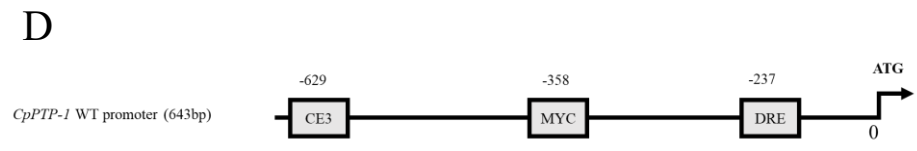


Fig. 10 Transient transformation of *CpPTP-1* wild-type (WT) promoter in *C. plantagineum* and *A. thaliana*. (A) GFP activity in *C. plantagineum* leaves. (B) GFP activity in *A. thaliana* leaves. (C) Relative promoter activity of *CpPTP-1* WT promoter during dehydration, ABA treatment and non-stress conditions. (D) Schematic map of the important *cis*-acting elements of the *CpPTP-1* WT promoter.

3.5.3.2 Evaluation of deletion construct 1 of the *CpPTP-1* promoter

The deletion construct 1 of the *CpPTP-1* promoter was generated as the shortest promoter::*GUS* construct with a length of 256 bp to examine the importance of the DRE *cis*-acting element in the absence of the other *cis*-acting elements. The resulting construct was co-bombarded with the pGJ280 plasmid on *C. plantagineum* and *A. thaliana* leaves. The results showed no promoter activity of the deletion construct 1 in neither *C. plantagineum* nor *A. thaliana* during dehydration, ABA treatment and non-stress conditions. The comparison of the deletion construct 1 and WT promoter of the *CpPTP-1* shows significant differences of promoter activity in *C. plantagineum* and *A. thaliana* (Fig. 11A,B). These data indicate that DRE *cis*-acting element is not capable of regulating the *CpPTP-1* promoter in the absence of other *cis*-acting elements and requires other partners to act as a dehydration responsive *cis*-acting element. This demonstrates that other *cis*-acting elements of *CpPTP-1* promoter such as CE3 or MYC may play an important role in activation of the *CpPTP-1* promoter.

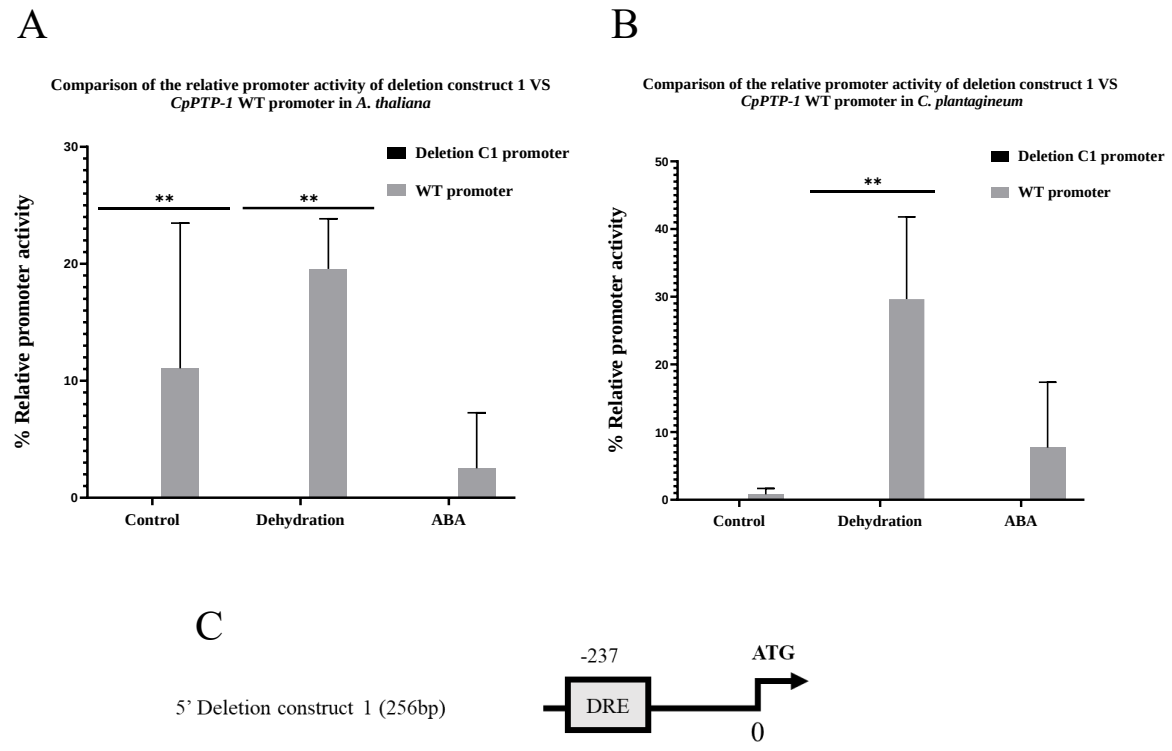


Fig. 11 Transient transformation of deletion construct 1 of the *CpPTP-1* promoter in *C. plantagineum* and *A. thaliana*. (A) Relative promoter activity of deletion construct 1 compared to WT *CpPTP-1* promoter during dehydration, ABA (1 mM) treatment and non-stress conditions in *A. thaliana* (B) Relative promoter activity of deletion construct 1 compared to WT *CpPTP-1* promoter during dehydration, ABA treatment and non-stress conditions in *C. plantagineum*. (C) Schematic map of deletion construct 1 of the *CpPTP-1* promoter. The graphs were drawn and data were statistically analyzed using GraphPad Prism 8. TWO-way analysis of variance (ANOVA) was applied to the relative promoter activity data. Signs of * and ** represent significance at 5% (*P<0.05) and 1% (**P<0.01) probability levels, respectively.

3.5.3.3 Evaluation of deletion construct 2 of the *CpPTP-1* promoter

The deletion construct 2 of the *CpPTP-1* promoter was generated to examine the importance of the coupling element 3 (CE3) and its contribution to the activity of the *CpPTP-1* promoter. For this purpose, the CE3 was deleted and a 447 bp fragment of the *CpPTP-1* promoter containing MYC and DRE *cis*-acting elements was fused to the pBT10gus plasmid. After deleting the CE3, a significant difference was observed in *CpPTP-1* promoter activity in *C. plantagineum* and *A. thaliana* under dehydration and ABA treatment in comparison to normal conditions (Fig. 12 A). The results showed higher promoter activity in *A. thaliana* compared to *C. plantagineum* during dehydration and ABA treatment. In *A. thaliana* and *C. plantagineum*, the highest promoter activity was observed after dehydration (Fig. 12 A). The comparison of the deletion construct 2 with WT *CpPTP-1* promoter in *C. plantagineum* showed a significant decrease after deleting the CE3 *cis*-acting element during dehydration. There was a slight decrease after deleting the CE3 *cis*-acting element after ABA treatment (Fig. 12 B). The results indicate that CE3 plays a crucial role in higher activity of the *CpPTP-1* promoter, particularly during dehydration in *C. plantagineum*. In *A. thaliana*, I observed higher promoter activity after deleting the CE3 *cis*-acting element during dehydration and ABA treatment (Fig. 12 C). The results indicate two different reactions of deletion construct 2 to dehydration and ABA treatment between *C. plantagineum* as a homologous system and *A. thaliana* as a heterologous system.

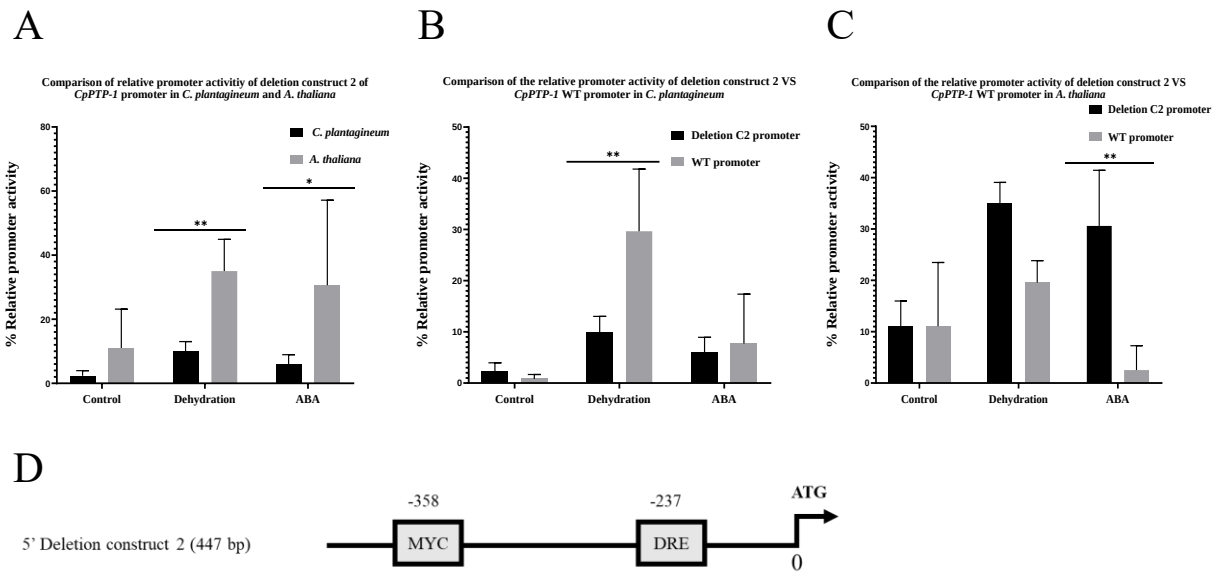


Fig. 12 Transient transformation of deletion construct 2 of the *CpPTP-1* promoter in *C. plantagineum* and *A. thaliana* during dehydration, ABA (1 mM) treatment and non-stress conditions. (A) Relative promoter activity of deletion construct 2 in *C. plantagineum* and *A. thaliana*. (B) Relative promoter activity of deletion construct 2 compared to WT *CpPTP-1* promoter in *C. plantagineum*. (C) Relative promoter activity of deletion construct 2 compared to WT *CpPTP-1* promoter in *A. thaliana*. (D) Schematic map of deletion construct 2 of the *CpPTP-1* promoter. The graphs were drawn and data were statistically analyzed using GraphPad Prism 8. TWO-way analysis of variance (ANOVA) was applied to the relative promoter activity data. Signs of * and ** represent significance at 5% (*P<0.05) and 1% (**P<0.01) probability levels, respectively.

3.5.3.4 Site-directed mutagenesis of the MYC *cis*-acting element

A single nucleotide mutation was generated in the MYC *cis*-acting element to investigate the role and effect of MYC *cis*-acting element in regulating the activity of the *CpPTP-1* promoter. The comparison of the deleted MYC-*CpPTP-1* promoter::*GUS* construct (MYC-*CpPTP-1*) showed a significant and higher promoter activity in *A. thaliana* than *C. plantagineum* under dehydration, ABA treatment and non-stress conditions (Fig. 13 A). I compared the activity of the MYC-*CpPTP-1* promoter against the wild-type promoter (WT *CpPTP-1* promoter). The result showed a dramatic decrease in the activity of the *CpPTP-1* promoter after deleting the MYC *cis*-acting element in *C. plantagineum* gene. The WT *CpPTP-1* promoter exhibited a significantly higher activity than MYC-*CpPTP-1* promoter under dehydration, ABA treatment and non-stress conditions in *C. plantagineum*. The *CpPTP-1* promoter carrying the deleted MYC *cis*-acting element showed a slight activity under dehydration but significantly lower activity than the wild-type promoter in *C. plantagineum* (Fig. 13 B). The results demonstrated that MYC plays an important role in the activity of the *CpPTP-1* promoter and consequently regulation of the expression of the *CpPTP-1* gene on the transcriptional level. On the other hand, the result indicated that MYC-*CpPTP-1* has still high promoter activity compared to WT *CpPTP-1* promoter in *A. thaliana* under dehydration and ABA treatment. The activity of the MYC-*CpPTP-1* promoter under dehydration was higher than ABA treatment and both conditions showed higher activity than the non-stress conditions (Fig. 13 C).

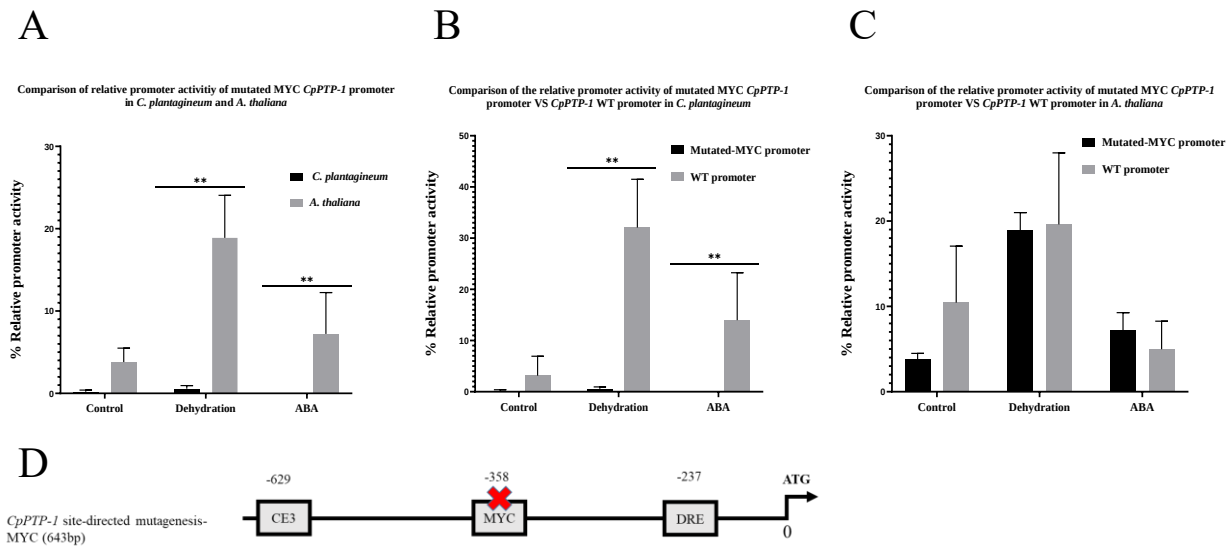


Fig. 13 Transient transformation of Site-directed mutagenesis of the MYC *cis*-acting element of the *CpPTP-1* promoter in *C. plantagineum* and *A. thaliana* during dehydration, ABA (1 mM) treatment and non-stress conditions. (A) Relative promoter activity of MYC-*CpPTP-1* promoter in *C. plantagineum* and *A. thaliana*. (B) Relative promoter activity of MYC-*CpPTP-1* promoter compared to WT *CpPTP-1* promoter in *C. plantagineum*. (C) Relative promoter activity of MYC-*CpPTP-1* promoter compared to WT *CpPTP-1* promoter in *A. thaliana*. (D) Schematic map of the MYC-*CpPTP-1* promoter. The graphs were drawn and data were statistically analyzed using GraphPad Prism 8. TWO-way analysis of variance (ANOVA) was applied to the relative promoter activity data. Signs of * and ** represent significance at 5% (*P<0.05) and 1% (**P<0.01) probability levels, respectively.

3.5.3.5 Site-directed mutagenesis of the DRE *cis*-acting element

To expand the understanding of the *CpPTP-1* promoter activity, the core sequence of the DRE *cis*-acting element was knocked out through generating a single nucleotide mutation as described in part 2.2.4.5.2. The comparison of the deleted DRE *cis*-acting element promoter::*GUS* construct (DRE-*CpPTP-1* promoter) in *C. plantagineum* and *A. thaliana* showed promoter activity after both dehydration and ABA treatment. I observed that the DRE-*CpPTP-1* promoter has lower promoter activity in *C. plantagineum* than *A. thaliana* under dehydration. On the other hand, during ABA treatment the DRE-*CpPTP-1* promoter showed slightly higher activity in *C. plantagineum* than in *A. thaliana* (Fig. 14 A). The activity of the DRE-*CpPTP-1* promoter was compared to activity of the WT *CpPTP-1* promoter. It was observed that mutation in the DRE *cis*-acting element causes a significant reduction of the *CpPTP-1* promoter activity under dehydration and ABA treatment in comparison to the wild-type promoter. This indicates that DRE plays a significant role in *C. plantagineum* and removing its effect causes a big decrease in *CpPTP-1* promoter activity under stress treatment (Fig. 14 B). According to the results, the DRE-*CpPTP-1* promoter compared to the WT *CpPTP-1* promoter showed relatively high promoter activity in *A. thaliana* which is in contrast to the results from *C. plantagineum* (Fig. 14 C).

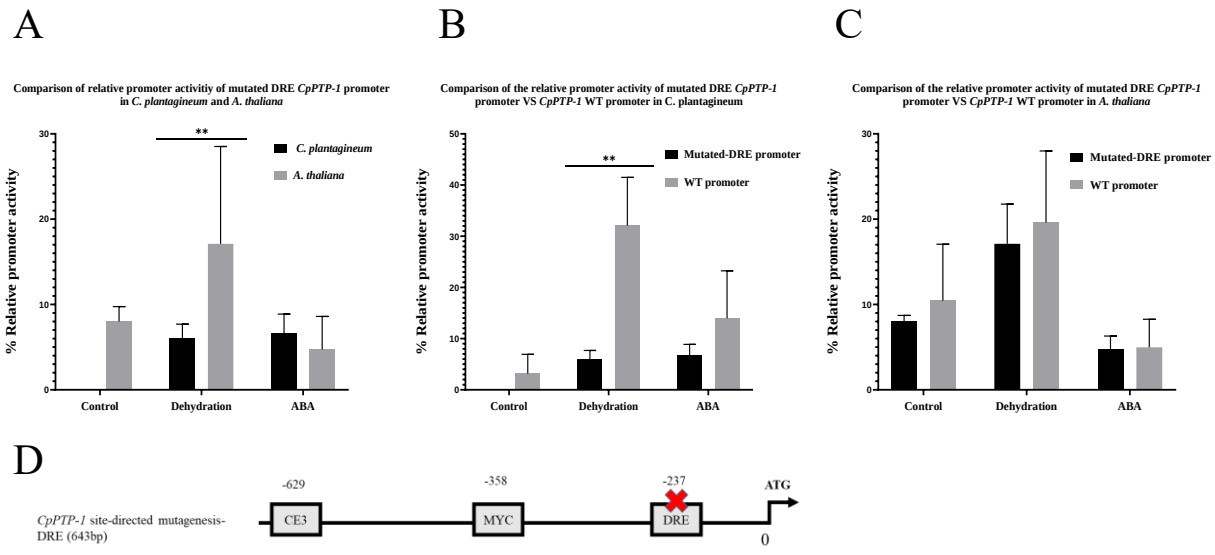


Fig. 14 Transient transformation of Site-directed mutagenesis of the DRE *cis*-acting element of the *CpPTP-1* promoter in *C. plantagineum* and *A. thaliana* during dehydration, ABA (1 mM) treatment and non-stress conditions. (A) Relative promoter activity of DRE-*CpPTP-1* promoter in *C. plantagineum* and *A. thaliana*. (B) Relative promoter activity of DRE-*CpPTP-1* promoter compared to WT *CpPTP-1* promoter in *C. plantagineum*. (C) Relative promoter activity of DRE-*CpPTP-1* promoter compared to WT *CpPTP-1* promoter in *A. thaliana.* (D) Schematic map of the DRE-*CpPTP-1* promoter. The graphs were drawn and data were statistically analyzed using GraphPad Prism 8. TWO-way analysis of variance (ANOVA) was applied to the relative promoter activity data. Signs of * and ** represent significance at 5% (*P<0.05) and 1% (**P<0.01) probability levels, respectively.

3.6 Overexpression of the CpPTP-1 protein

To overexpress the CpPTP-1 protein (PTP-1+6 N-His-tags 25.85 KDa), the coding sequence of the *CpPTP-1* was cloned into the expression vector pET28a+. The recombinant pET28a+-CpPTP-1 plasmid was transformed into the *E. coli* strains BL21 (DE3), RIPL and C43 as suitable strains tolerated to the toxic proteins. IPTG induction was carried out to induce the synthesis of the CpPTP-1 protein. 1 ml samples were taken before IPTG induction as t0 sample and 1 ml samples after 1 hour (t1), 3 hours (t3) and 5 hours (t5) of the induction. Total proteins of the collected samples were extracted using 1x Laemmli buffer (Laemmli 1970). The results showed no high protein expression after induction of different colonies (Fig. 15 A). Processing of the 1 ml-samples was done by sonication of the bacterial cells to find out if the target protein is accumulated in the insoluble protein fraction (pellets), mainly inclusion bodies or in the soluble protein fraction (supernatants) (Wingfield et al. 2001). The empty pET28a+ was used as control to distinguish the host proteins from CpPTP-1 recombinant proteins. A protein of around 25 KDa was observed, but the same band was detected in the empty pET28a vector as control in Coomassie stained SDS-PAGE gel and Western blot using His-tag antibody (Fig. 15B,C).

To overexpress the CpPTP-1 protein, the Gateway expression system was tested as described in part 2.2.5.2. Cloning of the CpPTP-1 protein (PTP-1+7 N-His-tags 25.85 KDa) in the gateway expression system was performed and the results showed no high protein expression. The recombinant PQlinkHD-CpPTP-1 plasmid was transformed into the *E. coli* strains BL21 DE3, RIPL and C43 as suitable strains tolerated to the toxic proteins. A comparison of empty *E. coli* strains BL21 as a control, pET28a- CpPTP-1 and PQlinkHD-CpPTP-1 was done (Fig. 15 D,E,F).

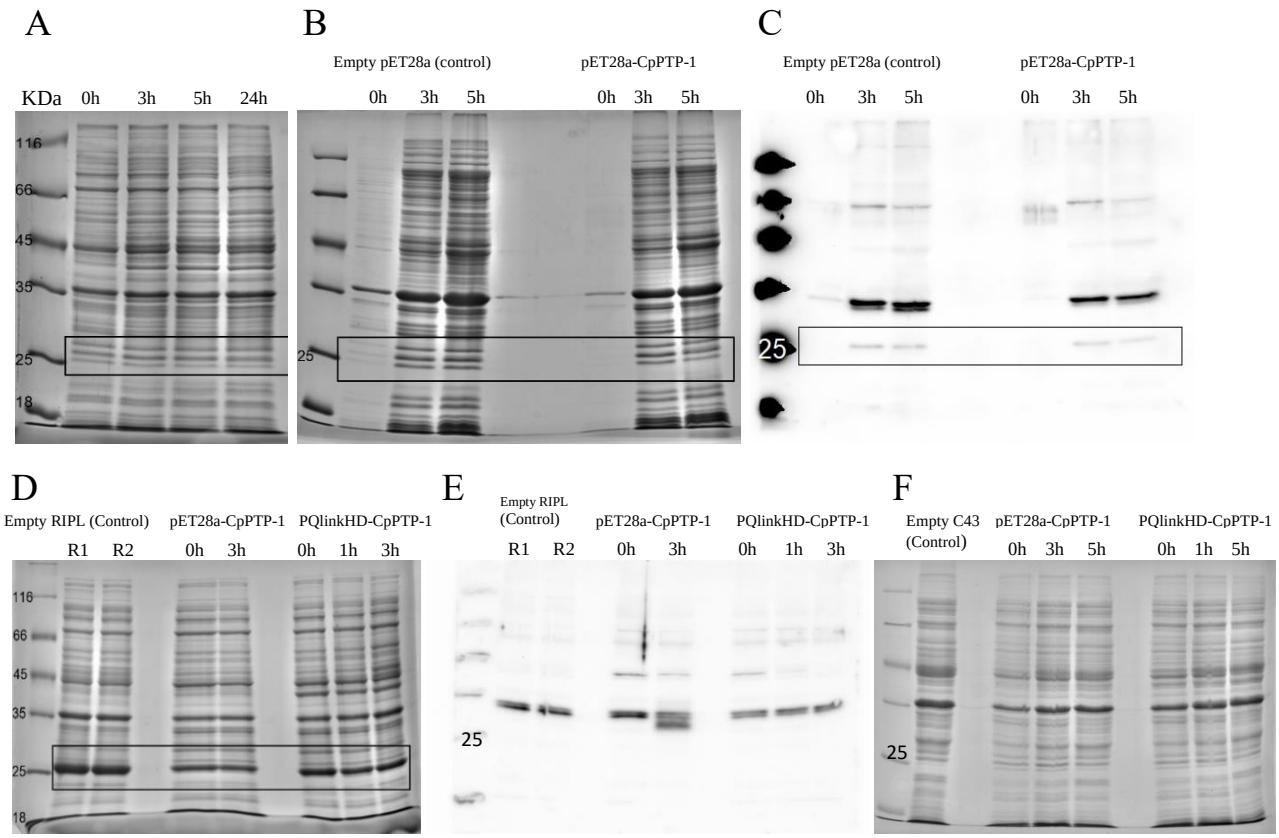


Fig. 15 Over expression of CpPTP-1 protein in pET28a+ and PQlinkHD expression systems. (A) Assessment of the CpPTP-1 protein induction by IPTG on the Coomassie stained SDS-PAGE gel. (B) Comparison of the pET28a-CpPTP-1 and empty pET28a (control) protein induction in *E. coli* strains BL21 DE3 on the Coomassie stained SDS-PAGE gel. (C) pET28a-CpPTP-1 and empty pET28a were separated via SDS-PAGE and transferred by electrophoresis to a nitrocellulose membrane for immunological analyses using His-tag antibody. (D) Comparison of the pET28a-CpPTP-1, PQlinkHD-CpPTP-1 and host proteins from empty E. coli strains BL21 RIPL (control) protein induction on the Coomassie stained SDS-PAGE gel. (E) pET28a-CpPTP-1, PQlinkHD-CpPTP-1 and host proteins from empty E. coli strains BL21 RIPL (control) were separated via SDS-PAGE and transferred by electrophoresis to a nitrocellulose membrane for immunological analyses using His-tag antibody. (F) Comparison of the pET28a-CpPTP-1, PQlinkHD-CpPTP-1 and host proteins from empty E. coli strains BL21 C43 (control) protein induction on the Coomassie stained SDS-PAGE gel. Replicates (R), induction by IPTG in hour (h).

To further test the overexpression of the CpPTP-1 protein, the signal peptide of the PTP protein was removed and the PTP protein without signal peptide was fused into PQlinkHD (PQlinkHD-CpPTP-1-no-SP) as a gateway expression system (PTP-1-no-SP+7 N-His-tags 20.35 KDa). The recombinant PQlinkHD-CpPTP-1-no-SP was transformed into the *E. coli* strains BL21 DE3 and C43 as suitable strains tolerated to the toxic proteins. IPTG induction was carried out to induce the synthesis of the CpPTP-1 protein. The proteins were separated and assayed through Coomassie stained SDS-PAGE gel and Western blot using His-tag antibody (Fig. 16A,B,C,D). The results did not show any overexpression of CpPTP-1 protein after IPTG induction in two different transformed colonies of BL21 DE3 and C43.

I cloned only the functional domain of the *CpPTP-1* into PQlinkHD (PTP-1-Functional domain (+301 to +603; 100 amino acids)+7 N-His-tags 13.42 KDa) to test if only the functional domain is able to overexpress in the *E. coli* strains BL21 DE3 and C43 as suitable strains tolerated to the toxic proteins. The results again did not show any overexpression of CpPTP-1 protein after IPTG induction in two different transformed colonies of BL21 DE3 and C43 (Fig. 16E,F).

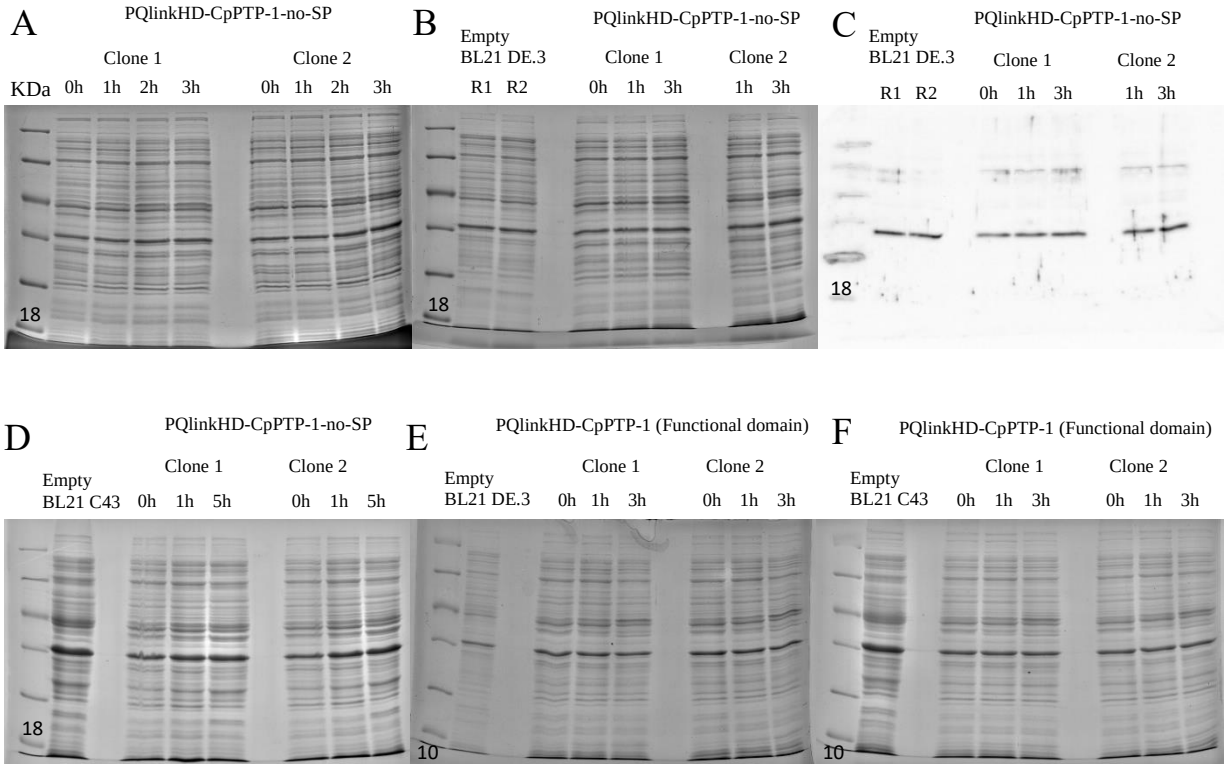


Fig. 16 Over expression of CpPTP-1 protein in PQlinkHD expression systems. (A) Assessment of the CpPTP-1 protein induction by IPTG on the Coomassie stained SDS-PAGE gel. (B) Comparison of the two colonies of PQlinkHD-CpPTP-1-no-SP and host proteins from empty *E. coli* strains BL21 DE.3 (control) after protein induction on the Coomassie stained SDS-PAGE gel. (C) Two colonies of PQlinkHD-CpPTP-1-no-SP and host proteins from empty *E. coli* strains BL21 DE.3 (control) were separated via SDS-PAGE and transferred by electrophoresis to a nitrocellulose membrane for immunological analyses using His-tag antibody. (D) Comparison of the two colonies of PQlinkHD-CpPTP-1-no-SP and host proteins from empty *E. coli* strains BL21 C43 (control) after protein induction on the Coomassie stained SDS-PAGE gel. (E and F) Comparison of the two colonies of PQlinkHD-CpPTP-1-Functional domain and host proteins from empty *E. coli* strains BL21 DE.3 and C43 (control) after protein induction on the Coomassie stained SDS-PAGE gel. Replicates (R), induction by IPTG in hour (h).

3.7 Protein-protein interaction

Identification of interacting proteins as partners of a known protein is a part of an understanding of a protein function. The interaction of CpPTP-1 with other proteins as partners was carried out using a yeast two-hybrid screening to identify proteins interacting with the CpPTP-1 protein.

3.7.1 Yeast two-hybrid screening (Y2H)

The pAS2-CpPTP-1 construct presents only the functional domain of the CpPTP-1 protein (+301 to +603; 100 amino acids) which was used as a bait to isolate any possible interacting protein as a prey from *C. plantagineum* pAD-GAL4 plasmid library. The bait and prey were transformed into the yeast Y190 strain. The transformation was confirmed on the SD agar plate without Leu and Trp as a selective medium 1 (Fig. 17 A). Forty colonies were selected and further investigated on the selective medium 2 without Leu, Trp and His including 30 mM 3-AT for the evaluation of expression of HIS3 gene as a reporter gene in a specific protein-protein interaction (Fig. 17 B). These forty colonies were further analyzed using PCR with pAD-GAL4 forward (pAD-Fwd) and reverse (pAD-Rev) primers to identify the proteins (Fig. 17 C).

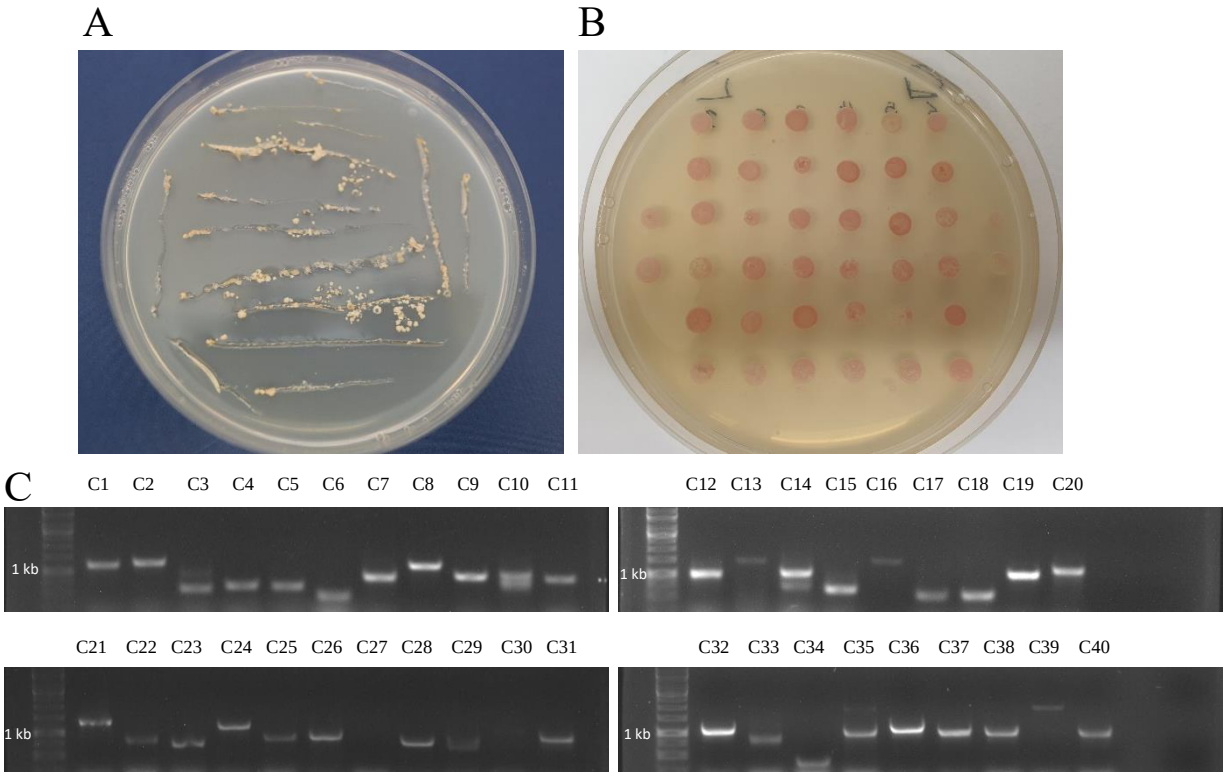


Fig. 17 Protein-protein interaction through yeast two-hybrid screening. (A) SD agar plate without Leu and Trp as a selective medium 1 to confirm the yeast transformation. (B) SD agar plate without Leu, Trp and His including 30 mM 3-AT as selective medium 2 to confirm the protein-protein interaction. (C) PCR products of forty colonies separated on the 1 % agarose gel. Clone (C).

3.7.2 Identification of interacting proteins

The PCR products of fourteen colonies out of the forty colonies from selective medium 2 were sequenced using pAD-GAL4 forward primer. The sequences were analyzed using the NCBI website. In this case, different interacting proteins were identified (Table 3). A protein from clone 12 which belongs to lipocalin protein family is similar to temperature-induced lipocalin (TIL) and chloroplastic lipocalin (CHL) proteins from *A. thaliana.* Phillips et al. (2002) reported that the PTP protein may protect chloroplast structures during dehydration. Another study showed that *A. thaliana* Chloroplastic lipocalin (AtCHL) protects thylakoid membrane lipids against reactive oxygen species (Levesque-Tremblay et al. 2009). On the basis of this matter that PTP and CHL proteins are involved in protection of chloroplast structures during stresses, clone 12 was chosen for further investigations.

Table 3 Fourteen interacting proteins with CpPTP-1 protein from yeast two-hybrid screening

Clone number	Protein name
2	SER/ARG-rich protein 34A
5	PLATZ transcription factor family protein
6	Heat shock factor 7
7	1-aminocyclopropane-1-carboxylate (ACC) oxidase
8	Adenosylhomocysteinase-like (SAHase)
12	Temperature-induced lipocalin or Chloroplastic lipocalin
16	Aldolase superfamily protein
20	late embryogenesis abundant protein pcC27-45
21	Eucalyptus grandis polyubiquitin-like
22	60S ribosomal protein (RP) L12
25	Histone H2B
32	60S ribosomal protein L10
33	UBC32, Ubiquitin-conjugating enzyme E2 32
36	Late embryogenesis abundant protein, putative / LEA protein

3.7.3 Interaction between CpPTP-1 and *C. plantagineum* Chloroplastic lipocalin (CpCHL)

A targeted yeast two-hybrid screening was performed to test the specific interaction between CpPTP-1 and CpCHL. The pAD-GAL4-CpCHL was constructed as a prey as described in part

2.2.6.5. The pAS2-CpPTP-1 construct was used as a bait for the yeast transformation. The yeast transformation of pAD-GAL4-CpCHL and pAS2-CpPTP-1 recombinant plasmids was considered as a positive control. The pAS2-CpPTP-1 and empty pAD-GAL4 were transformed into the yeast as a negative control. The yeast transformations of both positive and negative controls were confirmed by growing the yeast colonies on the SD agar plate without Leu and Trp as a selective medium 1 (Fig. 18A,B). The specific interaction of pAS2-CpPTP-1 and pAD-GAL4-CpCHL was tested by transferring the colonies from selective medium 1 to selective medium 2. Growth on the selective medium 2 without Leu, Trp and His including 30 mM 3-AT confirmed the specific interaction of pAS2-CpPTP-1 and pAD-GAL4-CpCHL. The results showed that yeast colonies (five colonies with three replicates) which contain prey and bait are able to grow on the selective medium 2 as positive control transformation (Fig. 18 C). On the other hand, no colonies were able to grow on the selective medium 2 in the negative control transformation (Fig. 18 D). The results indicate that there is a specific interaction between CpPTP-1 and CpCHL proteins in yeast, suggesting the same interaction in *C. plantagineum* probably during abiotic stress.

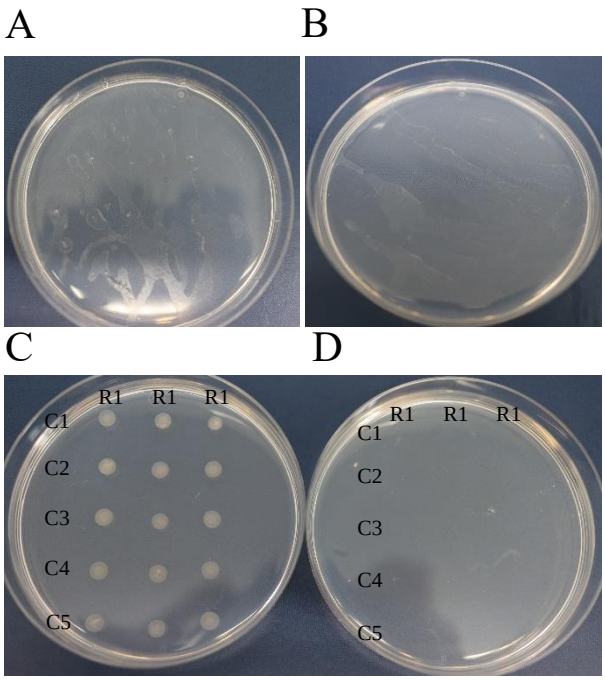


Fig. 18 specific protein-protein interaction through targeted yeast two-hybrid screening. (A) Transformation of positive control on the SD agar plate without Leu and Trp as a selective medium 1 to confirm the yeast transformation. (B) Transformation of negative control on the SD agar plate without Leu and Trp as a selective medium 1 to confirm the yeast transformation. (C) Positive control interaction on the SD agar plate without Leu, Trp and His including 30 mM 3-AT as selective medium 2 to confirm the protein-protein interaction. (D) Negative control interaction on the SD agar plate without Leu, Trp and His including 30 mM 3-AT as selective medium 2 to confirm the protein-protein interaction. Clone (C), Replicates (R).

3.8 Evaluation of *CpCHL* gene expression changes during desiccation and salt stress

The expression analysis of *CpCHL* on the transcript level was carried out under 150 mM and 250 mM NaCl treatment, desiccation and non-stress conditions in *C. plantagineum*. The *elongation factor 1a (EF1a)* was used as a reference gene to relatively normalize the expression data. The result showed a significant increase in expression of *CpCHL* in desiccation conditions compared to non-stress conditions. Treatment of *C. plantagineum* plants with 150 mM and 250 mM NaCl slightly increased the expression of *CpCHL* in comparison to non-stress conditions. Therefore, the *CpCHL* gene is induced under desiccation (Fig. 19A,B).

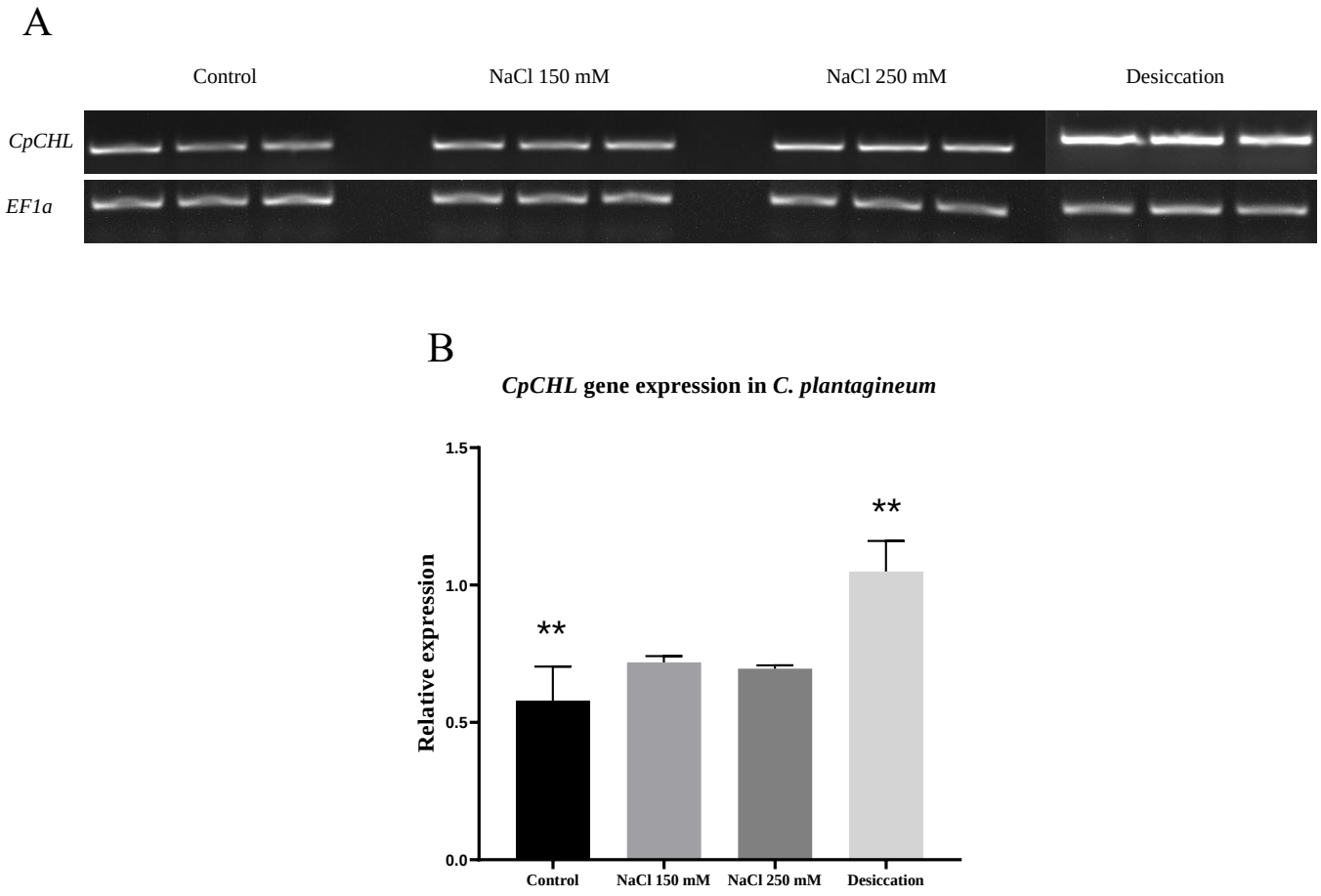


Fig. 19 Gene expression study of the *CpCHL* under NaCl 150 mM and 250 mM treatment, non-stress and desiccated *C. plantagineum* plants. *EF1a* gene was used as a reference gene. (A) RT-PCR of the *CpCHL*, and *EF1a* as a reference gene under different conditions. (B) Relative expression of the *CpCHL* under different conditions. The graphs were drawn and data were statistically analyzed using GraphPad Prism 8. One-way analysis of variance (ANOVA) was applied to the gene expression data. Signs of * and ** represent significance at 5% (*P<0.05) and 1% (**P<0.01) probability levels, respectively.

3.9 Subcellular localization of the CpCHL in *C. plantagineum*

The subcellular localization of the CpCHL was performed to further analyze the CpCHL in *C. plantagineum* leaves. For this, two GFP fusion constructs using the pGJ280 plasmid were generated. The CpCHL protein signal peptide (0 to +150; 50aa) was fused to the GFP sequence as the first GFP fusion construct (pGJ280-CpCHL-sp) and the second GFP fusion construct contained entire CpCHL gene (pGJ280-CpCHL). The recombinant pGJ280-CpCHL-sp and pGJ280-CpCHL were transiently introduced by particle bombardment into *C. plantagineum* leaves. Signal of green fluorescence in the chloroplasts of the transformed leaves was imaged using a confocal laser-scanning microscope (Fig. 20). The subcellular localization of the pGJ280-sp-CpCHL and pGJ280-CpCHL revealed that the signal peptide of the CpCHL translocated the GFP protein and CpCHL protein into the chloroplasts (Fig. 20A,B). This indicates that the Chloroplastic lipocalin of *C. plantagineum* is a plastid targeted protein.

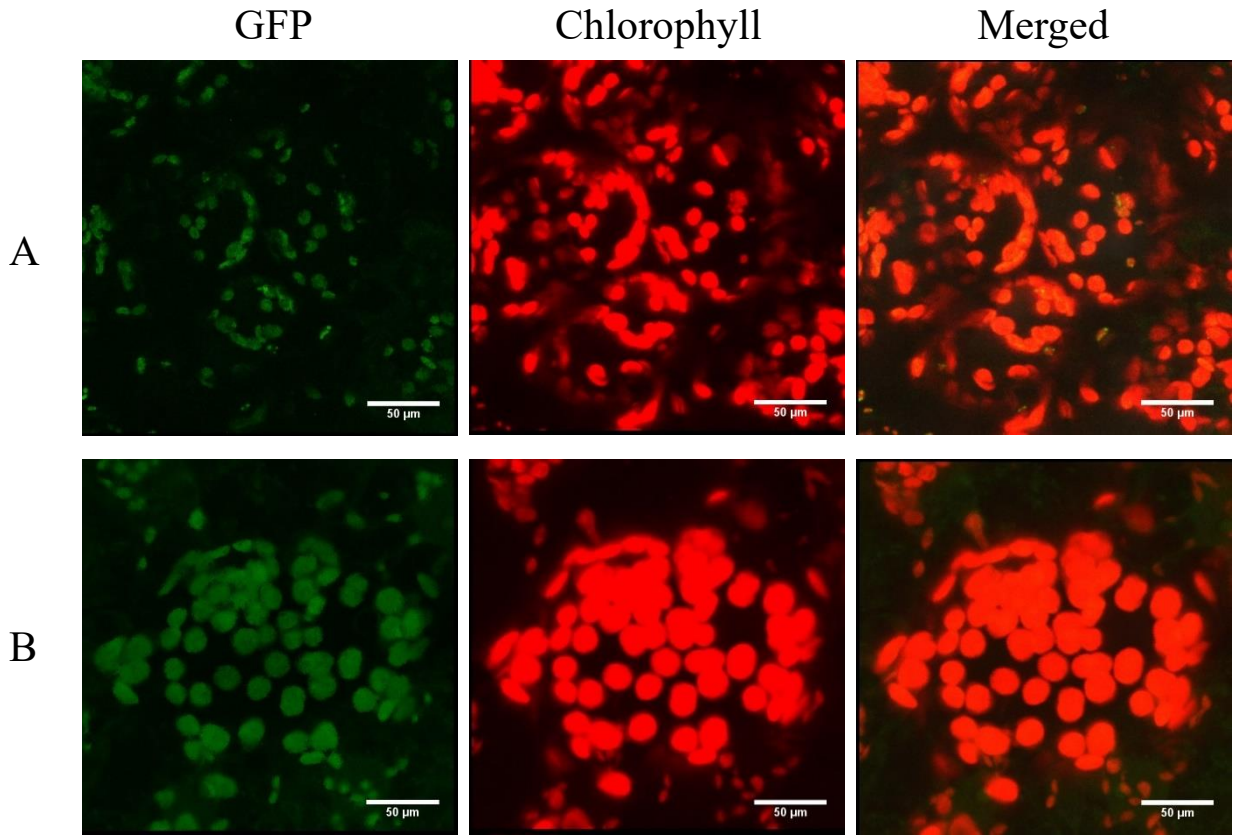


Fig. 20 Subcellular localization of the CpCHL protein. Fluorescence signals were visualized using confocal laser-scanning microscopy Leica DMi8. (A)The green fluorescent signals from the pGJ280- CpCHL-sp which resulted from a GFP fused to the signal peptide of the CpCHL. (B) GFP signals of the pGJ280-CpCHL which resulted from a GFP fused to the full-length CpCHL protein.

4. DISCUSSION

In this study, the transcriptional regulation and cellular function of a plastid-targeted protein (PTP) were investigated. Chloroplasts act as photosynthetic machinery and environmental sensors which optimize different cell functions in response to various stressors, but the mechanisms which are involved in plastids during stress conditions are still only partially understood (Tamburino et al. 2017). The chloroplast is a relatively autonomous organelle in terms of containing its own small genome which encodes up to 100 genes for its own crucial functions and most of the plastid-localized proteins are encoded by the nuclear genome and translocated into chloroplasts (Pfannschmidt et al. 2009). This indicates that there are complex communication mechanisms that optimize the expression of genes in the nucleus and plastids to balance the function of the cellular metabolism (Sun and Guo 2016; Joshi 2014). Phillips et al. (2002) reported the PTP protein family as plastid-targeted proteins that are responsive to ABA and dehydration and thus may protect chloroplasts during dehydration via protein-DNA interaction in *C. plantagineum.* The PTP protein most probably plays a key role in protection of the photosynthetic machinery in chloroplasts during stresses, but this role has not been characterized so far.

Proteins rarely play their role independently and 80 % of the proteins operate their functions in interaction with other proteins to form functional complexes (Yanagida 2002; Berggard et al. 2007). Phillips et al. (2002) provided evidences that PTP proteins form coiled-coil interactions which could be a clue to unravel the PTP protein functions. Mier et al. (2017) reported that coiled-coil domains mediate the physical protein-protein interactions. The study of the protein-protein interactions is a necessary step to unravel the functions of the PTP protein family in plastids.

In addition to studies of the PTP protein function during desiccation stress, it is also necessary to investigate key players in expression of the corresponding gene at the transcriptional level to have a broad understanding of the *CpPTP* gene family during abiotic stress. Therefore, it is indispensable to characterize the key elements in the activation and suppression of expression during transcriptional regulation of the genes coding for the PTP protein family. The promoter regions of the genes, and particularly the corresponding *cis*-acting elements control the transcriptional regulation of the genes (Zou et al. 2011). Characterization of the *CpPTPs* promoter contributing *cis*-acting elements during stress is an important step to obtain a better understating of the regulation of the expression of the *CpPTP* gene family during abiotic stress.

4.1 Phylogenetic analysis of the PTP protein family

As shown in Fig. 3 A, eight members of the PTP protein family in *C. plantagineum*, *Lindernia brevidens* and *Lindernia subracemosa* share conserved amino acid residues. The phylogenetic analysis showed that CpPTP-1 and CpPTP-3 have a more recent common ancestor compared to other members of the PTP protein family. Phillips et al. (2008) reported that *L. brevidens* is a desiccation tolerant plant and a close relative of *C. plantagineum* from the Linderniaceae family. As it is reported in Fig. 3 C, the Lbr_002697-RA protein from *L. brevidens* shares a more recent common ancestor with the *C. plantagineum* CpPTP proteins than with the other proteins in *L. brevidens* and *L. subracemosa*. I suggest that the Lbr_002697-RA in *L. brevidens* may have the same function as CpPTP protein family.

4.2 Coiled-coil domains of the CpPTP-1 protein

The MARCOIL online software (Zimmermann et al. 2018) confirmed the existence of two coiled-coil domains at the C-terminal domain of the CpPTP-1 protein (Fig. 3 A) which had already been reported by Phillips et al. (2002). The study of the protein function in different cellular processes requires annotation of coiled-coils as structural domains of the proteins that participate in different biological interactions (Ludwiczak et al. 2019; Lebar et al. 2020). The structure of coiled-coils is conserved and functionally involved in vesicle tethering, catalytic activity as molecular spacers, chromosome segregation, recognition and cleavage of DNA molecule, controlling centriole architecture, communication in conformational change, and acting as molecular rulers and scaffolds (Truebestein and Leonard 2016).

4.3 A unique Methyl-accepting chemotaxis domain

The PTP proteins from *C. plantagineum* uniquely comprise a Methyl-accepting chemotaxis-like conserved domain (chemotaxis sensory transducer) as bacteria and archaea receptor (Fig. 3 B). The observation from other publications shows that there is not any other gene with Methyl-accepting chemotaxis-like conserved domain from other plant species. Methyl-accepting chemotaxis proteins (MCPs) are involved in cell survival, pathogenesis, and biodegradation, and thus play crucial roles in the adaption of bacteria to different environmental conditions, the MCPs mediate chemotaxis to different environmental and intracellular signals (Salah Ud-Din and Roujeinikova 2017). There are studies that show various aspects of cellular activities such as biogenesis of toxin (Harkey et al. 1994), biofilm (Hickman et al. 2005),

encystment and flagellum (Berleman and Bauer 2005), exopolysaccharide (Black and Yang 2004) and degradation of xenobiotic compounds (Luu et al. 2015) are influenced by MCPs regulatory roles. *Rhodospirillum centenum* is a photosynthetic bacterium and a MCP mediates the encystment of this bacterium, while the biogenesis of the cyst is crucial for the survival of the bacterium under adverse environmental conditions (Berleman and Bauer 2005). Based on these results, a *Rhodospirillum centenum* deletion mutant line of MCP (cyst defective strains) showed susceptibility to heat and desiccation in resistant cyst cells and showed 1000-fold reduction of the total viable cyst cells compared to wild-type cells (Berleman and Bauer 2005). It is suggested that desiccation tolerance has been evolved from cyanobacteria and algae ancestors to angiosperm plants through cyanobacteria-related endosymbionts and other photosynthetic bacteria which led to evolution of desiccation tolerance in chloroplasts of plants, and other bacteria-related endosymbionts which led to evolution of desiccation tolerance in mitochondria of plants (Gaff and Oliver 2013).

Based on the previous studies, the MCP domain of the CpPTP protein family may have been evolved from photosynthetic bacteria like *Rhodospirillum centenum* to *C. plantagineum*.

4.4 The toxicity of the CpPTP-1 protein for *E. coli* strain BL21

As it is described in part 3.6, I tried to overexpress the CpPTP-1 protein in *E. coli* strains BL21 (DE3), RIPL and C43 as suitable strains tolerated to the toxic proteins. Different constructs harboring different parts of the *CpPTP-1* including the entire coding sequence, coding sequence without signal peptide, and functional domain of the CpPTP-1 protein were expressed in different *E. coli* strains. None of them showed overexpression of the CpPTP-1 protein. Since the Methyl-accepting chemotaxis domain is involved in biogenesis of cholera toxin (Harkey et al. 1994), I suggest that CpPTP-1 Methyl-accepting chemotaxis domain probably produces a toxin for the *E. coli* strains BL21 (DE3), RIPL and C43, and even toxicity tolerant strains of *E. coli* such as RIPL and C43 were not able to overexpress the CpPTP-1 protein.

4.5 The promoter region of *CpPTP-1* contains putative regulatory *cis*-acting elements

The *CpPTP-1* promoter contains putative *cis*-acting regulatory elements such as DRE, MYC and coupling element 3 (CE3) which play regulatory roles in response to different stresses (Fig. 6). It is demonstrated that dehydration induces the accumulation of ABA, and consequently

ABA triggers the regulation of many dehydration-responsive genes (Narusaka et al. 2003). The existence of the ABA-responsive *cis*-acting element (ABRE; CACGTGGC) in the promoter region of many ABA-inducible genes was reported by Guiltinan et al. (1990); Bonetta and McCourt (1998). Hobo et al. (1999) demonstrated that the coupling element 3 (CE3) (ACGCGTGTCCTG) with a core sequence of A/GCGT is similar to the ABRE (ACGTGG/TC) sequence. Yamaguchi-Shinozaki and Shinozaki (1994) reported a dehydration-responsive *cis*-acting element (DRE; TACCGACAT) from the *A. thaliana rd29A* gene. It has been shown that the core motif of the DRE (A/GCCGAC) regulates the same functions as the DRE *cis*-acting element and could act as a coupling element of the ABRE *cis*-acting element (Narusaka et al. 2003). Based on the previous studies, the CE3 and ABRE *cis*-acting element are similar to each other. The CE3 *cis*-acting element (A/GCGT) of the *CpPPT-1* is similar to ABRE (ACGT) of the *Cp13-62* which is presented by Giarola et al. (2018) and associated with desiccation tolerance in *C. plantagineum*, and thus either the DRE-core motif could act as a coupling element of the CE3 or the CE3 could act as a coupling element of the DRE. Tran et al. (2004) reported that the *A. thaliana ERD1* gene contains a motif called MYC *cis*-acting element (CATGTG) within its promoter region which plays a crucial role in induction of *ERD1* gene expression under dehydration conditions. Therefore, the MYC *cis*-acting element may have the same role and regulate the induction of *CpPTP-1* gene expression in *C. plantagineum* under dehydration conditions.

4.5.1 Stably transformed *A. thaliana* plants show high activity of the *CpPTP-1* promoter

The results revealed that seeds of the stably transformed *A. thaliana* exhibited high promoter activity which indicates that *A. thaliana* transcription factors regulate the activation of the *CpPTP-1* promoter during seed development in *A. thaliana* (Fig. 9 A). Giarola et al. (2018) hypothesized that activity of Cp, Lb, and *Ls13-62* promoters in seeds of stably transformed *A. thaliana* shows that *cis*-acting regulatory elements which are involved for seed-specific expression are conserved among species. Accordingly, the corresponding *cis*-acting elements of the *CpPTP-1* promoter are probably conserved between *C. plantagineum* and *A. thaliana.*
The results show that the *CpPTP-1* promoter is a highly active promoter in the first generation (T1) of transgenic *A. thaliana* leaves. The heterologous expression in *A. thaliana* is responsive to different conditions. Slightly higher promoter activity was observed in untreated samples compared to the dehydrated samples of the T1 lines which can be due to the suppression of the

CpPTP-1 promoter activity during dehydration (Fig. 7). Four-week-old seedlings and fully grown flowering plants of the second generation (T2) of *A. thaliana* lines showed high *CpPTP-1* promoter activity at the same level under both dehydration and non-stress conditions (Fig. 8). High promoter activity was observed before and after NaCl and ABA treatment (Fig. 9B,C). It is worth to mentation that there was a difference in activity of the *CpPTP-1* promoter in the T1 and T2 generations of *A. thaliana* lines. The activity of the *CpPTP-1* promoter in the T1 generation of *A. thaliana* lines in non-stress conditions was slightly higher than in stress conditions but the activity of the *CpPTP-1* promoter in the T2 generation of *A. thaliana* lines was equal in stress and non-stress conditions, and promoter maintained high constitutive activity at the same level at all developmental stages during stresses and non-stress conditions. According to the results, heterologous expression of the *CpPTP-1* promoter maintains high activity throughout the life cycle of *A. thaliana* in plant organs such as leaf, stem, root, flower, silique and seed of *A. thaliana*. The *CpPTP-1* promoter behaves like a native constitutive promoter. This indicates that the *Arabidopsis* transcription factors can bind to the *CpPTP-1 cis*-acting element and regulate the activity of the promoter regardless of the treatment conditions.

Plant viruses and plant housekeeping genes are the main source of constitutive promoters (Hernandez-Garcia and Finer 2014). The *CaMV35S* promoter (Odell et al. 1985) is one of the most widely used viral promoters for different studies in molecular biology. Jiang et al. (2018) reported a native constitutive promoter from *A. thaliana* which is called serine carboxypeptidase-like gene (*AtSCPL30*) and confers constitutive promoter activity in most of the developmental stages of stably transformed *Nicotiana benthamiana*. The native plant promoters reduce the chance of transgene silencing in comparison to *CaMV35S* and take advantage of higher biosafety (Jiang et al. 2018). The features of the *CpPTP-1* promoter indicate that this promoter could be considered as a constitutive promoter in a heterologous system like *A. thaliana*. A novel plant constitutive promoter like *CpPTP-1* promoter may be applied either for molecular studies in *A. thaliana* or for plant biotechnology to produce transgenic crops with improved traits.

4.5.2 The *CpPTP-1* promoter is induced by dehydration in transiently transformed *C. plantagineum* and *A. thaliana* leaves

As presented in part 3.5.3.1, dehydration and ABA induce the activity of the *CpPTP-1* promoter compared to non-stress conditions, but dehydration leads to higher promoter activity in *C. plantagineum* than the constitutive expression. Dehydration is the primary stimuli of the

CpPTP-1 promoter and ABA as one of the consequent products of dehydration had less influence on the *CpPTP-1* promoter. The *CpPTP-1* promoter in *C. plantagineum* showed almost no activity in non-stress conditions (Fig. 10 C) which is in contrast to the *CpPTP-1* promoter activity in *A. thaliana*.

The *CpPTP-1* promoter was induced upon dehydration in *A. thaliana*, but it showed higher promoter activity in non-stress conditions than ABA treatment (Fig. 10 C). As it is discussed above, the *CpPTP-1* promoter is a highly active promoter in the T2 stably transformed *A. thaliana* plants regardless of the treatment conditions but it presented reduction of promoter activity under ABA treatment in transiently transformed leaves (Fig. 10 C). This reduction suggests that the *CpPTP-1* promoter can be stable over generations, and a stable transformation is more efficient than a transient one to use.

Giarola et al. (2018) reported that a promoter of *C. plantagineum Cp13-62* gene is highly active during dehydration in transiently transformed *C. plantagineum* leaves. Giarola et al. (2018) hardly found promoter activity in stably transformed *A. thaliana* plants in non-stress conditions but some after salt treatment. In contrast, high activity of the *CpPTP-1* promoter was found in stably transformed *A. thaliana* after exposure to stresses and non-stress conditions. It is suggested that the *CpPTP-1* promoter is stronger than the *Cp13-62* promoter in a heterologous system like *A. thaliana*.

4.5.3 The DRE *cis*-acting element of the *CpPTP-1* promoter does not play its regulatory role alone and requires other *cis*-acting elements

It was observed that deletion construct 1 which only contains the DRE *cis*-acting element showed no promoter activity in both *A. thaliana* and *C. plantagineum* (Fig. 11). On the other hand, in the deletion construct 2 with a longer sequence of the *CpPTP-1* promoter but still lacking the CE3 *cis*-acting element (GCGT), higher promoter activity was observed than for the deletion construct 1, although the activity was still significantly lower than the WT promoter in *C. plantagineum* (Fig. 12). In both deletion constructs, the CE3 *cis*-acting element was not present. The data provide evidence that a short motif including the four nucleotides GCGT as the core-sequence of CE3 are required for the function of the DRE element. The CE3 *cis*-acting element may act as a coupling element of the DRE. The promoter activity in deletion construct 2 indicates that other *cis*-acting elements also influence the promoter activity during dehydration and ABA treatment in *C. plantagineum*. Missihoun et al. (2014) reported an aldehyde dehydrogenase 7B4 (*ALDH7B4*) gene promoter that contains an ACGT motif as a

necessary coupling element of a DRE *cis*-acting element in *A. thaliana* under dehydration, ABA and NaCl treatments.

In another study, Giarola et al. (2018) provided evidence that the *Cp13-62* promoter with a deleted DRE *cis*-acting element showed a big loss in promoter activity in response to dehydration compared to the WT *Cp13-62* promoter. Van den Dries et al. (2011) reported that the DRE *cis*-acting element is essential for the activity of the *Cp LEA-like 11-24* gene during different stresses and combined mutations in ABRE1, ABRE2, ABRE3 and DRE motifs could deactivate the *Cp LEA-like 11-24* promoter region in *C. plantagineum*. To further investigate the role of the DRE *cis*-acting element in the *CpPTP-1* promoter, the DRE *cis*-acting element was deleted through site-directed mutagenesis. The *CpPTP-1* promoter lacking the DRE *cis*-acting element showed lower promoter activity than the WT promoter after dehydration, ABA treatment and non-stress conditions (Fig. 14). This finding further proves that the DRE plays a crucial role in the regulation of the *CpPTP-1* promoter in *C. plantagineum*. The DRE motif is a binding site for the DREB (dehydration-responsive element binding protein) transcription factors to regulate the expression of a gene through an ABA-independent pathway (Nakashima et al. 2009). Therefore, the DRE *cis*-acting element of the *CpPTP-1* is probably involved in the ABA-independent regulatory pathway of *CpPTP-1* transcription.

4.5.4 A mutation in the MYC *cis*-acting element abolishes activity of the *CpPTP-1* promoter

As shown in Fig. 13 a single mutation in the MYC *cis*-acting element caused a dramatic loss in the activity of *CpPTP-1* promoter after dehydration and ABA treatment. This shows that the MYC is another crucial *cis*-acting element in both dehydration- and ABA-induced *CpPTP-1* promoter activity in *C. plantagineum.* Tran et al. (2004) reported that three drought, high salinity and ABA inducible NAC transcription factors ANAC019, ANAC055, and ANAC072 bind to the MYC motif (CATGTG) of *ERD1* gene in *A. thaliana.* Another study showed that a chilling-induced gene *PsMPT* did not respond to chilling after deleting a MYC motif in the *PsMPT* promoter region, thus suggesting that the MYC *cis*-acting element plays a key role in response to chilling of the *PsMPT* gene (Zhang et al. 2016). Based on previous studies, I suggest that the MYC *cis*-acting element of the *CpPTP-1* promoter is essential for stress inducibility and may act as a binding site of NAC and MYC transcription factors in *C. plantagineum* during different stresses. Although NAC transcription factors are involved in ABA-independent pathways (Hu et al. 2008), the MYC transcription factors are involved in ABA-dependent

regulatory pathways (Abe et al. 1997). Therefore, the MYC *cis*-acting element of the *CpPTP-1* promoter may be involved in both ABA-independent and ABA-dependent regulatory pathways of initiation of transcription.

4.5.5 A combination of *cis*-acting elements is involved in regulation of the *CpPTP-1* promoter

The data indicate that the DRE or other *cis*-acting elements in the first 256 bp of the *CpPTP-1* promoter region are not sufficient for the promoter activity and other *cis*-acting elements are required for the promoter activity in response to stress (Fig. 11). Extension of the length of the *CpPTP-1* promoter resulted in a construct containing DRE and MYC but lacking CE3. This construct led to an increase in promoter activity but still lower than the WT promoter (Fig. 12). This shows the importance of the CE3 *cis*-acting element as a coupling element of either MYC or DRE. The importance of the DRE *cis*-acting element was demonstrated by deleting the DRE *cis*-acting element (Fig. 14). According to the observation of this study, I suggest that the DRE *cis*-acting element is essential but not sufficient for the high activity of the *CpPTP-1* promoter. Supporting data are derived from the fact that a mutation within the MYC *cis*-acting elements shuts down the *CpPTP-1* promoter activity (Fig. 13). Therefore, the MYC *cis*-acting element plays an even more important role than the DRE *cis*-acting element for the activity of the *CpPTP-1* promoter during dehydration and ABA treatment.

Taken together, I suggest that a combination of *cis*-acting elements including MYC, DRE and CE3 is involved in regulating transcription of the *CpPTP-1* promoter. Mutations in regulatory motifs of desiccation-related genes have (re-) evolved desiccation tolerance in vegetative plant tissues (Van den Dries et al. 2011). Mutations during evolutionary events may have organized this combination of *cis*-acting elements within the *CpPTP-1* promoter region through generating or deleting novel inducible regulatory *cis*-acting elements leading to develop desiccation tolerance in *C. plantagineum* under environmental pressure.

4.6 The CpPTP-1 protein participates in protein-protein interactions

To obtain further knowledge of the function of the CpPTP-1 protein, a yeast two-hybrid screening approach was performed to provide evidence of interaction of the CpPTP-1 protein with other proteins. Gaining knowledge of functions and properties of an unknown protein can be facilitated by identifying the interacting proteins, whose function and properties are already

well characterized. In most cases, proteins play their roles in contact with other proteins in functional complexes. It has been demonstrated that most of the proteins which interact with each other are involved in the same cellular functions (Yanagida 2002; Berggard et al. 2007; von Mering et al. 2002). Many studies have provided evidence that the interaction between proteins regulates crucial functions such as signal transduction, sensing the environmental stimuli, enzymatic signaling, maintaining the cellular metabolism, energy transmission within the cell structure, trafficking and scaffolding (Braun and Gingras 2012; Lebar et al. 2020).
The CpPTP-1 protein contains two coiled-coil domains at its C-terminal domain. Phillips et al. (2002) reported that the coiled-coil domains of the CpPTP may protect the structure of the chloroplasts through protein–DNA interaction. Jeong et al. (2003) reported MFP1 a nuclear-encoded protein from *A. thaliana* which binds to the various regions of chloroplast protein-DNA complexes (nucleoids) via coiled-coil structure. It is worth to note that the MFP1 is located in thylakoid membranes. Despite the role of coiled-coil domain in protection of chloroplast DNA, Mier et al. (2017); Lebar et al. (2020) reported that the coiled-coil domains mediate protein-protein interactions.

The yeast two-hybrid screening suggests that the CpPTP-1 protein interacts with a protein from the lipocalin protein family. Two lipocalin proteins including a temperature-induced lipocalin (TIL) as a plasma membrane localized protein and a chloroplastic lipocalin (CHL) as a chloroplast-localized protein have been described as stress-inducible proteins in *A. thaliana* (Abo-Ogiala et al. 2014; Levesque-Tremblay et al. 2009). The transcript of this interacting protein matched to a gene *Cpl_154816* from the unpublished *C. plantagineum* genome sequence.

4.6.1 The interacting lipocalin protein is localized in chloroplasts

Subcellular localization of the Cpl_154816 from the lipocalin protein family demonstrated that this protein is localized within the chloroplasts of *C. plantagineum* leaves (Fig. 20). Charron et al. (2005) reported a chloroplast-localized protein named TaCHL which is an abiotic stress-induced protein which enhances freezing tolerance of wheat. Charron et al. (2005) further provided evidence that lipocalin proteins are associated with desiccation tolerance in the red algae *Porphyra yezoensis* and cryotolerance in marine yeast *Debaryomyces hansenii*. On the basis of the subcellular localization of the CpPTP-1 interacting protein, this protein was named a *C. plantagineum* chloroplastic lipocalin (CpCHL).

4.6.2 Evaluation of gene expression during desiccation and salt stress

It is shown in Fig. 4 C and Fig. 19 B that the transcript level of *CpPTP-1* and *CpCHL* increased under desiccation conditions. This indicates a role for both genes during desiccation. NaCl treatment did not affect the expression level of *CpPTP-1* and *CpCHL* as much as desiccation. The expression level of *CpPTP-1* was reduced significantly under 150 mM NaCl treatment, but increased after 250 mM NaCl treatment. A relatively high expression was observed for both *CpPTP-1* and *CpCHL* transcripts in non-stress conditions, and this even increased in desiccation. High expression of stress-related genes in non-stress conditions was also found by Casaretto et al. (2016), they reported that maize *OsMYB55* over-expression line caused high expression of stress-related genes under normal conditions, the same genes were up-regulated during heat stress in WT plants.

I suggest that the expression of *CpCHL* and specially *CpPTP-1* in non-stress-conditions may be associated with desiccation tolerance in *C. plantagineum* as a defense system. Based on the discussion for the roles of Methyl-accepting chemotaxis-like conserved domain in bacterium, I suggest that the MCP domain of the CpPTP-1 protein may mediate chemotaxis to different environmental and intracellular signals as a part of the regulatory networks in cellular protection mechanism either in stress or non-stress conditions.

4.6.3 CpPTP-1 and CpCHL proteins may protect thylakoid membranes of chloroplasts of *C. plantagineum* leaves

A targeted yeast two-hybrid screening has confirmed the interaction between CpPTP-1 and CpCHL proteins in yeast (Fig. 18). Therefore, the same interaction is suggested in *C. plantagineum* in planta. Azzarito et al. (2013); Watkins et al. (2015) reported that protein-protein interaction is mediated by helical structures of proteins such as short α-helices or long twisted α-helices as coiled-coils. Therefore, it is suggested that the interaction between CpPTP-1 and CpCHL proteins is most probably mediated by coiled-coil domains of the CpPTP-1 protein and the α-Helix of the CpCHL protein (Fig. 21).

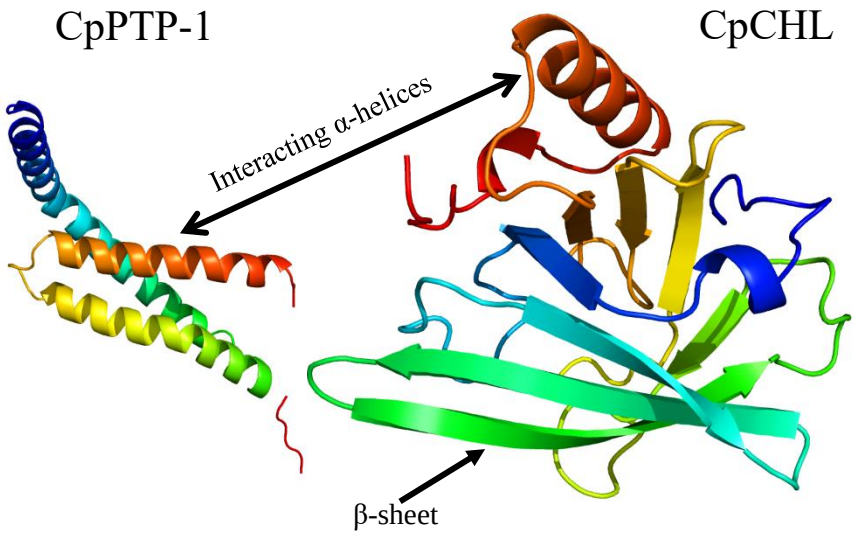


Fig. 21 Secondary structure of CpPTP-1 and CpCHL proteins. 3-D modeling was performed using Phyre2 web portal for protein modeling, prediction and analysis (Kelley et al. 2015). The interaction between CpPTP-1 and CpCHL is mediated by α-helical structures.

Abo-Ogiala et al. (2014) reported that less chlorophyll b was degraded under salt stress in the *A. thaliana* line in which a temperature-induced lipocalin from the salt resistant *Populus euphratica* is overexpressed. In contrast, the degradation of chlorophyll b and accumulation of chloride and sodium in chloroplasts were higher in *Attil1-1* and *Attil1-2* knock out and knock down lines of *A. thaliana* compared to WT plants under salt stress (Abo-Ogiala et al. 2014). Lipocalin proteins bind to iron and channel proteins. Therefore, Abo-Ogiala et al. (2014)

suggested that the *temperature-induced lipocalin* (*TIL*) is involved in salt-induced trafficking and regulation of ion homeostasis may play a central role in protection of chloroplasts during salt stress. Wahyudi et al. (2018) reported that knockdown tomato lines of *SlTIL1*, *SlTIL2* and *SlCHL* showed high accumulation of ROS such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), while they were low in tomato lines over-expressing *SlTIL1*, *SlTIL2* and *SlCHL* suggesting that lipocalins are involved in tolerance of tomato to oxidative stress.

Levesque-Tremblay et al. (2009) reported a *chloroplastic lipocalin AtCHL* from *A. thaliana* which is induced under drought stress, ABA treatment, exposure of high light and paraquat and is specifically localized in the lumen of thylakoids. They suggested that other proteins are probably involved in the transport of *AtCHL* into the lumen via protein-protein interaction. It was also shown by Levesque-Tremblay et al. (2009) that the knockout *A. thaliana* line of *AtCHL* showed high levels of hydroxy fatty acids as products of lipid peroxidation which is caused by singlet oxygen ($^1O_2$) as an oxidative damage under drought, high light and paraquat. In contrast, an *Arabidopsis* line overexpressing *AtCHL* tolerated those stresses and did not show high levels of lipid peroxidation. Levesque-Tremblay et al. (2009) further suggested that AtCHL protein protects thylakoid membrane lipids by preventing lipid peroxidation which is mediated by reactive oxygen species, particularly singlet oxygen. Another study by Boca et al. (2014) confirmed that AtTIL and AtCHL proteins play important roles in protection of lipids by preventing lipid peroxidation during abiotic stress. (Boca et al. 2014) found out that the AtCHL protein plays its role against lipid oxidation by protecting lipophilic antioxidants such as tocopherol as scavenger of $^1O_2$ and zeaxanthin which is involved in non-photochemical energy quenching (NPQ) as a process of dissipation of excess absorbed light energy in photoprotection.

Malnoe et al. (2018) renamed the chloroplastic lipocalin (*CHL*) as plastid lipocalin (*LCNP*). They reported a chloroplast-localized membrane protein from *A. thaliana* named suppressor of quenching1 (*soq1* or *qH*) which is involved in photoprotection through NPQ. It has been shown that LCNP is a necessary protein for qH either directly or indirectly and qH plays its photoprotection role via decreasing lipid peroxidation under cold and high light stress conditions, suggesting that LCNP enhances sustained NPQ in peripheral antenna (LHCII) by preventing singlet oxygen damage which protects thylakoid membranes of chloroplasts under stress conditions (Malnoe et al. 2018).

4.7 Conclusions

In Fig. 22, a schematic summary is illustrated to display the *CpPTP-1* gene action from transcription to protein production, and its interacting protein CpCHL roles under desiccation stress. The findings of this study suggest that the *CpPTP-1* promoter activity is correlated with transcript expression levels under dehydration and a combination of *cis*-acting elements including CE3, MYC and DRE is involved in regulating expression of the *CpPTP-1* gene on the transcriptional level in *C. plantagineum* under stress conditions (Fig. 22 A).

Based on the study of Levesque-Tremblay et al. (2009), the AtCHL protein is localized in the lumen of thylakoids and other proteins are probably involved in the transport of the AtCHL protein into the lumen via protein-protein interaction. Accordingly, the same function may occur in *C. plantagineum* and the CpPTP-1 and CpCHL proteins probably interact with each other and move inside the lumen of the thylakoid (Fig. 22 B).

On the basis of this study and previous studies, I suggest that the CpPTP-1 and CpCHL proteins form a complex to protect thylakoid membrane lipids of chloroplasts either by preventing lipid peroxidation through protecting lipophilic antioxidants such as tocopherol and zeaxanthin or by elevating sustained NPQ by preventing ROS damage (Fig. 22 B). According to this study and the study of Phillips et al. (2002), I suggest that the CpPTP-1 protein has a dual role and interacts with chloroplast DNA and lipocalin protein in parallel to probably play a protective role in chloroplasts during desiccation stress.

I suggest that the MCP domain of the CpPTP-1 protein may mediate chemotaxis to different environmental and intracellular signals in cellular protection mechanisms.

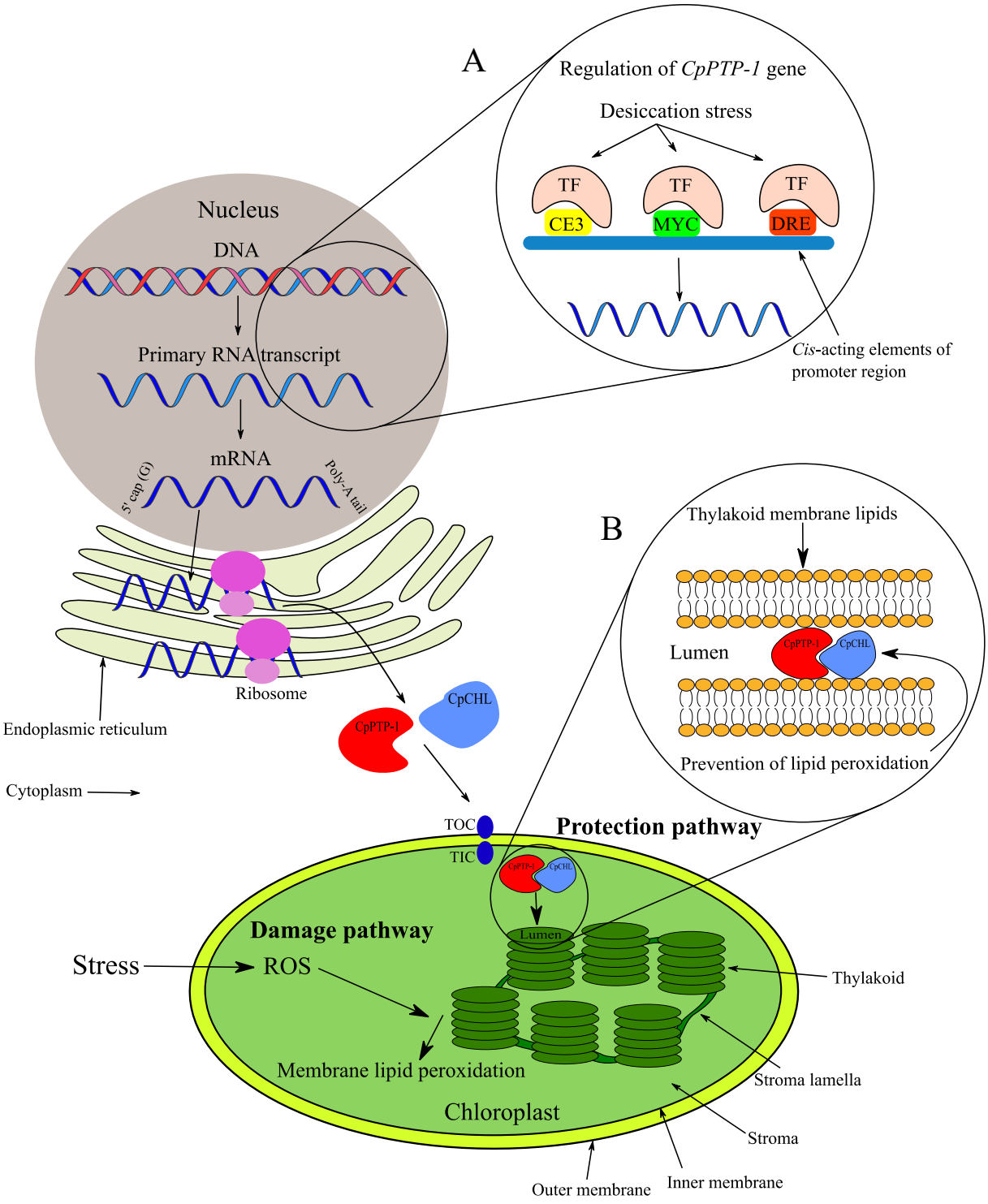


Fig. 22 A summary of CpPTP-1 protein cellular function in *C. plantagineum*. (A) Transcriptional regulation of the *CpPTP-1* gene and corresponding *cis*-acting regulatory elements of the promoter region. A combination of CE3, MYC and DRE *cis*-acting elements controls the regulation of promoter under stress conditions. (B) The CpPTP-1 protein is translocated inside the chloroplast and interact with CpCHL protein. Abiotic stress conditions promote production of ROS and ROS cause lipid peroxidation as an oxidative damage, but interaction of the CpPTP-1 and CpCHL proteins provide a protection mechanism to prevent lipid peroxidation of thylakoid membrane under oxidative stresses. Protein translocon at the outer envelope membrane of chloroplasts (TOC), protein translocon at the inner envelope membrane of chloroplasts (TIC) (Jarvis and Soll 2001). Transcription factor (TF). The schematic summary was drawn using Inkscape software.

5. ACCESSION NUMBERS

Gene names	NCBI GenBank accession
CpPTP-1	AF356001.1
CpPTP-2	AY028426.1
CpPTP-3	AY028427.1
CpPTP-4	AY028428.1

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IV. DECLARATION

I hereby declare that this PhD dissertation is my own work, except where explicitly stated otherwise in the text or in the bibliography.

V. CONFERENCES

2019 Poster: Ahmad Mollazadeh Taghipour, Oluwaseun Johnson Akinlade, Dorothea Bartels. Functional promoter study of plastid-targeted protein 1 (PTP-1) associated with desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. Joint 30th International Conference on Genome informatics (GIW) & Australian Bioinformatics and Computational Biology Society (ABACBS) Annual conference (GIW/ABACBS 2019). 9-12 December 2019, Sydney, Australia.

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