Characterization of polygalacturonase inhibitor 1-like protein (CpPGIP) from *Craterostigma plantagineum* and analysis of stress inducible *pcC13-62* promoters to understand desiccation tolerance in Linderniaceae

Dissertation

Zur Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn, 2020

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

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Tag der Promotion: 10. August 2020

Erscheinungsjahr: 2020

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Abbreviations

3-AT	3-amino-1,2,4-triazole
ABA	abscisic acid
ABRE	ABA responsive element
APS	ammonium persulfate
bp	nucleotide base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CDS	coding sequence
Cp	*Craterostigma plantagineum*
CTAB	Cetyl trimethyl ammonium bromide
d	days
Da	dalton
DEPC	diethylpyrocarbonate
DMF	N, N-Dimethylformamid
DNA	deoxyribonucleic acid
dNTP	deoxy ribonucleotide triphosphate
DREB	DRE-binding protein
dH_2O	distilled “milli–Q" water
EDTA	ethylene diamine tetra acetic acid
EtBr	ethidium bromide
g	gram
GUS	β–glucuronidase
h	hour(s)
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
His–tag	histidine–affinity tag
HsP	heat-shock protein
IPTG	Isopropyl β- d-1-thiogalactopyranoside
KOH	potassium hydroxide
LB	Luria and Bertani medium
LEA	late embryogenesis abundant
Lb	*Lindernia brevidens*
Ls	*Lindernia subracemosa*
LiCl	lithium chloride
M	molar
mA	milli amperes
min	minute(s)
$MgCl_2$	magnesium chloride
MOPS	3-(N-morpholino)propanesulfonic acid
ml	milliliter
MS	Murashige and Skoog
NaCl	sodium chloride
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered salt solution
PCR	polymerase chain reaction
PG	polygalacturonase
PGIP	polygalacturonase inhibitor 1-like protein
pH	pondus Hydrogenii
RNA	ribonucleic acid
RNase	ribonuclease
Rpm	rounds per minute
RT	room temperature
RT–PCR	reverse transcriptase–polymerase–chain reaction
SDS	sodium dodecylsulfate
SOC	super optimal broth
TAE	tris-Acetate-EDTA
Taq	*Thermophilus aquaticus*
TBS	tris– buffered salt solution
TCA	trichlor acetic acid
TEMED	tetramethylethylendiamine
Tris	tris(hydroxymethyl)–aminomethane
TWEEN	Polyoxyethylene (20) sorbitan monolaurate
V	volts
[v/v]	volume/volume
[w/v]	weight/volume
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

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Summary

Desiccation tolerance (DT) is a very complex multifactorial process comprising a combination of physiological, morphological, cellular, genomic, transcriptomic, proteomic, and metabolic processes. Drought and salinity are the most severe abiotic stresses limiting agricultural production worldwide (Liu *et al.,* 2019). *Craterostigma plantagineum* is an extensively studied resurrection plant and a member of the Linderniaceae family (Bartels, 2005; Rahmanzadeh *et al.*, 2005). This thesis, is an attempt to understand structural, physiological and cellular responses involved in desiccation tolerance in the resurrection plant *C. plantagineum*. The thesis is divided into two major parts. The first part is the characterization of an important cell wall protein "polygalacturonase inhibitor 1-like protein (CpPGIP)" and in the second part we studied "stress inducible *pcC13-62* promoters" to understand regulatory promoter elements triggering gene expression related to desiccation tolerance in Linderniaceae.

The polygalacturonase inhibitor 1-like protein (CpPGIP) is an important cell wall protein. PGIP's are leucine-rich repeat proteins produced by plants against polygalacturonase, a key virulence agent in pathogens. PGIP expression is induced by many biotic or abiotic stimuli, including fungi, insects, mechanical damage, salicylic acid, methyl jasmonate, and oligogalacturonic acid (Liu *et al.,* 2016). In this thesis, the *CpPGIP* gene was cloned and the corresponding recombinant protein was purified. Quantitative analysis of the expression of the gene revealed that *CpPGIP* transcripts accumulate at a much higher level when the plant is under dehydration stress than without stress. The recombinant CpPGIP protein was purified and was studied during various stress conditions to understand its role and importance when the plant is subjected to stress. A yeast-two-hybrid assay identified potential candidates which might be interacting with the CpPGIP protein by forming complexes and thus helping in running the plant machinery on the cellular level. We hypothesize that PGIP might play an important role in the cell wall maintenance of *C. plantagenium*. These findings will contribute towards the understanding of the role of PGIPs and in screening potential combat proteins with novel recognition specificities against evolving pathogenic factors for counteracting pathogen invasion as well as its role in helping the plant in combating drought stress. Severe drought has been shown to elicit a whole-plant response guided by key phytohormones, which not only respond to water

stress but also play a critical role in the response of plants to biotic stress imposed by herbivores and pathogens (Hossain *et al.,* 2018). Our ability to predict how *C. plantagenium* responds to pathogens while simultaneously encountering the consequences of drought remains limited.

The pcC13-62 protein was first identified from the resurrection plant *C. plantagineum* and it has been suggested to be involved in plant desiccation tolerance (Giarola *et al.,* 2018; Zha *et al.,* 2016; Rodriguez *et al.,* 2010). Since *13-62* plays an important role in the desiccation mechanism, *pcC13-62* was chosen as a candidate gene for our study. In this study, the regulation of *pcC13-62* has been investigated in the closely related species *C. plantagineum, L. brevidens and L. subracemosa*. The expression of the gene *pcC13-62* was analysed in *C. plantagineum* and in the closely related desiccation-tolerant species *L. brevidens* and the desiccation sensitive species *L. subracemosa*. The study demonstrated a correlation between promoter activity and gene expression levels, suggesting transcriptional regulation of gene expression. A detailed expression pattern of the *13-62* promoter was determined by histochemical *β*-glucuronidase (GUS) staining of transgenic plants that harboured a *pcC13-62* promoter::GUS reporter construct. Comparison of promoter sequences identified a dehydration-responsive element in the promoters of the tolerant species which is required for dehydration-induced β-glucuronidase (GUS) accumulation. We hypothesize that variations in the regulatory sequences of the *pcC13-62* gene might be required for desiccation tolerance. Comparison of the promoter regions revealed that promoters have different activities, but some functional *cis*-acting elements are conserved between *C. plantagineum, L. brevidens and L. subracemosa*.

The data from the study of both the candidates (*CpPGIP* and *pcC13-62*) provide more insight into the evolution of the molecular basis of desiccation tolerance in the plant *C. plantagineum.*

1. Introduction

1.1 Importance of water for plants

Water is one of the most crucial elements responsible for plant growth and development. Many important biological processes like photosynthesis and nutrient uptake require water. Because of the important role which water plays in maintaining cellular homeostasis, water deficiency is critical for plant growth and development. Plants have adopted several strategies to avoid the stress during water deficiency or flooding. One of the strategies includes the closing of stomata which decreases transpiration and minimizes the loss of water. However, closed stomata prevent the plant to take up more carbon dioxide from the atmosphere and this affects the rate of photosynthesis (Gechev *et al.*, 2012).

When a plant is no longer able to extract water from the soil it starts to wilt. Water plays a very important role for the maintenance of the turgor (= inner cell pressure). Almost all proteins within the cell are hydrated in order to function properly. In case of water deficiency this hydration layer gets disrupted thereby altering the structure of proteins and therefore lowering or even abolishing their activity. Hence, water deficit has a negative effect on plant growth and biomass production (McDowell *et al.*, 2008).

Even though, most plants are able to withstand a mild water deficit, a prolonged period of severe drought is lethal for most plants. In order to adapt to the environment, the plants adopt different strategies for example a thicker cuticle, modified stomata, or a change in the carbon metabolism (McDowell *et al.*, 2008).

As seen in **Fig. 1** water shortage is already a worldwide problem. Water availability is already becoming scarce in many parts of the world, especially in developing countries. Predicted climate changes will have a negative impact on water resources and could make the situation of water shortage even worse (FAO, 2008).

1. Introduction

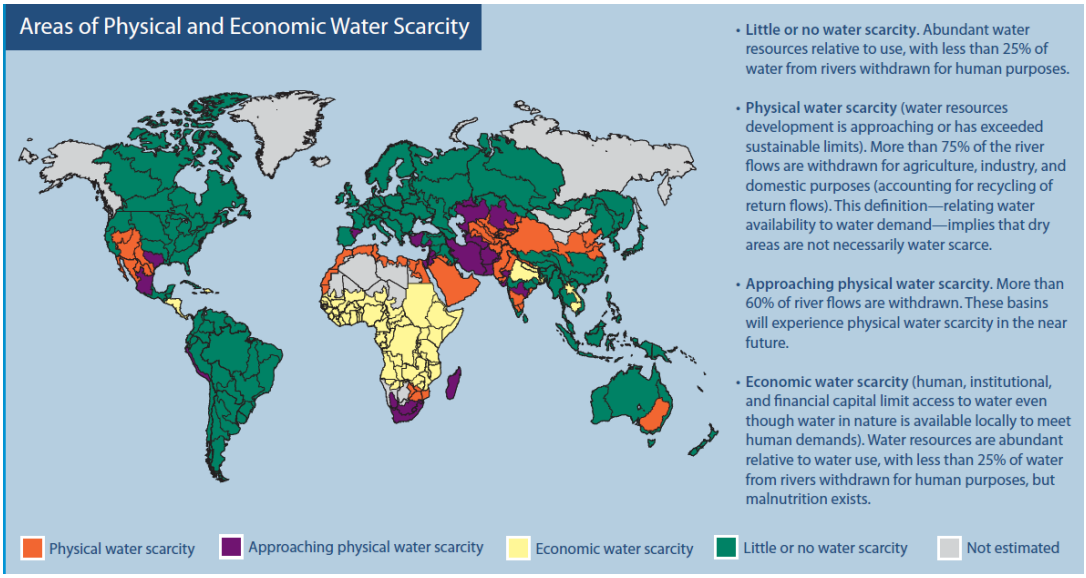

Fig. 1. Physical and economic water scarcity in different regions of the world. (Source: Comprehensive Assessment of Water Management in Agriculture, 2007).

Knowledge about plant responses to water stress is essential to be able to successfully enhance dehydration tolerance of crops. Understanding of mechanisms of desiccation tolerance is of great value for the development of dehydration tolerant crops.

1.2 Resurrection plants and evolution of desiccation tolerance in plants

The most useful experimental systems to study the mechanism of desiccation tolerance are the resurrection plants, as they can survive desiccation of their vegetative tissues (Gaff, 1971). Most resurrection plants grow in areas where the water availability is seasonal and many desiccation tolerant plants are found in semi-arid regions of Southern Africa (Moore *et al.*, 2009). During the dry conditions, resurrection plants have the ability to equilibrate the water content in their leaves to the relative humidity of the air. Some of the resurrection plants even have the ability to withstand complete desiccation and survive in dry air with a relative humidity of 0% (Gaff, 1987). During desiccation, resurrection plants can curl their leaves and keep them in an air-dried state until water becomes available. After rehydration, desiccated resurrection plants are able to recover and resume all their physiological activity.

Resurrection plants can be distinguished in two categories: homoiochlorophyllous and poikilochlorophyllous, respectively (Tuba *et al.*, 1998). The homoiochlorophyllous desiccation tolerant plants retain their chlorophyll and keep their photosynthetic

structures intact during desiccation. This gives them the ability to recover rapidly when water becomes available. Poikilochlorophyllous resurrection plants degrade their chlorophyll during dehydration. Therefore, poikilochlorophyllous desiccation tolerant plants need to resynthesize their photosynthetic system upon rehydration. Most resurrection plants are also sensitive to the rate at which cellular water is lost (Oliver and Bewley, 1997). It is thought that the mechanism of desiccation tolerance has been evolved by primitive plants such as bryophytes which are non-vascular land plants. Therefore, gradual dehydration is an important factor for most resurrection plants to protect themselves against damage caused by desiccation. This indicates that resurrection plants need time to switch to a protective gene expression program (Moore *et al*., 2009).

Bryophytes can be classified in three separate groups: (1) liverworts, (2) hornworts and (3) mosses. Bryophytes were the first plants that moved from water and colonized the land (Mishler and Churchill, 1985). On moving to the land, the bryophytes adapted to live in a relatively dry environment which resulted in evolutionary changes (Charron and Quatrano, 2009; Floyd and Bowman, 2007).

The initial evolution was essential for bryophytes to colonize the land. The earliest mechanisms of desiccation tolerance were very similar to those found in current desiccation tolerant bryophytes. It has been hypothesized that mechanisms of desiccation tolerance that are observed in seeds have evolved from these primitive mechanisms of vegetative desiccation tolerance (Oliver *et al*., 2000; Giarola *et al.,* 2017). There appears to be a trade-off between desiccation tolerance and growth. Desiccation tolerance requires a heavy amount of energy and therefore the higher plants eventually lost their ability to tolerate desiccation in their vegetative tissue. Another reason for the loss of desiccation tolerance was that it was no longer required or was not efficient to maintain (Alpert, 2006).

Compared to the desiccation sensitive plants the metabolic rates in desiccation tolerant plants are much lower. However, it is thought that desiccation tolerance re-evolved independently in some plant species which are known as the modern-day resurrection plants (Proctor *et al*., 2007). This hypothesis means that genes involved in the

acquisition of desiccation tolerance are also present in the genomes of most desiccation sensitive plant species.

During desiccation, resurrection plants have to cope with different types of stress, which have been divided into three categories: (1) mechanical stress caused by the loss of turgor pressure, (2) disturbance of cell membrane integrity through the disruption of phospholipids in cell membranes and (3) interruption of metabolism caused by oxidative stress (Vicrè *et al*., 2004).

1.3 The role of the cell wall during desiccation

The cell wall is a highly dynamic compartment that evolves during cell growth and cell differentiation and in response to biotic and abiotic stresses. The cell wall provides a protective barrier and consists mainly of cellulose microfibrils, hemicelluloses, pectins, and “structural” glycoproteins such as extensin and arabinogalactan proteins (Vicre *et al*., 2004; Jones *et al*., 2004; Moore *et al*., 2013). Plant cell walls are divided into a primary cell wall and a secondary cell wall. The primary cell wall is present in almost all cells of the plants, whereas the secondary wall is visible only in differentiated tissues. Two types of cell walls can be differentiated. Type I primary walls found in the eudicotyledons, non-common in monocotyledons and gymnosperms (Minic *et al*., 2006), are composed of a network of cellulose microfibrils, mostly cross-linked with xyloglucans (XyG) and embedded in a matrix of pectic polysaccharides. Type II primary cell walls, characteristic of monocotyledons (grasses and rushes), are composed of glucuronoarabinoxylans (GAXs) and mixed- linkage (1→3),(1→4)-*β*-D-glucan (*β*-glucan) polymers that link cellulose microfibrils (Vogel *et al*., 2008; Le Gall *et al*., 2015). Pectic polysaccharides and XyG are generally poorly represented in type II primary cell walls. Furthermore, ferulic and *p*-coumaric acid arabinosyl esters can cross-link GAX in type II primary walls.

Mechanical stress is one of the more challenging stresses that resurrection plants have to overcome to survive desiccation (Iljin *et al*., 1957). As water is lost from the cell, plasmolysis occurs resulting in plasma membrane tearing from the more rigid cell wall and cell death. Resurrection plants have developed strategies to minimize the impact of mechanical stress during desiccation and to avoid irreversible damage (Farrant *et al*., 2003). During dehydration of the resurrection plants *C. wilmsii* and *C.*

*plantagenium*a decrease of about 78% of the cellular volume occurs in foliar tissues (Farrant *et al*., 2000). This extensive reduction of mesophyll cells is due to a strong folding of cell walls. In *S. lepidophylla*, an important event of folding of the cell walls and plasmalemma with continuous apposition to the cell wall is visualized during desiccation (Thomson *et al*., 1997). In *M. flabellifolius*, the folding of the cell wall is less distinct, and it is not observed in all cell types (Farrant *et al*., 2000). The folding of the cell wall is considered as a strategy developed by cells of DT plants to maintain the contacts between the plasma membrane and the cell wall during dehydration and to avoid the tearing between these structures and hence cell lysis and death. Cell wall modifications do occur in DT plants in response to dehydration (**Fig. 2**).

Name	Cell wall modification
Boea hygrometrica	(i) Extensive cell wall folding accompanied by protoplasmic shrinkage (ii) An increase of pectin and wax/suberin events occurred mainly during the rehydration phase (iii) The contents of cell wall-associated lignin were reduced in desiccated leaves (iv) Transcripts encoding cell metabolism were induced in rehydrated acclimated plants, indicating cell wall loosening during rehydration
Craterostigma plantagineum	(i) A marked reduction of the demethylesterification of HG in the dry state (ii) An upregulation of gene expressions corresponding to expansin and XyG synthesis (iii) CpGRP1-CpWAK1 complex could be inducing morphological changes (iv) A role for CpCRP1 in the leaf cell wall prior to dehydration stress and in mechanisms which are required for the successful recovery from desiccation (v) The transcripts encoding proteins involved in ion transport such as membrane-associated carriers together with proteins involved in cell wall plasticity are abundant in fully hydrated conditions in *C. plantagineum*
Craterostigma wilmsii	(i) Decrease about 78% of the cellular volume (ii) A strong folding of the cell wall (iii) A modification in the sugar composition of hemicellulosic fraction (iv) An increase of epitopes recognized by the XyG-directed monoclonal antibodies
Eragrostis nindensis	(i) Arabinoxylans and xylans are involved in the regulation of mechanical properties of cell walls (ii) Ferulic acid can cross-link neighbouring arabinoxylan molecules or arabinoxylans to enhance cell wall stiffening
Haberlea rhodopensis	(i) Upregulated transcript *Hrh*DR35 encoding an XyG endotransglucosylase/hydrolase (ii) Downregulation of many cell wall-related genes including XyG endotransglucosylases and pectate lyases
Lindernia brevidens	(i) A strong folding of the cell wall
Myrothamnus flabellifolius	(i) Arabinose-enriched primary cell wall (ii) AGP is a contributor in ensuring flexibility and to facilitate the rehydration
Ramonda serbica	(i) Activities of nonspecific peroxidases play a role in cell wall remodelling
Selaginella bryopteris	(i) Phospholipase A1 gamma-like protein and glucan endo-1,3-alpha-glucosidase Agn1 have been reported to play a structural role in reinforcing the cell wall during stress
Selaginella lepydophylla	(i) A strong folding of the cell wall (ii) Plasmalemma with continuous apposition to the cell wall
Sporobolus stapfianus	(i) A strong folding of the cell wall (ii) Transcripts encoding enzymes involved in cell wall remodelling are increased in abundance during dehydration (iii) A late accumulation of ferulate and caffeate, precursors of cell wall lignin and cross-linking compounds, could enhance cell wall extensibility
Xerophyta spp.	(i) Highly arabinosylated xylans and arabinogalactan proteins

Fig. 2. Cell wall modifications of desiccation tolerant plants. (Source: Shivaraj *et al.*, 2018).

1.4 Molecular mechanisms of drought and salt tolerance in plants

As sessile organisms, plants are exposed to many types of environmental stresses such as drought, cold, salinity, high temperature and others. Among the various abiotic stresses, water stress caused by drought and salt is the most prevalent abiotic stress that challenges plants. Both drought and salt stress largely affect plant physiology and metabolism and result in numerous changes. Physiological changes including leaf wilting or abscission, reduction in leaf area, stimulation of root growth, changes in relative water content (RWC), *etc.* occur in plants thereby affecting the plant metabolism. At the cellular level, drought and salt stress causes osmotic stress and removal of water out of the cytoplasm thereby leading to cellular dehydration. These stresses also cause the accumulation of reactive oxygen species (ROS) in the cells, which then in turn cause oxidative damage and affect cellular structures and metabolism negatively. ROS disrupt cellular homeostasis by reacting with lipids, proteins, pigments, and nucleic acids resulting in lipid peroxidation (LP), membrane damage, and the inactivation of enzymes, thus affecting cell viability (Bartels and Sunkar 2005; Lata and Prasad 2011).

Although most of the changes have serious consequences of stress injury, plants have evolved sophisticated mechanisms to adapt to drought and salt stress. Except for the ionic component in salt stress, responses to drought and salt stresses are largely identical. These similarities include metabolic processes such as a decrease in photosynthesis and increase in the levels of stress-related plant hormones like abscisic acid and jasmonic acid (Jian-Kang Zhu, 2002). High intracellular concentrations of sodium and chloride ions are an additional problem of salinity stress. According to Zhu (2002), the adaptive responses can be generally grouped into three control aspects: 1) homeostasis which is mainly relevant to salt stress, namely reestablishment of cellular homeostasis under stress conditions; 2) stress damage control or detoxification to repair stress damage; 3) growth control through coordinate cell division and expansion to levels suitable for the particular physiological conditions. Molecular and cellular responses to drought and salt stress include stress signal perception, signal transduction, gene expression and finally metabolic changes leading to stress tolerance (Xiong *et al.,* 2002; Bartels and Sunkar 2005; Agarwal *et al.,* 2006; Lata and Prasad 2011).

1.5 Desiccation tolerance in the Linderniaceae family and comparative studies within this family

The dicotyledonous South African resurrection plant *Craterostigma plantagineum* Hochst. has been the main model for studying desiccation tolerance in plants at both the molecular and physiological level (Bartels and Salamini, 2001; Bartels, 2005; Rodriguez *et al.*, 2010; Dinaker and Bartels, 2013). *C. plantagineum* is a member of the Linderniaceae family in the order Lamiales (Rahmanzadeh *et al*., 2005). Several other members within the family of Linderniaceae are also tolerant to desiccation (Fischer *et al*., 1992).

Amongst the most notable changes occurring in the leaves of *C. plantagineum* during water deficit stress leads to a remarkable, and reversible, interconversion between octulose (Oct) and sucrose (Suc). It has been proposed that transketolases (key enzymes of the reductive and oxidative pentosephosphate pathways, responsible for the synthesis of sugar phosphate intermediates) contribute to the Suc-to-Oct interconversion during rehydration of *C. plantagineum* (Egert *et al.,* 2015). At least two transketolasegenes have been demonstrated to be transcriptionally upregulated during rehydration (Bernacchia *et al.*, 1996; Willige *et al.,* 2009). The accumulation of sucrose appears to be a ubiquitous response to water deficiency in the leaves of resurrection plants.

It has been reported that *Lindernia brevidens* Skan, which is a close relative of *C. plantagineum* has the ability to recover after extreme desiccation (Phillips *et al*., 2008). *L. brevidens* is endemic to tropical rainforests in eastern Africa (Fischer *et al*., 1992) and is restricted to the rainforests of the Usambara Mountains and the Taita hills, whereas *C. plantagineum* has colonized areas with limited seasonal water availability from Niger to Sudan, Ethiopia and East Africa, extending to Angola, Namibia and South Africa (**Fig. 3**). Therefore, it is surprising that *L. brevidens* (**Fig. 6**) exhibits desiccation tolerance, although in its habitat it is never exposed to prolonged periods of drought. It was proposed that *L. brevidens* retained desiccation tolerance through genome stability (Phillips *et al*., 2008). In the same study by Phillips *et al*., 2008, it was shown that *L. brevidens* uses similar mechanisms as *C. plantagineum* to protect itself against desiccation. As shown in **Fig. 3** even the flower morphology is similar between both.

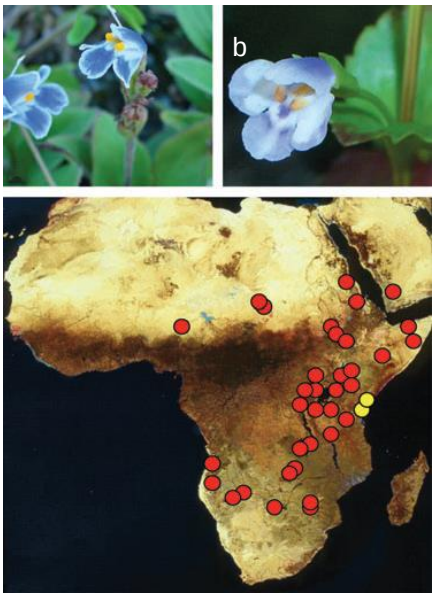

Fig. 3. Morphology and distribution of *Lindernia brevidens* and *Craterostigma plantagineum* (a) *C. plantagineum* and (b) *L. brevidens* share similar floral morphology. (c) Distribution of *L. brevidens* (yellow dots) *C. plantagineum* (red dots). (Source: Phillips *et al*., 2008).

The majority of the *Lindernia* species are sensitive to desiccation. One of these desiccation sensitive *Lindernia* species is *L. subracemosa* (**Fig. 7**). Also, shown by the phylogenetic analysis in **Fig. 4**, *L. brevidens* and *L. subracemosa* are closely related to *C. plantagineum*. The phylogenetic tree was inferred from sequences of the chloroplast maturase gene (MATK). It was shown that both *L. brevidens* and *L. subracemosa* are in the same branch as the members of the Craterostigma genus (Phillips *et al*., 2008) (**Fig. 5**).

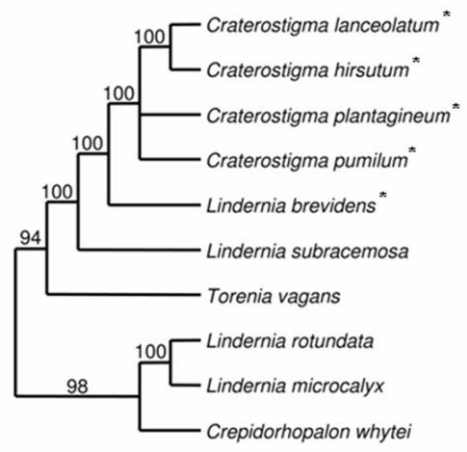

Fig. 4. Phylogenetic relationship between selected members of the Linderniaceae family. The desiccation-tolerant species are marked with an asterisk. The numbers above the branches are the bootstrap percentages. (Source: Phillips *et al*., 2008).

1. Introduction

Only a few studies have been made on the mechanisms involved in the acquisition of desiccation tolerance in the plant *L. brevidens* (Phillips *et al.*, 2008; Smith-Espinoza *et al*., 2007). Therefore, further studies need to be made in order to gain information and understanding about desiccation tolerance mechanism in Linderniaceae family.

Fig. 5. Effect of drought stress in *Craterostigma plantagenium.* The figure (a) shows untreated *C. plantagenium*. The figure (b) shows the effect of extreme dehydration on *C. plantagenium*. The figure (c) shows that upon rehydration, the plant recovers and resumes normal physiological activity.

Fig. 6. Effect of drought stress in *Lindernia brevidens.* The figure (a) shows untreated *L. brevidens*. The figure (b) shows the effect of extreme dehydration on *L. brevidens*. The figure (c) shows that upon rehydration, the plants recover and resume normal physiological activity. Hydrated and rehydrated leaves appear structurally similar. (Source: Phillips *et al*., 2008).

Because of the close relationship of *L. subracemosa, C. plantagineum* and *L. brevidens* these plants can be used for comparative studies on mechanisms of desiccation tolerance between desiccation tolerant and desiccation sensitive plants.

Fig. 7. Effect of drought stress in *Lindernia subracemosa*. The figure (a) shows untreated desiccation sensitive *L. subracemosa*. The figure (b) shows the effect of extreme dehydration on *L. subracemosa*. The figure (c) shows that upon rehydration, the plant does not recover and resume normal physiological activity. (Source: Phillips *et al*., 2008).

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

1.6 *Cis* - elements involved in dehydration responsiveness in plants and the role of the *13-62* plant proteins

There are sometimes changes happening in the nucleotides within the non-coding DNA sequences. The non-coding DNA represents a large portion of the genome and the changes in the genetic material lead to evolution (Ludwig, 2002). A large part of non-coding DNA is involved in the regulation of transcription and hence in the nucleotide changes in non-coding regions of the DNA.

The functionality of the existing *cis*-acting regulatory elements can be changed by mutations occurring in the genome. Gene duplication events may also change the spacing and distribution of *cis*-acting regulatory elements and can affect the regulation of transcription. Point mutations within the promoter sequences can generate new *cis*-acting elements (Stone and Wray, 2001). Hence, sequence alterations in the promoter region play an important role in the evolution of the *cis*-acting elements.
A study on the short term evolutionary dynamics of *cis*-regulatory elements in *A. thaliana* has been made (de Meaux *et al*., 2005). A putative promoter region was

examined within the species for nucleotide variation. The region of the enzyme chalcone synthase (CHS) was determined for the nucleotide and functional variation of the *cis* regulatory region. The 5'- upstream region of *CHS* from 28 different *A. thaliana* accessions was analyzed and various nucleotide polymorphisms were found in the *CHS* promoter region. Distinct parental promoter alleles were paired within F1 heterozygotes and functional *cis*-regulatory variation was analyzed. In heterozygous F1 individuals, parental alleles are analyzed in a common *trans*-regulatory environment.

The relative expression levels of parental-specific alleles were determined and the influence of different stimuli (dark, light and insect-feeding) on CHS transcription was analyzed. Functional variations were found, which seem to be independent of the trans regulatory background. De Meaux *et al.*, 2005 showed that a few point mutations could already cause functional changes in promoters. The study indicated that changes in *cis*-acting regulatory elements can have major impacts on gene regulation.

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

The plant desiccation-related proteins (DRPs) are a group of proteins that play an important role in desiccation tolerance (Wang *et al.*, 2003; Karlson *et al.*, 2004). The pcC13-62 is one such DRP which was first identified in 1990 from the resurrection plant *C. plantagineum* (Zha *et al.,* 2013; Giarola *et al.,* 2018). The DRP proteins are relatively small and have been suggested to be characteristic of resurrection plants (Piatkowski *et al.*, 1990). They were found to be abundantly expressed in the resurrection plants such as Xerophyta *humilis* (Collett *et al*., 2004), *X. viscosa* (Ingle *et al.*, 2007) and *Selaginella lepidophylla* (Iturriaga *et al.*, 2006). They were also identified from non-resurrection plants such as *Gossypium barbadense* (Zuo *et al.*, 2005), *Oryza sativa*

(Swarbrick *et al.*, 2008), *Arachis hypogaea* (Payton *et al.*, 2009), and *hybrid poplar* (Bae *et al.*, 2010) but were not abundantly expressed in them.

The pcC13-62 proteins were found to be present in chloroplasts by immunocytochemical analysis (Schneider *et al.*, 1993). The expression of the pcC13-62 proteins is induced by desiccation, abscisic acid (ABA) treatment as well as by the other stresses. The expression is down-regulated when plants transit from a dormant state to a metabolically active state during rehydration (Zha *et al.*, 2013). Therefore, the pcC13-62 may be characteristic for the resurrection-type of plants and could encode proteins missing in desiccation-intolerant plants (Bartels *et al.*; 1990). So far, the pcC13-62 plant DRPs have not been purified at the protein level, nor their structure has been determined.

1.7 Polygalacturonase inhibitor proteins (PGIPs)

Successful colonization of plant tissues by microbial pathogens requires them to overcome the cell wall. Therefore, pathogens produce a wide array of plant cell wall degrading enzymes, among which endo-polygalacturonases are secreted at very early stages of the infection process (Have *et al.*, 1998). PGs cleave the α-(1–4) linkages between the D-galacturonic acid residues of homogalacturonan, the main component of pectin, causing cell separation and maceration of the host tissue. To counteract the activity of PGs, plants deploy the cell wall polygalacturonase inhibiting proteins (PGIPs) that inhibit the pectin-depolymerizing activity of PGs. No plant species or mutants totally lacking PGIP activity have been characterized so far.

The structure of PGIPs is typically formed by 10 imperfect leucine-rich repeats (LRRs) of 24 residues each, which are organized to form two β-sheets, one of which (sheet B1) occupies the concave inner side of the molecule and contains residues crucial for the interaction with PGs (Di Matteo *et al.*; 2003). In addition to PG inhibition, the interaction between PGs and PGIPs promotes the formation of oligogalacturonides (OGs), which are elicitors of a variety of defence responses (Cervone *et al.*, 1989; Ridley *et al.*, 2001; Ferrari *et al.*, 2013).

Early characterization of a polygalacturonase-inhibiting activity was reported in 1970s (Albersheim and Anderson, 1971) and the first *pgip* gene was isolated 20 years later

in French bean (Toubart *et al.*, 1992). Since then, several PGIPs and a large number of *pgip* genes have been characterized. Up to now more than 170 complete or partial *pgip* genes from dicot and monocot plants have been deposited in nucleotide databases (e.g., http://www.ncbi.nlm.nih.gov/). Most of these genes have been identified as *pgip* genes on the basis of sequence identity but only a few of them have been shown to encode proteins with PG-inhibitory activity.

Genome analysis has shown that *pgip* genes did not undergo a large expansion and may exist as single genes, as in diploid wheat species (Di Giovanni *et al.*, 2008). These might be organized into gene families, the members of which are organized in tandem and can vary from two, as in *Arabidopsis thaliana* (Ferrari *et al.*, 2003), to sixteen, as in *Brassica napus* (Hegedus *et al.*, 2008). The majority of *pgip* genes are intronless, however, some of them can contain a short intron as in *Atpgip1* and *Atpgip2* (Ferrari *et al.*, 2003). *pgip* genes can be inactivated by transposable elements as in cultivated and wild wheat where the occurrence of *Copia*-retrotransposons and *Vacuna* transposons has been reported (Di Giovanni *et al.*, 2008).

At the protein level, members of a *pgip* family show both functional redundancy and sub-functionalization (De Lorenzo *et al.*, 2001; Federici *et al.*, 2006). As suggested previously, these features likely have an adaptive significance for combating more efficiently a broad array of pathogens (Ferrari *et al.*, 2003) or responding more rapidly to diverse environmental stimuli (D'Ovidio *et al.*, 2004b). In support of this view, a recent analysis of the genomic organization and composition of the legume *pgip* families suggested that the forces driving the evolution of the *pgip* genes follow the birth-and-death model (Kalunke *et al.*, 2014), similarly to what proposed for the evolution of NBS-LRR-type *R* genes (Michelmore and Meyers, 1998). This possibility is based on genomic features that include inferred recent duplications, diversification as well as pseudogenization of *pgip* copies, as found in soybean, bean, barrel clover and chickpea (Kalunke *et al.*, 2014). The organization of the *pgip* families therefore supports the view that tandem duplications are frequent in stress-related genes and are beneficial for survival in challenging environments (Oh *et al.*, 2012).

1.8 PG-PGIP Interaction

PG–PGIP complexes are considered a model protein– protein interaction system in the backdrop of plant pathogen interactions (Villamil and Van der Hoorn, 2008). Although three-dimensional structures of many PGs have been elucidated to date (Pickersgill *et al.*, 1998; Federici *et al.*, 1999; Bonivento *et al.*, 2008), the only PGIP whose crystal structure has been solved is that of *Pv*PGIP2 from *Phaseolus vulgaris* (Di Matteo *et al.*, 2003). Most of the data available on the PG–PGIP interactions has been a result of studies involving *Pv*PGIP2. Previous studies employed targeted mutation of *pg* and *pgip* genes, and investigated the *in vitro* inhibition behaviour of the protein variants synthesized to identify the amino acid residues involved in the protein–protein interactions (Leckie *et al.*, 1999; Mattei *et al.*, 2001; Raiola *et al.*, 2008). Amide-exchange mass spectrometry in combination with protease protection and fluorescence spectrometric analysis was employed to deduce the amino acids of *An*PGII, a PG isoform II from *Aspergillus niger*, required for interaction with *Pv*PGIP2 (King *et al.*; 2002). The availability of advanced bioinformatics tools for protein homology modelling and docking have been exploited in in-depth analysis of PG–PGIP complexes *in silico* and found to be in conformity with the experimental results (Lim *et al.*, 2009; Maulik *et al.*, 2009).

In contrast to the magnitude of literature available on dicot PGIPs, information available in case of monocots is meagre. Although PGIPs from wheat and rice have been tested for inhibition against various PGs (Jang *et al.*, 2003; Kemp *et al.*, 2003; Janni *et al.*, 2006, 2013), no efforts have gone into understanding their mode of inhibition and the underlying structural basis of their interaction with PGs.

2. Aims and structure of the work

The current study has been performed studying the desiccation tolerant plants *C. plantagenium*, *L. brevidens* and the desiccation sensitive plant *L. subracemosa*. The studies were focused on drought and salt stress-induced gene and protein expression. The work is divided into two main sections.

1. Characterization of polygalacturonase inhibitor 1-like protein (CpPGIP) from *Craterostigma plantagineum*

This section describes the characterization of the polygalacturonase inhibitor 1-like protein (CpPGIP) from *C. plantagineum.* The following experiments were performed in order to study the importance of this protein for maintaining the cell wall intact during dehydration and rehydration and to understand its expression under stress.

- **Localization studies:** To confirm the CpPGIP localization in the plant cell wall.

- **Analysis of CpPGIP expression:** Cloning of CpPGIP into pET28-a vector and overexpressing it, purification of the protein and development of a polyclonal antiserum to investigate protein expression and to study the interaction of CpPGIP and CpPG (polygalacturonase).

- **Identification interacting partners of CpPGIP:** Yeast two-hybrid screening to identify interacting proteins against the protein.

2. Identification and analysis of *pcC13-62* promoters

Desiccation tolerant plants *C. plantagenium, L. brevidens* and the desiccation sensitive plant *L. subracemosa* were used as experimental systems in this study. The *13-62* putative promoter regions in both the plant species were identified and analyzed to test if structural differences in the promoter sequence of the *13-62* are responsible in determining the different transcript accumulation in tolerant and sensitive species. The work was divided into the following tasks:

2. Aims and structure of the work

- **Identification of the promoter:** To identify*13-62* putative promoter regions in the plant species and analyze if structural differences in the promoter sequence of the *13-62* are responsible in determining the diverse transcript accumulation in tolerant and sensitive species.

- **Preparation of promoter-GUS constructs and analysis of the promoter activity using transient transformation:** To investigate functionality of promoters by identifying *cis*-acting regulatory elements. The promoter activity in plants was analysed by transient transformation.

- **Promoter mutagenesis:** The conserved *cis*-acting elements were mutated to confirm the importance of these elements in the context of tolerant and sensitive plant species.

- **Localization studies of Cp 13-62:** To confirm the Cp13-62 localization in the plant cell wall.

3. Materials and methods

3.1 Materials

3.1.1 Plant material

All plants were established in the Institute of Molecular Physiology and Biotechnology of Plants (IMBIO) and were grown and maintained by the lab technician C. Buchholz. Three to four-week-old *in vitro C. plantagineum*, *L. brevidens* and *L. subracemosa* plants were used for transient expression experiments. The plants were maintained under the controlled short-day conditions (eight hours of light at 22°C and 16 hours of darkness at 20°C). For relative water content measurement, genomic DNA extraction and RNA preparation 8 – 10-week-old plants were used from the pot.

Wild-type *Nicotiana benthamiana* seeds were sowed on wet, Lizetan® (Bayer, Leverkusen, Germany)-treated soil and grown under short-day conditions. After two weeks of growth, the plants were singularized and were transferred into separate pots. Plants which were 6-8 weeks old were then used for *A. tumefaciens* transformation.

3.1.2 Chemicals

The chemicals used were ordered from the following companies:

- Sigma-Aldrich Chemie GmbH (Munich, Germany)
- Carl Roth GmbH (Karlsruhe, Germany)
- AppliChem GmbH (Darmstadt, Germany)
- Apollo Scientific (Ltd Bledsbury, Czech Republic)
- Merck AG (Darmstadt, Germany)
- Invitrogen (Karlsruhe, Germany)
- Fermentas (St. Leon–Rot, Germany)
- Bio-Rad (Munich, Germany)
- Biomol (Hamburg, Germany)
- GE Healthcare (Freiburg, Germany)
- Dushefa Biochemie B.V. (Haarlem, Netherlands)
- Stratagene (Heidelberg, Germany)

3.1.3 Equipment

- Centrifuges 5415D / 5417R/ 5810R (Eppendorf, Hamburg, Germany)
- Heating block MHR11 (HLC BioTech, Bovenden, Germany)
- PCR thermocycler T3000 (Biometra, Göttingen, Germany)
- SmartSpec 3000 spectrophotometer (Bio-Rad, USA)
- Incubator shaker G25 (New Brunswick Scientific, New Jersey, USA)
- Typhoon scanner 9200 (Amersham, Freiburg, Germany)
- Image scanner c300 (Azure biosystems, Dublin, California, USA)
- BioSpec-nano spectrophotometer (Shimadzu Biotech, Japan)
- Blotting chamber for proteins: Criterion Blotter (Biorad, Munich, Germany)
- Desalting columns: PD–10 (GE Healthcare, Freiburg, Germany)
- Particle Gun: Biolistic (Bio-Rad, Hercules, USA)
- pH–meter (SCHOTT GLAS Mainz, Germany)
- Sonification water bath: Sonorex Super RK102P (Bandelin electronics, Berlin, Germany)
- Gel electrophoresis chambers: minigel (Biometra, Göttingen, Germany) and EasyCast (Owl, Portsmouth, USA)
- Lyophilisator: LDC–2 (Christ, Osterode am Harz, Germany)
- Electroporation system: GenepulserII Electroporator (Bio-Rad, Hercules, USA)
- Confocal Laser Scanning Microscope: ZE2000 (Nikon, Düsseldorf, Germany)
- Chemiluminescence detector: Intelligent Dark Box II, (Fujifilm, Tokyo, Japan)
- Blotting chamber for proteins: Criterion Blotter” (Bio-Rad, Munich, Germany)
- Binocular microscope: SMZ-800 (Nikon, Düsseldorf, Germany)

3.1.4 Consumable supplies

Consumables such as pipette tips, falcon tubes (15 ml and 50 ml), cryo tubes, PCR tubes and centrifugation tubes (1.5 ml and 2 ml) were ordered from Sarstedt AG, Nümbrecht, Germany.

3.1.5 Enzymes and markers

DNA–marker (New England Biolabs, Ipswich, USA), Restriction enzymes and Pfu–DNA–polymerase (Fermentas, St. Leon–Rot, Germany), Taq DNA–polymerase (Ampliqon, Skovlunde, Denmark), Taq DNA–polymerase (isolated and provided by Tobias Dieckmann, IMBIO).

3.1.6 Kits

- CloneJET PCR cloning Kit. Thermo Scientific (St-Leon-Rot, Germany)
- NucleoSpin Gel and PCR clean-up. Macherey-Nagel (Düren, Germany)
- Plasmid DNA purificationNucleoBond® Xtra Midi / Maxi. Macherey-Nagel (Düren, Germany)
- RevertAid First Strand cDNA Synthesis Kit. Fermentas (St. Leon–Rot, Germany)
- GenomeWalkerTM universal kit (Clontech, Heidelberg, Germany)

3.1.7 Membranes and Whatman papers

The nitrocellulose membranes for the protein blots were Protran premium 45 μm (GE Healthcare Life Sciences, Amersham). Whatman paper filter WH10311897 (Schleicher & Schuell, Dassel, Germany) and Whatman 3 mm Chr paper (GE Healthcare, Buckinghamshire, UK) were used for yeast colony colorimetric assay and blotting assays.

3.1.8 Medium, supplements and buffers

SOC–medium: 2 % (w/v) Tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$.

LB–medium: 1.0 g/ l Tryptone, 10 g/l NaCl, 5 g/ l yeast extract, pH 7.0.

LB-agar: 15 g/ l Select–Agar was added to LB-medium.

YEB-medium: 5 g Beef extract, 5 g peptone, 5 g sucrose, 1 g yeast extract, pH 7.0. After autoclaving filter-sterilized $MgCl_2$ solution to a final concentration 2 mM was added.

10 X DNA loading buffer (10 ml): 25 mg bromophenol blue, 1 ml 1 X TAE; 25 mg xylencyanol; 3 ml glycerol; 6 ml sterile water.

1 X TE buffer: 10 mM Tris-HCl; 1 mM EDTA (pH 8.0).

X-Gluc staining solution: 10 mg X-Gluc salt (dissolved in 100 µl DMF) per 20 ml 50 mM $NaPO_4$ buffer.

YPAD-medium: 4 % (w/v) Pepton, 2 % (w/v) yeast extract, 10 % (v/v) glucose solution (40 % w/v), pH 6.5.

SD-medium: 0.67 % (w/v) YNB, 5 % (v/v) glucose (40 %), 10 % (v/v) drop-out solution, pH 5.8 Media was autoclaved for 20 min at 121°C and 1.2 bar.

AS medium: 1 M $MgCl_2$; 50 µM acetosyringone in DMSO; 1 M MES (KOH adjusted pH 5.6), sterile H_2O.

RNase A (made as stock solution): 10 mg of RNase A was dissolved in 1 ml sterile water and heated for 5 min at 95°C. Stored in aliquots at -20°C.

Ampicillin stock solution: 100 mg/ ml in dH_2O. Dilution: 1:1000.

Kanamycin stock solution: 50 mg/ ml in dH_2O. Dilution: 1:1000.

Gentamycin stock solution: 25 mg/ ml in dH_2O. Dilution 1:1000.

Spectinomycin stock solution: 50 mg/ ml in dH_2O. Dilution 1:1000.

Rifampicin stock solution: 50 mg/ ml in DMSO (dimethyl sulfoxide). Dilution: 1:500.

3.1.9 Bacterial and yeast strains

***Escherichia coli* DH10B** (Lorow & Jessee, 1990): Strain was used for cloning.

***Escherichia coli* BL21** (Pharmacia, Freiburg, Germany): Strain was used for the over-expression of proteins.

***Agrobacterium tumefaciens* GV3101**: Strain used for transient transformations (Koncz and Schell 1986).

***Saccharomyces cerevisiae* Y190:** This yeast strain has been used for yeast two-hybrid screening. It contains mutations in different genes, such as *try3*, *his3*, and *leu2*. Because of these mutations, the strain loses the ability to synthesize the corresponding enzymes or amino acids. Thus, tryptophan, leucine, and histidine can be used as selection markers (Durfee *et al.*, 1993).

3.1.10 Vectors

Molecular details of the vectors are shown in the appendix. All the vectors used in this work are kept as plasmids at -20°C or in glycerol stock as bacteria strains at -80°C. All the microorganisms including bacteria and yeasts are stored in glycerol at -80°C (Department of Molecular Physiology, Institute of Molecular Physiology and Biotechnology of Plants (IMBIO), University of Bonn).

pJET1.2/blunt (Thermo Scientific, St. Leon-Rot, Germany): Plasmid used for blunt-end cloning of PCR-fragments.

pET–28a (Novagen): This plasmid encodes for N/C–terminal histidine–tags (His–tag) and possesses an inducible promoter. The vector was used for the expression of His-tagged proteins.

pBIN19 (Novagen, Darmstadt, Germany): This plasmid comprises a binary vector system that facilitates replication in *Agrobacterium tumefaciens*.

pBT10-GUS: This vector contains the coding sequence of the reporter gene β-glucuronidase (GUS/ *uidA*). It was used to generate promoter-GUS fusion constructs. The vector contains a *β-lactamase* gene that confers ampicillin resistance for selection.

pGJ280: This plasmid contains a dual CaMV35S promoter and encodes for the Green Fluorescent Protein (GFP) and was used for over-expression and localization studies. The vector was originally constructed by Dr. G. Jach (Max-Planck-Institute, Cologne, Germany) and was used for protein localization analysis (Willige *et al.*, 2009).

pAS2-1: This binary vector system can replicate autonomously in *E. coli* and *S.*

cerevisiae. The gene of interest is fused to the GAL4 DNA binding domain and was therefore exploited for the generation of bait-fusion construct for the yeast-two hybrid assays. The TRP1 gene allows auxotroph growth on tryptophan-depleted media.

pACT2: This binary vector system can replicate autonomously in *E. coli* and *S. cerevisiae*. The cDNA library is fused to the GAL4DNA activating domain and was therefore exploited for the amplification of the cDNA library in the yeast-two-hybrid assays. The LEU2 gene allows auxotroph growth on leucine-depleted media.

pAD-GAL4: A eukaryotic system to detect protein–protein interactions in vivo, provides a method for the rapid identification of genes encoding proteins that interact with a given protein (i.e., a bait protein).

pDONR 207: Gateway Donor vector.

pEarleyGate 103: Gateway compatible plant transformation vector with GFP and 6xHis C-terminal tags.

pMpGWB228-TagRFP: The pMpGWB vectors allow genes to be fused to a variety of reporters and tags through a simple and uniform procedure using Gateway cloning technology.

3.1.11 Software, programs and online tools

- Blastp – protein blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
- ClustalW2 (www.ebi.ac.uk/clustalw/)
- Reverse Complement (www.bioinformatics.org)
- SNAPgene
- Vector NTI (Invitrogen, USA)
- SigmaPlot Version 12.3
- ImageQuant Version 5.2 software
- Mega for windows Version 5.0
- Microsoft Office package 2015
- Primer3web (http://primer3.ut.ee/)
- PLACE Web Signal Scan (http://www.dna.affrc.go.jp/PLACE/signalscan.html)

- TRANSFAC® Professional Suite from BIOBASE (http://www.biobase-international.com)
- PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)

3.1.12 Primer list

Primer pairs were designed with the help of the Primer3 program. A primer with a guanine/cytosine (G/C) content of 45-55 % was considered most stable, with a higher ratio of GC in terminal nucleotides. Primer pairs were further selected based on similar melting temperatures (TM) in the range of 55°C-65°C and low self-complementarity of the sequences. Mutagenesis primers were designed according to "QuikChange II Site-Directed Mutagenesis Kit (Agilent technologies, USA)". Primers were obtained from Sigma–Aldrich and Eurofins Genomics. All primers were stored at -20°C in 100 mM concentrations. The following primers were used in this thesis:

Table 1: List of primers used in this thesis.

Name	Sequence (5'-3')
pJET1.2 forward	CGACTCACTATAGGGAGAGCGGC
pJET1.2 reverse	AAGAACATCGATTTTCCATGGCAG
Oligo (dT)18	TTT TTT TTT TTT TTT TTT
pGJ280_rev	TGTGCCCATTAACATCACCA
T7–Promoter	TAATACGACTCACTATAGGG
T7–Terminator	GCTAGTTATTGCTCAGCGG
GUS-sense	CGTCCTGTAGAAACCCCAACC
GUS-rev	GATAGTCTGCCAGTTCAGTTCG
5'-pBT10-GUSfwd	AATACGCAAACCGCCTCT
GUS-Start	GGTTGGGGTTTCTACAGGACG

pAS2-1 fwd	TCATCGGAAGAGAGTAG
pAS2-1-rev	CTGAGAAAGCAACCTGAC
pACT2_fwd	TAAAAGAAGGCAAAACGATGT
pACT2_Gal4AD	GCGTTTGGAATCACTACAGG
9-1_pAD_GSP1	ACCCCATACATACAACACATCCAT
9-1_pAD_GSP2	TCTCCCGACAGCTGATTGTTC
CpPIP1-attB1	AAAAAGCAGGCTTAACCATGAAGTCTTCAACAACT
CpPIP1-attB2	AGAAAGCTGGGTACTTGCAATCGCGCAAAGG
TagRFP_R	CCTCGACCACCTTGATTCTC
PGIP_RT_cons2_Fwd	GCAGCAAAAGTGGAAGCTCA
PGIP_RT_cons2_Rev	CAACGAAGACGAAGACAACG
CpPGIP_Nco1_F	CCATGGCTTCTTCTTCCAAAGCCACCGACTT
CpPGIP_Xho1_R	CTCGAGCTTGCAATCGCGCAAAGGTG
M13 Forward	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC
pEarleyGate 103 forward	CGCGCTCGAGATCACAAGTTTGTACAAAAAAGC
CpPGIP1_Y2H_F	CGCGCTCGAGCACCACTTTGTACAAGAAAG
CpPGIP_Y2H_R	CCATGGTCCCTTCTTCCAAAGCCACC
CpGRP1XhoI_Y2H_R	GGATCCTTACTTGCAATCGCGCAAAGG
Cp13-62GW_iso1_R1	CTCGAGTTAAGGATTATTGACGGCCGCC
Cp13-62GW_iso1_R2	GAAGAACTCGGCTTCAAGCAACTC
Cp13-62GW_iso2_R1	GTTCAAAGGGAACTCTAAGAGACTAA

Cp13-62GW_iso2_R2	GGGTTGGAATCTTGGCATAGCTC
Cp13-62g_iso1_F	GCCAATATGGAAGTGCTGCAAAG
Cp13-62g_iso2_F	CAAACGGAGGGCTAGATTCAT
Cp13-62g_iso1+2_rev	CCTTCTTCGAAGCTTCATCAC
Lb13-62g_F	CTAAAACACACAGCCATGTACGA
Lb13-62g_R	ACTCCACCGGTCCACTTCTTC
Lb13-62g2_F	TTCGGTAAATCGTGGGAGAAAAGAGAG
Ls13-62g_F	GTGAAGATCATCTAGATCAACGTACGAG
Ls13-62g_R	CAACCCCTCGAGAAACCTAA
Cp13-62p_iso1_c845t_F	CATGCATGCAAGACGATTATT
Cp13-62p_iso1_c845t_R	GGTTCTACACCTGTCAGGCTTTCGACGGAGG
Cp13-62p_iso2_c904t_F	CCTCCGTCGAAAGCCTGACAGGTGTAGAACC
Cp13-62p_iso2_c904t_R	CTCCGGCCAAAGCCTGACAGCTGTAGAAC
Lb13-62_c662t_R	GTTCTACAGCTGTCAGGCTTTGGCCGGAG
Lb13-62_c662t_F	AGTTTTTACACGTGTCAGGCTGTGGCGGAAGG
Lb13-62_c662t_F	CCTTCCGCCACAGCCTGACACGTGTAAAAACT
Ls13-62_c623t_F	TCGACACCTGTCGGGCTGCCGCCG
Ls13-62_c623t_R	CGGCGGCAGCCCGACAGGTGTCGA

3.2 Methods

3.2.1 Stress treatment

The untreated plants were watered every 3 days. Drought treatment was performed by withholding water till the desired relative water content was reached (48 – 72 hours, RT). The partial dehydration was given to the plants by withholding water for 8 - 10 hours at RT. Rehydration was done by submerging the plants in water for 48 - 72 hours.

For osmotic stress treatments, the water was replaced by the appropriate solutions (concentrations from 100 mM – 400 mM) of mannitol, ABA and sodium chloride. The leaves were detached from the plants and were submerged in the respective solutions for 24 – 48 hours.

3.2.2 Relative water content

The second leaf of each plant (5 cm – 7 cm) was cut and weighed on a balance (fresh weight). The leaves were then transferred to a 50-ml falcon containing water and were incubated overnight in the dark. Next day, the leaves were removed from the falcon and were weighed after removing the excess water on a tissue paper (turgid weight). The leaves were then dried in an oven at 80°C for three days and were then weighed on a balance (dry weight). At least, 3 - 4 pots were used for each treatment. Relative water content was calculated according to the following equation and the mean was calculated for the values in the end.

$$RWC = \frac{(Fresh\ weight - Dry\ weight)}{(Turgid\ weight - Dry\ weight)} \times 100$$

3.2.3 Growth of microorganisms

The *E. coli* (Dh10B) strains were cultured on solid LB agar plates at 37°C, liquid LB medium was used to grow *E. coli* cultures on a shaker at about 180 - 200 *g*. The media contained antibiotics for selection if necessary.

The *A. tumefaciens* strain (GV3101) was grown on solid YEB-agar or in liquid YEB medium at 28°C at 250 *g*.

The *Saccharomyces cerevisiae* strain (Y190) was grown at 30°C on solid YEPD/ SD-agar or in liquid YEPD/ SD-medium at 250 *g*. Amino acid drop out SD medium were used as selection markers, if required.

3.2.4 Extraction of nucleic acids

- **Genomic DNA extraction from plants (CTAB) (Rogers and Bendich 1985)**

About 500 mg of leaf material was weighed and transferred to a 15 ml falcon tube. Plant material was re-suspended in 2.5 ml of 2xCTAB (PVP40 was also added in the CTAB buffer) at 65°C and incubated for one hour. The mixture was vortexed briefly and centrifuged for 10 min at 10,000 *g* in a tabletop centrifuge. After centrifugation, the supernatant was transferred to a new 15 ml falcon tube and 1 ml of chloroform-isoamyl alcohol (24:1) was added to it. The mixture was mixed vigorously for 30 seconds and centrifuged again at 10,000 *g* at room temperature for 10 min. The supernatant was transferred to a new 15 ml tube and the volume transferred was noted down. Then 2 volumes of precipitation CTAB solution was added to it and mixed. This was then incubated at room temperature for 1 hour. The tubes were centrifuged after one hour at 10,000 *g* and the supernatant was removed. The pellet was dissolved in 1 ml of 1.2 M NaCl and moved to a 2-ml tube. To this 1 ml of chloroform was added and the tube was centrifuged at maximum speed for 10 min at room temperature. The upper phase was collected after the centrifugation and the volume was noted down then, 0.6 volumes of propane-2-ol were added to the upper phase and centrifuged at maximum speed for 10 min at 4°C. The supernatant was removed and 500 µl of 70% (v/v) ethanol was added to the pellet in the tube. The tube was then centrifuged again for 10 min at maximum speed at room temperature. The supernatant was removed and the pellet was left to dry. The DNA was re-suspended in 50 µl of T.E+ 1 µl of RNase A (10 mg/ml) per sample and incubated for 20 min at 65°C in order to completely dissolve the DNA.
CTAB-buffer: 0.1 M Tris-HCl, pH 8.0; 40 g/l CTAB (4X); 20 mM EDTA, pH 8.0; 1.4 M NaCl; 2% (w/v) polyvinylpyrrolidone (PVP-40); 2% (w/v) ß-mercaptoethanol.

Precipitation solution CTAB: 5 g/l of CTAB; 0.04 M NaCl.

TE buffer: 10 mM Tris (pH 8.0); 1 mM EDTA (pH 8.0).

- **Purification and precipitation of DNA**

To purify a DNA sample from protein residues and other contaminants, the sample was brought to 400 µl with T.E buffer, 200 µl of chloroform-isoamyl alcohol (24:1) and 200 µl of phenol were added and the sample was centrifuged for 3 min, 14,000 *g* at RT. The upper phase was transferred into a fresh 1.5 ml tube and 0.1 volume 3 M sodium acetate (pH 4.5) and 0.7 volume isopropanol were added to it then centrifuged (20 min, 14,000 *g*, 4°C). The pellet was washed with 1 ml 70% (v/v) ethanol, air-dried and dissolved in 20 µl sterile T.E buffer.

- **DNA fragments extraction from agarose gels**

DNA fragments of PCR products or from enzymatic digestions of plasmid DNA constructs were isolated from agarose gels using the NucleoSpin® Extract II Kit (2.1.6). The extraction and purification were done after excising the bands from the agarose gel following the instructions of the kit manufacturer.

- **Alkaline lysis (Birnboim and Doly 1979)**

The bacteria from the overnight culture were centrifuged in 15 ml tube (3 min; 8000 *g*; RT) or three following time in 2 ml tubes (2 min; max speed; RT). The supernatant was discarded. An additional centrifugation step was performed and then with the help of a pipette the remaining liquid was completely removed. 250 µl of B1 added to the bacterial pellet and vortexed to re-suspend. The bacteria then moved to 2 ml tube if 15 ml tubes were used in the first step and 250 µl of B2 was added and mixed by inverting the tube 4-5 times. The tubes were incubated for 3 min at the RT. Thereafter 350 µl of B3 added and tubes were gently mixed and centrifuged (10 min, 14,000 *g*, 4°C). The supernatant was transferred to a new 1.5 ml tube and under the chemical hood, 300 µl of chloroform and 300 µl of phenol were added. The tubes were vortexed for 30 seconds and then centrifuged (3 min, 14,000 *g*, RT). The upper phase was moved to new tubes and 0.7 volumes of isopropanol were added to the tubes; tubes were mixed by inversion and centrifuged (15 min, 14,000 *g*, 4°C). The pellet was washed with 1 ml of 70% (v/v) ethanol (5 min, 14,000 *g*, 4°C). The ethanol was discarded and the pellets were allowed to air-dry. Finally, the pellets were re-suspended in 40 µl TE/ RNAse A buffer.

B1: 50 mM Tris-HCl, pH 7.5; 10 mM EDTA, pH 8.0; 100 µg/ml RNAse A.

B2: 0.2 M NaOH; 1% (w/v) SDS. Prepare immediately before use.

B3: 0.9 M KAc pH 4.8 (set with acetic acid).

- **Phenolic RNA extraction (Valenzuela-Avendaño *et al.* 2005)**

Frozen plant material (200 mg) was subjected to 1.5 ml RNA extraction buffer. The suspension was vortexed, centrifuged (10,000 *g*, 10 min, RT) and the supernatant was mixed with 300 µl of chloroform–isoamylalcohol (24:1) vortexed for 10 seconds and centrifuged (10,000 *g*, 10 min, 4°C). After centrifugation, the upper phase was precipitated with 375 µl of ice-cold isopropanol and 375 µl 0.8 M sodium citrate/ 1 M sodium chloride solution. The mixture was incubated (10 min, RT) and subsequently centrifuged (14,000 *g*, 10 min, 4°C). Supernatant was discarded and the pellet gets washed 2 times with 70% ethanol (-20°C). After each washing step the sample is again centrifuged like before. The RNA pellet was air-dried (5-10 min) and re-suspended in 100 µl of DEPC-treated water and 167 µl of lithium chloride. An incubation step is performed for 2 hours on ice and the sample gets centrifuged (14,000 *g*, 20 min, 4°C). Pellet is again washed with ethanol and dried at RT and the re-suspended in 20 µl DEPC-treated water. Concentration is determined with the Nano-Drop and quality of RNA is checked by loading 1 µl on agarose gel.

RNA extraction buffer: 38% phenol (v/v), 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate (pH 5.0), 5% glycerol (v/v), autoclaved prior to use (20 min, 121°C, 1.2 bar). Phenol is added after autoclaving and always to the amount required for use.

DEPC-water: 1 ml Diethylpyrocarbonate added per 1000 ml of water, incubation for 12 hours at 37°C, autoclaved prior to use (20 min, 121°C, 1.2 bar).

- **Plasmid DNA mini-prep from yeast**

Yeast cells were collected into a 1.5 ml tube either from liquid culture and re-suspended in 200 µl lysis buffer. Then 200 µl phenol/chloroform (24:1) was added together with 0.3 g of acid-washed glass beads (425-600 µm). The suspension was vortexed vigorously for 5-10 min to break the cell wall and centrifuged (5 min, 14,000

g, RT). The top aqueous phase was transferred to a fresh tube and mixed with 2.5 volume of 100% ethanol and 1/10 volume of 3 M NaAc (pH 5.2). Plasmid DNA was precipitated by centrifuging (10 min, 14,000 *g*, RT) and washed with 70% ethanol. The plasmid DNA was then re-suspended in 20 µl TE buffer and 2 µl was used for transforming *E. coli*.

Lysis buffer: 2% (v/v) Triton X-100; 1% (w/v) SDS; 100 mM NaCl; 10 mM Tris-HCl pH 8.0; 1 mM EDTA.

3.2.5 DNA fragment cloning

- **Gateway cloning**

A fusion construct for *CpPGIP* and *Cp13-62* was generated in the Gateway cloning vector pDONR207, subsequently transformed and sequenced in the entry vector pMpGWB228-TagRFP pEarleyGate 103. The two-step gateway PCR reaction was prepared as follows:

Table 2: PCR reaction mix preparation and PCR conditions for step 1 of gateway cloning.

Step 1: PCR reaction mix (50 µl)		Step 1: PCR conditions		
Water	32.5 µl	Initial denaturation	98°C; 2 minutes	
5 X Phusion buffer	10 µl	Denaturation	98°C; 30 seconds	x10
attB1-primer	2.5 µl	Annealing	55°C; 30 seconds	
attB2-primer	2.5 µl	Elongation	72; 30 seconds	
dNTPs (10 mM)	1 µl	Final elongation	72°C; 5 minutes	
Template DNA (1 µg/ µl)	1.0 µl	Storage	4°C	
Phusion DNA-polymerase	0.5 µl			

Table 3: PCR reaction mix preparation and PCR conditions for step 2 of gateway cloning.

Step 2: PCR reaction mix (50 µl)		Step 2: PCR conditions		
Water	19 µl	Initial denaturation	98°C; 2 minutes	
5 X Phusion buffer	8.0 µl	Denaturation	98°C; 15 seconds	x5
attB1 adapter	4.0 µl	Annealing	45°C; 30 seconds	
attB2 adapter	4.0 µl	Elongation	72°C; 15 seconds	
dNTPs (10 mM)	4.0 µl	Denaturation	98°C; 15 seconds	x20
PCR product (step 1)	10 µl	Annealing	55°C; 30 seconds	
Phusion DNA-polymerase	1.0 µl	Elongation	72°C; 15 seconds	
		Final elongation	72°C; 5 minutes	
		Storage	4°C	

The PCR product from step 2 was purified from the gel and eluted in a final volume of 20 µl**.**

BP reaction mixture was prepared in the final volume of 5 µl as follows: purified PCR product (50-90 ng/ µl), vector pDONR 207 (75 ng/ µl), TE buffer (pH 8.0) (1 µl), BP clonase mix (1 µl). Reaction was incubated for 1-5 hours at 25°C. After 1 hour 0,5 µl Proteinase K was added and the reaction was incubated at 37°C for 10 min. 2 µl of the reaction mixture was used for transformation. The positive clone was identified by sequencing and the plasmid of the positive clone was further used in the **LR reaction** as follows: plasmid (entry clone) (25-75 ng/ µl), destination vector (75 ng/ µl), TE buffer (pH 8.0) (1 µl), LR clonase mix (1 µl). The reaction was incubated for 1 h at 25°C. After 1 hour 0,5 µl Proteinase K was added and the reaction was incubated at 37°C for 10 min. 2 µl of the reaction mixture was used for transformation. The construct was identified by sequencing and was further used for localization studies.

➢ Amplification of DNA fragments by PCR (Mullis & Faloona, 1987)

Table 4: Standard PCR reaction and the standard PCR program (30 cycles) used for the amplification of fragments using plasmid DNA and cDNA as template.

PCR reaction mix (20 µl)		PCR conditions		
Water	12.4 µl	Initial denaturation	98°C; 30 seconds	
5 X Phusion buffer	4.0 µl	Denaturation	98°C; 10 seconds	x29
Forward-primer	1.0 µl	Annealing	60°C; 15 seconds	
Reverse-primer	1.0 µl	Elongation	72; 30 seconds	
dNTPs (10mM)	0.4 µl	Final elongation	72°C; 10 minutes	
Template DNA (1 µg/ µl)	1.0 µl	Storage	4°C	
Phusion DNA-polymerase	0.2 µl			

➢ Agarose gel electrophoresis

PCR products or DNA and RNA samples from plants or bacteria were analysed in an agarose gel (0.8-1.5% (w/v)). DNA or RNA was loaded on the gel and separated by electrophoresis (small-size gel chamber: 65-90 mA, 20-60 min; mid-size gel chamber: 100-140 mA, 30-60 min) in 1× TAE using a 1 kb DNA ladder as reference when required. DNA fragments were visualized under UV light using ethidium bromide staining.

Agarose gel: 0.8-1.5% (w/v) agarose in 1× TAE buffer.

Ethidium bromide solution: 1 mg/ l ethidium bromide in 1× TAE buffer.

➢ Restriction digestion (Sambrook *et al.,* 1989)

Restriction digests were performed at 37°C for 1-3 hours or overnight. To avoid spontaneous re-ligation of compatible ends of single-digested, linearized plasmids, de-phosphorylation of plasmids was performed after the restriction digest using alkaline phosphatases. The SAP-enzyme (Thermo Fisher Scientific) was added to the restriction digest reaction (final concentration 1 unit/ µl) and incubated for 10 min at

37°C. Inactivation of the phosphatases was achieved by heating the samples for 15 min at 65°C.

- **Ligation**

Ligation was normally carried out at 22°C for 3 hours or at 4°C overnight. The number of insert molecules in the reaction should be minimum 3 folds higher than the number of the opened vector molecules. The amount of vector and insert was calculated by the following formula:

$$Insert\ mass\ in\ ng = 3 \times \left(\frac{insert\ length\ in\ base\ pairs}{vector\ length\ in\ base\ pairs}\right) \times vector\ mass\ in\ ng$$

A standard ligation reaction consisted of the following parts: Insert and vector as per the calculation,1 µl T4 ligase buffer and 0.3 µl T4 ligase. Then water was added to make the final volume of the reaction to 10 µl. The ligation mixture was further used for transformation or stored at -20°C.

3.2.6 Transformation methods

- **Preparation of competent *E. coli* (RbCl method)**

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

TFB I: 30 mM KAc; 100 mM RbCl; 10 mM $CaCl_2.2H_2O$; 50 mM $MnCl_2.4H_2O$; 15% (v/v) Glycerol. Adjust pH to 5.8 using 0.2 M acetic acid and filter sterilize.

TFB II: 10 mM MOPS; 75 mM $CaCl_2.2H_2O$; 10 mM RbCl; 15% (v/v) Glycerol. Adjust

pH to 6.5 using KOH and filter sterilize.

- **Transformation of competent *E. coli***

For one transformation, 50 µl of chemically competent cells were thawed on ice. The ligation mixture (3 µl to 5 µl) was added to the cells and the mixture was incubated on ice for 5 min. Subsequently, cells were heat-shocked on a heat block at 42°C for 50 seconds. Immediately after the transformation, cells were placed on ice for 5 min. Then, 450 µl of SOC medium was added to the cell mixture and the bacteria were incubated on a shaker (200 *g*) at 37°C for 1 hour, 200 µl and 300 µl of this cell mixture were plated on LB agar plates containing selective antibiotics and incubated overnight at 37°C (16 to 18 hours).

- **Preparation of electrocompetent *A. tumefaciens* via electroporation (Tung and Chow 1995)**

A single colony of *A. tumefaciens* containing the helper plasmid GV3101 was inoculated in 3 ml YEB-rifampicin medium and grown overnight at 250 *g*. Next day, the suspension was diluted in 50 ml of fresh YEB-rif medium and further cultured until the OD_{600} reached 0.5. Afterwards, the cell culture was centrifuged for 30 min at 5000 *g*. The supernatant was discarded and the pellet was re-suspended in following solutions with centrifugation for 10 min (5,000 *g*, 4 °C) between each washing step.

Resuspension solution: 25 ml 1 mM Hepes pH 7.5, 12.5 ml 1 mM Hepes pH 7.5, 10 ml 10% (v/v) glycerol, 1 mM Hepes pH 7.5, 5 ml 10% (v/v) glycerol, 1 mM Hepes pH 7.5, 2 ml 10% (v/v) glycerol, 1 ml 10% (v/v) glycerol.

After adding of each solution, the suspension was centrifuged for 10 min at 4°C and at 5000 *g*. Finally, 1 ml of 10% (v/v) glycerol was added. The competent cells were divided in 50 µl aliquots, frozen in liquid nitrogen and stored at - 80°C.

- **Transformation of A. tumefaciens via electroporation (Tung and Chow 1995)**

The transformation was done using the electroporation method in which an electric pulse rips tiny holes into the cell membrane allowing plasmid DNA to enter. An aliquot of *Agrobacterium* competent cells was taken and thawed on ice for 7-10 min. Meanwhile a dilution of the plasmid DNA containing the promoter fragment cloned in

pBIN19 vector (1:200) was diluted with water. An electroporation cuvette (2 mm) was precooled on ice or in the freezer. 1-2 µl of plasmid DNA was added to the cells. After mixing the cells were transferred into the cooled electroporation cuvette and the electroporation was carried out. Right after the electroporation the cells were regenerated with 1 ml of YEB and transferred for 2 hours on a shaker (180 *g*, 28˚C) in a growth tube. After regeneration cells were plated on YEB+RIF+KAN plates for the selection of successfully transformed cells. The plates were incubated at 28˚C for two days.

Capacitance: 25 µF, voltage: 2.5 kV, resistance: 400.

- **Transient transformation via particle gun bombardment (Sanford *et al.* 1993)**

30 mg gold particles (1.6 µm diameter) which were used as micro-carriers were weighed into a 1.5 ml Eppendorf tube and washed with 1 ml 100% ethanol with vigorously vortexing for 5 min. After sedimentation of the particles, the supernatant was carefully pipetted off and discarded. The gold particles were washed three times as follows: add 1 ml sterile water vortex for 1 min and wait until particles have settled again. Take off supernatant and discard. Repeat the washing step three times and finally dissolve gold particles in 500 µl sterile 50% (v/v) glycerol. Prepared gold particles (60 mg/ml) were stored at 4 °C in 50 µl aliquots for up to one month without decrease in transformation efficiency. One aliquot of the gold particles was used for coating: 25 µg plasmid-DNA, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 100 mM freshly prepared spermidine were in this order added to the gold suspension rapidly while vortexing for 5 min at maximum speed. The suspension was briefly centrifuged and the supernatant was discarded. The particles were then washed twice with 140 µl 70% and 100% ethanol, respectively. The covered gold particles were finally suspended in 50 µl 100% ethanol. 25 µl of the gold suspension were used for each bombardment. Bombardment was performed according to the instruction of PDS-1000/He manufacturer. Briefly, a plastic macro-carrier disk with 25 µl of DNA-coated gold particle (micro-carrier) suspension was placed into the macro-carrier holder along with a stopping metal grid. The system macro-carrier and stopping grid was placed into the launch assembly unit as described by the manufacturer. Healthy *C. plantagineum*, *L. brevidens* and *L. subracemosa* leaves or fresh onion epidermises were well arranged in the centre of a 1/2 MS solid medium plate and placed at 5-10 cm below the stopping screen. Vacuum was then applied to increase the gas pressure within the

bombardment chamber. The release of the pressure led to the burst of the rupture disk and allowed the macro-carrier to eject at high velocity the DNA-coated gold particles into the leaves or onion epidermal cells. The particles were accelerated with a helium pressure of 1150 pounds per square inch (psi) under a vacuum of 27 mm Hg (3.6 MPa). The leaves or onion epidermis were incubated on 1/2 MS plates for 12-48 hours and analysed under a confocal laser microscope.

- **Yeast competent cell preparation**

Liquid YPD medium (4-5 ml) was inoculated with yeast and allowed to grow overnight. The pre-culture was then diluted to the OD_{600} of 0.2 with fresh YPD medium and allowed to grow up to the OD 0.8 - 1.0 (approximately 4 - 6 hours). The cells were then harvested (1 min, 1700 *g* in 2ml tubes/ 5 min, 1500g in falcon). The supernatant was discarded and the cells were placed in ice and re-suspended in 500 µl TE/ LiAc buffer for centrifugation (1 min, 1700 *g*, 4˚C (repeat this step 2 or 3 times)). Finally, the cells get re-suspended in 150 – 300 µl TE/ LiAc buffer (depending on the number of transformations). Yeast competent cells cannot be stored and should be prepared every time fresh prior to transformation.

YPD medium (2X): 4% (w/v) bacto peptone; 2% (w/v) yeast extract; 92% (v/v) deionised water. Adjust pH to 5.6 then add 80 mg/ l adenine hemisulphate. Autoclave and after cooling down add glucose to 4% (v/v) using a 50% (w/v) glucose solution which has been autoclaved separately.

For plates 1X YPD agar was used and for the cultures 2X YPD broth was used.

- **Transformation of yeast (Gietz and Schiestl (1995) with some modifications by Gabriel Schaaf and Loque)**

The competent cells were pipetted into the PCR tubes in the following order: 100 µl of PEG/ LiAc buffer, 16.5 µl competent cells, 200 – 500 ng plasmid and 3.5 µl salmon DNA (8 mg/ µl). The salmon DNA should be denatured for 3 min at 95˚C and then placed on ice. The complete mixture was then incubated for 30 - 40 min at RT (to increase the efficiency shake the tubes at 500 *g*). The tubes were the placed in a thermocycler at 42˚C for 20 min. Everything was plated on the selective media.

Salmon sperm DNA: Add to 400 mg DNA sterile TE/ LiAc buffer (pH 7.5) to 50 ml

final volume. Incubate at 4˚C for 24 – 48 hours while rotating on a rotor, aliquot and store at -20˚C.

LiAc stock solution: 1 M LiAc (adjust pH to 7.5 with acetic acid).

TE/ LiAc buffer: (1X T.E, 0.1 M LiAc) in a sterile falcon tube take 40 ml sterile water, 5 ml LiAc stock solution and 5 ml 10X TE.

PEG/ LiAc buffer: Add 10 ml LiAc stock solution and 10 ml 10X TE to 80 ml of the 50% PEG.

3.2.7 Screening methods

- **Transformed bacterial clones (colony PCR)**

Colony PCR was used for quick screening of the plasmids containing a desired insert directly from bacterial colonies obtained after transformation. The PCR was prepared and performed as described in the following table:

Table 5: PCR reaction mix preparation and PCR conditions for a standard colony PCR.

PCR reaction mix (20 µl)		PCR conditions		
Water	16.33 µl	Initial denaturation	98°C; 3 minutes	
10 X PCR buffer	2.04 µl	Denaturation	98°C; 30 seconds	x24
Forward-primer	0.41 µl	Annealing	60°C; 60 seconds	
Reverse-primer	0.41 µl	Elongation	72°C; 30 seconds	
dNTPs (10 mM)	0.41 µl	Final elongation	72°C; 10 minutes	
Template	Bacterial colony	Storage	4°C	
Taq-polymerase	0.41 µl			

- **Screening of yeast clones**

Yeast colonies that were present on selective plates (SD-His-Leu-Trp) were picked to perform colony PCR using library specific primers to amplify DNA inserts. The PCR product was sent for sequencing to identify the interaction partners. Also, yeast colonies present on selective plates (SD- His-Leu-Trp) were re-suspended in 50 µl sterile water and then 5 µl were inoculated onto the same fresh selection plates (SD-His-Leu-Trp + 25 mM 3-AT). Colonies which grew well at 25 mM concentration of 3-AT plates and rapidly generated a high amount of the blue compound as compared with the bait in β-galactosidase assays were taken as positive clones. Beta-galactosidase assays were first performed for all plates for the Y2H library screening, because one white colony in a β-galactosidase assay in an auto-activation test was selected as bait. Colonies that became blue were re-suspended in 50 µl sterile water and 5 µl were spread onto SD-Try-Leu-His + 25 mM 3-AT plates and SD-Try-Leu-His + 50 mM 3-AT plates.

- **Colony colorimetric assay for β-galactosidase activity**

A Whatman filter WH10311897 that fits the Petri dishes was prepared and placed onto the yeast colonies grown on a SD or YPD plate. The Whatman filter was lifted from the YPD or SD plate carefully using forceps to make sure every yeast colony was transferred onto the Whatman filter. Place the filter yeast side up in a liquid nitrogen bath for 10 sec. The frozen filter was placed with the yeast facing upwards onto two layers of Whatman 3 MM Chr paper which had been soaked completely in the Z-buffer in a new Petri dish. Air bubbles between the Whatman filter and the Whatman papers were removed quickly while the Whatman filter thaws. The plate was then incubated at 37°C. Blue colouring was regularly checked over a maximum period of 24 h. Pictures were taken to document the amount of blue compound generated by each yeast lysate.

Z-buffer: 60 mM Na_2HPO4; 40 mM $NaH_2PO_4.2H_2O$; 10 mM KCl; 1 mM $MgSO_4.7H_2O$; pH adjusted to 7.0 with 10 N NaOH.

Reaction solution: 6 ml Z-buffer; 100 µl 4% (w/v) X-Gal; 11 µl β- mercaptoethanol. Prepared freshly.

- **Sequencing**

Sequencing was performed by Eurofins (Eurofins Genomics, Ebersberg) using the Sanger sequencing method. The sequencing reaction was prepared as follows in sequencing tubes: 15 µl plasmid/ PCR product (50-100 ng/ µl for plasmid DNA and 1-10 ng/ µl for purified PCR product) and 2 µl primer (forward/ reverse 10 pmol/ µl).

3.2.8 Preparation of glycerol stocks for bacteria and yeast

500 µl of an overnight bacterial culture was mixed with 500 µl of autoclaved 100% (v/v) glycerol in a cryo-tube and immediately frozen in liquid nitrogen. The tubes were stored at -80°C.

To store the yeast 500 µl of yeast culture was mixed with 100% (v/v) glycerol and the appropriate medium (250 µl each) in a cryo-tube and immediately frozen in liquid nitrogen. The tubes were stored at -80°C.

3.2.9 Reverse transcription polymerase chain reaction

- **DNase treatment and synthesis of cDNA**

Treatment with DNaseI is performed to degrade the remaining DNA in the sample. After that the RNA can be converted into cDNA. A typical DNase treatment reaction in a final volume of 20 µl is prepared as follows:

Table 6: Reaction mix for DNase treatment.

RNA	x µl (1 µg)
10x DNase1 reaction buffer with $MgCl_2$	2 µl
Sterile water	Up-to 20 µl
RNase free DNase1	2 µl

The reaction is incubated for 30 min at 37°C. 2 µl of 25 mM EDTA is added to the reaction and is incubated for 10 min at 65°C. After EDTA treatment the reaction is cooled on ice and DNase treated samples are divided into two tubes (10 µl in each tube). The synthesis of the first strand cDNA is performed using an oligo-dT primer. A

control reaction is prepared without reverse transcriptase. For each positive sample 1 µl oligo-dT primer (100 pmol/ µl) to DNase-treated RNA is added. The final volume is reached up to 13.5 µl with water. The samples are incubated at 65°C for 5 min and then cooled on ice. To each sample 4 µl of 5x first strand buffer, 2 µl dNTP (10 mM) and 0.5 µl reverse transcriptase are added. The reaction mixture is incubated at 42°C for 1 h. The reaction is terminated by heating at 70°C for 5 min. The reaction mixture is then used directly for the PCR or stored at -20°C until further use.

- **RT- PCR**

To analyse the expression of genes under different conditions the cDNA of interest is amplified. The PCR reaction mix and conditions used are as follows:

Table 7: PCR reaction mix preparation and PCR conditions for RT PCR.

PCR reaction mix (20 µl)		PCR conditions		
Water	15.4 µl	Initial denaturation	98°C; 3 minutes	
10 X PCR buffer	2.04 µl	Denaturation	98°C; 30 seconds	x34
Forward-primer	0.41 µl	Annealing	60°C; 30 seconds	
Reverse-primer	0.41 µl	Elongation	72°C; 60 seconds	
dNTPs (10 mM)	0.41 µl	Final elongation	72°C; 10 minutes	
cDNA template	1.0 µl	Storage	4°C	
Taq-polymerase	1.0 µl			

3.2.10 Protein extraction from plant tissues (Laemmli 1970)

Total soluble plant proteins were extracted using “Laemmli” sample buffer. 50-100 mg powdered plant material was homogenized with 150-200 µl of Laemmli buffer by vortexing in a 1.5 ml tube. The extract was boiled for 5 min in a water bath or heating block and centrifuged for (5 min, 13,000 *g*, RT) to separate insoluble material. The supernatant containing crude total proteins was collected in a fresh tube and stored at -20°C. Samples were heated at 95°C for 5 min before loading on the gel.

Laemmli buffer (1×): 62.5 mM Tris-Cl (pH 6.8); 10% (v/v) glycerol; 2% SDS (w/v); 0.1% (w/v) bromophenol blue and 0.7 M (≈ 5%) β-mercaptoethanol (added freshly).

3.2.11 Cell wall protein extraction (Feiz *et. al*., 2006)

Step1: Tissue preparation

The tissues (5 g) were ground in liquid nitrogen to obtain a fine powder, and then 20 ml of buffer A was added to them. The slurry was transferred in a 50 ml tube and left on a shaker (24 Hz, 2 min). After that the tubes were transferred on a rocking platform at 4°C overnight. Next day, the tubes were centrifuge (4000 *g*, 15 min, 4°C). The supernatant was discarded and the pellet was re-suspended in10 ml of buffer B, then again placed on a rocking platform for 30 min at 4°Cand centrifuge (4000 *g*, 15 min, 4°C). The supernatant and the pellet were re**-**suspended in 10 ml of buffer C, and placed on a rocking platform for 30 min and centrifuged **(**4000 *g*, 15 min, 4°C**)**. The supernatant was discarded and the pellet was again re-suspended in 10 ml of buffer D, placed on a rocking platform for 30 min at 4°C and centrifuged (4000 *g*, 15 min, 4°C). The step with buffer D was repeated twice and the pellet was finally transferred in a 30 ml tube for step 2 (the pellet is ground in liquid nitrogen first and stored at -80°C when step 2 is made the following day).

Buffer A: 5 mM Na acetate, 0.4 M sucrose, pH 4.6, 4°C

Buffer B: 5 mM Na acetate, 0.6 M sucrose, pH 4.6, 4°C

Buffer C: 5 mM Na acetate, 1 M sucrose, pH 4.6, 4°C

Buffer D: 5 mM Na acetate, pH 4.6, 4°C

Step 2: Cell wall proteins extraction

The pellet is re-suspended in 7.5 ml of buffer AC then placed on a rocking platform (30 min, 4°C) after which it is centrifuged (10000 *g*, 15 min, 4°C) and the supernatant is collected. The step is repeated twice and the supernatants are pooled together and stored at -20°C (calcium fraction)**.** The same pellet is again re-suspended in 10 ml of buffer AE and shaken vigorously at 37°C for 1 h and then centrifuge (10000 *g*, 15 min, 4°C). The step is repeated thrice and the supernatants are pooled together and stored at -20°C (EGTA fraction)**.** The pellet is finally re-suspended in 15 ml of buffer AL and placed on a rocking platform at 4°C overnight. Next day the slurry is centrifuged (10000 *g*, 15 min, 4°C), and the supernatant is stored at -20°C (lithium fraction)**.** All steps to be done at 4°C**.**

Buffer AC: 5 mM Na acetate, 200 mM $CaCl_2$, pH 4.6, 4°C

Buffer AE: 5 mM Na acetate, 50 mM EGTA, pH 4.6, 4°C

Buffer AL: 5 mM Na acetate, 3 M LiCl, pH 4.6, 4°C

Step 3: Concentration

The three fractions (Ca, EGTA, Li) are transferred separately to a concentrator (15 ml Amicon ultra centrifugal filter 1000 MWCO) to a volume of 200 µl. ReadyPrep 2-D cleanup from Bio-Rad was used according to manufacturer's directions. Protein concentration was determined using the Bradford method and was checked on SDS-PAGE.

Step 4: Acetone precipitation (desalting)

Acetone precipitation was performed to eliminate acetone soluble interferences from protein extracts. To 1 volume of protein solution 4 volumes of cold acetone (keep acetone for at least 3 h or overnight at -20°C) was added. The solution was mixed and kept at least 20 min/ 3 hours at -20°C and then spinned for 15 min at 4°C in microfuge at maximum speed (15000 *g*). Supernatant was discharged carefully and the pellet was retained. The tubes were dried by inversion on tissue paper (as the pellet was difficult to see) and then air-dried for 30 min to eliminate any acetone residue. For SDS-PAGE, the samples were re-suspended in 50 µl of sample buffer.

3.2.12 Overexpression and Extraction of recombinant proteins from *E. coli* cells

Recombinant proteins were extracted from *E. coli* BL21 clones to check the induction efficiency and to see whether they were secreted or sedimented as "inclusion bodies". The expression of the recombinant proteins was induced by adding 1 mM IPTG when the bacteria reached OD_{600} of 0.6 and were further cultured at 22-26 °C for 5 hours in the dark. 1 ml culture sample was collected before and every hour after IPTG added, then centrifuged (2 min, 14,000 *g*, 4°C). The supernatants were discarded and the pellets were stored at -20°C. The bacteria pellets were re-suspended in 200 µl ice-cold PBS, 5mM DTT and 1% (v/v) Triton X-100. The suspensions were sonicated on ice (4× 20 sec) for complete lysis and centrifuged for 10 min (12,000 *g*, 4°C). 50 µl of the supernatant (soluble proteins) were diluted with one volume 2× sample buffer while the pellets were suspended in one volume 1× sample buffer. These samples were heated at 95°C for 10 min and analysed by SDS-PAGE or stored at -20°C.

PBS: 8 g/ l NaCl; 0.2 g/ l KCl; 1.44 g/l Na_2HPO_4; 0.24 g/ l KH_2PO_4.

2× sample buffer: 4% SDS; 20% (v/v) glycerol; 120 mM Tris, pH 6.8; 0.01%

Bromphenol blue, 0.2 M DTT (added freshly before use).

3.2.13 Extraction and purification of recombinant proteins by His-tag affinity-chromatography

Soluble His-tagged recombinant proteins were purified by metal ion chromatography on His- tag binding columns under native conditions. The bacterial pellets from 100 ml IPTG-treated culture were re-suspended in 5 ml buffer A plus 1 mg/ ml lysozyme, incubated on ice for 30 min and sonicated until the cell suspension became translucent (6× 20 sec). The cell suspension was centrifuged for 30 min (12,000 *g*, 4 °C) and the supernatant was filtered through a 0.45 µm membrane. Before loading the supernatant onto the column, the column was washed with 3-bed volumes dH_2O, charged with 5-bed volumes 50 mM $NiSO_4$, 3-bed volumes of dH_2O to remove the free $NiSO_4$ and equilibrated with 3-bed volumes of buffer A. The filtered supernatant was then loaded onto the column and allowed to drain freely by gravity. The column was washed with 10-bed volumes buffer A and 8-bed volumes buffer B. The protein was eluted with the buffer C in 500 µl fractions. The purity of the protein fractions was verified using SDS-PAGE analysis and the quantity was estimated using the Bradford assay. Aliquots of the non-purified supernatant and of the flow-through were analysed along with the protein fractions. The column was regenerated with strip buffer. All buffers and solutions used for the assay were prepared with autoclaved H_2O.

Buffer A: 50 mM NaH_2PO_4, pH 7.4; 300 mM NaCl; 250 mM imidazole; 10% (v/v) glycerol; 0.1% (v/v) Triton X-100; 1 mM β-mercaptoethanol (added freshly), pH was adjusted to 8.0 with NaOH.

Buffer B: 50 mM NaH_2PO_4 (pH 7.4); 300 mM NaCl; 30 mM imidazole; 10% (v/v) glycerol; 0.1% (v/v) Triton X-100; 1 mM β-mercaptoethanol (added freshly), pH was adjusted to 8.0 with NaOH.

Buffer C: 50 mM NaH_2PO_4, pH 7.4; 300 mM NaCl; 5 mM imidazole; 10% (v/v) glycerol; 0.1% (v/v) Triton X-100; 1 mM β-mercaptoethanol (added freshly), pH was adjusted to 8.0 with NaOH.

Strip buffer: 100 mM EDTA, pH 8.0; 500 mM NaCl; 20 mM Tris-HCl, pH 7.9.

The protein purified from the column was then lyophilised and the powder was used to raise protein specific polyclonal antibodies in rabbits (Seqlab (Sequence Laboratories Göttingen GmbH, Göttingen, Germany; www.seqlab.de)). The concentration of the protein powder was checked on a SDS- gel against different concentrations of BSA.

3.2.14 Quantification of proteins (Bradford 1976)

To load the same amount of each protein sample to an SDS- gel it is important to determine the protein content in each sample. This is done by Bradford assay. SDS strongly affects the protein measurement and therefore it is important to remove SDS from the sample before. To do that 1-5 µl of the sample is mixed with 100 µl potassium phosphate buffer by vortexing. Samples are incubated at RT for 10 min and centrifuged at maximum speed to pellet SDS. The supernatant can then be used to measure protein concentration. Supernatant is mixed with 700 µl of water and 200 µl Bradford-reagent (Roti-Quant, Carl Roth, Karlsruhe, Germany). After 5 min incubation, the OD_{595} is measured against the blank, consisting of 700 µl water, 200 µl Bradford- reagent and 100 µl potassium phosphate buffer. By measuring the absorption, one calculates the protein concentration in the sample by comparing the OD value to a BSA standard curve.

Potassium phosphate buffer: 0.1 M potassium phosphate (pH 6.8).

3.2.15 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970)

Separation of proteins is done using the method first described by Laemmli. For all the results in this thesis 15% SDS- gel was used. For a gel run Tris/ Glycin running buffer is needed. After preparing the gel chamber the gel pockets are cleaned using an injection needle. The protein samples are heated at 95˚C for 10 min and then centrifuged at maximum speed for 10 min at RT. For protein separation, the same amount of each sample is loaded. Six µl of protein ladder U7 is loaded as reference. The gel run is performed for 1.5 - 2 hours at 200 V, 15 mA (stacking gel)/ 20 mA (separation gel).

Table 8: Components needed to prepare stacking gel and separating gel for SDS-page.

Component	Stacking gel (ml)	Seperating gel (ml)
Water	2.89	3.4
30% Bisacrylamid	0.53	7.5
1.5M Tris (pH 8.8 or 6.8)	0.5 (pH 6.8)	3.8 (pH 8.8)
10% SDS	0.04	0.15
10% Ammonium persulphate	0.04	0.15
TEMED	0.004	0.006

3.2.16 Coomassie Blue staining of SDS-PAGE gels

SDS gels were stained using the triphenylmethane Coomassie G-250 protein dye. Coomassie has a sensitivity of 10-50 ng protein. As a first step the SDS gel is transferred and incubated in fixation solution for at least 1 hour. The gel is then washed three times with water for 10 min. Staining in Coomassie Brilliant Blue is performed with staining solution overnight at room temperature with gentle shaking. De-staining is done with water.

Fixation solution: 10% acetic acid, 40% methanol

Staining solution stock: 100 g/ l ammonium sulphate, 1% (v/v) phosphoric acid, 0.1% (w/v) Coomassie G-250

Staining solution: 4 volumes of staining solution, 1 volume of methanol

3.2.17 PonceauS red staining

After blotting the membrane is stained with PonceauS to check the effectiveness of transfer. The PonceauS red interacts reversible with the positive charged groups of the protein residues. Membrane is incubated in PonceauS staining solution for 20 min. The unbound dye is removed by washing the membrane with water for a few seconds. Membrane is then analysed by immunodetection. The protein ladder should be marked with a pencil after the PonceauS stain.

Staining solution: 0.2% PonceauS in 3% (w/v) TCA

3.2.18 Western blot

For detection of specific proteins using antibodies the proteins are transferred from the SDS gel to a nitrocellulose membrane using (Wet-) Western blot technique. Towbin buffer is needed to fill the chamber. The blotting sandwich is composed in the following order: Support grid (in the direction of cathode): Whatman paper, SDS gel, nitrocellulose membrane, support grid.
Blotting chamber is placed on a magnetic stirrer in the cold room. Blotting takes 45 min using a voltage of 70 V, 400 mA and a temperature of 4˚C.
Towbin buffer: 25 mM Tris, 192 mM Glycine, 20% (v/v) methanol

3.2.19 ECL detection

The membrane is blocked on a shaker for 1 h at RT or overnight at 4˚C using the blocking solution. After blocking the membrane is washed 3 times with TBST for 10 min. Membrane is incubated using the primary antibody (CpPGIP, 1:5000 in TBST) overnight at 4˚C. The membrane is washed again with TBST 3 times and then incubated in secondary antibody (IgG-Peroxidase, 1:5000 in TBST) at RT for 45 min. The washing step is repeated again and *Thermo Pierce ECL Western Blotting substrate* is used to develop the signal. ECL solution A (Luminol) and solution B (H_2O_2) are mixed 1:1. The blot is taken out of the blocking solution and placed on a glass plate. Using a pipette ECL solution is transferred to the membrane. Plastic film is used to cover the surface so that the solution is distributed uniformly and there are no air bubbles, the membrane is incubated for 1 min. Tissue is used to suck off the excess ECL solution. Chemiluminescence signals were detected by using the luminescence image analyser.
Blocking solution: 4%(w/v) milk powder in TBST
10x TBS: 200 mM Tris-Hcl, pH 7.5, 1.5 M NaCl
TBST: 1x TBS, 0.1% (v/v) Tween-20, pH 7.5

4. Results

4.1 Characterization of polygalacturonase inhibitor 1-like protein (CpPGIP) from *C. plantagineum*

4.1.1 Preparation of the polygalacturonase construct using gateway cloning

- **Amplification of the full-length coding sequence including the signal peptide**

The full length PGIP cDNA sequence was provided by Dr. Valentino Giarola. In order to include the signal peptide sequence GSP1 and GSP2 primer sequences were designed at the 5´end of the sequence and the sequence was amplified by cDNA walking using λcDNA as template. The amplified sequence was eluted from the gel in a final volume of 15 µl using elution buffer and cloned into the pJET1.2vector (**section 3.2.4**). Positive clones were confirmed by colony PCR and sequencing of the insert. The signal peptide was 26 bp long and the overall length of the complete cDNA sequence was 800 bp (**Fig. 8**).

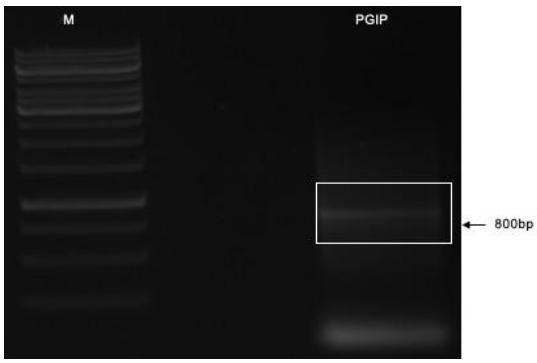

Fig. 8. Amplification of PGIP from cDNA using a combination of GSP1 and GSP2 primers**.** Lane 1: molecular-weight size markers (M), lane 2: *PGIP* amplified from cDNA (PGIP).

- **Preparation of the construct using the gateway method**

The plasmid preparation of the full-length sequence including the signal peptide was used as the template for the gateway cloning (**3.2.5, Fig. 9**). The correct PGIP sequence containing the signal peptide was identified by sequencing. The PCR band obtained after the two-step gateway reaction was eluted in a final volume of 15 µl using T.E buffer (pH: 8.0). This elution was further used in LR and BP reactions. The vector pDONR207 was used as the “entry vector” and the two “destination vectors” used were pEarleyGate and pMpGWB228. The plasmid obtained from the construct containing the full-length sequence in pEarleyGate 103 and pMpGWB228 was used further to

infiltrate *N. benthamiana*. The PGIP sequence cloned into the destination vectors was confirmed by sequencing using the specific primer combinations.

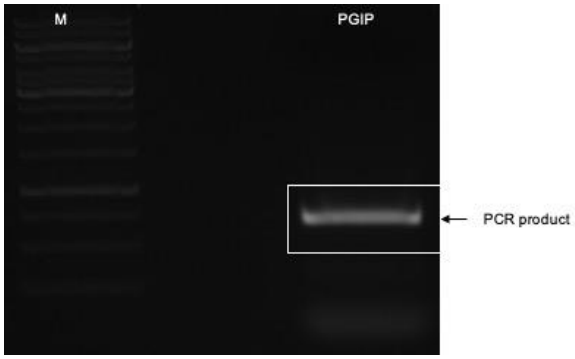

Fig. 9. Amplification of *PGIP* after the two-step gateway PCR using attB1 and attB2 primer combination for the primary PCR and attB1 adaptor and attB2 adaptor for the secondary PCR. Lane 1: molecular-weight size markers (M), lane 2: *PGIP* amplified from the step 2 PCR using PCR product from step 1 as template (PGIP). For step 1 reaction the plasmid from 3.2.1.1 was used as template.

4.1.2 Sub cellular localization of CpPGIP

- **Transformation of *Nicotiana benthamiana***

A Single colony of transformed *A. tumefaciens* was picked and inoculated in 7 ml of YEB medium with appropriate antibiotics (rifampicin and spectinomycin (**section 3.1.8**)) and incubated overnight on a shaker (180 *g*, 28 °C). The cells were collected by centrifuging at room temperature at 4000 *g* for 15 min. The Supernatant was discarded and cells were diluted with AS medium (**section 3.1.8**) to OD_{600} 0.1 - 0.3. The suspension was then incubated at 28 °C in the dark for 2 h with continuous shaking (35 *g*). The 6-8 weeks-old, regularly watered wild type *N. benthamiana* plants were used for infiltration (**Fig. 10**). The fluorescence was observed one to two day's (48 hours) post-infiltration under the confocal laser microscope.

Fig. 10. *N. benthamiana* leaf infiltration with the construct at the abaxial side using a syringe. 2-3

leaves per plant were infiltrated.

➢ Protein localization via confocal microscopy

The CpPGIP protein localization in the transformed *N. benthamiana* was analysed by laser confocal inverted microscope (Nikon Eclipse TE 2000-U) using EZ-C1 3.80 software with appropriate filters. Samples of the infiltrated *N. bentamiana* leaves were fixed on the glass slides with water. The cells were also treated for plasmolysis with 1 M sucrose for 20 min. The protein was found to be localized in the cell wall of the leaves. After plasmolysis, the protein started to form aggregates and the separation of the cytoplasmic strands could be clearly seen (**Fig. 11, 12**).

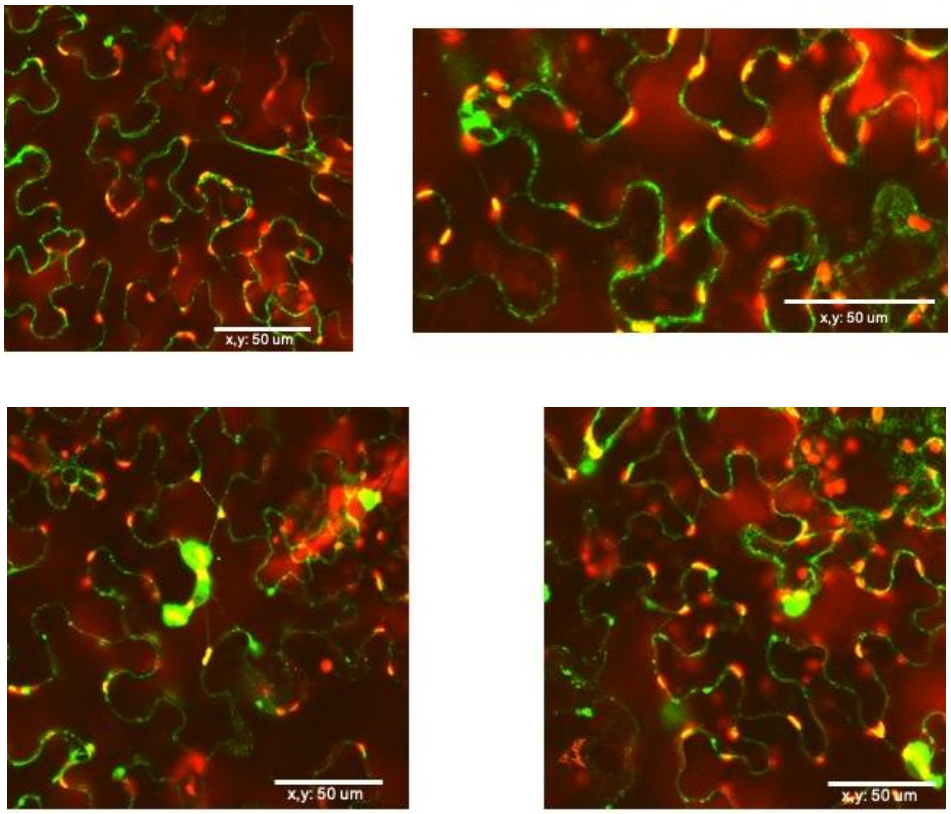

Fig. 11. CpPGIP protein localization (pEarleyGate 103 construct). (Top) Untreated *N. benthamiana* leaves. (Bottom) *N. benthamiana* after plasmolysis treated with 1 M sucrose for 20 min.

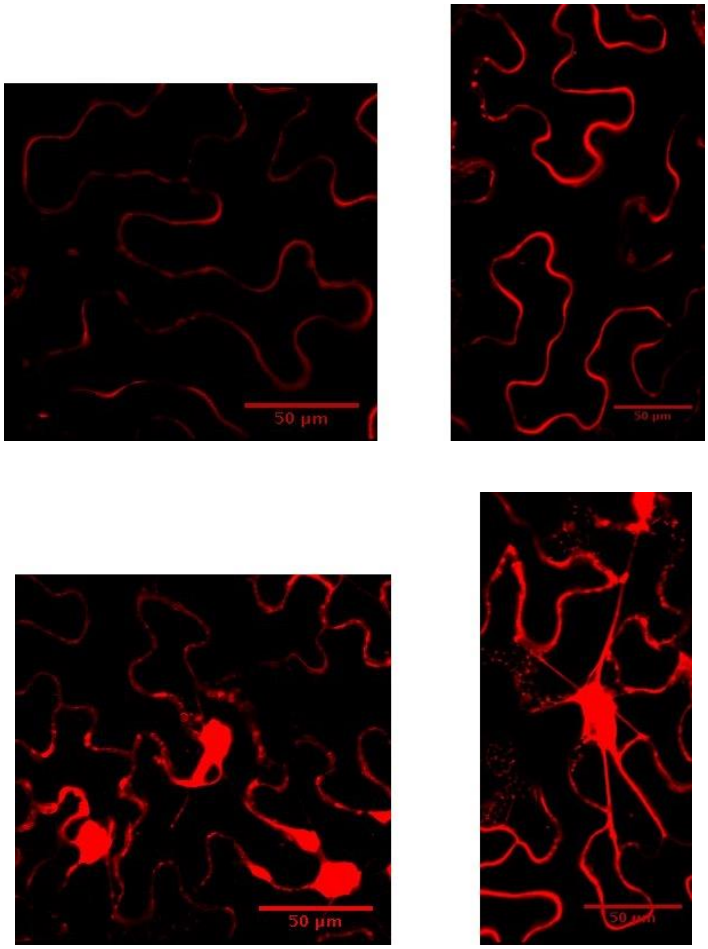

Fig. 12. CpPGIP protein localization (pMpGW228construct). (Top) Untreated *N. benthamiana* leaves. (Bottom) *N. benthamiana* after plasmolysis treated with 1 M sucrose for 20 min.

4.1.3 Expression profile of *CpPGIP*gene under different stress conditions in leaves and roots

Transcript and protein expression patterns vary in different developmental stages of the plant, in different plant tissues and can change upon stress treatments. For the characterization of the gene, it is required to study the expression profiles. The transcript analysis of *CpPGIP* gene was done by RT- PCR (**section 3.2.9**). The first step was to treat the *C. Plantagenium* (**Fig. 13**) by withholding the water and measuring the relative water content (**section 3.2.2**). RNA was isolated (**section 3.2.4**) from roots and leaves of the plants with different relative water contents and cDNA was synthesised. This cDNA was used as a template in the PCR amplification along with the gene specific primer combinations. Elongation factor 1α (EF1α) and pCC11-24 were used as controls in the PCR (**Fig. 14**). The primer sequences are mentioned in the **section 3.1.12**.

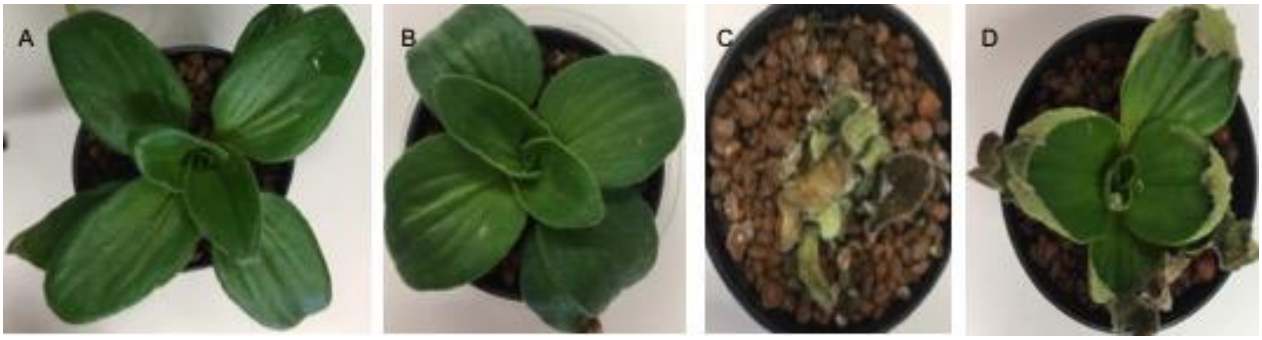

Fig.13. Treatment of *C. plantagenium* for the measurement of the relative water content (RWC). A) Control plants were watered regularly (RWC= 92%).B) Water was progressively withheld for partially dehydrated plants until the water content in the pots declined to the permanent wilting point (RWC= 53%). C) Completely dehydrated plants were left without water for 7 days (RWC= 8%). D) After the dehydration treatments, the pots were re-watered for a rehydration treatment up to 72 hours (RWC= 70%). Samples were harvested after each treatment for the calculation of the RWC (**3.2.2**).

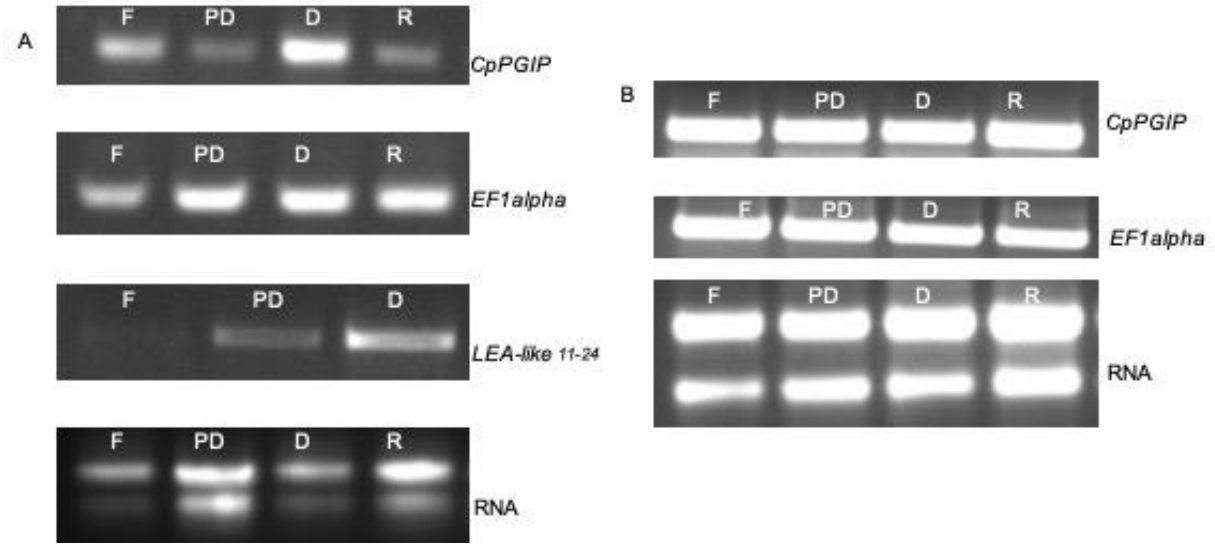

Fig.14. Expression of *CpPGIP* in response to dehydration. A) RT-PCR analysis of the *CpPGIP* transcript using 1 µg total RNA extracted from untreated (F), partially dehydrated (PD), dehydrated (D) and rehydrated (R) plant samples from leaves (A) and roots (B). RT-PCR was performed for 35 cycles for each PCR. Primers are listed in section 3.1.12.

The transcript expression was found to be highly upregulated in the dehydrated plant samples from the leaves. In case of the root samples the transcript, level was equal for all the four treatments. In the control PCR *EF1 alpha* and *11-24* were used. The *EF1aplha* transcript was equally expressed in all the four treatments of *Cp* and *11-24* transcript is upregulated in response to dehydration (Ataei *et al.*, 2016).

4.1.4 Generation of polyclonal antibody against the CpPGIP protein

- **Mutagenesis and cloning of CpPGIP into the E. coli expression vector pET28-a**

In a first step the full-length amplified fragment of *PGIP* (800 bp) cloned into the pJET1.2vector (**section 4.1.1**), was used as the template for mutagenesis PCR (**section3.2.5**). By using the mutagenesis primers CpPGIP_Nco1_F and CpPGIP_Xho1_R (**Fig. 15**), the restriction sites *NcoI* and *XhoI* were introduced. The PCR product was eluted from the gel in a final volume of 15 µl (**Fig. 16**).

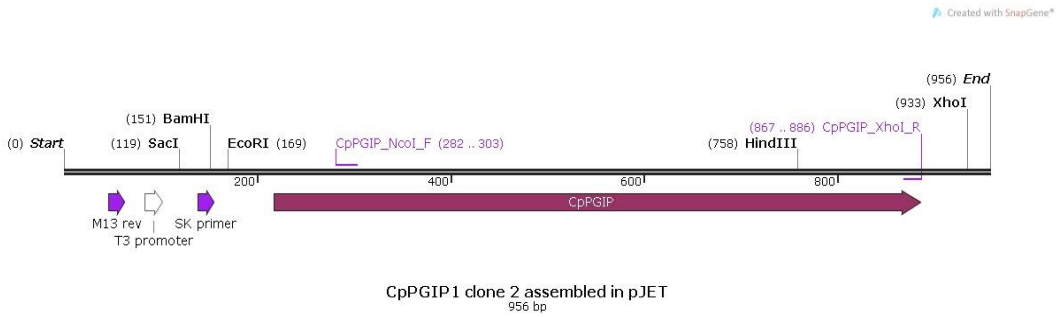

Fig. 15. Introduction of the restriction sites in the full length *CpPGIP* sequence (956 bp) using SnapGene software.

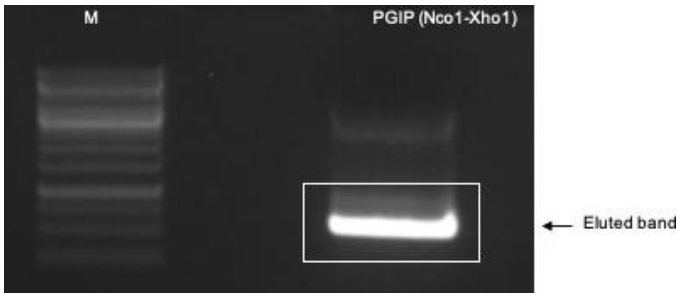

Fig. 16. Amplified *CpPGIP* fragment containing the restriction sites (*Nco1* and *Xho1*)**.** Lane 1: molecular-weight size markers (M), lane 2: Amplified *CpPGIP* fragment containing restriction sites which was eluted from the gel.

The eluted PCR product containing the *CpPGIP* fragment with restriction sites was transformed into *E.coli* Dh10B cells and ligated to pJET1.2vector. The insertion of the restriction sites into the sequence was confirmed by sequencing. The plasmid preparation was used for subsequent cloning steps into the expression vector pET28-a (supplementary data). After restriction digests of the fragment and the empty pET28-a vector, the amplicon was ligated into the expression vector. Successful ligation was confirmed by sequencing with T7-promoter and T7-terminator primers.

4. Results

➢ Expression of CpPGIP

The pET28a-CpPGIP constructs were transformed into *E. coli* BL21 cells. BL21 competent *E. coli cells* are widely used for transformation and protein expression. Since the constructs were already confirmed by sequencing before, they were not sequenced after the second transformation. The pET-28a vector carries an N-terminal HisTag. Two positive colonies identified by sequencing were used for overexpression, which was induced by the application of IPTG (final concentration 1 mM) for five hours. An aliquot of the culture was also collected in small 1 ml samples right before adding IPTG and then in the first, third and fifth hour. The protein was best expressed five hours after induction (**Fig. 17 (b)**).

To verify the correctness of the protein, the overexpressed protein was also run on an SDS gel and a Western blot was performed. For this the 1 ml samples, which were collected during the overexpression were used. The membrane was incubated against the HisTag antibody since a HisTag is present in pET28a vector to which the construct was ligated. The CpPGIP protein which was bound to pET28a was detected with a molecular mass of around 25 kDa on the nitrocellulose membrane using HisTag antibodies (**Fig. 17 (a, c)**).

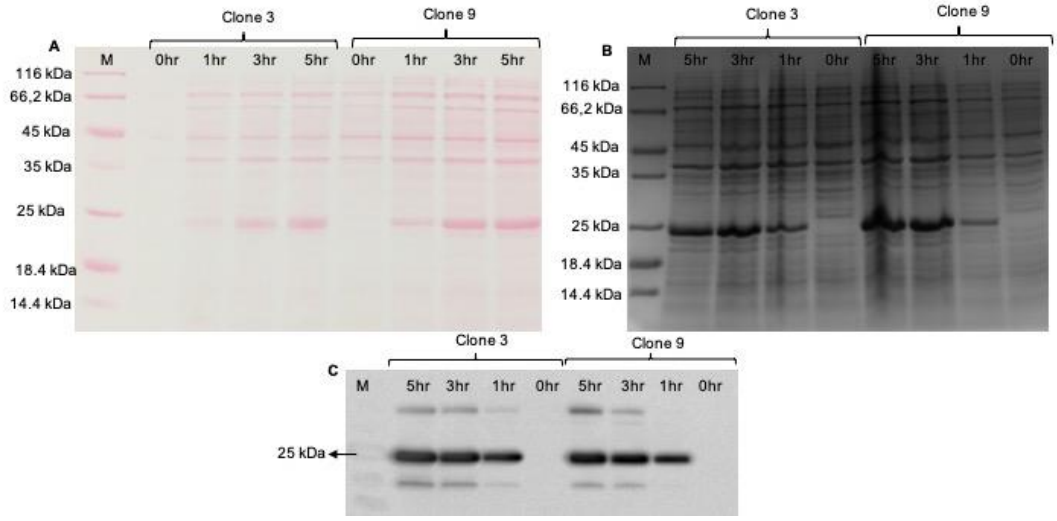

Fig. 17. Over-expression of the CpPGIP: protein detection by Comassie staining and Western blot**.** A) Ponseau stained nitrocellulose membrane after the western blot. B) Coomassie stained SDS gel showing the overexpression of the CpPGIP protein at 0 hour and one hour, three hours and five hours after induction. C) The CpPGIP (25 kDa) protein was detected after the Western blotting using a HisTag antibody.
(M) Molecular-weight size markers

For detailed analysis, small samples (1 ml) were taken and processed as described in **section 3.2.12**. Soluble protein fractions (S0, S1, S2 and S3) and insoluble proteins (P0, P1, P2 and P3) were separated on SDS-PAGE gels (**Fig. 18**). The protein was found to be present in inclusion bodies (pellet). Hence, after this verification the inclusion bodies were used from the big culture for protein purification on a large scale.

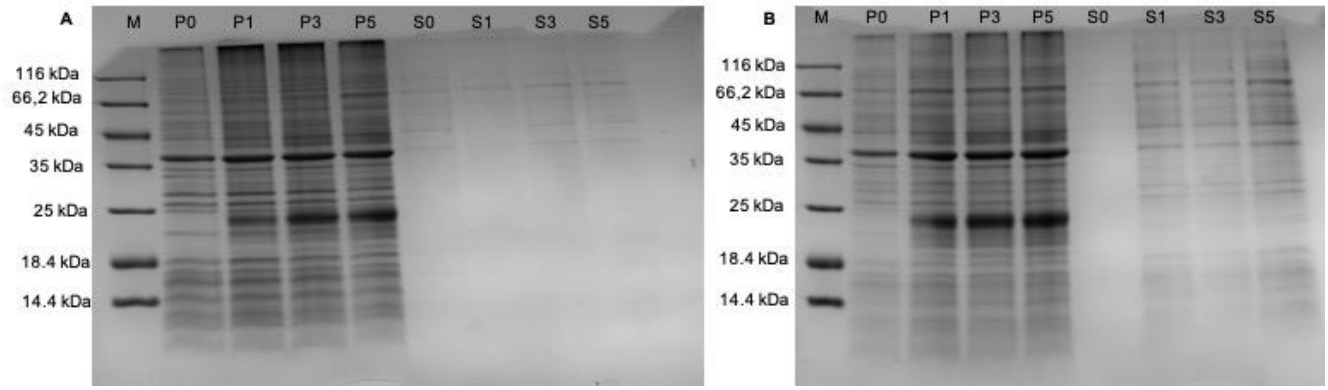

Fig. 18. Comassie stained SDS gels showing the presence of the CpPGIP protein in the inclusion bodies**.** A) Overexpressed 1 ml samples from clone 3. B) Overexpressed 1 ml samples from clone 9. (M) Molecular-weight size markers, (P0, P1, P3 and P5) inclusion body samples collected after 0, 1, 3 and 5 hours of induction respectively, (S0, S1, S3 and S5) soluble protein fractions collected in 0, 1, 3 and 5 hours respectively.

- **Protein purification for antibody production**

The culture containing the overexpressed recombinant protein was prepared as described in **section 3.2.13**. As depicted in **Fig. 19**, the protein fraction F0 contains all proteins, while only unbound proteins (Ft) are eluted from the column by mild washing steps. The CpPGIP containing protein fractions F1-F7 display a strong protein band at 25 kDa. Isolation and purification of the recombinant protein CpPGIP by His-tag affinity chromatography was followed as described in **section 2.11.** The elution profiles show maximum protein contents in fractions F2 and F3. Although CpPGIP protein bands are highly enriched after the purification, a few unspecific proteins are detected (**Fig. 19**).

Fractions F1-F4 and F5-F7 were pooled together and binding of unspecific proteins was further reduced by demineralization of the protein samples with PD-10 desalting columns (GE Healthcare). Samples were run on an SDS gel along the overexpressed samples to confirm the correct size of the protein (**Fig. 20 (a)**). The purified proteins were detected at 25 kDa by Coomassie brilliant blue staining.

The collected protein samples were lyophilised to remove all liquid content. The protein powder was obtained after 4 days of continuous lyophilisation and was stored at -20°C until further use.

The lyophilised protein powder obtained was then run against different BSA concentrations (**Fig. 20 (b)**) to determine the concentration of the purified protein. The final protein concentration was found to be close to 4 μg. This powder was then packed in a falcon tube and sent to the Seqlab (Sequence Laboratories Göttingen GmbH, Göttingen, Germany; www.seqlab.de) for the production of the polyclonal antibodies against the CpPGIP protein in rabbits. It took around 2 months to receive the third bleeding.

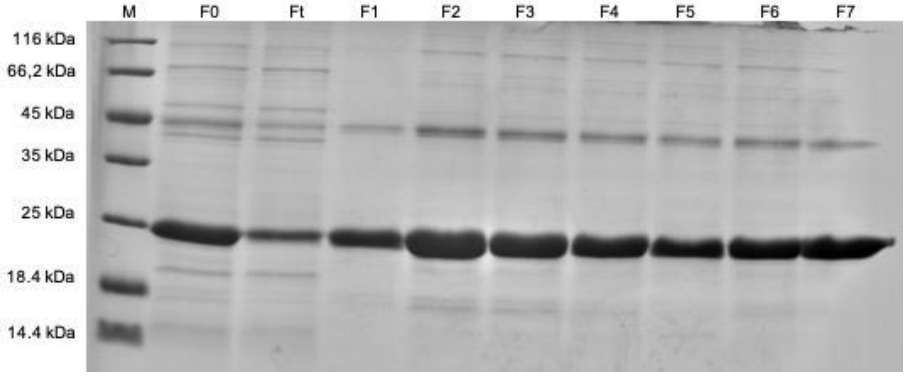

Fig. 19. The purified protein fractions of CpPGIP (25 kDa) obtained using Ni-NTA columns on a Coomassie stained SDS gel**.** (M) Molecular-weight size markers. (F0) Total proteins before affinity chromatography; (Ft) Flow- through. (F1–F7) Eluted His-tag fractions.

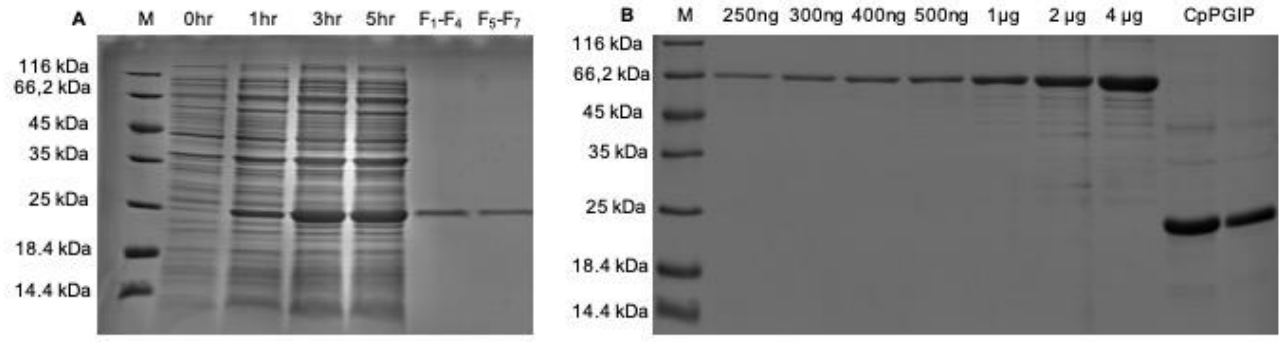

Fig. 20.A) Comparison of CpPGIP overexpression clone 3 and eluted His-tag fractions protein fractions (F1-4 & F5-7) obtained from NI-NTA columns by Coomassie staining. No unspecific bands were detected in the pooled protein fractions (F1-F7).

Fig. 20.B) Coomassie stained SDS gel showing the purified CpPGIP protein compared with different BSA concentrations. (M) Molecular-weight size markers. (CpPGIP) purified protein.

4.1.5 Antibody testing

To increase the binding specificity, monospecific antibodies against CpPGIP were produced as described in **section 4.1.4**. **Fig. 21 and 22** show the specific signal bands for CpPGIP in a protein blot. The antibody tested here was from the third bleed. The antibody blot was compared against the pre-immune serum blot. The samples used for the blots were purified protein, empty vector and 5th hour protein from *E. coli* overexpressing CpPGIP. The blot incubated in pre-immune serum did not show any specific bands whereas the blot incubated in the serum of the third bleeding showed specific protein bands detected at 25 kDa (**Fig. 21**). Protein extracts from untreated *C. plantagenium* leaves and roots were also analysed in an immunoblot (**Fig. 22**). For the root samples the protein migrated slightly higher than 25 kDa. For the untreated leaf sample a signal was obtained at 25 kDa. Again, no specific bands were observed for the pre-immune serum.

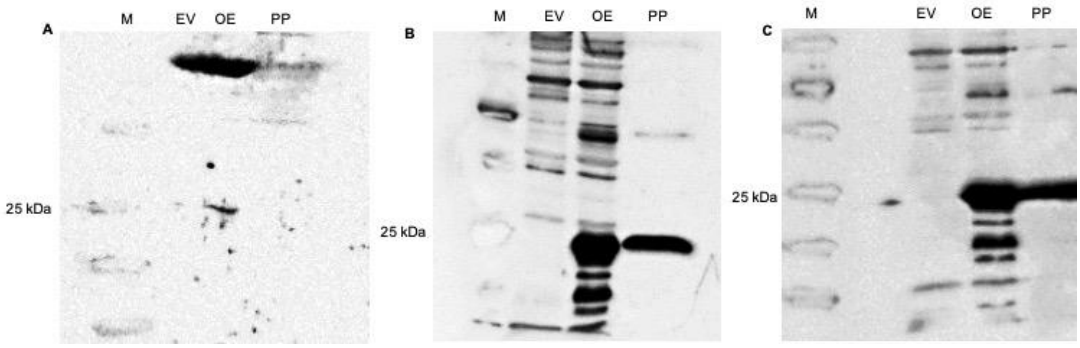

Fig. 21. Test of monospecific antibodies. A) Pre-immune serum (1:5000). B) CpPGIP antibody (1:5000). C) CpPGIP antibody (1:10,000)**.** (M) Molecular-weight size markers. (EV) Empty vector. (OE) 5th hour over-expressed recombinant protein sample. (PP) Purified protein.

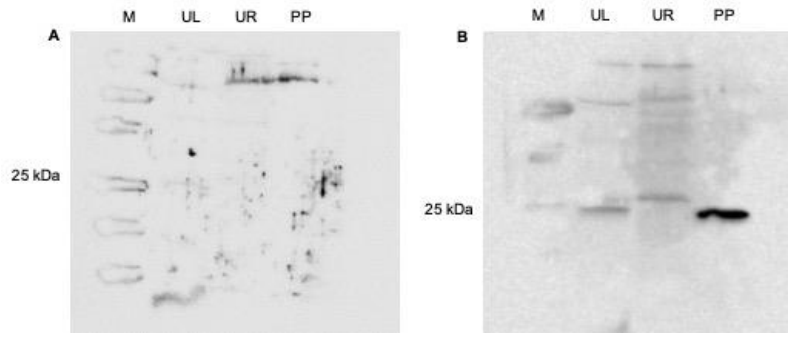

Fig. 22. Test of monospecific antibodies. A) Pre-immune serum (1:5000). B) CpPGIP antibody (1:10,000). (M) Molecular-weight size markers. (UL) Proteins from untreated *Cp* leaves. (UR) Untreated *Cp* roots. (PP) Purified protein.

The antibody was found to be specific for the CpPGIP protein.

4.1.6 Expression of CpPGIP upon water limiting conditions using total protein extracts from plants

To determine whether expression of CpPGIP is regulated in response to water stress, fully-turgescent *C. plantagenium* plants were compared to dehydrated plants (relative water contents of 92-08 % (**Fig. 13**)). The ground plant material obtained from the plants treated for various dehydration stresses was used for this study. The plant material was processed as described in **section 3.2.10**. The quantification was done using the Bradford method.

As shown in **Fig. 23**, dehydration stress significantly affected protein expression in leaves and roots of the plants. The protein expression was observed to be most up-regulated during the dehydration stress in both leaves and root samples and was even higher in roots compared to leaves. In samples derived from untreated plants protein expression was also high but less when compared to dehydrated samples. The lowest expression was observed in the rehydrated and partially desiccated plant samples (**Fig. 23**). The protein expression pattern was identical to that of transcript expression (**Fig. 14**).

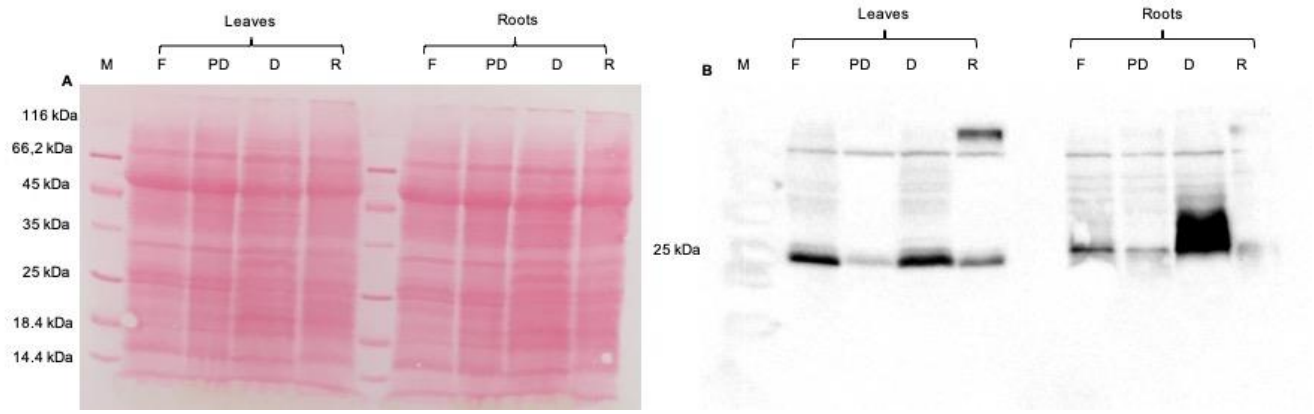

Fig. 23. Protein expression of CpPGIP in response to different dehydration treatments**.** A) Ponceau stained membrane. B) Protein immunoblot. M) Molecular-weight size markers. (F) Untreated. (PD) Partially dehydrated. (D) Dehydrated. (R) Rehydrated.

As a control experiment the plant samples were also tested with antiserum against the CpCeDet11-24 (**Fig. 24**) since the expression of 11-24 is already documented (Petersen *et al*, 2012). The antibody was diluted 1:5000 and incubation was done

overnight. The expression of 11-24 protein was up-regulated in response to stress.

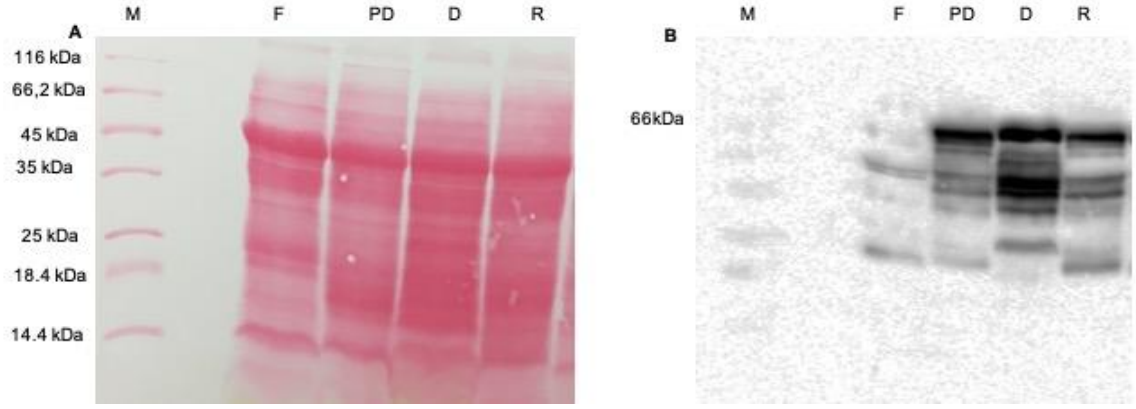

Fig. 24. Protein expression for Cp11-24 upon different dehydration stresses. A) Ponceau stained membrane. B) Protein blot. M) Molecular-weight size markers. (F) Untreated. (PD) Partially dehydrated. (D) Dehydrated. (R) Rehydrated.

4.1.7 Expression of CpPGIP in the cell wall extracts upon water limiting conditions

Since PGIP is a protein which is mainly located in plant cell walls, proteins extracted from plant cell walls were blotted and analysed on an immunoblot to check the protein expression in various water limiting conditions. Cell wall proteins were extracted according to a three-step extraction procedure using sequentially $CaCl_2$, EGTA, and LiCl-complemented buffers as described in **section 3.2.11**. The extracts were prepared only for the leaf samples of *C. plantagenium*. Since $CaCl_2$ is the first fraction it contained the maximum amount of protein (**Fig. 25**) followed by EGTA and LiCl fractions respectively (**Fig. 26**). The expression of the protein was found to be highly up-regulated during dehydration in all three extractions.

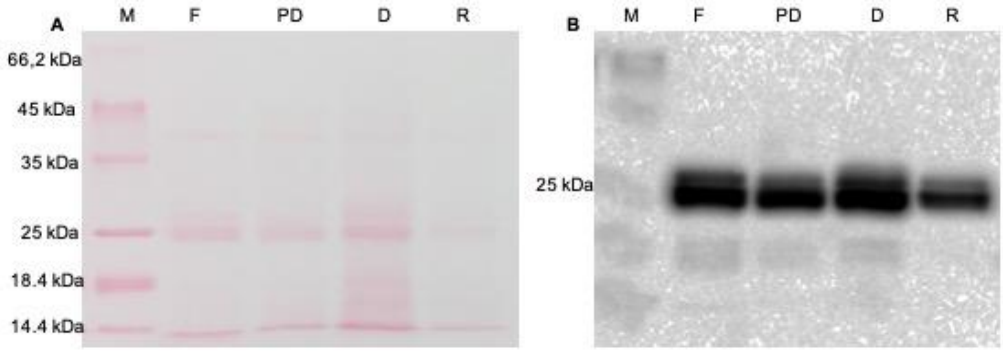

Fig. 25. Protein expression in $CaCl_2$ fractions upon different dehydration stresses. A) Ponceau stained membrane. B) Protein blot. M) Molecular-weight size markers. (F) Untreated. (PD) Partially dehydrated. (D) Dehydrated. (R) Rehydrated.

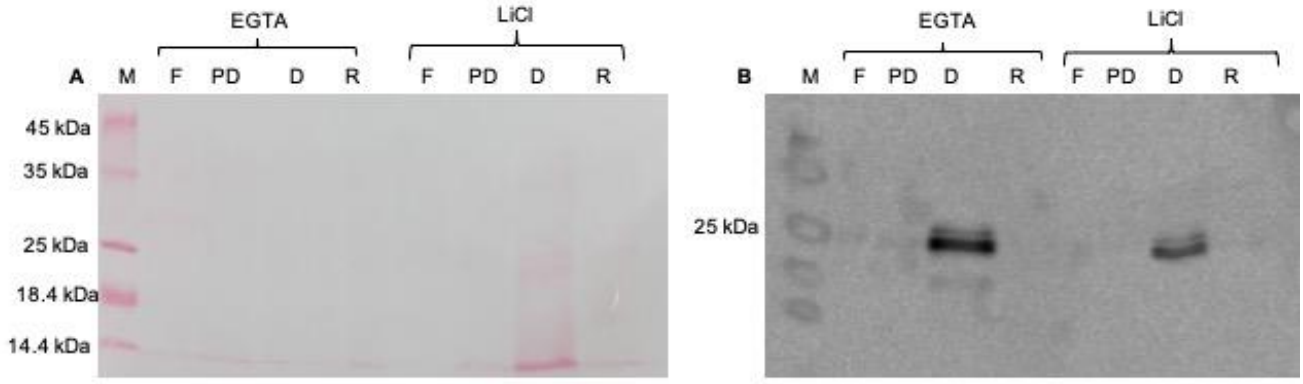

Fig. 26. Protein expression for EGTA and Licl fractions upon different dehydration stresses. A) Ponceau-stained membrane. B) Protein blot. M) Molecular-weight size markers. (F) Untreated. (PD) Partially dehydrated. (D) Dehydrated. (R) Rehydrated.

4.1.8 Protein expression under the salt stress

Different dehydration stresses and ABA and salt stress were compared. The untreated *C. plantagenium* (RWC 92%) leaves were incubated in 1 M ABA solution for 24 and 48 h respectively.

As shown in **Fig. 27**, dehydration stress significantly affected the protein expression. The protein amount was higher in the 24 h ABA-treated leaves than in the 48 h ABA treated leaves. The protein expression was most abundant in dehydration stress.

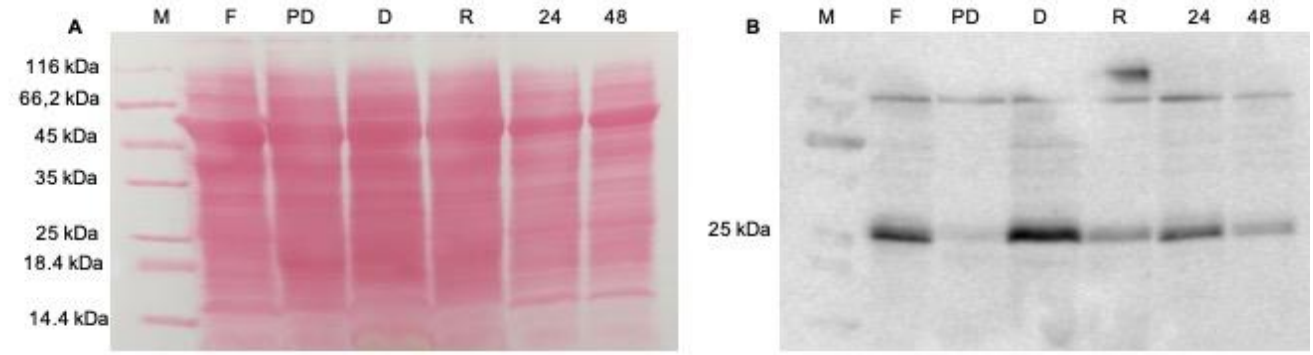

Fig. 27. Comparison of protein expression for CpPGIP upon different dehydration stresses including ABA salt stress. A) Ponceau stained membrane. B) Protein blot. M) Molecular-weight size markers. (F) Untreated. (PD) Partially dehydrated. (D) Dehydrated. (R) Rehydrated. (24) Leaves incubated in ABA solution for 24 hours. (48) Leaves incubated in ABA solution for 48 hours.

4.1.9 Identification of CpPGIP interacting partners using a yeast-2-hybrid screening

The yeast-two-hybrid system is a frequently used technique for the identification of protein-protein interactions *in vivo*. It is based on the activation of down-stream reporter genes by binding of a specific transcription factor (e.g. GAL4) to an upstream activating sequence (UAS) (Keegan *et al.*, 1986). The transcription factor is composed of two (or more) domains: A DNA-binding domain (BD) that interacts with the upstream activating sequence (UAS) and an activator domain (AD) that promotes activation of transcription (Keegan *et al.*, 1986). Generation of a bait construct that expresses the protein of interest fused to the BD and a cDNA library encoding putative interacting partners fused to the AD (prey), respectively allows interaction of bait and prey leading to merging of AD and BD, which can exert their function in close vicinity of each other without direct binding. The reconstitution of the functional transcription factor activates downstream reporter genes that can be monitored.

- **Generating yeast two-hybrid protein-bait strains and yeast auto-activation test**

The coding sequence of *CpPGIP* was amplified using the CpPGIP plasmid present in the pJET1.2vector (**section 4.1.1**) using the primers CpPGIP_Y2H_Fwd and CpPGIP_Y2H_Rev. The amplified construct was then cloned in the pJET1.2vector and the correctness of the construct was verified by Sanger sequencing. The plasmid was digested with the restriction enzymes *NcoI* and *XhoI* and then cloned into the *NcoI* and *XhoI* digested pAS2- 1 vector to generate the CpPGIP-GAL4-BD fusion construct (**Fig. 28**). The construct was confirmed by DNA sequencing and subsequently transformed into the yeast strain Y190. Positive colonies were selected from SD-Try medium and confirmed by PCR using the primers pAS2_1fwd and pAS2_1rev. Then they were tested for auto activation. Each colony was re-suspended in 50 µl sterile H_2O and 5 µl of the mixture was pipetted to SD-Try-His medium with 0 mM, 1 mM, 7.5 mM, 15 mM, 25 mM and 50 mM 3-AT to test autoactivation of the HIS3 reporter gene. Similarly, the wild-type yeast strain was also tested for auto-activation by plating it on the YPAD selection medium and SD-Trp and SD-Leu medium. The growth of Y190 was found to be completely inhibited at 25 mM 3-AT and in the SD medium (**Fig. 29**).

The auto-activation test of the *HIS3* reporter showed that growth of positive yeast clones was inhibited by 25 mM 3-AT. Finally, a positive clone was chosen as the

optimal bait because it grew well under the non-3-AT conditions and its growth is completely inhibited by 25 mM 3-AT (**Fig. 30**).

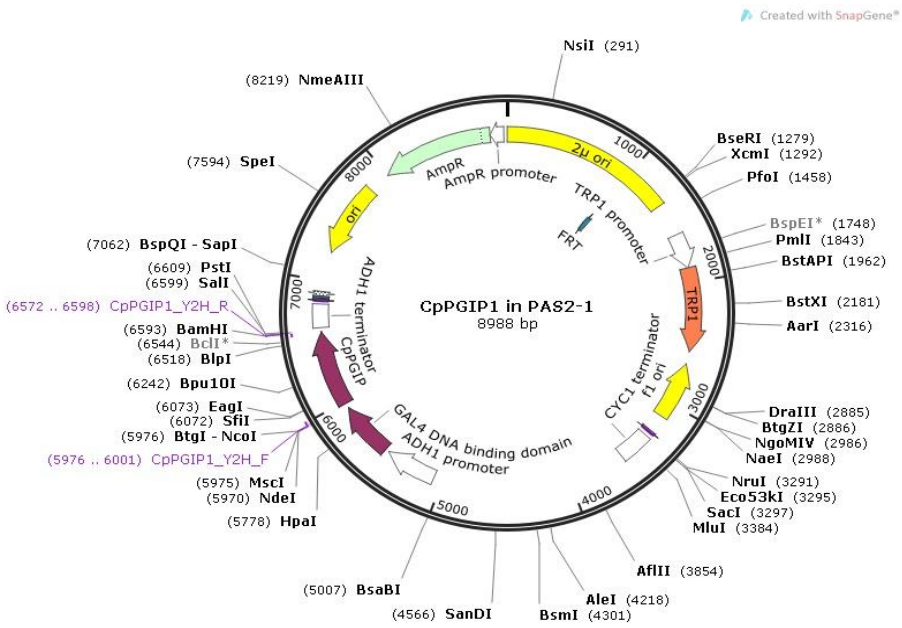

Fig. 28. PAS2-1 vector containing the CpPGIP fragment.

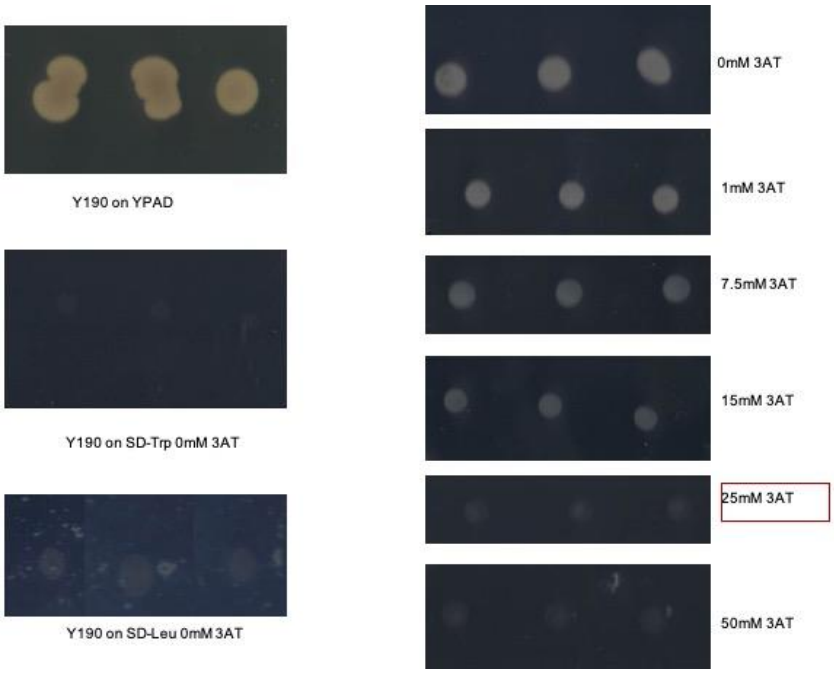

Fig.29. Yeast (Y190) auto-activation test. (On the left) The growth of wild type yeast strain on YPAD, SD-Try and SD-Leu medium after 3 days of incubation at 30°C. (On the right) The six panels show the growth status of Y190 3 days on YPAD medium containing progressively higher concentrations of 3-AT (0 mM, 1 mM, 7.5 mM, 15 mM, 25 mM and 50 mM).

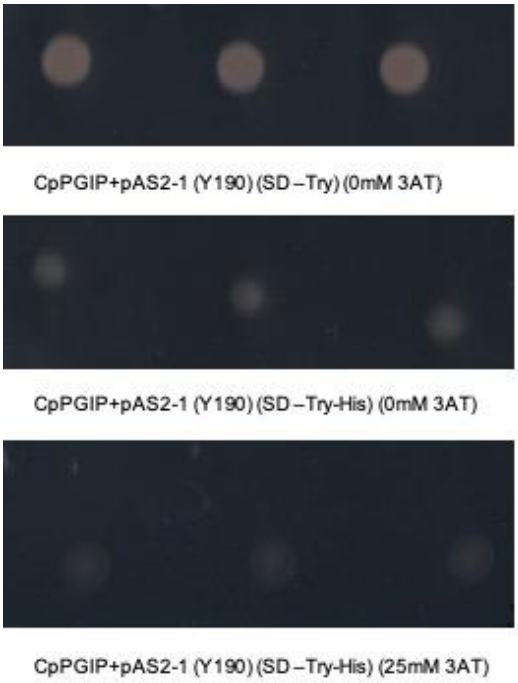

Fig. 30. Auto-activation test for the HIS3 reporter gene in the CpPGIP-GAL4-BD fusion construct (bait) after 3 days of incubation at 30°C. The positive colonies could grow on the SD-Trp medium but the growth was completely inhibited in the medium containing SD-Trp-His and SD-Trp-His-25 mM 3AT medium.

Hence, it was shown that the bait construct and the wild-type yeast were not able to activate the reporter gene on their own.

- **Prey library transformation and identification of protein-protein interactors**

For the prey construct the yeast library construct (prepared by Sarah Jane Dulitz) was used. The already prepared yeast library was re-grown and the plasmid was isolated. The library plasmid (prey) along with the CpPGIP-PAS2-1 plasmid (bait) were used together to transform the yeast (**section 3.2.6**). The transformed cell suspension was spread on SD medium lacking leucine, tryptophan and histidine plus 25 mM 3-AT for screening. After 5-7 days of incubation at 30°C, more than 60 colonies were obtained (**Fig. 31**). To rapidly identify colonies that contain interacting preys, a colorimetric β-galactosidase assay was performed directly with these colonies (**Fig. 32**). The colonies that generated blue stain in β-galactosidase assays were picked and re-suspended in 50 µl sterile H_2O. Five microliter of each colony suspension was replicated on different SD medium to further confirm that they contain the interacting preys from the *HIS3* reporter. In total, 8 positive yeast colonies were obtained from the colorimetric assay.

Positive colonies were also selected and confirmed by colony PCR using the PAD fwd and PAD rev primers. Although only 8 colonies were tested positive in the β-galactosidase assay but in the colony PCR most of the colonies showed a positive band (**Fig. 33**). Most of the colonies which tested positive in the PCR were also sent for sequencing to identify the PGIP interaction partners. For sequencing the PCR product was used which was directly eluted from the gel.

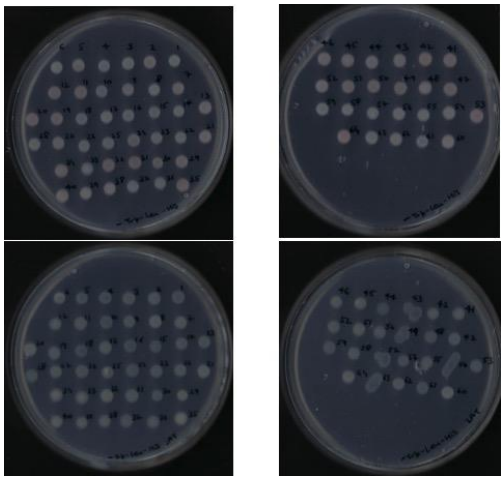

Fig. 31. The growth of yeast colonies that contain putative interacting partners on SD-Trp-Leu (25 mM 3-AT) medium after 5-day incubation at 30°C.

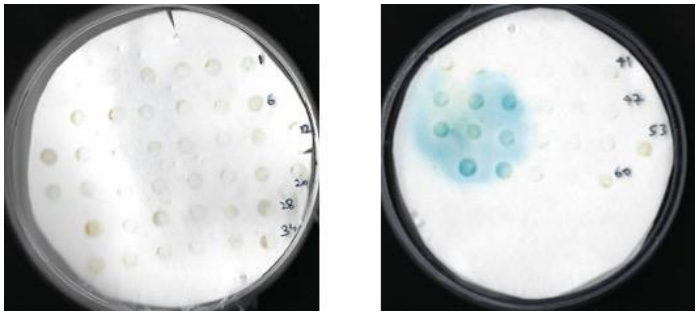

Fig. 32. Theβ-galactosidase assay result with yeast interactor colonies after 12 h incubation at 37°C**.** Colonies that accumulated blue compound were replicated on different SD medium and were sent for sequencing.

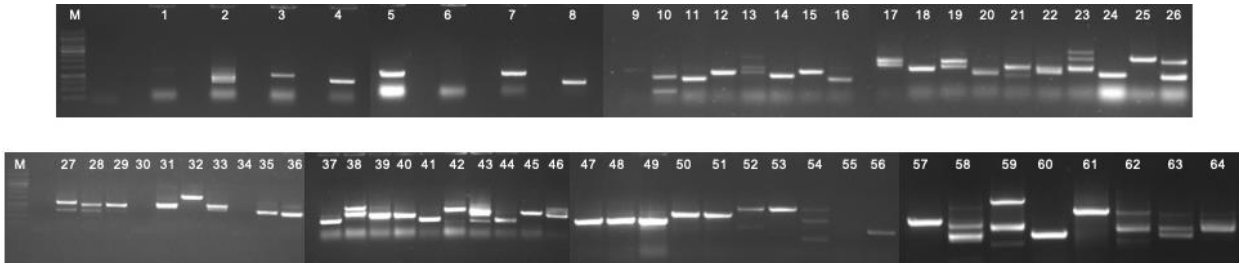

Fig. 33. PCR products of positive yeast colonies in CpPGIP yeast two-hybrid screening. The PCR product for the positive clones was eluted and sent for sequencing. (M) Molecular-weight size markers.

4. Results

The protein interaction partners identified by the yeast two hybrid screen are listed in the following table.

Table 9: Isolated proteins that interact with CpPGIP as identified by yeast two hybrid assay.

Identified protein partner	Description
Clone 12 (560 bp)	Unnamed protein product
Clone 15 (604 bp)	Photosystem II 10 kDa polypeptide, chloroplastic-like
Clone 25 (481 bp)	Late embryogenesis abundant protein ECP63-like [
Clone 32 (440 bp)	LexA-Gal4 chimeric protein
Clone 37 (598 bp)	ricin B-like lectin R40G3
Clone 42 (679 bp)	cytochrome P450 94B3-like
Clone 50 (684 bp)	Activator of 90 kDa heat shock protein ATPase
Clone 53 (595 bp)	unknown [Yeast two-hybrid vector pAB8]
Clone 7 (555 bp)	transmembrane protein 208 [*Sesamum indicum*]
Clone 18 (727 bp)	ribulose bisphosphate carboxylase small chain, chloroplastic-like [*Jatropha curc*as]
Clone 29 a (477 bp)	GDBD-H4-Gcn5(F221A)-HA fusion protein [Yeast two-hybrid vector pDG4]
Clone 29 b (479 bp)	protein CWC15 homolog [Olea europaea var. sylvestris]
Clone 47 (943 bp)	acyl-protein thioesterase 2 isoform X2 [*Nicotiana sylvestris*]
Clone 51 (686 bp)	activator of 90 kDa heat shock protein ATPase [*Dorcoceras hygrometricum*]
Partner 7 (612 bp)	dynein light chain 2, cytoplasmic [*Sesamum indicum*]
Partner 8 (575 bp)	apyrase 6 [*Erythranthe guttata*]
Partner 9 (1276 bp)	cinnamoyl-CoA reductase 1-like [*Nicotiana tomentosiformis*]

Partner 13 (1409 bp)	subtilisin inhibitor-like [*Nicotiana attenuata*]
Partner 23 a (985 bp)	Outer envelope pore protein 16-2, chloroplastic, partial [*Capsicum chinense*]
Partner 23 b (818 bp)	Outer envelope pore protein 16-2, chloroplastic, partial [*Capsicum chinense*]
Partner 24 (1278 bp)	cinnamoyl-CoA reductase 1-like [*Nicotiana tomentosiformis*]
Partner 25 (878 bp)	F-box protein PP2-B10-like [*Capsicum annuum*]
Clone 96 (1249 bp)	Heat shock protein SSC1
Clone97 (1210 bp)	transmembrane protein 208
Clone 43 (442 bp)	protein NETWORKED 4B
Clone 64 (758 bp)	carbapenemase type 2
Clone 67 (707 bp)	Carbepenem-hydrolyzing beta-lactamase KPC precursor
Clone 79 (745 bp)	carbapenemase type 2
Clone 69 (712 bp)	beta-lactamase KPC-2
Clone 74 (364 bp)	unnamed protein product
Clone 76 (476 bp)	unnamed protein product
Clone 71 (465 bp)	unnamed protein product
Clone 57 (1092 bp)	SEC14 cytosolic factor
Clone 33a (554 bp)	Rhodanese-related sulfurtransferase
Clone 35 (684 bp)	protein transport protein SFT2-like
Clone 82 (551 bp)	unnamed protein product

4.1.10 Interaction of CpPG (polygalacturonase) with CpPGIP

Polygalacturonase inhibiting proteins (PGIPs) are cell wall proteins that inhibit the pectin-depolymerizing activity of polygalacturonases secreted by microbial pathogens and insects. Since there was no known bacterial or fungal polygalacturonase which was available we decided to test if CpPGIP protein interacts with its own polygalacturonases. The polygalacturonase sequence for *C. plantagenium* was provided by Dr. Valentino Giarola from the transcriptomic data bank. The two chosen sequences were labelled as *PG2101* and *PG5326*. According to the data the *PG2101* was highly expressed during partially desiccated stage and *PG5326* was highly expressed without any water stress (untreated *Cp* plants). The partially desiccated cDNA from *C. plantagenium* was used to amplify the 2101 polygalacturonase sequences using 2101 forward and 2101 reverse primer combination (**Fig. 34**). The PG5326 could not be amplified even after many attempts.

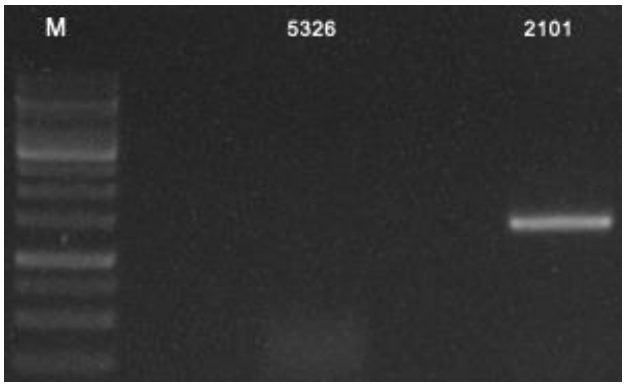

Fig.34. Amplification of PG5326 and PG2101 using the untreated and partially dehydrated cDNA respectively from *C. plantagenium*. Lane 1: molecular-weight size markers (M). Lane 2: *PG 5326* which could not be amplified. Lane 3: Amplified *PG2101* which was later eluted.

The *PG2101* band was eluted from the gel and ligated to pJET1.2vector. The plasmid was prepared and the sequence was confirmed by Sanger sequencing. The plasmid was confirmed to contain the insert and was then digested using *XbaI* and *NcoI* enzymes and was ligated to the pAD-GAL4 vector which was also digested using *XbaI* and *NcoI* enzymes.

The wild-type yeast (Y190) was transformed using the CpPGIP-PAS2-1 and PG2101-pAD-GAL4 and this was the test to check if the two proteins interact with each other in the yeast system. Unfortunately, no colonies were observed on the SD-trp-leu yeast plates. Hence, it was concluded that the protein of interest (CpPGIP) and the particular polygalacturonase protein (CpPG2101) did not interact with each other.

4.2 Identification and analysis of *pcC13-62* promoters

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

4.2.1 Identification of the promoter

Genomic DNA was extracted from leaves and was used to prepare genome walking libraries with the GenomeWalkerTM universal kit (Clontech, Heidelberg, Germany). Genomic fragments corresponding to the 5'*13-62* gene sequence were amplified from *C. plantagineum* genome walking libraries with gene-specific and library-specific (AP1 and AP2; GenomeWalkerTM universal kit manual) primers (**Fig. 35**). Using the primers four different polymerase chain reactions were set up as explained in chapter 3.2.2.1. The 5 µl of the PCR product was saved to perform the secondary PCR and the remaining 45 µl were loaded on the agarose gel (**section 3.2.5**). The amplification from *L. brevidens* libraries was unsuccessful and thus primers designed to match conserved sequence regions of *C. plantagineum* and *L. subracemosa* genomic fragments (Lb13-62g_F and Lb13-62g2_R; were used to obtain the 5'*13-62* sequence from *L. brevidens* genomic DNA. For *L. subracemosa* the promoter fragment was amplified by Niklas Udo Jung.

The sequence between the β-galactosyltransferase and the *13-62* translational start codons from *C. plantagineum* (962 bp for isoform1 and 1029 bp for isoform2) and *L. subracemosa* (723 bp) was used as the *13-62* promoter sequence in the promoter analyses. A transposon was found to be inserted between the β-galactosyltransferase and the *13-62* genes in *L. brevidens*, and thus the sequence between the transposon terminal repeats and the *13-62* translational start codon was used as the promoter sequence for this species. The promoter sequences were screened for *cis*-acting regulatory elements using the PLACE databases.

(https://www.dna.affrc.go.jp/PLACE/?action=newplace)

The genomic regions of *Cp13-62iso1*, *Cp13-62iso2*, *Lb13-62*, and *Ls13-62* were isolated and analysed to identify regulatory motifs that cause high expression of *13-62* transcripts in vegetative tissues of desiccation tolerant species. The genomic regions of the 13-62 locus were conserved and similarly organized: the β-galactosyltransferase protein coding sequence was upstream of the *13-62* gene, and the *13-62* coding sequence had the same exon-intron structure (**Fig. 36**). The distance between the β-galactosyltransferase coding sequence and the *13-62* coding sequence varied among the different species. The distance was ~700 bp in *L. subracemosa* and nearly 1000 bp in *C. plantagineum*, whereas it was more than 4000 bp in *L. brevi*dens (**Fig. 36**). The large difference was due to the insertion of a transposable element ~800 bp upstream of the *Lb13-62* gene translational start codon. The transposable element contained features similar to the maize P instability factor (PIF) such as short terminal inverted repeat sequences and the coding sequence for a transposase (Zhang *et al.*, 2001).

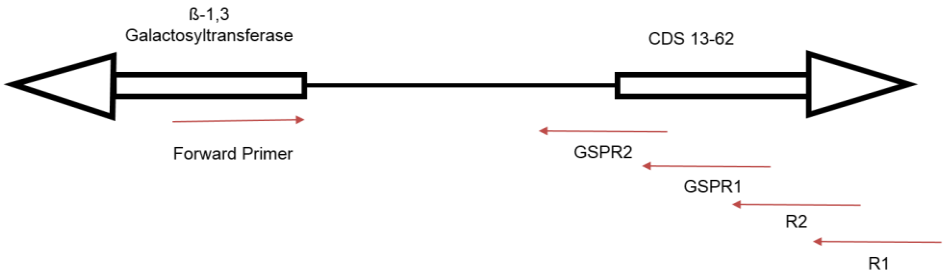

Fig. 35. Scheme of the forward and reverse primers designed on the cDNA sequence of *Lindernia brevidens* in order to obtain the *13-62* promoter region.

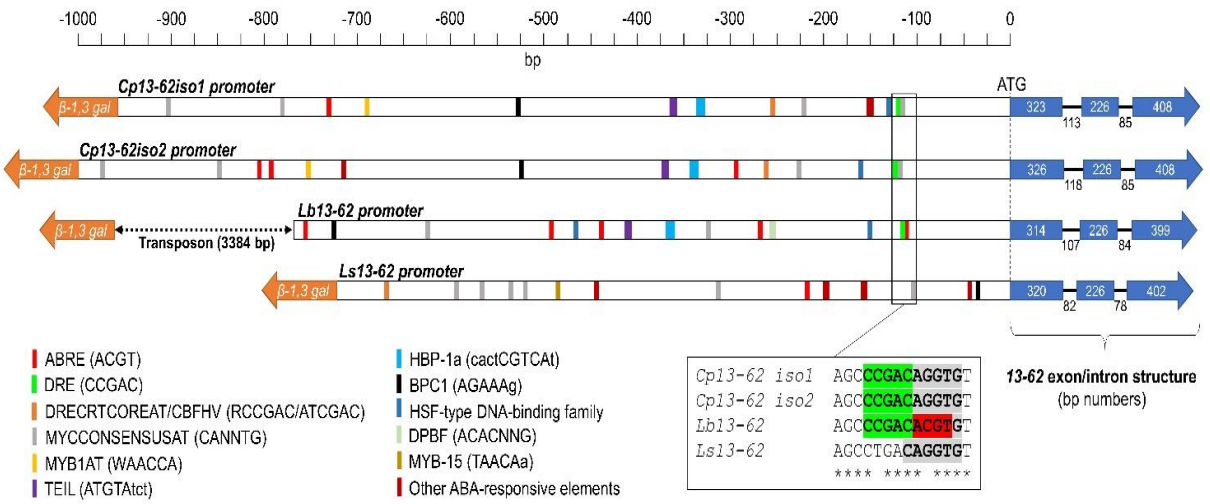

Fig. 36. Structure of the 13-62 gene locus in *C. plantagineum*, *L. brevidens*, and *L. subracemosa*. Putative *cis*-acting regulatory elements associated with abscisic acid (ABA)- and dehydration-responsive gene expression are indicated by coloured boxes. The box indicates the position of the conserved drought-responsive element (DRE) in the promoter of desiccation-tolerant species.

Elements related to ABA and drought promoter responsiveness were predicted upstream of the *Cp, Lb*, and *Ls13-62* gene translational start sites (**Fig. 36**). Only a few structural elements are conserved in the desiccation tolerant species. The promoter of the desiccation sensitive species *L. subracemosa* showed almost no conservation of *cis*-elements. Most of the sequence conservation was observed within the first 170 bp upstream of the translational start site. In this region, we identified a putative consensus sequence (CCGAC) for a DRE and binding sites for Myc factors. The putative DRE motif was conserved in the desiccation tolerant species *C. plantagineum* and *L. brevidens* but not in the desiccation-sensitive species *L. subracemosa*, in which a single-nucleotide variation was found (**Fig. 36**).

4.2.2 Preparation of promoter-GUS constructs and analysis of the promoter activity using transient transformation

To test which promoter sequences are essential for transcriptional activation of the *13-62* genes, the above-described promoter regions were fused to *GUS* as a reporter gene (**Fig. 37**) and the promoter activities were determined. A reporter-plasmid pBT10GUS harbouring the ß-glucuronidase (GUS) reporter gene was used (Sprenger-Haussels and Weisshaar, 2000). The pBT10-GUS plasmid was obtained by the alkaline lysis (**section 3.2.4**). The restriction digestion was performed as explained in **section 3.2.5**. The restriction enzymes used in case of *L. subracemosa* were *NcoI* and

XbaI. In case of *L. brevidens* the *NcoI* and *SalI* were used. All the digested vector was loaded and run on the agarose gel.

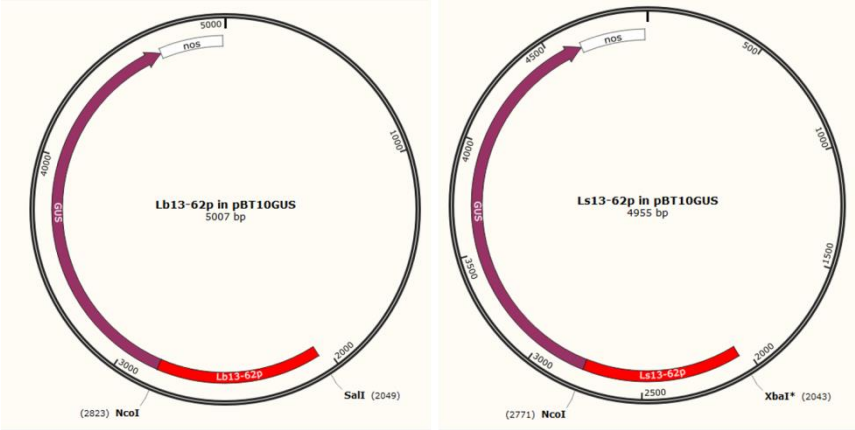

Fig. 37. Fusion of the *13-62* promoter fragment to the pBT-10 GUS vector. The figure on the left is for *L. brevidens* (marked in red) ligated to the pBT-10 GUS vector. The figure on the right shows the same for *L. subracemosa.*

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

The response of the *Cp13-62iso1*, *Cp13-62iso2*, *Lb13-62*, and *Ls13-62* promoters to dehydration was evaluated by monitoring GUS accumulation in dehydrated leaf tissues transiently transformed with *Cp*, *Lb*, and *Ls13-62* promoter::*GUS* constructs. Only

promoters from the desiccation-tolerant species (*C. plantagineum* and *L. brevidens*) showed activities in response to dehydration when tested in the corresponding species (**Fig. 38**).

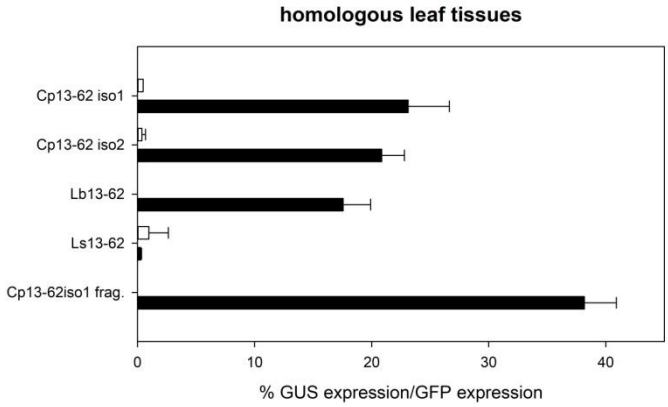

Fig. 38. Activity of the *13-62* promoter in response to dehydration. The *Cp13-62p* and *Lb13-62p* are inducible by dehydration. The *Ls13-62p* does **not** respond to dehydration

The activity of the *Ls13-62* promoter in *C. plantagineum* and the *Cp13-62* promoter in *L. subracemosa* were analysed to determine whether the lack of *Ls13-62* promoter activity upon dehydration was due to the promoter structure or the absence of transcription factors in the desiccation-sensitive species. The *Cp13-62* promoter was functional in the *L. subracemosa* background, which suggested that *trans*-acting factors are present in *L. subracemosa.* However, the *Ls13-62* promoter did not function in the *C. plantagineum* background, which indicates a lack of *cis*-elements essential for promoter activation in response to dehydration (**Fig. 39**).

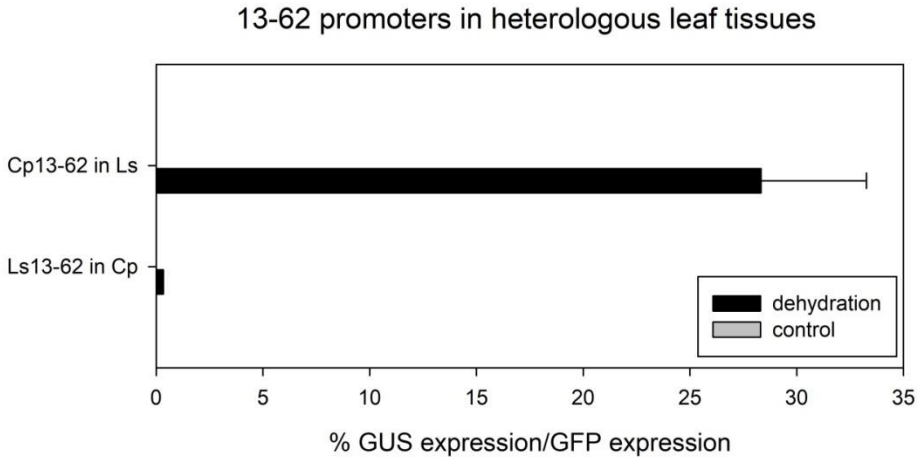

Fig. 39. Response to dehydration of *Cp13-62iso1* and *Ls13-62* promoters in heterologous tissues. *Ls* has transcription factors to activate the promoter. The *Ls*-promoter (**no *DRE*-element**) shows no activity in *Cp.*

4.2.3 Promoter mutagenesis

Mutagenesis of predicted *cis*-elements in the promoter sequences was done using the Quick-change II Site-Directed Mutagenesis kit (Stratagene, Heidelberg, Germany).

It was tested whether the conserved 170 bp region from the desiccation-tolerant promoters could drive *GUS* expression in response to dehydration. A promoter::*GUS* construct containing this region derived from the *Cp13-62iso1* promoter was sufficient to obtain dehydration-induced promoter activation in *C. plantagineum* (**Fig. 38**).This fragment contains the DRE motif conserved in the tolerant species (**Fig. 36**). Thus, the functionality of this element was tested; promoter::*GUS* constructs with mutated DRE motifs (C*C*GAC mutated to C*T*GAC in the tolerant species, or C*T*GAC into C*C*GAC in the sensitive species) were created and used in expression experiments. The PCR product was eluted and ligated to the pJET1.2vector and the presence of the mutation was confirmed by Sanger sequencing. The plasmid containing the mutation was ligated to the pBT10GUS vector and the construct was used for transient transformation.

The mutated promoters from the desiccation tolerant species were unable to drive dehydration-induced *GUS* expression, suggesting that this motif was essential for the dehydration response (DRE-; **Fig. 40**). By contrast, when the DRE element of the desiccation-sensitive species *L. subracemosa* was restored to C*C*GAC, a small increase of promoter activity was measured in response to dehydration (DRE+; **Fig. 40**). The activity increased when the *Ls13-62*(+DRE) promoter was tested in the *C. plantagineum* background, but it did not reach the same level as observed for the wild-type promoters of desiccation tolerant species (**Fig. 40**). Taken together, these results suggested that the DRE element is essential but not sufficient for dehydration-induced activation of the *13-62* promoter.

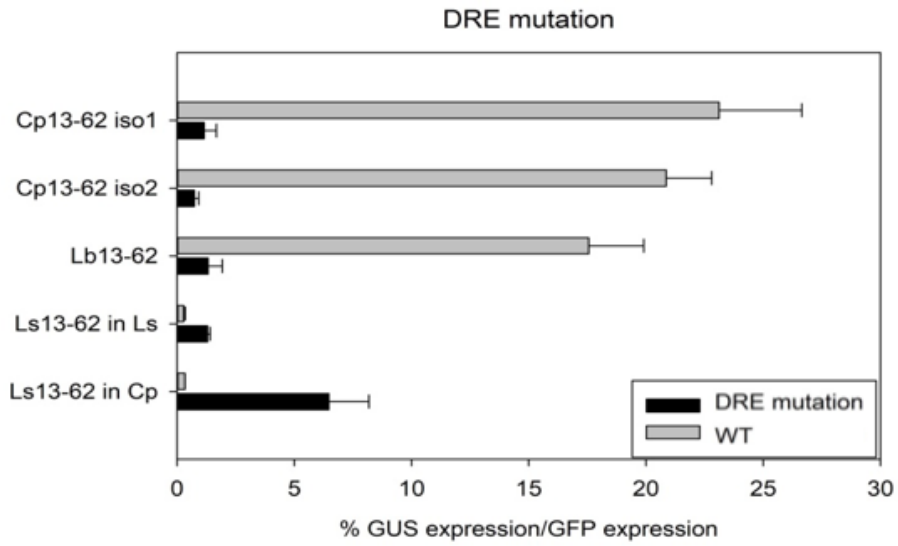

Fig. 40. Effect of mutation of the DRE motif on activity of the *13-62* promoter in homologous and heterologous tissues. The DRE consensus sequence was either impaired (-DRE; promoters of desiccation-tolerant species) or restored (+DRE; promoter of desiccation- sensitive species) and the response to dehydration was compared using wild-type and mutated promoters.

4.2.4 Localization studies of Cp 13-62

The 13-62-pEarleyGate 103 and CpPGIP-pMpGW228 fusion expression constructs were prepared and infiltrated into 6-8 weeks old, regularly watered wild-type *N. benthamiana* plants to study the Cp13-62 protein localization. The fluorescence was observed one to two days (48 h) post-infiltration. under the laser confocal inverted microscope (Nikon Eclipse TE 2000-U) using EZ-C1 3.80 software with appropriate filters. Samples on glass slides were fixed with water. The cells were also treated for plasmolysis with 1 M sucrose for 20 min. The 13-62 protein was found to be localized in the cell wall of the leaves. After plasmolysis, the protein started to form aggregates especially in the pMpGW228 construct (**Fig. 41 and 42**).

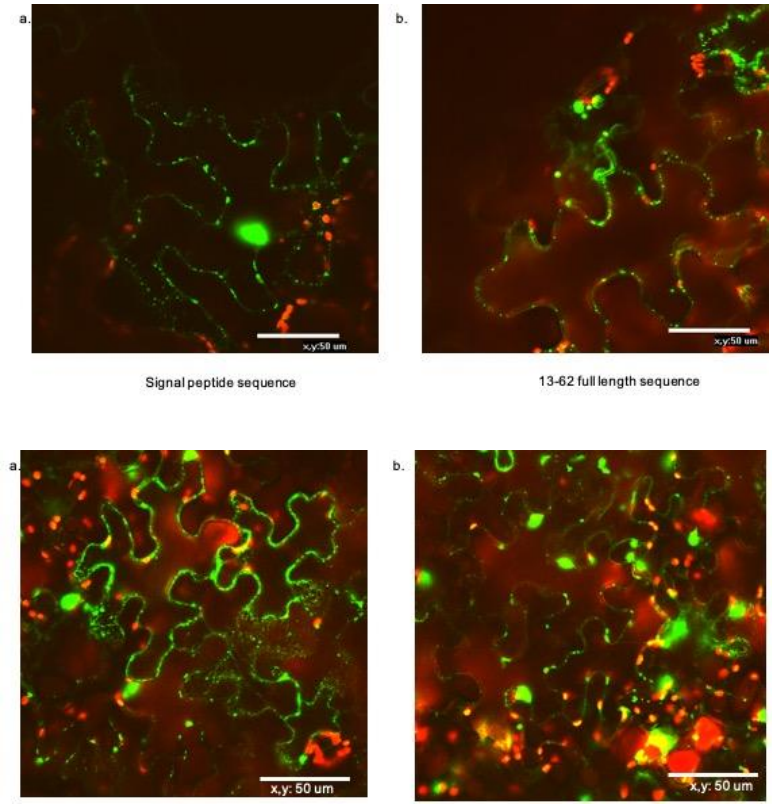

Fig. 41. Cp13-62 protein localization (pEarleyGate 103 construct). (Top) Untreated *N. benthamiana* leaves. (Bottom) *N. benthamiana* after plasmolysis with 1 M sucrose for 20 min.

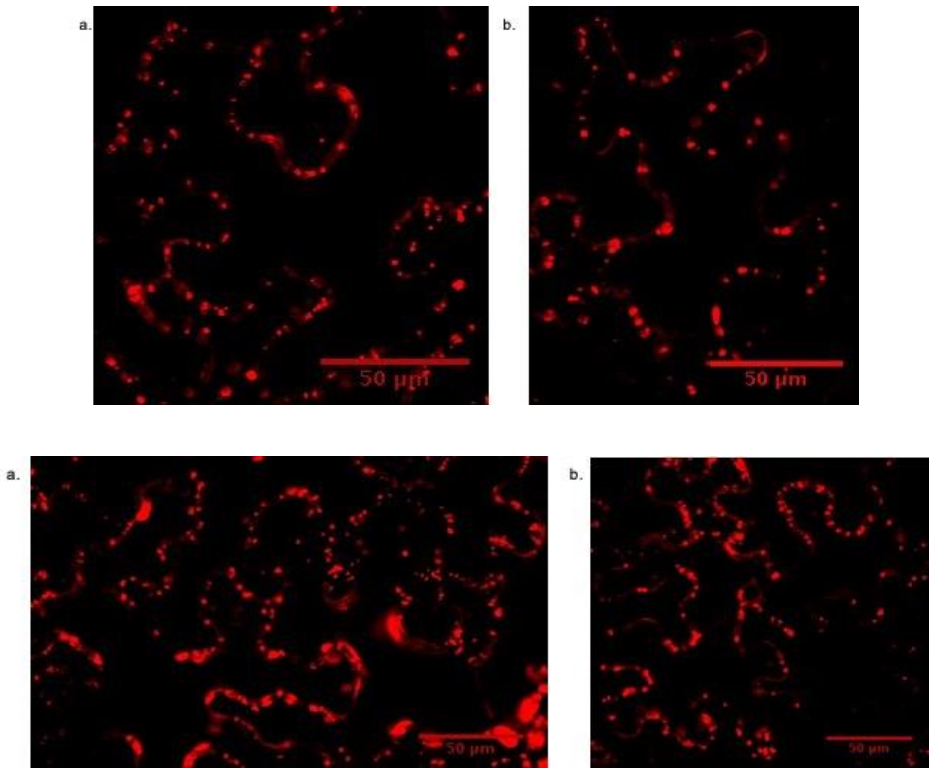

Fig. 42. Cp13-62 protein localization (pMpGW228). (Top) Untreated *N. benthamiana* leaves. (Bottom) *N. benthamiana* after plasmolysis with 1 M sucrose for 20 min.

5. Discussion

The resurrection plant *C. plantagineum* is able to withstand desiccation of its vegetative tissues and is found in areas with variable water availability (Chen *et al.,* 2020; Dries *et al.,* 2011). As resurrection plants dry, protein synthesis is necessary for the production of the proteins associated with implementation of desiccation tolerance and for the specific proteins regulating the implementation. The major changes in the protein complement during induction of desiccation tolerance imply changes in gene expression and turnover of degraded proteins. In resurrection plants protein synthesis continues to surprisingly low water contents. In the resurrection monocot *Xerophyta villosa* protein synthesis rates increase markedly below 60% RWC before declining again at 30% RWC (Blomstedt *et al.,* 2018).

Both CpPGIP and pcC13-62 are cell wall localized (**Fig. 11, 12, 41 and 42**) and could contribute to acquisition of desiccation tolerance. Hence in this thesis the regulation of the desiccation-related *pcC13-62* gene at the promoter level and CpPGIP at the protein and gene level was studied. Studying both these candidates could help us to unravel their protective role during desiccation tolerance and the data could provide more insight into the evolution of the molecular basis of desiccation tolerance.

5.1 Expression profile of the *CpPGIP* gene and subcellular protein localization

Investigation of the expression of the *CpPGIP* transcript under drought stress allowed a comparison of the expression profiles under different water limiting conditions. This study may provide further insight into understanding drought tolerance of *C. plantagenium*. In this study, the *CpPGIP* expression was high for the untreated leaf and root samples and it decreased in the partially dehydrated sample. The expression level in the rehydrated sample was the same as that observed in the untreated sample. The highest expression level was observed in the late dehydrated sample (RWC< 8%). The elongation factor 1alpha (EF1alpha) was used as a control and all the expression levels in the control were observed to be the same (**Fig. 14**). Hence from the study of the transcript profiles of *CpPGIP* it can be concluded that the *CpPGIP* gene gets activated when the plant is subjected to drought stress which could mean that the *CpPGIP* gene product could be playing a similar role as other drought induced genes such as *11-24* and *pcC13-62*. The *pcC13-62* transcripts are expressed at a high level

in response to dehydration in the desiccation tolerant species *C. plantagineum* and *L. brevidens* (Giarola *et al.,* 2018). Similarly, the *LEA-like 11-24* gene is a desiccation-related gene that is abundantly expressed in response to dehydration in leaves of *C. plantagineum* and *L. brevidens* (Phillips *et al.*, 2008). The high accumulation of the CpPGIP transcripts under severely dehydrated conditions indicates that the regulation of *CpPGIP* gene expression appears to play an essential role in the acquisition of desiccation tolerance.

However, it should be noted here that we simulated dehydration shock in the plants by limiting the water intake for up to 72 h under controlled laboratory conditions. The investigation of gene expression should also be examined in the natural plant habitats to confirm the results. It is also important to note that plants face multiple abiotic stress factors in reality, which can interact and influence the response of the plant at the physiological and molecular level. Therefore, this transcript analysis suggests that gene expression needs definitely to be tested for each new stress condition such as salt stress in combination with drought stress. In addition to that the promoter study of the *CpPGIP* gene should also be done to find out which *cis*- elements might be involved in activating the gene during drought stress.

It has been reported that PGIPs occur in the cell walls of a variety of dicotyledonous plants and in several monocotyledonous plants (De Lorenzo *et al.*, 2001; Kemp *et al.*, 2003). The analysis carried out here confirmed that the CpPGIP protein is mainly localized in the cell wall (**Fig. 11, 12**). This could be studied further by using cell wall markers along with the PGIP protein to see where the protein is exactly located in the cell wall. The CpPGIP protein itself might play an important role in influencing the *C. plantagenium* cell wall structure during drought by interacting with other proteins. This needs to be studied further by studying transformed tobacco/ *C. plantagineum* cells under control and drought conditions with the PGIP protein in order to deeply understand CpPGIP's role in the maintenance of the *C. plantagenium* cell wall.

5.2 Analysis of the CpPGIP protein

The function of a protein is tightly linked to its expression in different tissues, developmental stages and sub-cellular compartments. These diverse expression patterns are highly regulated within the protein biosynthesis process and take place on the transcriptional, post-transcriptional, translational and post-translational level (Halbeisen *et al.*, 2008). Export and degradation of proteins counteract the synthesis process and delineate a counterbalance in these complex regulatory networks. The interplay of different proteins in both control mechanisms of the protein biosynthetic pathway, results in dependency from one protein on the other (Dever *et al.*, 2016).

The mature PGIP protein is characterized by the presence of a Leu-rich repeat (LRR) domain that comprises more than two-thirds of the protein. PGIPs interact with the active site amino acid residues of PGs through the amino acid exposed outside of the LRR motif, thereby, inhibiting their activity (Zhang *et al.*, 2016). The protein sequence of CpPGIP was analysed using “charmm” software (http://www.charmm-gui.org). The LRR repeats were analysed in the PGIP sequence from *C. plantagenium* to be sure that PGIP from *C. plantagenium* is constituted of the same structure since this protein had not been studied before (**Fig. 43**).

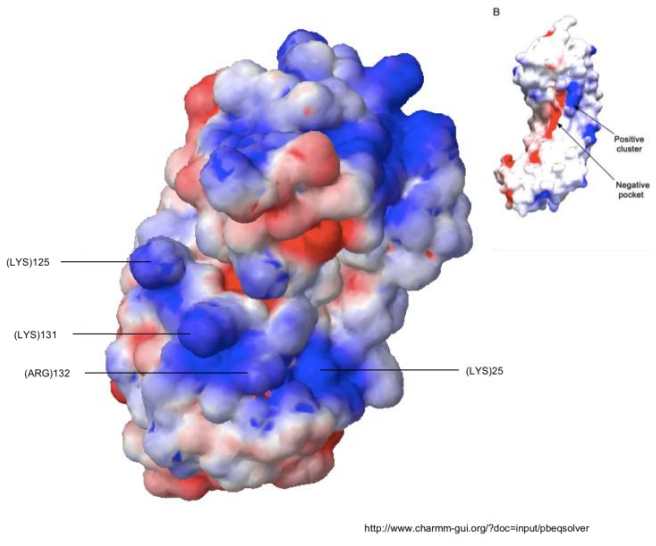

Fig. 43: A three-dimensional structure of CpPGIP with a motif of four clustered arginine and lysine residues was identified.

5. Discussion

The expression of the CpPGIP protein was studied using the PGIP antibody which was developed after overexpression of the recombinant protein and protein purification (**Fig. 17, 18, 19 and 20**). The results showed that the protein expression levels of the CpPGIP protein in *C. plantagineum* were affected upon different abiotic stresses and environmental conditions, such as salinity and dehydration (**Fig. 23 and 24**).

The immunological analysis of total leaf and root protein extracts from different water-controlled stages revealed strong expression in the dehydrated sample in both leaves and roots (**Fig. 23 and 34**). The protein expression abundance was the same as for the transcript (**Fig. 14**) which suggests that PGIP expression is mainly regulated on the transcriptional level.

To confirm this observation additional biological repeats were carried out. The blot with the leaf samples had the protein positioned at a molecular weight of 25 kDa. Interestingly, the root samples showed a protein positioned a bit higher than 25 kDa which indicates a possible isoform of the protein in the roots (**Fig. 23**). In some of the blots with the leaves two protein bands at 25 kDa and at 27 kDa were also observed indicating the presence of two CpPGIP isoforms. However, if the higher band is an isoform or not needs to be studied in detail. This study could be performed using protein mass spectrometry.

Along with the total proteins also the cell wall extracts were prepared to find out which cell wall fraction had the highest protein expression. The dehydrated leaf samples had the highest expression also in the cell wall extract (**Fig. 25 and 26**). The cell wall extracts were prepared using $CaCl_2$, EGTA and LiCl buffers. Out of the three the $CaCl_2$ extract had the highest expression of the CpPGIP protein (**Fig. 25 and 26**). Again, when cell wall extract blots were compared the expression was strongest in the dehydrated sample followed by untreated and rehydrated sample and was the lowest in the partially dehydrated sample. This supports the notion that PGIP might play an important role when *C. plantagenium* wilts during drought and then resurrects conditions when water is again available. The cell wall of *Cp* has evolved to counteract stresses imposed during desiccation and subsequent rehydration and PGIP might be involved in assisting the cell wall during stress conditions.

Since PGIP is mainly a cell wall protein it could contribute to maintain the cell wall structure in *C. plantagenium* in a similar way as the apoplastic glycine-rich protein (CpGRP1) and cell wall-associated protein kinase (CpWAK1) which form a complex (CpGRP1–CpWAK1) during dehydration-induced morphological changes in the cell wall during dehydration in *C. plantagineum* (Jung *et al.*, 2019*)*. The mechanism in which CpPGIP interacts with other cell wall proteins in assisting the plant's desiccation tolerance mechanism needs to be further investigated by identification of the potential protein interacting partners to elucidate the function of the CpPGIP protein.

5.3 Identification of CpPGIP interaction partners

Protein-protein interactions are essential in almost all biological processes, extending from the formation of cellular macromolecular structures and enzymatic complexes to the regulation of signal transduction pathways (Thaminy *et al.*, 2004). Localization analysis showed that CpPGIP is mainly targeted to the cell wall (**Fig. 11 and 12**). Even though PGIP's are broadly known for their role as polygalacturonase inhibitors (Liu *et al.,* 2016) our data from transcript analysis and protein study suggested that the protein might also be influencing the *C. plantagineum* during dehydration.

We hypothesize that the CpPGIP might be working in co-ordination with other proteins and a yeast two hybrid assay was the approach to identify possible CpPGIP interaction partners. The results from the yeast two-hybrid screening are summarized in **Table 9** which shows the list of the potential proteins which could be interacting with the PGIP. We selected proteins such as heat shock protein SSC1, F-box protein and late embryogenesis abundant protein which supports the theory that CpPGIP could be involved in dehydration stress responses of *C. plantagenium* as well. It has already been reported before that apart from pathogen attack PGIP expression can also be induced by many biotic or abiotic stimuli (Liu *et al.,* 2016) and our results support the same hypothesis.

A possible model for the regulation of the processes of *CpPGIP* is depicted in **Fig. 44**.

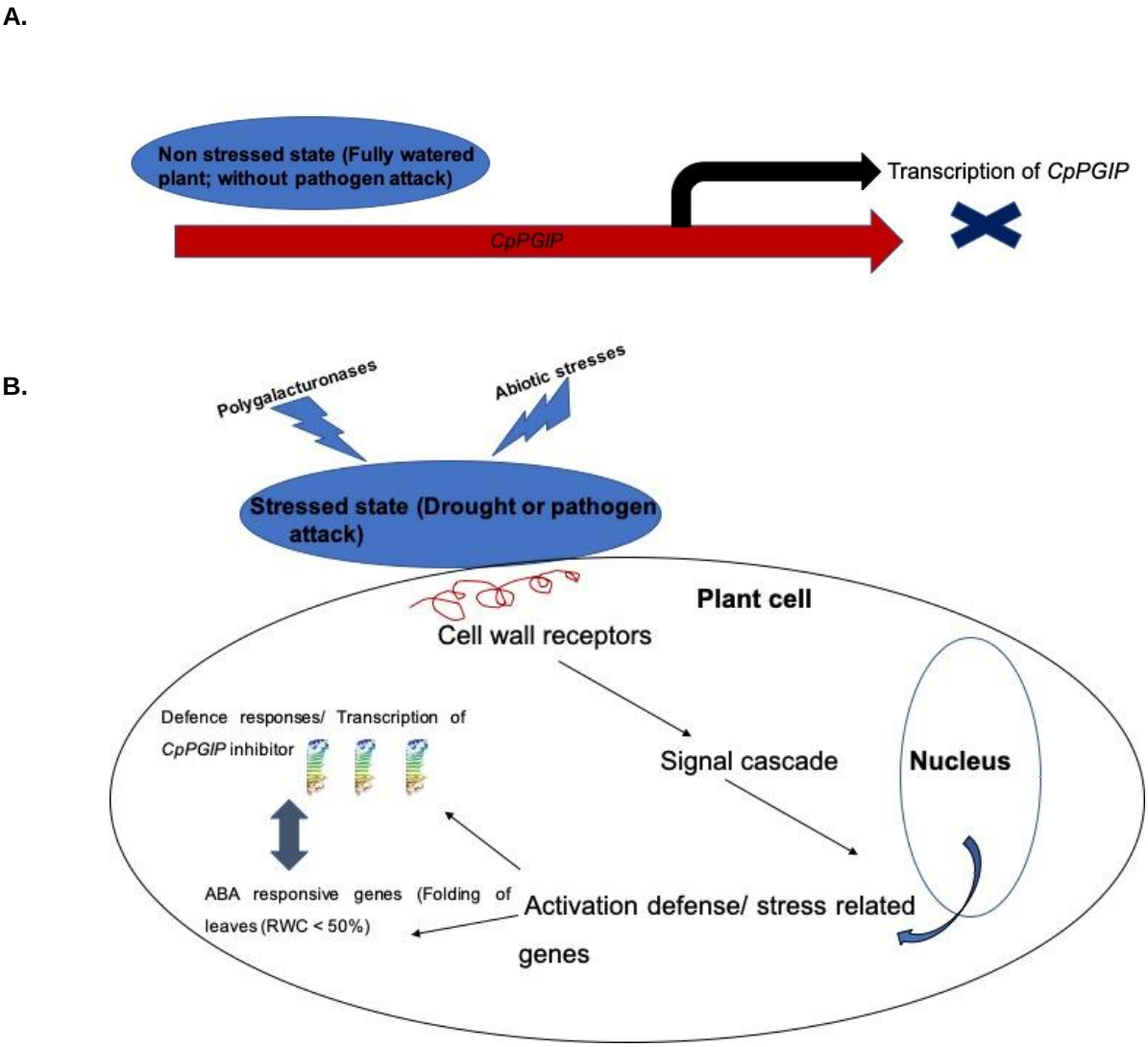

Fig. 44: Model for the regulation of expression of *CpPGIP*.

A. Under turgescent conditions, transcription of *CpPGIP* is prevented.

B. Water-limiting conditions and pathogen attack promote the *PGIP* gene activity, resulting in the increased production transcription factors which increase protein biosynthesis. Since the transcript of *CpPGIP* is also very elevated from the drought stress (**Fig. 14**) we hypothesize that PGIP gene is also activated in water-limiting conditions and its interaction with other stress inducible genes confers *Cp* protection against drought and also helps in conserving the cell wall structure. Nevertheless, the mechanism that regulates *CpPGIP* gene expression and how the CpPGIP protein interacts with other proteins to form complexes is still not very well understood. We still need to research *PGIP* interaction with other drought inducible genes.

5.4 Promoter analysis of stress inducible *pcC13-62* genes

Numerous genes involved in desiccation tolerance have been characterized in *C. plantagineum* (Michel *et al.*, 1994; Velasco *et al*., 1994; Ditzer *et al.*, 2006; van den Dries *et al.*, 2011). Since promoters are the main regulatory elements for enhancing the transcriptional efficiency, the focus of this study was to analyse the *13-62* promoter sequence involved in desiccation tolerance in closely related plant species *C. plantagenium, L. brevidens* and *L. subracemosa* which differ in desiccation tolerance.

We compared the expression and promoter activity of the DRP (Plant desiccation-related proteins) gene *pcC13-62* in closely related desiccation-tolerant and desiccation-sensitive Linderniaceae. It was found that the accumulation of *13-62* transcripts is controlled at the transcriptional level and involves specific promoter *cis* elements such as a DRE motif that occurs only in tolerant species (Giarola *et al.*, 2018). The data from the promoter comparison (**Fig. 36**) shows the importance of DRE motifs for the regulation of desiccation-tolerant genes in resurrection plants and suggests the contribution of nucleotide variations in regulatory regions for the establishment of desiccation tolerance. LEA genes are the most abundant group of protective genes activated in desiccation-tolerant tissues. The name LEA was assigned to these genes as they were initially discovered to be abundantly expressed during the final stage of cotton seed development (Dure *et al.*, 1981). However, these genes are also expressed during dehydration in vegetative tissues of both desiccation-tolerant and desiccation-sensitive plants. Conserved amino acid motifs and sequence similarities have been used to divide LEA proteins into different subclasses (Hundertmark and Hincha, 2008; Jaspard *et al.*, 2012). In our study, it was shown that the *Cp*, *Lb*, and *Ls*13-62 promoters contain conserved structural features (**Fig. 36**).

Intriguingly, a transposable element similar to members of the PIF/Harbinger superfamily was identified and was found to be inserted between the *Lb13-62* and β-1,3 galactosyltransferase coding sequences. The presence of this transposable element was interesting since it was only found to be present in *Lb13-62*. The *Lb13-62* promoter sequence used for the analysis in this study was restricted in its length to the region between the predicted transposon terminal inverted repeat sequence and the ATG start codon of *Lb13-62*. The analysis showed that regulatory *cis*-elements

important for dehydration-induced activation appear to be within this sequence (**Fig. 36**). The transposon had similarities to that of the P instability factor (PIF) found in maize. It is a class 2 (DNA) element which can transpose via a DNA intermediate and usually has short terminal inverted repeats (TIRs) (Zhang *et al*., 2001 and 2004). The maize *PIF* encodes two ORFs (ORF1 and ORF2), of which ORF2 is the TPase while the function of the other ORF1 is unknown (Grzebelus *et al*., 2006). This transposon insertion has probably taken place during evolution in *L. brevidens* but its role in desiccation tolerance is not clear.

The *13-62* promoter from *C. plantagineum* was active in dehydrated *L. subracemosa* leaves but the *L. subracemosa* promoter was not functional in dehydrated *C. plantagineum* leaves, the lower activity in *L. subracemosa* can be explained by the lack of essential *cis*-acting elements (**Fig. 38**). *In silico* analysis revealed the presence of several ABA- and dehydration-related *cis*-elements in the *Cp*, *Lb*, and *Ls13-62* promoters. However, the spatial organizations of these *cis*-elements differ, indicating limited conservation of the promoter architecture among the three species (**Fig. 36**).

Some elements, such as TEIL, HBP-1a, HSF, DRE, and MYC, were found in the *13-62* promoters of the two desiccation-tolerant species *C. plantagineum* and *L. brevidens*. This promoter sequence contains the only DRE element in the desiccation-tolerant species *C. plantagineum* and *L. brevidens*, and the deletion of the DRE elements nearly completely abolished dehydration-induced promoter activation (**Fig. 39**). In *L. subracemosa*, a single-nucleotide mutation in the corresponding promoter region suppressed part of the dehydration responsiveness.

When the DRE element was introduced into the *L. subracemosa* promoter, dehydration responsiveness was partially restored (**Fig. 39**). Therefore, we assume that other *cis*-acting elements in the first 170 bp are possibly missing in the *L. subracemosa* promoter and may work as coupling elements (working in co-ordination with each other) for the DRE element (**Fig. 39 and 40**). The importance of the DRE element in the promoter of desiccation-tolerant species has previously been demonstrated for the *CDeT11-24* promoter (Dries *et al.*, 2011).

5.5 Localization of the 13-62 protein

Localization analysis showed that the 13-62 protein is mainly localized in the cell wall (**Fig. 41 and 42**). The protein might regulate gene expression by forming a complex with other proteins. However, how these protein complexes are formed and how they function needs to be further investigated. This could be achieved by finding the interaction partners by techniques such as yeast two hybrid assay. Both CpPGIP and pcC13-62 are cell wall localized (**Fig. 11, 12, 41 and 42**) and could be interesting candidates to help us to understand the mechanisms involved in desiccation tolerance in *C. plantagenium*.

6. Future perspectives

The extensive characterization of CpPGIP in this thesis provides insights in the regulation and function of a novel protein (CpPGIP) in *C. plantagenium* which in future might help us to understand the complex cell wall of the plant. Additional promoter regulation data is required to support the suggested expression profiles in response to drought and salt stress. A possible expansion of stress-treatments such as salinity and osmotic stress or wound stress on the expression of CpPGIP should be investigated in order to further understand the role of the polygalacturonase inhibitor 1-like protein in the involvement of the drought tolerance mechanism of *C. plantagenium*. Furthermore, experiments such as yeast two hybrid assay and co-immunoprecipitation should be performed to identify other interaction partners of CpPGIP protein.

To unravel more functions of the second candidate gene *pcC13-62* it needs to be studied at the protein level. The pcC13-62 protein homologue, designated as ‘‘MS-desi’’ was studied by Zha *et al.* in 2013 where they hypothesized that, MS-desi might also play an important role in plant defence against infection by pathogens. Efforts should be made to identify the interaction partners of 13-62 to understand its function at the protein level. In case of the localization experiment, it can be performed again with the help of cell wall markers to exactly determine the localization of the protein within the cell wall.

7. References

Ataei S., Braun V., Challabathula D., Bartels D. 2015. Differences in *LEA-like 11-24* gene expression in desiccation tolerant and sensitive species of Linderniaceae are due to variations in gene promoter sequences. Functional Plant Biology 43(7) 695-708.

Blomstedt C., Gaff D., Griffiths C., Donald F., Hamill J., Neale A. 2018. Plant Desiccation Tolerance and its Regulation in the Foliage of Resurrection "Flowering-Plant" Species. Agronomy 8(8), 146.

Chen P., Jung N., Giarola V., Bartels D. 2020. The Dynamic Responses of Cell Walls in Resurrection Plants During Dehydration and Rehydration. Frontiers in Plant Science 10.3389, 1698.

Dries N., Facchinelli F., Giarola V., Phillips J., Bartels D. 2011. Comparative analysis of *LEA-like 11-24* gene expression and regulation in related plant species within the Linderniaceae that differ in desiccation tolerance. New Phytologist 190: 75–88.

Farrant J., Willigen V., Loffell D. 2003. An investigation into the role of light during desiccation of three angiosperm resurrection plants. Plant Cell and Environment 26 (8), 1275–1286.

Farrant J. 2000. A comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plant species. Plant Ecology 151 (1), 29–39.

Giarola V., Jung N., Singh A., Satpathy P., Bartels D. 2018. Analysis of *pcC13-62* promoters predicts a link between *cis*-element variations and desiccation tolerance in Linderniaceae. Journal of Experimental Botany 69, 3773–3784.

Giarola V., Hou Q., Bartels D. 2017. Angiosperm Plant Desiccation Tolerance: Hints from Transcriptomics and Genome Sequencing. Trends in Plant Science 22(8).

Gechev T., Dinakar C., Benina M., Toneva V., Bartels D. 2012. Molecular mechanisms of desiccation tolerance in resurrection plants. Cellular and Molecular Life Sciences 69:3175-3186.

Grzebelus D., Yau Y., Simon P. 2006. Master: a novel family of PIF/ Harbinger-like transposable elements identified in carrot (*Daucus carota L*.). Mol Gen Genomics 275: 450–459.

Gall H., Philippe F., Domon J. 2015. Cell wall metabolism in response to abiotic stress. Plants, 4 (1), 112–166.

Höll J., Vannozzi A., Czemmel S., D'Onofrio C., Walker A., Rausch T., Lucchin M., Boss P., Dry I., Bogs J. 2013. The R2R3-MYB Transcription Factors MYB14 and MYB15 Regulate Stilbene Biosynthesis in *Vitis vinifera*. The Plant Cell 25: 4135–4149.

Hibi T., Kosugi S., Iwai T., Kawata M., Seo S., Mitsuhara I., Ohashi Y. 2007. Involvement of *EIN3* homologues in basic PR gene expression and flower development in tobacco plants. Journal of Experimental Botany 58: 3671–3678.

Hobo T., Asada M., Kowyama Y., Hattori T. 1999. ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. The Plant Journal 19(6): 679-689.

Hossain M., Veneklaas E., Hardy G., Poot P. 2019.Tree host–pathogen interactions as influenced by drought timing: linking physiological performance, biochemical defence and disease severity. Tree Physiology 39 (1), 6-18.

Iljin W. 1957. Drought resistance in plants and physiological processes. Annual Review of Plant Physiology, 8 (1), 257–274.

Jung N., Giarola V., Chen P. 2019. *Craterostigma plantagineum* cell wall composition is remodelled during desiccation and the glycine-rich protein CpGRP 1 interacts with pectins through clustered arginines. The Plant Journal 100(4), 661–676.

Jones L., McQueen-Mason S. 2004. A role for expansins in dehydration and rehydration of the resurrection plant *Craterostigma plantagineum*. FEBS Letters 559 (1–3), 61–65.

Kim S., Ma J., Perret P., Li Z., Thomas T. 2002. Arabidopsis ABI5 Subfamily Members Have Distinct DNA-Binding and Transcriptional Activities. Plant Physiology 130: 688–697.

Liu N., Ma X., Zhou S., Wang P., Sun Y., Li X., Hou Y. 2016. Molecular and functional characterization of a polygalacturonase-inhibiting protein from *Cynanchum komarovii* that confers fungal resistance in Arabidopsis. PLoS ONE 11(1): e0146959.

Liu J., Wang F., Yu G., Zhang X., Jia C., Qin J., Pan H. 2015. Functional Analysis of the Maize C-Repeat/DRE Motif-Binding Transcription Factor CBF3 Promoter in Response to Abiotic Stress. International Journal of Molecular Sciences 16: 12131-12146.

McDowell N., Pockman W., Allen C., Breshears D., Cobb N., Kolb T., Plaut J., Sperry J., West A., Williams D., Yepez E. 2008. Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? New Phytologist 178: 719–739.

Moore J., Nguema-Ona E., Gibouin M. 2013. Arabinose-rich polymers as an evolutionary strategy to plasticize resurrection plant cell walls against desiccation. Planta 237 (3), 739–754.

Minic Z., Jouanin L. 2006. Plant glycoside hydrolases involved in cell wall polysaccharide degradation. Plant Physiology and Biochemistry 44 (7–9), 435–449.

Petersen J., Eriksson S., Harryson P. Pierog S., Colby T., Bartels D., Röhrig H. 2012. The lysine-rich motif of intrinsically disordered stress protein CDeT11-24 from *Craterostigma plantagineum* is responsible for phosphatidic acid binding and protection of enzymes from damaging effects caused by desiccation. Journal of Experimental Botany 63 (13), 4919-4929.

Phillips J., Fischer E., Baron M., Dries N., Facchinelli F., Kutzer M., Rahmanzadeh R., Remus D., Bartels D. 2008. Lindernia brevidens: a novel desiccation-tolerant vascular plant, endemic to ancient tropical rainforests. The Plant Journal 54: 938–948.

Piatkowski D., Schneider K., Salamini F., Bartels D. 1990. Characterization of Five Abscisic Acid-Responsive cDNA Clones isolated from the Desiccation-Tolerant Plant *Craterostigma plantagineum* and Their Relationship to Other Water-Stress Genes. Plant Physiology 94: 1682-1688.

Rodriguez C., Edsgärd D., Hussain S., Alquezar D., Rasmussen M., Gilbert T., Nielsen H., Bartels D., Mundy J. 2010. Transcriptomes of the desiccation-tolerant resurrection plant *Craterostigma plantagineum*. The Plant Journal 63, 212–228.

Simonini S., Kater M. 2014. Class I BASIC PENTACYSTEINE factors regulate HOMEOBOX genes involved in meristem size maintenance. Journal of Experimental Botany 65: 1455–1465

Trivedi D., Ansari M., Tuteja N. 2013. Multiple abiotic stress responsive rice cyclophilin (OsCYP-25) mediates a wide range of cellular responses. Communicative & Integrative Biology 6:5.

Tabata T., Nakayama T., Mikami K., Iwabuchi M. 1991. HBP-1a and HBP-1b: leucine zipper-type transcription factors of wheat. The EMBO Journal 10: 1459 – 1467.

Thomson W., Platt K. 1997. Conservation of cell order in desiccated mesophyll of Selaginella lepidophylla. Annals of Botany 79 (4), 439–447.

Vogel J. 2008. Unique aspects of the grass cell wall. Current Opinion in Plant Biology 11 (3), 301–307.

Vicre´ M., Farrant J., Driouich A. 2004. Insights into the cellular mechanisms of desiccation tolerance among angiosperm resurrection plant species. Plant Cell & Environment 27 (11), 1329–1340.

7. References

Willige B., Kutzer M., Tebartz F., Bartels D. 2009. Subcellular localization and enzymatic properties of differentially expressed transketolase genes isolated from the desiccation tolerant resurrection plant *Craterostigma plantagineum*. Planta 229 (3), 659-666.

Young Kim S., Ma J., Perret P., Li Z., Thomas T. 2002. Arabidopsis ABI5 Subfamily Members Have Distinct DNA-Binding and Transcriptional Activities. Plant Physiology 130: 688–697.

Zha H., Liu T., Zhou J., Sun H. 2013. MS-desi, a desiccation-related protein in the floral nectar of the evergreen velvet bean (*Mucuna sempervirens Hemsl*): molecular identification and characterization. Planta 238: 77-89.

Zhang X., Jiang N., Feschotte C., Wessler S. 2004. PIF- and Pong-Like Transposable Elements: Distribution, Evolution and Relationship with Tourist-Like Miniature Inverted-Repeat Transposable Elements. Genetics 166: 971–986.

Zhang X., Feschotte C., Zhang Q., Jiang N., Eggleston W., Wessler S. 2001. P instability factor: An active maize transposon system associated with the amplification of Tourist-like MITEs and a new superfamily of transposases. PNAS 98: 12572–12577.
.

8. Supplementary data

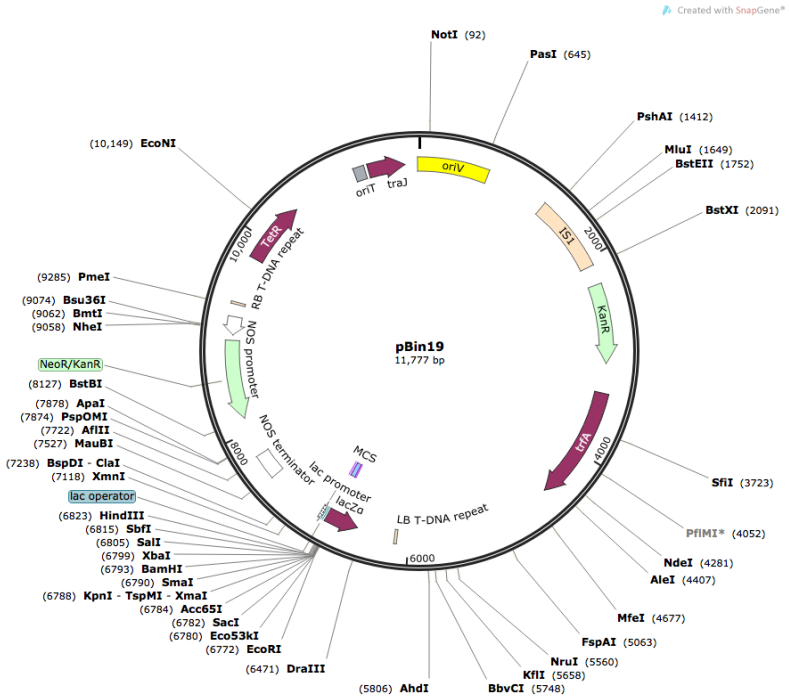

Fig. 45. ThepBIN19 vector map with restriction enzyme digestion sites.

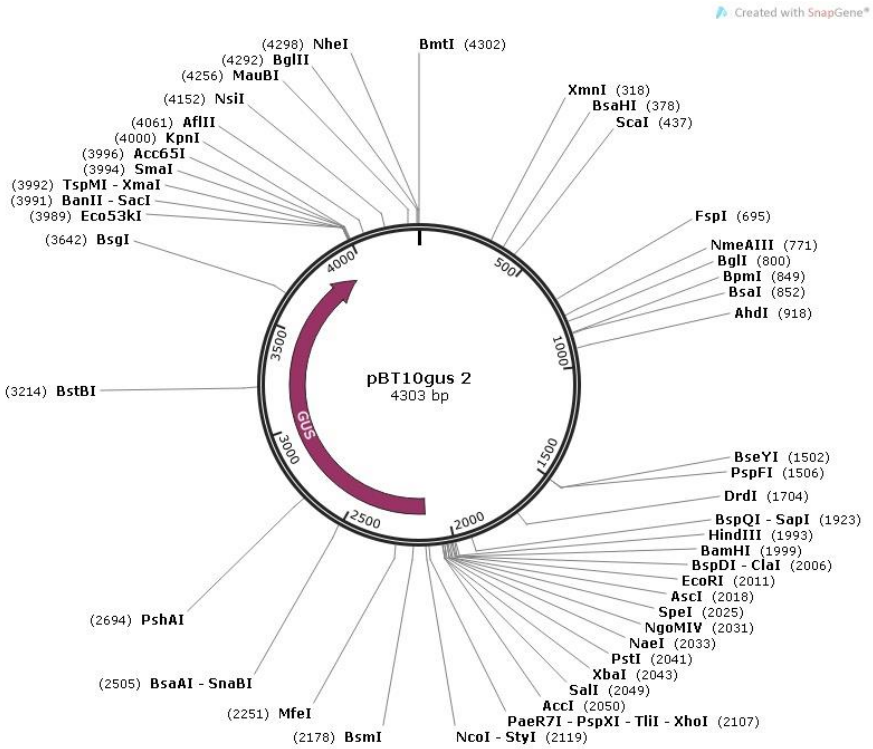

Fig. 46. The pBT10-GUS vector map with restriction enzyme digestion sites.

8. Supplementary data

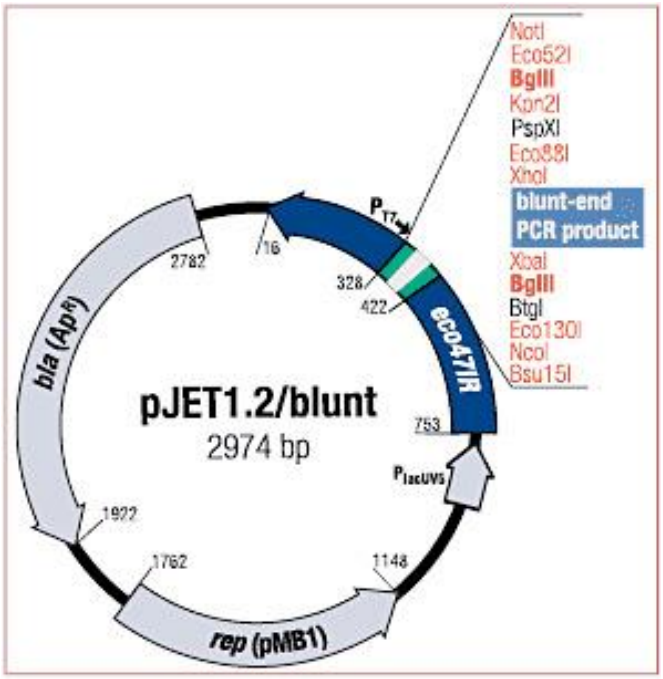

Fig. 47. ThepJET1.2vector map with restriction enzyme digestion sites.

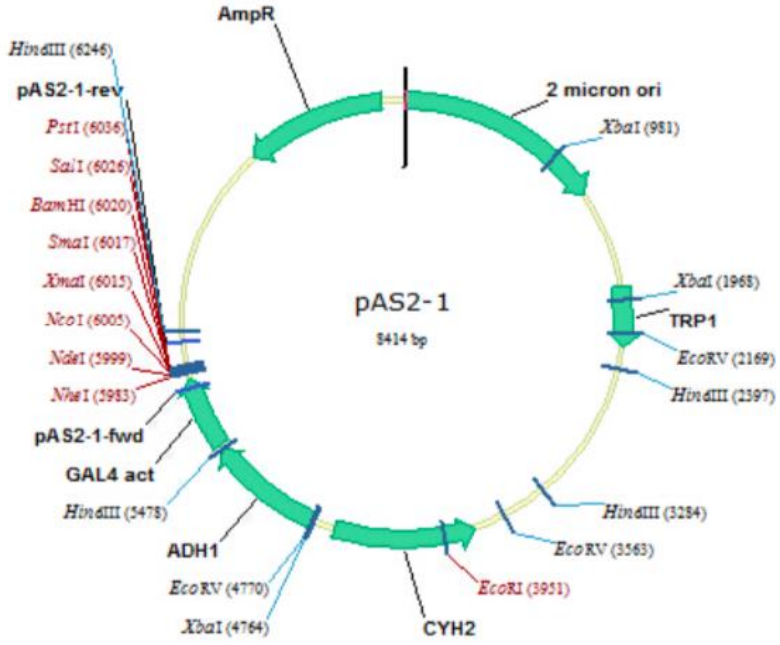

Fig. 48. ThepAS2-1 vector map with restriction enzyme digestion sites.

8. Supplementary data

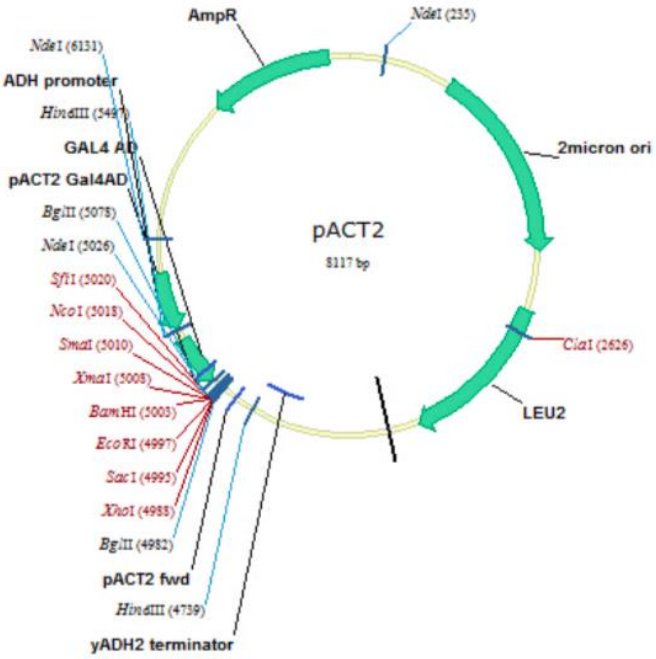

Fig. 49. The pACT2 vector map with restriction enzyme digestion sites.

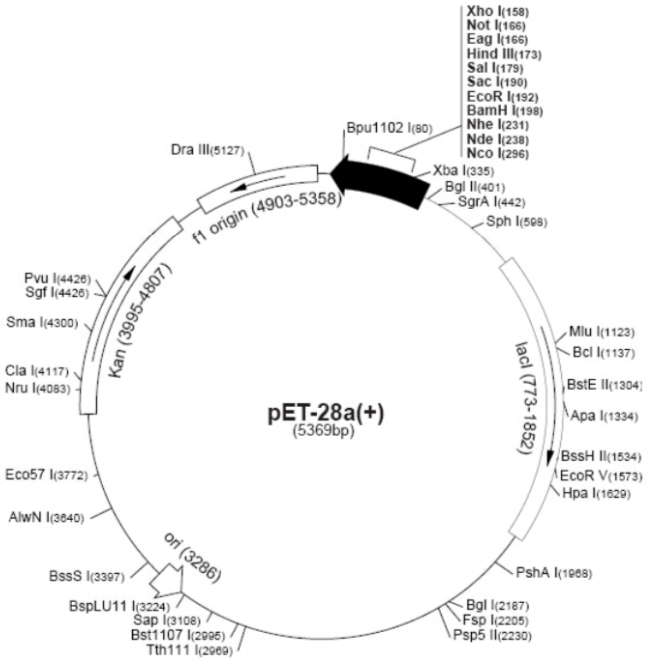

Fig. 50. The pET-28a vector map with restriction enzyme digestion sites.

8. Supplementary data

CpPGIP clone 5 in pJET

GGCACGAGGAAGAAAGCTAAGTAAAAAATCACGATGAAGTCCTCAACAACTCTCCTCTTAATAACCCTTTCTCTC
CTACCATTATTACACTACATCCCTTCTTCCAAAGCCACCGACTTTCCGCCGTGCAACGCCCAAGACAAGGCGGTC
CTTCTCAAAGTCAGAGACCACTTCGGCGGCCCAAACGGCCGACTCTCCGATTGGGACAACAACACAGATTGCTGT
TCGGAATGGAGTTTCGTAGGTTGCAGCAGCAAAAGTGGAAAATCGTACGGACGCATAGACACGGTGACTTTTAGC
CGTTCTTGGGGACTTTCGGGCACAATACCTTCGGACTTTGGAGATCTTCCTTACCTTAGCTTCTTCATTTTGGCC
GACAACATAGGCGTTAATGGCCCTATCCCCAAGGCATTTGGTAATCTCAAAAGGCTCTATCACATCGAACTCGAC
TCTAATGCCTTGACTGGGCCGATTCCTAAGGAGCTCTTACAGCTGAAGAACCTCCAAGTAGTTGATCTTTCGAAC
AATAAGCTGTCGGGAGAAATTCCAGAGTTGTCTTCGTCTTCGTTGGGAAAGCTTACGCAGTTCAACGTTTCGTAC
AATCAGCTCTGTGGGTCGATTCCTTCTGGGCTGAGCAAGTTTGGGAAGGCGAGCTTTGATCATAATAAGTGCCTC
TGCGGTCCACCTTTGCCCGATTGCAAGTAATTATGCAATGGATGTGTTGTATGGTANGGNAATCTTT

CpPGIP clone 2 in pJET

GGCACGAGGCCACAACTTACAAAAAGAAAAGTAAAAAAACACGATGAAGTCTTCAACAACTCTCCTCTTAATAAC
CCTTTCTCTCCTACCATTATTACACTACATCCCTTCTTCCAAAGCCACCGACTTTCCGCCGTGCAACGCCCAAGA
CAAGGCGGTCCTTCTCAAAGTCAGAGACCACTTCGGCGGCCCAAACGGCCGACTCTCCGATTGGGACAACAACAC
AGATTGCTGTTCGGATTGGAGTTTCGTAGGTTGCAGCAGCAAAAGTGGAAGCTCATACGGACGCATAGACACGGT
GACTTTTAGCCGTTCTTGGGGACTTTCGGGCACAATACCTTCGGACTTTGGAGATCTTCCTTACCTTAGCTTCTT
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CGAACTCGACTCTAATGCCTTGACTGGGCCGATTCCTAAGGAGCTATTACAGCTGAAGAACCTCCAAGTAGTTGA
TCTTTCGAACAATCAGCTGTCGGGAGAAATTCCAGCGTTGTCTTCGTCTTCGTTGGGAAAGCTTACGCAGTTCAA
CGTTTCGTACAATCAGCTCTGTGGGTCGATTCCTTCTGGGCTGAGCAAGTTTGGGAAGGCGAGCTTTGATCATAA
TAAGTGCCTCTGCGGTCCACCTTTGCGCGATTGCAAGTAATAATGCAAGGATGTGTTGTATGTATGGGGATCTTG
CTGAAAAACTCGAGCCATCCCGAATTTTCCGC

Lb13-62p in pBT10GUS clone 2 (SalI-NcoI)

GTCGACCATCTAGATCAACGTACGAGTGTTTACGACATGAAAATTTCAGAAAGAAAAAGCTTATAGACAAAACGA
GATATATTGAAAATAAATTATATAAATTCGGTGAGATCATGTTATTTTCCCGTATGTATTATTTCTATTATCACA
TTTGTAATGAAATTTTGATCATATCATATTTTAGTTATGAATAATAATATTTTTTTCTGACAGTAGTGAAGTCTA
TTTTCAAATATTAAATGGCCTGAAACTTAATTGCCCGTTCATTTATTATTGTACGCCACGTCCGTTCAATTATTT
TCAAAATACTATTATTTCAAGAAATAACCTCCCTAGACGTGTAATGTAATAATAACAGTGTTAATGTATCTAGAC
GAAACGGTCGAAAGAGTTCTTAATGACAAACTCCCTCGTCATAACTTGGACGGACAATTTAAATTTTAACTCACT
CAACTGTCGTAATCCCATCTCGAATTTTCTTCCAACGCCTTACGAGGCCATGCGTATACGTATTCCACCACACAA
GGCAACTTACTTTTGCAACAAAACACATCACCTCTAATCACATCCTTCAAAAAATAAAAAAAAAAGAAACATAAT
TTACTCGTCAGCCATCCACCTTTCACTACAGCCACCTCTCCACGATCCTTCCGCCACAGCCCGACACGTGTAAAA
ACTTTCGGACACCTATCGAAGCTATAAATAGGAGCAACCAACAAAGCAACACCAAGAAACGACAGAAAACAAGAA
GCATTTTTCAAGCCGCAGCATCAACCATGG

Ls13-62p in pBT10GUS clone 2 (XbaI-NcoI)

TCTAGATCTCCCACTTCTTTGCTTCAAAATAACTTCTTTTGCTCCATTTTTACACTCACCGACGATGAATACATT
GAAGAAGACAAGCAAGCACTAGAGCAGATATTTCCATACTCCGCAGCACTTTCCAATTCAAATGGGCAGACCCTT
AAATTAGATTCAACTGTTAGAAGTATGTAACTTCACTTTTTCACTTGTACTGATGAGCAGCTGAGCAACAGAGCG
GTGAAAGATTCACCGATTAACAAGAGGAGTTCAGACAAAAACAACCGAGTAAAGCTCAAACCCAATCTAACTCCT
TTCCTATTTAGGTCAATAAATTAGTACACTAATAGTAAAAAGCTTTCTCTTCTAAAATGAGGTTAGACTGAGAAT
TTACAAAAGTATATAGAGTTCAGTTATAGAGCGAAGATTCCATGTGTACCCCTCTAATAATATAAGGTCAAAACT
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ACACGATCTTTCGGCGGCAGCCTGACAGGTGTCGAAACTTGCGGACACCTATTCGAGCTATAAATTGCATGTCCT
CAAAGAGCACGACCCAGCAAAGAAAGAAAACGAACCATTTCACGCGCCGCGCACCATGG

8. Supplementary data

Cp13-62p in pBT10GUS clone 13 (XbaI-NcoI)

TCTAGATCTCCCACTTCTTCTTTTTCATCATTGCTTCAAAAAATCTTCTTTTGCTCCATTTGCACACTCACTGAC
GATGAATACATTGAAGAAGACAAGCACTATAAAAGGGTCCCATTTTTTGCAGCACTTTCATATTGGCAAACCCTA
AAATCTCCATTATCGCCTCCTGAAATTAGATTCGTTCAACTGTAAAAAGATGTAACTTTTTAACTTTGTCCTAAT
GACTCCGTGAAAACGTCGTCAATGATTCAACGATTGACAGAAGTTCAGACAAAAACCACCAAAGTAAAATAGTCG
CTGAAGGGGTGATATTGTAATCAACGCATAGTTACATGCTTTCAGTAAAATCGAAATATTACATTCTTACATCTC
TAACTTCAAAATCAAAACTTTTAATATTGTCTTTTTTTCTTTTCAAATTTGTGTCTTATCCACTCAGAAAGATTG
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AATTAGCGTTTCGAAAAATAACTTCAAAATATCGTCTTTTTGGCCGATATTATTTAGAGAACAACAAAAAAAAGA
GGTCGTATGTATCTAGCAAGGATCTCGACTAAAAACACTCGTCATCGGTACGACGGTTCACTCTGTCCTAATCTT
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TATGTCCTCCCAACTAATCACAACCCTTAAAAGCTATTACTCGTCTGAAAATAATCCACTTTTCATTACAACCGC
CTCGCCACGATCCTCCGTCGAAAGCCCGACAGGTGTAGAACCTTGCTCAAACCTATCCGAGCTATAAATAGCAGC
CATGCAACTAAGCGAAGACATAGAAANGAAANTAAAAAANAAANNGCCATCTTCACACACAGCAGCAAACCATGG

Cp13-62p in pBT10GUS clone 4a (XbaI-NcoI)

TCTAGATCTCCCACTTCTTCTTTTTAACCATTGCTTCAAAAAAGCTTCTTTTGCTCCATTTGCACACTCACTGAC
GATGAATACATTGAAGAAAACTATAAAAGGGCCCAATCTTTGCAGCACTTCCATATTGGCAAACCCTTAAATCTC
CATTATCGCCTCGTGAAATTAGATTCGTTCAACTGTAGAAAGACGCAGCTTTTTAACTTTCTCCTAATGACTACG
TGAGGAAAAACGTCGTCAATGATTCAACGATTAACAGAAGTTCAGATAGAAACCACCAAAGTAAAAGAGCTATGC
CAAGATTCC**AACCCAGTAAAAGAGCTCTGCTATGCTAATTGCTAATCACTGCAGGGGTGA**TATTGTAATTAGATA
ATAGTAATAAAGGAGTACATGCCATCAATAGTAAAATCGAAATATCACATTCTTACATCTCCAACTTCAAAACTT
TTAATCTTGTCTTCTTTTCTTCTTTTCAAATTTGTGTCTTATATCCAACTCAGAAAGATTGAATGTTATTAAATG
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CTCGACTAAAAACACTCGTCATAGGCACGACGGTTCACTCTGTCCTAATCTTATCTCTCGACGTTTCTAGTTAAC
GCACTGAATATATTTAATCGACGCAGTCAATGTTGTCATCACAATACATACAGATGTCCTCCCAACTAATCACAA
CCCTCCAAAGCTATTACTCGTCTGAAAATAATCCACTTTTCACTACAACCAACTTTCCACGATCCTCCGGCCAAA
GCCCGACAGCTGTAGAACCTTGCTGAAACCTAGCCGAGCTATAAATAGCAGCCATGCAACTAAGCGAAGAACCAG
AGAAAAGAAAATAATAGAAAAAAAGCCATCTTCACACACAGCAGCAAACCATGG

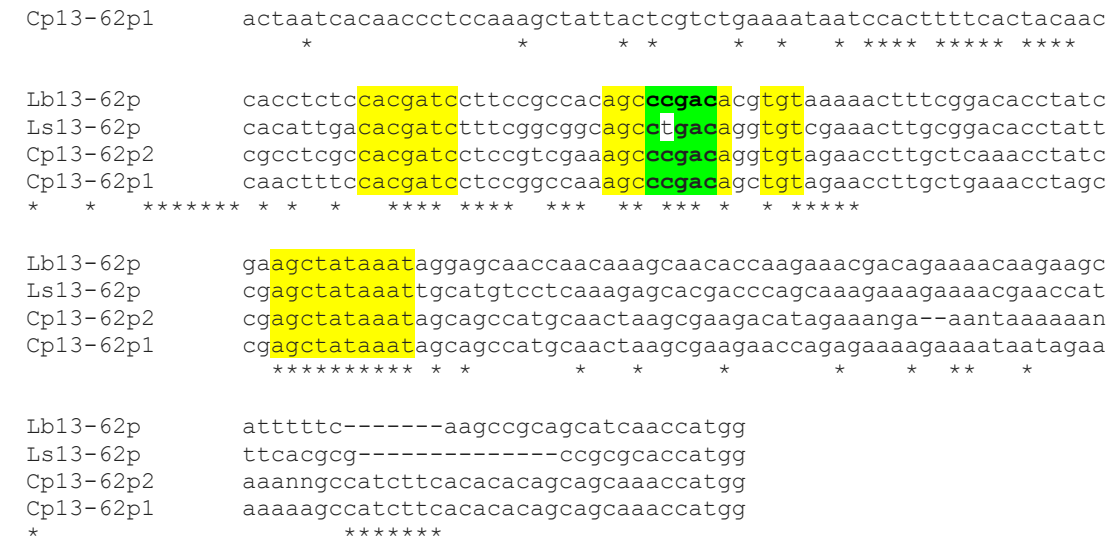
Multiple sequence alignment for the *13-62* promoter sequences in *C. plantagineum*, *L. brevidens* and *L. subracemosa* with the *cis*-elements.

```
Lb13-62p      ------------------------------------------------------------
Ls13-62p      ------------tctagatctcccacttctttgcttcaaaataacttcttttgctccatt
Cp13-62p2     Tctagatctcccacttcttctttttcatcattgcttcaaaaaatcttcttttgctccatt
Cp13-62p1     Tctagatctcccacttcttctttttaaccattgcttcaaaaaagcttcttttgctccatt


Lb13-62p      ------------------------------------------------------------
Ls13-62p      tttacactcaccgacgatgaatacattgaagaagacaagcaagcactagagcagatattt
Cp13-62p2     tgcacactcactgacgatgaatacattgaagaagacaagcactataaaagggtcccattt
Cp13-62p1     tgcacactcactgacgatgaatacattgaagaaaactat-------aaaagggcccaatc


Lb13-62p      ------------------------------------------------------------
Ls13-62p      ccatactccgcagcactttccaattcaaatg----------------ggcagacccttaa
Cp13-62p2     ttt------gcagcactttcatattggcaaaccctaaaatctccattatcgcctcctgaa
Cp13-62p1     ttt------gcagcacttccatattggcaaacccttaaatctccattatcgcctcgtgaa


Lb13-62p      ------------------------------------------------------------
Ls13-62p      attagattcaactgttagaagtatgtaacttcacttttcacttgtactgatgagcagct
Cp13-62p2     attagattcgttcaactgtaaaa-agatgtaacttttttaactttgtcctaatgactccgt
Cp13-62p1     attagattcgttcaactgtagaa-agacgcagctttttaactttctcctaatgactacgt
```

8. Supplementary data

```
Lb13-62p       Gtcgaccatctagatcaacgtacgagtgtttacgacatgaaaatttcagaaagaaaaagc
Ls13-62p       gagcaacagagcggtgaaagattcaccgattaacaagag--gagttcagacaaaaacaac
Cp13-62p2      ga----aaacgtcgtcaatgattcaacgattgacagaag--tt---cagacaaaaaccac
Cp13-62p1      gaggaaaaacgtcgtcaatgattcaacgattaacagaag--tt---cagatagaaaccac
               *      *      * ** *    *  * **   *   *       **** * ***   *

Lb13-62p       ttataga-------------------------caaaacgagatatattgaaaataaatta
Ls13-62p       cgagtaa---------------agctcaaacccaatctaactccttttcctatttaggtca
Cp13-62p2      caaagtaaaatagtcgctgaaggggtga--------------------------------
Cp13-62p1      caaagtaaaagagctatgccaagattccaacccagt**aaaagagctctgctatgc**taattg
                 *   *

Lb13-62p       tataaat---tcggtgagatcatgttattttcccgtatgtattatttctattatcacatt
Ls13-62p       a------------------taaattagtacactaatagtaaaaagctt------------
Cp13-62p2      -------------------tattgtaatcaacgcatagttaca-------------tgct
Cp13-62p1      ctaatcactgcaggggtgatattgtaattagataatagtaataaaggagtacatgccatc
                                 *    *  *       **   *

Lb13-62p       tgtaatgaaattttgat---------------------------catatcatattttagt
Ls13-62p       ------------------------------------------------------------
Cp13-62p2      ttcagtaaaatcgaaatattacattcttacatctctaacttcaaaatcaaaacttttaat
Cp13-62p1      aatagtaaaatcgaaatatcacattcttacatctccaacttca------aaacttttaat


Lb13-62p       tatgaataataatatttttttctgacagtagtgaa-----gtctattttcaaatattaaa
Ls13-62p       ------------------------------------------------------------
Cp13-62p2      attgtct---tttt--ttcttttcaaatttgtgtctt---atccactcagaaagattgaa
Cp13-62p1      cttgtcttcttttc--ttcttttcaaatttgtgtcttatatccaactcagaaagattgaa


Lb13-62p       tggcctgaaacttaattgcccgttcatttattattgtacgccacgtccgttcaattattt
Ls13-62p       ------------------------------------------------------------
Cp13-62p2      tgttattaaac---------gaatctattaggctttctatttaggctttttatctcttg
Cp13-62p1      tgttattaaat---------gaatc--------------tattaggctttttatctcttg


Lb13-62p       tcaaaatactat----------------tatttcaagaaataacctccctagacgtgtaa
Ls13-62p       ------------------------------------------------------tctct
Cp13-62p2      taaatttatttttattaatgaaaattagcgtttcgaaaaataacttcaaaata-tcgtct
Cp13-62p1      taaatctatttttattaatgaaaattagcgttttgaaaaataacttcaaaata-tcgtat
                                                                       *

Lb13-62p       tgtaataataacagtgtt-aatgtatctagacgaaacggtcgaaagagttcttaatga--
Ls13-62p       tctaaa-----atgaggttagactgagaatttacaaaagtatatagagttca--gttata
Cp13-62p2      ttttggccgatattatt-----tagagaaca----acaaaaaaaaagaggtcgtatgtatc
Cp13-62p1      ttttggcttagtattatttagagagaaacaa----aaangaaaaagaggtcgtatgtatc
               * *                              *      * **** **      *

Lb13-62p       --caaactccctcgtcataact--tggac---g--gacaatttaaattttaactcactca
Ls13-62p       gagcgaagattccatgtgtacccctctaataatataaggtcaaaactgtaacttcgcggg
Cp13-62p2      tagcaaggatctcgactaaaaa-cactcg---tcatcggtacgacgg--------ttcac
Cp13-62p1      taacaaggatctcgactaaaaa-cactcg---tcataggcacgacgg--------ttcac
                    *      *      *                       *

Lb13-62p       actgtcgtaatcccatctcgaattttcttccaacgccttacgaggccatgcgtatacgta
Ls13-62p       cctgtccaaatcccatctctgactttt---------cttctaacgctt------------
Cp13-62p2      tctgtcctaatcttatctctcgacatt-------tctagttaacgcattgaatatattta
Cp13-62p1      tctgtcctaatcttatctctcgacgtt-------tctagttaacgcactgaatatattta
                *****  ****  *****      *              * **

Lb13-62p       ttccaccacacaaggcaacttacttttgcaacaaaacacatcacctctaatcacatcctt
Ls13-62p       ----------------------cgaa----ctcaaacgta----atccatcgtttgactga
Cp13-62p2      atcg----acgcagtcaatgttatc----atcgcaatgc----atacatatgtcctccca
Cp13-62p1      atcg----acgcagtcaatgttgtc----atcacaatac----atacagatgtcctccca
                                          *  *          *        *

Lb13-62p       caaaaaataaaaaaaaaagaaacataatttactcgtcagccatccacctttcactacagc
Ls13-62p       caggaaaaggcaaccctcgtaattgcaaattgtctgagaatcaaccacctttcattacaaa
Cp13-62p2      actaatcacaacccttaaaagctattactcgtctgaaaataatccacttttcattacaac
```

8. Supplementary data



Cp13-62isoform 1: 4
Cp13-62isoform2: 13

Acknowledgements

I would like to express my deep gratitude to my advisor Professor Dorothea Bartels, for her professional and enthusiastic guidance and commitment, for her motivation and immense knowledge throughout this research work. The work on this thesis was supported by the DFG (Deutsche Forschungsgemeinschaft) project Smartwall and was performed in collaboration with Luxembourg Institute of Science and Technology (LIST).

Special thanks to Dr. Valentio Giarola for all the theoretical and technical support during my research project that helped me to widen my research from various perspectives.

I would like to thank my friend Dr. Ilona Juszczak-Debosz for helping me with my thesis corrections despite of her very busy schedule. I am also very thankful towards Prof. Dr. František Baluška for him agreeing to be my second supervisor.

Advice and technical help given by Sarah Jane Dulitz and Xun Liu regarding the yeast-two-hybrid assay was greatly appreciated. I would like to thank Tobias Dieckmann and Christiane Buchholz for their invaluable and time-consuming support.

I wish to thank my family for their support and encouragement throughout my study. I am very grateful to my husband Prashant for his continuous patience, support and love during the past years.

Finally, I would like to thank my friends and colleagues for the time we shared in our extracurricular activities. Thanks to Patricija, Pooja, Niklas, Srijana, Xiaomin, Xun, Pelei, Ahmad Ammar, Ahmad, Selva and Aziz for all the support and motivation.