Analysis of ABA and Drought Stress Mediated Gene Expression in the Desiccation Tolerant Resurrection Plant *Craterostigma plantagineum*

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Abbreviations

A_x	Absorbance at a given "x" wavelength
ABA	abscisic acid
Amp	ampicillin
bp	base pair
CaMV	Cauliflower Mosaic Virus
cDNA	complementary deoxyribonucleic acid
Ci	Curie
cpm	counts per minute
CTAB	cetyltrimethylamonium bromide
d	day
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	1,4-dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
x g	gravity constant (980 cm/s^2)
h	hour
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
kb	kilobase
mM	millimole
MOPS	3-(N-Morpholino)propane sulfonic acid
mRNA	messenger ribonucleic acid
ODx	optical density at a given "x" wavelength
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIPES	1,4-Piperazine-N,N'-bis[2-ethane sulfonic acid]
RNA	ribonucleic acid

rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit (enzymatic activity)
% (v/v)	percentage (volume/volume)
% (w/w)	percent (weight/weight)

I Introduction

Water deficit is a common environmental stress experienced by plants. It affects both normal development and growth and has a major effect on plant productivity. Mankind has overcome this limitation to crop yield by irrigation, if water is available, or by growing plants in areas with high rainfall. The growing scarcity and competition for water, however, is a major threat to future advances in agriculture (Barker, 2000).

The transient and moderate drought stress described in studies of crop species is the most common form of dehydration that plants are likely to encounter. Research has so far enabled a picture of the possible factors involved in drought tolerance to emerge and crop lines are available with different degrees of tolerance to water deficit. However, food production is likely to be adversely affected particularly in semi-arid regions where, despite the apparently wide range of potentially useful technologies, efforts to increase productivity and alleviate poverty have so far met with limited success. To get to know why plants survive in certain habitats or which traits enable plants to either survive or grow in water-limited environments are important topics of research in order to establish the basis for the development of suitable technologies, for managing natural plant communities in a sustainable way or for the improvement of crop management.

Under water-stress conditions plant cells lose water and decrease turgor pressure. The plant hormone abscisic acid (ABA) increases as a result of water stress and has important roles in plant stress tolerance. A number of genes that respond to water stress at the transcriptional level have been described. Some genes respond very rapidly, whereas others are induced slowly after ABA accumulation. The use of physiological, biochemical and molecular techniques have advanced our understanding of how plants respond to water deficit. However, there are many more questions that need to be addressed to achieve a clear view of plant adaptation to water deficit such as: how is the stress perceived and how does it lead to cellular whole-plant signal transduction pathways?, how is ABA recognized? and which water deficit induced genes are required for tolerance to water loss?. In the following text, an overview of the current approaches used to study water stress and achievements in the field are presented.

1.1 Approaches to study desiccation tolerance

The molecular basis of dehydration tolerance has been investigated using three main approaches in plants: (a) examining tolerant systems, such as seeds and resurrection plants; (b) analysing mutants from genetic model species; and (c) analysing the effects of stress on agriculturally relevant plants.

Physiological research in tolerant systems has concentrated on seeds and desiccation-tolerant species such as resurrection plants, mosses and ferns (Oliver, 1984). At the molecular level, plants respond to water deficit by the induction of specific genes (Bartels *et al*., 1990; Guerrero *et al.,* 1990). Some of these genes are also expressed during the normal embryogenesis program when seeds desiccate and embryos become dormant. Following this developmental stage, seeds become viable for long periods of time in a desiccated state. Both environmental and developmental processes have in common the involvement of ABA.

1.1.1 Resurrection plants: *Craterostigma plantagineum*

Resurrection plants are a group of desiccation tolerant plants that have adapted to environments where the rainfall is extremely sporadic. Desiccation-tolerant species can be found in most major classes of plants. Most of them belong to the lower taxae, such as algae, bryophytes, and lichens (Bewley and Krochko, 1982; Oliver, 1984). They also occur among angiosperms, but no desiccation-tolerant plants have been reported for the gymnosperms. These plants are capable to withstand severe drought stress, retaining less than 5% of their total water and then reviving completely, recommencing normal metabolism and growth within approximately 24 h of watering (Gaff, 1971; 1989). In the majority of higher plants only the mature seed and the pollen grain have this characteristic, and some of the drought-responsive genes isolated from the vegetative tissue of resurrection plants (Bartels and Nelson, 1994; O'Mahony and Oliver, 1999) are also believed to be associated with desiccation tolerance in embryos of many angiosperms. These characteristics make resurrection plants excellent model systems to study the mechanisms of dehydration tolerance.

The desiccation tolerant resurrection plant *C. plantagineum* (*Scrophulariaceae*) has been used as an experimental system in the study of molecular basis of desiccation tolerance (Bartels *et al*., 1990; Ingram and Bartels 1996). It was described that, in order to tolerate desiccation, the physiological stage of the plants is certainly important. For instance, it was observed that young *C. plantagineum* plants are desiccation tolerant, whereas senescent plants do not readily recover from a severe dehydration. The speed of water loss is also critical, plants would not recover if water loss

occurs too fast. This observation is important, as it suggests that this is an active process during which the plant is preparing the metabolism for the extreme stress by redirecting gene expression.

Another advantage of this model plant is that desiccation tolerance can be investigated in both whole plants and undifferentiated callus cultures: tolerant callus of *C. plantagineum* is obtained by pre-treatment with ABA. In callus tissue, and to a certain extent in whole *C. plantagineum* plants, the transition to the tolerant state is largely free of complications of development or other adjustments inherent in seeds or other plant systems.

Some desiccation-induced genes characterised from *C. plantagineum* share similarities to genes expressed in seeds of other species. This set of RNAs encodes the so-called late embryogenesis abundant (LEA) proteins (see section 1.2.1), regulated mainly at the transcriptional level. Several of the desiccation/ABA responsive transcripts in *C. plantagineum* have been cloned and characterised but very little is known about the molecular signals that lead to the activation of their expression (Ingram and Bartels, 1996; Phillips and Bartels, 2000).

1.2 Stress specific responses

Physiological drought also occurs during cold, heat and salt stress, when the main damage caused to the living cell is related to water deficit. Dehydration often leads to irreversible destructive events in proteins and cellular membranes (Crowe *et al.*, 1983, 1998). Evaporation and transpiration remove water (as vapor) from the soil, and this water loss concentrates solutes in the soil.

Crop plants are very sensitive to NaCl: the 0.15 M concentration found in animal fluids is toxic to many crops, such as fruit trees, cereals and horticultural plants. Only salt tolerant or halophytic plants can tolerate 0.5 M NaCl. The effect of salt on plant cells has two components: osmotic stress and ion toxicity. The osmotic component is not specific for NaCl and results from dehydration and loss of turgor*. Dissolved solutes in the rooting zone generate a negative osmotic potential that lowers the soil water potential. The general water balance of plants is thus affected because leaves need to develop a more negative water potential to maintain a "downhill" gradient of water potential between the soil and the leaves. This effect of dissolved solutes is similar to that of a

* Because plants have rigid walls, they can build up a large positive internal hydrostatic pressure, called turgor pressure (T). It can be obtained from the following relation: $T = \varphi_w + \pi$; where φ_w is the water potential and π the osmotic pressure of a solution (or total number of dissolved particles). Turgor is an essential factor for plant cell growth, which is based on cell wall loosening and a turgor-driven increase in volume (Ray, 1987).

soil water deficit. Thus, osmotic stress results from desiccation and therefore it is a common component of drought and salt stress (Serrano and Gaxiola, 1994).

Ion toxicity results from both the increase in concentration of normal intracellular ions (mostly K^+) during water loss and the uptake of Na^+ and Cl^-. The intracellular enzymatic systems of eukaryotes have evolved to function in a very narrow range of ionic conditions, mostly 0.1 to 0.2 M K^+ and <50 mM Na^+ and Cl^-. An excess of K^+ caused by cell shrinkage and the uptake of external NaCl results in toxicity to many intracellular enzymes (Serrano and Gaxiola, 1994). In addition, excess of Na^+ may disturb mineral nutrition by inhibiting the uptake of essential cations such as K^+ and Ca^{2+} (Greenaway and Munns, 1980). Plant cells exposed to salt undergo osmotic adjustment via a double mechanism, accumulation of NaCl in the vacuole and accumulation of organic solutes (proline, glycine betaines, polyols) at the cytoplasm in order to protect enzymes and membranes against damage from high salt concentrations (McCue and Hanson, 1990; Nuccio *et al.*, 1999).

Recently, salt specific responses were identified. A mutation in the *SOS3* (salt overly sensitive 3) gene renders *A. thaliana* plants hypersensitive to Na^+-induced growth inhibition. *SOS3* encodes an EF hand calcium-binding protein that shares significant sequence similarities with an animal neuronal calcium sensors (NCS) and the yeast regulatory subunit (CnB) of the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (Liu and Zhu, 1998). In yeast, calcineurin is a central component in the signalling pathway that regulates Na^+ and K^+ homeostasis (Nakamura *et al.,* 1993; Mendoza *et al*, 1994). Salt stress, like drought, elicits a rapid rise in the cytosolic Ca^{2+} concentration (Knight *et al*, 1997). This rise in Ca^{2+} presumably initiates a signalling cascade, resulting in plant adaptive responses. The sequence similarity between SOS3, CnB and NCS suggests that SOS3 responds to the Ca^{2+} signal by activating a protein phosphatase or inhibiting a protein kinase (or by doing both) that then regulates K^+ and Na^+ transport systems. Although there do not appear to be conspicuous differences between the cytosolic Ca^{2+} signals elicited by drought and salinity (Knight *et al.*, 1997), subtle differences in their kinetics and subcellular spatial arrangement could result in drought- or salinity-specific responses. These results suggest that intracellular calcium signalling through a calcineurin-like pathway mediates the beneficial effect of calcium on plant salt tolerance. Moreover, the specific role of SOS3 in the tolerance of the ionic but not the osmotic component of salt stress (Liu and Zhu, 1997) strongly supports the existence of ionic stress-specific calcium signalling.

A major transcription system that controls abscisic-acid-independent gene specific expression in response to dehydration and low temperature has been identified. The system includes the DRE/CRT (dehydration-responsive element/C-repeat) *cis*-acting element and its DNA-binding protein, DREB/CBF (DRE-binding protein/C-repeat binding factor), which has an AP2/EREBP domain (Stockinger *et al.,* 1997; Liu *et al.,* 1998). DREB/CBF contains two subclasses, DREB1/CBF and

DREB2, which are induced by cold and dehydration, respectively, and control the expression of various genes involved in stress tolerance. Recent studies are providing evidence of differences between dehydration-signalling and cold-stress-signalling cascades, and of cross-talk between them (Shinozaki and Yamaguchi-Shinozaki, 2000).

1.2.1 Late Embryogenesis Abundant proteins (LEA)

The main achievement of molecular studies of desiccation in seeds has been the identification and characterisation of late embriogenesis abundant proteins. LEA-protein mRNAs were first described from research into genes abundantly expressed during the final stage of desiccation during seed development (Dure, 1989). LEA transcripts first appear at the onset of desiccation, dominate the RNA population in dehydrated tissues, and gradually declive several hours after the embryos begin to imbibe water (Skriver and Mundy, 1990). cDNAs encoding LEA proteins are consistently represented in differential screens for transcripts with increased levels during drought. Circumstantial evidence for their involvement in dehydration tolerance is strong. The genes are similar to many of those expressed in vegetative tissues of drought-stressed plants, and desiccation treatments can often induce precocious expression in seeds. ABA can also induce the *LEA* genes in seeds and vegetative tissues (Mundy and Chua, 1988; Bartels *et al.,* 1990; Rock and Quatrano, 1995).

LEA proteins appear to be located in many cell types and at variable concentrations. Within the cell they appear to be predominantly, but not exclusively, cytosolic. The concentrations in the cell are characteristically very high. A general structural feature of the LEA proteins is their biased amino acid composition, which results in highly hydrophilic polypeptides with just a few residues providing 20-30% of their total complement. For example, D19 protein from cotton contains 13% glycine and 11% glutamic acid. Most LEA proteins lack cysteine and tryptophan residues (Baker *et al.,* 1988).

It has been hypothesised that LEA proteins confer a structural role in cellular protection to desiccation damage by either stabilising cytoplasmic structures, binding water to maintain hydration at specific sites, or by acting as an ion trap (Dure *et al*, 1993a,b). However, direct biochemical proof evidence that LEA proteins protect specific cellular structures or ameliorate the effects of drought stress has not been reported yet. Their high concentrations in the cell and biased amino acid compositions suggest that they do not function as enzymes (Baker *et al.,* 1988). The randomly coiled moieties of some LEA proteins are consistent with a role in binding water. Total desiccation is

probably lethal, and therefore such proteins could help maintain the minimum cellular water requirement. A major problem under severe dehydration is that the loss of water leads to crystallisation of cellular components, which in consequence damages cellular structures. This may be counteracted by LEA proteins, and some of the LEA proteins could essentially be considered compatible solutes, which supports the likely role of sugars in maintaining the structure of the cytoplasm in the absence of water.

Classification of LEA proteins originated from a dot matrix analysis with proteins from cotton. A group was assigned on the basis of one cotton LEA protein showing regions of significant homology with at least one protein from another species (Dure, 1993a,b). The cotton proteins used for this classification were LEA D19 (Group 1), predicted to have enhanced water-binding capacity. LEA D11 (Group 2, also termed dehydrins) (Close *et al.,* 1989), which possible functions as chaperon, preserving protein structures. LEA D7 (Group3) is predicted to play a role in the sequestration of ions that are concentrated during cellular dehydration (Dure, 1993a,b). And LEA D29 (Group 5) is also predicted to sequester ions during water loss. The cotton proteins LEA D113 and LEA D95 define two additional classes. Two of these classes of protein have been shown to have a functional role in stress tolerance: HVA1, a group 3 LEA protein from barley; and LE25, a group 4 LEA protein from tomato. Overexpression of *HVA1* improves drought and salinity resistance in transgenic rice plants (Xu *et al.,* 1996). The LE25 protein was expressed in yeast and found to confer improved resistance to high salinity and freezing (Imai *et al.,* 1996). Although these experiments show that the organism in which the protein is overexpressed can better withstand the stress, few additional clues to the mechanism of protein function are provided.

1.3 Role of ABA

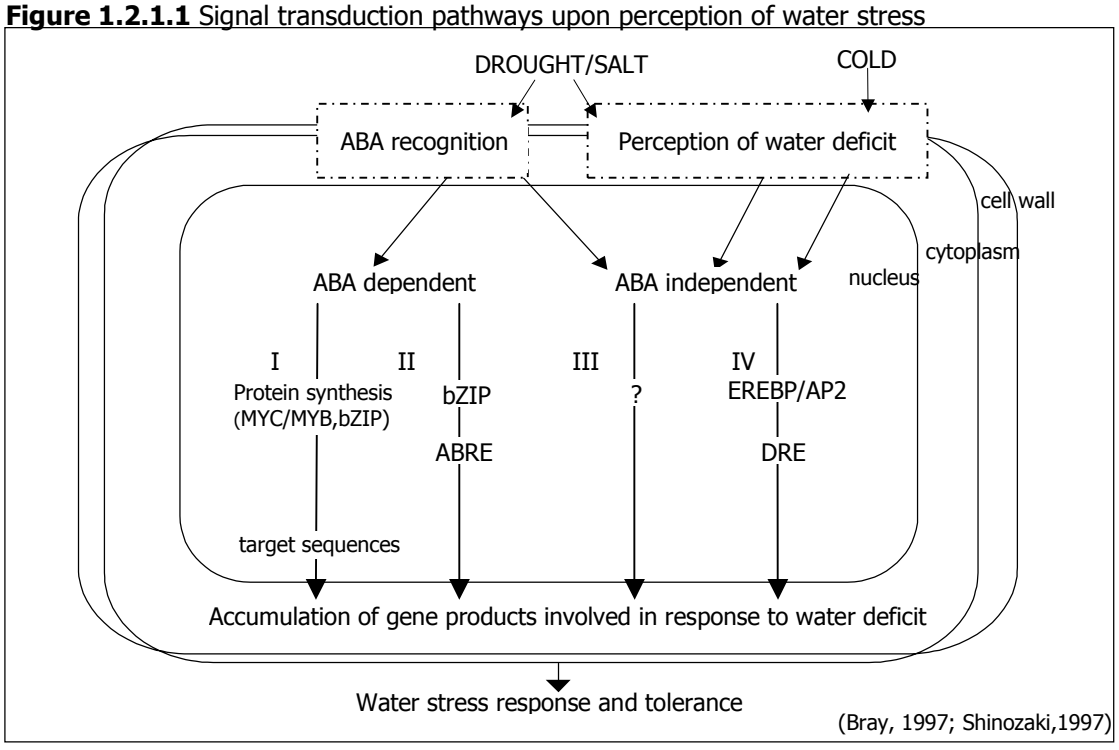
One major signal operating during drought stress is ABA. ABA is a sesquiterpene derived from mevalonate and strong evidence indicates that stress-induced ABA is derived from a C-40 precursor (Zeevart and Creelman, 1988). This plant hormone is present in all higher plants. It was originally described as a dormancy-inducing and abscission-accelerating substance. Further studies demonstrated that it is implicated in the control of several essential physiological processes (seed and bud dormancy) (Creelman, 1989) and plant responses to environmental stress (stomatal closure, growth inhibition) (Zeevaart and Creelman, 1988). Although many plant responses are mediated by phytohormones and these responses may be due to either a normal plant developmental program or stress conditions.

When plants wilt, ABA levels typically rise as a result of an increase in the rate of synthesis. It was shown that ABA concentration does not change in response to wilting in the presence of inhibitors of transcription and translation (Quarrie and Lister, 1984). These results suggest that the ABA system is regulated at the level of gene expression.

Although insights into the mechanisms for the recognition of ABA in the cell have recently being described (see section 1.3.3), the location, number and type of receptors for ABA are not known. However, there is evidence that ABA can be recognised both inside and outside the cell, these, and the response of gene expression to structural analogues of ABA (Davies and Jones, 1992), may indicate that there are multiple receptors for ABA.

Most of the genes that respond to drought, salt, and cold, stress are also induced by exogenous application of ABA, although other genes that are induced by water stress are not responsive to exogenous ABA treatment. These findings suggest the existence of both ABA-independent and ABA-dependent signal transduction cascades between the initial signal of drought or cold stress and the expression of specific genes. A simplified view of water deficit stress response in plants and the current knowledge on the signalling network is summarised in figure 1.2.1.1 (Bray 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). After the signal is perceived, by unkown mechanisms represented as dashed lines in figure 1.2.1, two types of responses are initiated, ABA dependent or ABA independent. In the scheme, ABA-dependent pathway I requires protein synthesis to activate transcription factors MYC/MYB (Abe *et al.,* 1997) and/or bZIP, which bind to DNA regions other than ABREs (ABA-responsive elements).

Figure 1.2.1.1 Signal transduction pathways upon perception of water stress



ABA-dependent pathway II activates bZIP (Hollung *et al.,* 1997; Nakawaga *et al.,* 1996), a transcription factor that turns on gene expression through binding to ABREs. ABA-independent pathway IV induces gene expression through activation of DREBP/CBF1 (see section 1.2), which binds to the DRE motif and leads to induction of cold- and drought- induced genes. ABA independent pathway III is not yet well understood (Shinozaki and Yamaguchi-Shinozaki, 1997) and includes several drought-inducible genes that do not respond to either cold or ABA treatment.

1.3.1 ABA related mutants

Progress in understanding the role of ABA in dehydration tolerance has been achieved by characterising mutants. For example, lines that are less sensitive to ABA than the wild type have been found in maize (*vp*) *a*nd in *A. thaliana* (*aba*). Embryos of the biosynthetic *vp* mutants are viviparous (Robertson, 1955; McCarty, 1991, 1995) and seeds of the ABA-deficient *aba* mutants from *A. thaliana* (Koorneef *et al.,* 1982) and *N. plumbaginifolia* (Marin *et al.,* 1996) fail to become dormant.

Recently, the wild type allele of the *aba* tomato mutant notabilis (LeNCED1, 9-cis-epoxycarotenoid dioxygenase) was ectopically expressed and progeny of one transformant had significantly higher bulk leaf ABA content compared to the wild type (Thompson *et al.,* 2000). The increased seed dormancy was reversed by addition of the carotenoid biosynthesis inhibitor norflurazon. These data provided strong evidence that NCED is a key regulatory enzyme in ABA biosynthesis in leaves, and demonstrate for the first time that plant ABA content can be increased through manipulating NCED (Thompson *et al*., 2000).

Unlike biosynthetic mutants, mutants that are altered in their responsiveness to ABA do not have reduced endogenous ABA content, and their phenotypes cannot be reversed to wild type by exogenous supply of ABA. Multiple mutations that either increase or decrease ABA sensitivity have been characterised in *A. thaliana*. Mutations in the enhanced response to ABA (*ERA1*) locus confer to germinating seeds a hypersensitivity to applied ABA (Cutler *et al.,* 1996). Moreover, the five distinct ABA-insensitive (ABI) loci (*ABI1-5*) were identified by selecting for seeds capable of gemination in the presence of inhibitory ABA concentrations (Koorneef *et al.,* 1989; Finkelstein, 1994).

The detailed genetic information available for *A. thaliana* facilitated the isolation of this mutants. The *ERA1* corresponding protein encodes for the β-subunit of a protein farnesyl transferase that is proposed to catalyse the modification of a receptor or component of the signal transduction pathway for membrane localisation. These signalling proteins work by attachment of a hydrophobic

farnesyl group to C-terminal target sequences. ERA1 is a negative regulator of ABA signal transduction in seeds (Cutler *et al.*, 1996) and guard cells (Pei *et al.*, 1998), which suggests farnesylation-dependent membrane targeting of soluble, negative regulators of ABA signalling.

The *ABI1* and *ABI3* genes were isolated by positional cloning (Giraudat *et al.*, 1992; Leung *et al.,* 1994; Meyer *et al.*, 1994). *ABI3*, and its maize ortholog *VP1,* are specifically expressed in seeds and encode a transcription factor able to activate *LEA* genes (McCarty *et al.,* 1991, 1995; Parcy *et al*, 1994). Genetic and molecular studies have shown that the ABI3 protein plays a prominent role in the control of seed maturation.

ABI1 and *ABA2* genes encode homologous protein phosphatases (Leung *et al,* 1994, 1997; Meyer *et al.,* 1994). *In vitro* assays and functional complementation studies in yeast confirmed that the ABI2 protein is an active protein phosphatase 2C (PP2C)(Leung *et al.,* 1997). *In vitro* enzymatic assays indicated that a loss of ABI1 PP2C activity leads to an enhanced responsiveness to ABA. Thus, the wild-type ABI1 and ABI2 phosphatases are negative regulators of ABA responses (Leung, 1998; Gosti *et al.,* 1999). A number of differences between the two mutants in adaptive responses to stress have been reported, thus, the homologues ABI1 and ABI2 phosphatases appear to assume partially redundant functions in ABA signalling, which may provide a mechanism to maintain informational homeostasis.

The *ABI4* and *ABI5* genes were isolated by positional cloning (Finkelstein *et al.,* 1998; Finkelstein and Lynch, 2000). The *ABI4* protein product showed homology to a plant-specific family of transcriptional regulators characterised by a conserved DNA binding domain, the AP2/EREBP domain. Expression analyses showed that despite the seed-specific nature of the mutant phenotype, *ABI4* expression is not seed specific. *ABI5* gene encodes for a member of a basic leucine zipper (bZIP) class of transcriptional regulator (Finkelstein and Lynch, 2000). Expression analysis showed that, like *ABI4*, *ABI5* was expressed in vegetative as well as seed tissues, although at much lower levels, and it regulates a subset of *LEA* genes during both developmental stages. In addition, *ABI5* expression appears to be regulated by ABA, by most of the other known *ABI* genes, and possibly by itself (Finkelstein and Lynch, 2000).

1.3.2 ABA signalling through activation tagging

Activation tagging allowed the identification of intermediates in the ABA signal transduction pathway leading to desiccation tolerance in *C. plantagineum* (Furini *et al., 1997*). This direct way to induce dominant mutations, called activation tagging, was developed by Walden and colleagues

(Hayashi *et al.,* 1992), who constructed a T-DNA vector with four copies of an enhancer element from the constitutively active promoter of the cauliflower mosaic virus (CaMV) 35S gene (Odell *et al,* 1985). With this approach, transcriptional enhancers from the cauliflower mosaic virus (CaMV) 35S promoter are randomly inserted in the genome with transferred DNA (T-DNA) of the soil bacteria *Agrobacterium tumefaciens*. These enhancers can cause transcriptional activation of nearby genes, causing dominant mutations which can be selected in the primary generation of transformants.

Based on a similar approach the *C. plantagineum* desiccation tolerant-1 gene, *CDT-1*, was isolated. It encodes a signalling molecule in the ABA transduction pathway (Furini *et al.,* 1997). Constitutive expression of *CDT-1* leads to desiccation tolerance in the absence of ABA and to the constitutive expression of characteristic transcripts. The ABA content in wild-type and transformed callus indicated that the integrated T-DNA did not affect ABA accumulation. Thus, the gene tagged by T-DNA integration must act downstream of the initial ABA signal.

CDT-1 represents a novel gene with unusual features in its primary sequence. Its mRNA does not possess a long ORF starting with an AUG and no protein product was detected by *in vitro* translation. However, it was proposed that the *CDT-1* mRNA could be translated into a short peptide of 22 amino acids. This regulatory gene resembles in several features SINE retrotransposons and it was anticipated that retroposition of *CDT-1* may ultimately reveal a mechanism by which acquisition of desiccation tolerance has been acquired progressively during evolution (Furini *et al.,* 1997).

Activation tagging is an emerging technology in plant functional genomics (Weigel *et al.,* 2000). Recently, this approach had permitted the identification of several new genes. One example is the *ORCA3* (Octadecanoid-derivative responsive *Catharanthus* AP2-domain protein), from *Catharanthus roseus*, which is involved in the jasmonate response pathway (van der Fits and Memelink, 2000). The *ORCA3* gene codes for a jasmonate-responsive AP2/EREBP-domain transcription factor and its overexpression resulted in enhanced expression of several metabolite biosynthetic genes. Regulation of metabolite biosynthetic genes by jasmonate-responsive AP2-domain transcription factors may link plant stress responses to changes in metabolism.

1.3.3 ABA Signal Transduction

ABA triggers closing of stomata* thus reducing water loss. Guard cell signalling integrates water status, hormonal stimuli, light, CO_2 levels and other environmental conditions to regulate

*The stomatal pore closing/aperture is defined by the turgor of the two surrounding guard cells. Guard cells are located at the epidermis of plant leaves, and in pairs surround stomatal pores.

stomatal apertures. Guard cells are a well-developed model system for understanding how components interact within a signalling network in a single cell. They are well suited for dissecting the functions of genes and proteins in signalling cascades. Consequently, powerful techniques have been developed for guard cell signalling studies, enabling combined molecular genetic, cell biological, biophysical and genomic analyses (Schroeder *et al.,* 2001).

Guard cell volume is controlled osmotically mainly by large influxes (stomatal opening) or effluxes (stomatal closure) of K^+ and anions. A variety of single-cell techniques have established that ABA causes rapid (within minutes) alterations in the activity of K^+ and anionic channels in the plasma membrane of guard cells via pH- and Ca^{2+} -sensitive signalling cascades (Blatt and Thiel, 1993; Ward *et al*., 1995). In wild-type *A. thaliana* guard cells, ABA, oxidative stress, cold, and external calcium elicited cytosolic calcium ($[Ca^{2+}]_{cyt}$) oscillations of differing amplitudes and frequencies induce stomatal closure. In guard cells of the vacuolar ATPase (V-ATPase) mutant *det3*, external calcium and oxidative stress elicits prolonged calcium increases, which do not oscillate abolishing stomatal closure. Conversely, cold and ABA elicits calcium oscillations in *det3*, and stomatal closure occurs normally (Allen *et al*., 2000). Moreover, in *det3* guard cells, experimentally imposing external calcium-induced oscillations rescues stomatal closure (White, 2000).

Another factor influencing ABA stomatal closing is the action of reactive oxygen species (ROS) activated Ca^{2+} channels. The activation of this mechanism mediates both influx of Ca^{2+} in protoplasts and increases in $[Ca^{2+}]_{cyt}$ in intact guard cells (Pei *et al.,* 2000). ABA induces the production of H_2O_2 in guard cells. If H_2O_2 production is blocked, ABA-induced closure of stomata is inhibited. Activation of Ca^{2+} channels by H_2O_2 and ABA- and H_2O_2-induced stomatal closing are disrupted in the recessive ABA-insensitive mutant *gca2* (Pei *et al.,* 2000).

Impairment of ABA-induced $[Ca^{2+}]_{cyt}$ increases in the ABA-insensitive *Arabidopsis* mutants *abi1-1* and *abi2-1* provides genetic evidence for the importance of $[Ca^{2+}]_{cyt}$ elevations in ABA signalling (Allen *et al.,* 1999). Moreover, phosphorylation events are central mediators of ABA signalling in guard cells (Armstrong *et al*., 1995; Li *et al.,* 2000). Some related protein kinase (Li *et al.,* 2000) and phosphatase genes (Leung and Giraudat, 1998) have been cloned. Both positively and negatively regulating protein kinases, and positively and negatively regulating PP2C have been proposed to function in ABA signalling (see figure 1.3.3.1) (Allen *et al*., 2000; Armstrong *et al.,* 1995; Leung and Giraudat, 1998; Pei *et al.,* 1997). ABA signalling can be transduced by several types of positively regulating protein kinases (Li *et al.,* 2000; Sheen, 1996). For example, biochemical analyses have led to the hypothesis that Ca^{2+} dependent protein kinases (CDPKs) may function upstream of ABA-activated Ca^{2+} independent kinases in maize protoplasts and *Vicia* guard cells (Mori and Muto, 1997; Sheen, 1996).

ABA stimulates production of the second messengers such as inositol-3-phosphate ($InsP_3$) and phospholipase D (PLD) (Jacob *et al.,* 1999). The level of phosphatidic acid (PtdOH) generated by PLD, transiently increases after ABA treatment of aleurone cells and guard cells (Jacob *et al.,* 1999). This promotes partial stomatal closure, inhibits stomatal opening and partially inactivates K^+_{in} channel currents. PtdOH does not elicit a $[Ca^{2+}]_{cyt}$ increase in guard cells (Jacob *et al.,* 1999) and PLD is therefore likely to act downstream of, or parallel to, ABA-induced $[Ca^{2+}]_{cyt}$ elevations.

A simplified model of proposed functions of positive and negative regulators mediating guard cell ABA signal transduction is presented in the figure 1.3.3.1, based on the model reported by Schroeder (2001). The sequence of events among regulators remains largely unknown and will require in-depth analyses.

Figure 1.3.3.1 ABA signalling in the guard cell

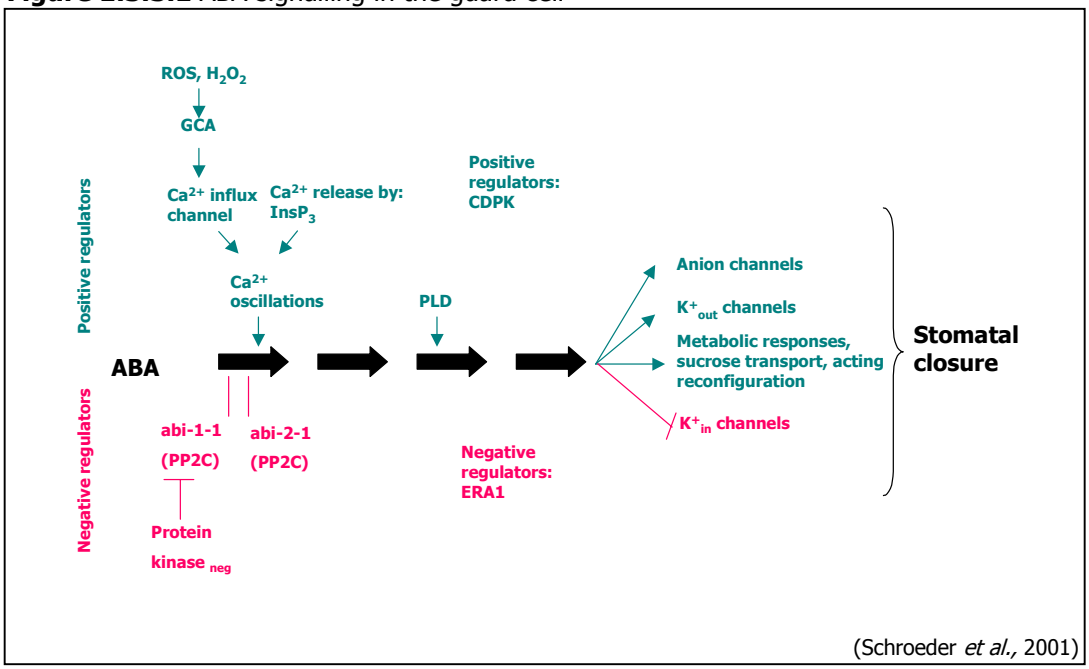


Figure 1.3.3.1 Positive ABA-activated regulators (top, in green) and negative ABA-signalling regulators (bottom, in red) in guard cells are shown. Some parallel signalling branches are excluded for simplicity.

1.4 Aims and objectives

Aims

Understand the molecular basis of desiccation tolerance using the resurrection plant *Craterostigma plantagineum* as a model system.

1a- To determine which genes/gene profiles lead to desiccation tolerance.

1b- To identify intermediates in the ABA signalling cascade that are essential for desiccation tolerance.

Objectives

Both objectives take advantage of the observation that *C. plantagineum*:
i) is salt sensitive and ii) requires ABA to survive desiccation at the callus level.

1a- Establish the molecular profiling technique to determine the transcript accumulation patterns that occur in response to dehydration in the desiccation tolerant plant *C. plantagineum* as compared to sodium chloride treatment. In doing so, those genes/gene profiles that are part of the desiccation tolerance programme, but not a general stress response, will be identified.

1b- Create and characterise dominant mutants that are able to survive desiccation without an ABA pre-treatment, thus identifying molecules involved in the ABA signalling network.

II Materials and methods

2.1 Materials

2.1.1 Buffers and solutions

Antibiotics

name	**stock**	**storage**	**final concentration**	**organism**
Ampicilin	water	-20°C	100 μg/mL	*E. coli*
Carbenicilin	water	-20°C	100 μg/mL	*E. coli/A.tumefaciens*
Gentamycin	water	-20°C	25 μg/mL	*A. tumefaciens*
Hygromycin	phosphate buffer saline	4°C	15 μg/mL	*C. plantagineum*
Kanamycin	water	-20°C	25 μg/mL	*E. coli/A. tumefaciens*
Rifampicin	methanol	fresh	100 μg/mL	*A. tumefaciens*
Tetracycline	ethanol	-20°C	100 μg/mL	*E. coli*

DNA loading buffer (10 X) 30% (v/v) glycerol, 1 X TAE, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene-glycol. Stored at 4°C.

Ethidium bromide stock 5 mg/mL ethidium bromide in sterile deionized water. Stored at 4°C.

Extraction buffer: plant DNA and RNA

nucleic acid	**composition**
DNA	100 mM Tris-HCl pH 9.5, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PEG 6000.
RNA (poly A^+)	Buffer 1: 0.1 M NaCl, 0.05 M Tris-HCl pH 9, 0.01 M EDTA, 2% SDS, pH 9. Buffer 2: 0.01 M Tris-HCl pH 7.5, 0.4 M NaCl, 0.2% SDS. Buffer 3: 0.01 M Tris-HCl pH 7.5, 0.1 M NaCl. Buffer 4: 0.01 M Tris-HCl pH 7.5.

Extraction buffer: plasmid DNA

organism	composition
E. coli	200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA pH 8, 0.5% SDS, 0.25 M NaOH.
A. tumefaciens	Buffer 1: 4 mg/mL lysozyme, 50 mM glucose, 10 mM EDTA pH 8, 25 mM Tris-Cl pH 8. Buffer 2: 1% SDS, 0.2 N NaOH. Freshly prepared.

Hybridisation buffer

nucleic acid	composition	conditions
cDNA (arrays)	0.5 M Na-Phosphate pH 7.2, 7% SDS, 1 mM EDTA pH 8, 100 μg/mL salmon sperm DNA.	65°C
DNA	0.6 M NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA pH 8.5, 1% SDS, 10 X Denhardt's, 10 μg/mL salmon sperm DNA.	65°C
Oligonucleotide.	6 X SSC, 1 X Denhardt's, 0.1% SDS, 0.05% Na-pyrophosphate, 10 μg/mL salmon sperm DNA	
RNA	5 X SSC, 0.1 M PIPES, 1 X Denhardt's, 0.1% SDS, 50% deionized formamide, 10 μg/mL salmon sperm DNA.	42°C

IPTG stock — 40 mg of isopropyl-β-D-thiogalacto-pyranoside in 1 mL of deionized water (141 μM). Filter sterilised then stored at –20°C. 1 mL in 1 L of media.

MOPS 5x — 0.2 M MOPS, 50 mM NaOAc, 50 mM EDTA. pH 7.5 with NaOH. Autoclaved.

OLB labelling buffer — 100 μL solution **A**: 1mL (1.25 M Tris-HCl pH 8, 0.125 M $MgCl_2$), 5 μL of 20 mM [dATP, dGTP and dTTP], 18 mL 2β-mercarptoethanol, 250 μL solution **B:** 2 M HEPES, pH 6 with NaOH. 150 μL solution **C:** Hexadeoxyribonucleotides resuspendend in 3 mM Tris-HCl, 0.2 mM EDTA pH 7. The mix was stored at –20°C.

Proteinase K stock — 0.2 mg in 1 mL water, stored at –20°C.

RNase A	10 mg/mL RNase A in 5 mM Tris-HCl pH 8. Boiled 10 min to remove DNase activity.
RNA loading buffer (10 X)	1 X MOPS, 1.75% formaldehyde, 0.5% deionized formamide, 0.4% (w/v) bromophenol blue. Made up in DEPC treated water. Stored at –20°C.
Stripping buffer (cDNA arrays)	5 mM Na-phosphate pH 7.2, 0.1% SDS.
SSC buffer 20 X	3 M NaCl, 300 mM sodium citrate.
TAE buffer	400 mM Tris-HCl, 200 mM NaOAc, 18 mM EDTA pH 7.8 with glacial acetic acid.
X-Gal stock	40 mg 5-bromo-4chloro-3-indolyl-β-D-galactoside (X-Gal) in 1 mL of dimethyl formamide (100 μM). Add 1 mL in 1 L of media.

2.1.2 Bacteria growth media

Luria broth (LB)	1% tryptone, 0.5% yeast extract, 1% NaCl pH 7.5. 15 g/L bactoagar were added for solid medium and autoclaved.
NZY	5 g bacto yeast extract, 5 g NaCl, 2 g $MgSO_4.7H_2O$, 10 g caseamino acids, pH 7.5. 15 g/L bactoagar were added for solid medium and autoclaved.
Top Agar	NZY, 10 mM $MgSO_4$ and 0.7% agarose.
YEB	0.5% (w/v) beef extract, 0.5% (w/v) caseamino acids, 0.5% (w/v) sucrose, 0.1% (w/v) yeast extract, pH 7.0. 15 g/L bactoagar were added for solid medium and autoclaved. 14 mM $MgCl_2$ added after autoclave.

2.1.3 Plant growth media

Hormone solutions

name	final concentration (M)	stock[1]
ABA (abscisic acid)	1 E-4	ethanol
BAP (6,benzylamino purine)	2.2 E-6	water
IAA (3,indoleacetic acid)	1.14 E-5	water
2,4-D (2,4-diclorophenoxiacetic acid)	2.2 E-6	50% ethanol
Kinetin	1 E-6	solved in NaOH,
NAA (naphtaleneacetic acid)	4.5 E-7	water
9-iP [6(γ,γ–dimethylallylaminopurine riboside)	6 E-6	solved in 0.5 N HCl, final pH 5.8 with 1 M KOH.

MS medium — 4.6 g Murashige and Skoog (MS) micro and macro elements (Duchefa), 2% sucrose, 250 mg/L antioxidant mix (see MSAR stock solutions), 3.8 g Gelrite (Roth), pH 5.8 with KOH 1 M in 1 L, then autoclaved. When the solution had reached 50°C, 2 mL of vitamin[2] solution were added.

MSAR medium — as a replacement of regular MS medium. 50 mL macroelements, 1 mL microelements, 6.8 mL Fe-EDTA, 5.8 mL $CaCl_2$, 2 mL KI and 3% sucrose. For solid medium 3.8 g Gelrite were added, pH 5.8 with KOH in 1 L water, then autoclaved. When the solution had reached 50°C 2 mL of vitamin solution were added.

MSAR solutions

solution	components for 1L	storage
Macro Elements	20 g NH_4NO_3; 40 g KNO_3; 7.4 g $MgSO_4 \cdot 7H_2O$; 3.4 g KH_2PO_4; 2 g $Ca(H_2PO_4)\,2\,H_2O$.	-20°C
Micro Elements	6.2 g H_3BO_4; 16.9 g $MnSO_4 \cdot H_2O$; 8.6 g $MnSO_4 \cdot 7H_2O$; 0.25 g $Na_2MoO_4 \cdot 2H_2O$; 0.025g $CuSO_4 \cdot 5H_2O$; 0.025 g $CoCl_2 \cdot 6H_2O$.	4°C.
Fe-EDTA	Solve separately 5.56 g $FeSO_4 \cdot 7H_2O$ (apply heat) and 7.46 g $Na_2EDTA \cdot 2H_2O$; mix the two solutions.	4°C

[1], [2] - All hormone and vitamin solutions were filter sterilised.

solution	components for 1L	storage
$CaCl_2$	7.5 g $CaCl_2 \cdot 2H_2O$	4°C
KI	375 mg KI.	4°C
Vitamins	5g myo-inositol (solved with 1 M NaOH); 250 mg thiamine HCl; 0.5 g nicotinic acid; 0.5 g pyridoxine HCl.	4°C
Antioxidant Mix	250 mg of: 150 mg ascorbic acid and 100 mg citric acid.	4°C

Plant transformation solutions

media[3]	replacement	hormones	use
MSAR1	MSAR	IAA 2 mg/L 2,4-D 0.5 mg/L kinetin 0.2 mg/L 9-iP 0.2 mg/L	plant transformation, callus initiation and proliferation.
MS	MSAR1 or MS	the above	callus maintenance
MSAR1a	MSAR or MS	0.5 mg/L BAP, 0.1 mg/L NAA	shoot induction
MSAR	MSAR or MS	none	plant formation

2.1.4 Chemicals

Laboratory reagents were obtained from Boehringer Manheim, Merck GmbH and Sigma-Aldrich unless otherwise stated. All were reagent grade, if not otherwise stated. Filter paper was obtained from Whatman, Hybond N-membranes and radioisotopes were from Amersham Buchler. XR films were from Eastman Kodak Co. Tissue culture chemicals were from DUCHEFA, Merck, and Boehringer. Plant solidifying agents, Gelrite, were purchased from Carl ROTH GmbH Co.

[3] Hygromycin was kept constant at 15 μg/mL during the plant transformation procedure

2.1.5 Oligonucleotides

Oligonucleotides were purchased from Life Technologies, Gibco BRL or MWG Biotech. Resuspended in sterile water. F, forward; R, reverse.

1. *Amp* AMP F 5'd[AACACTGCGGCCAACTTACTTCTGACA]3'
2. *Amp* AMP R 5'd[GGTCCTCCGATCGTTGTCAGAAGTAAGTT]3'
3. *Hn* HPT F 5'd[GGGTAAATAGCTGCGCCGATGGTT]3'
4. Universal F 5'd[GTAAAACGACGGCCAGTGA]3'
5. Reverse R 5'd[GGAAACAGCTATGACCATG]3'
6. *GUST7* gpaf F 5'd[TAATACGACTCACTATAGGGAGGTGGACGATATCACC]3'
7. *GUSpolyA* gpar R 5'd[TTTTTTTTTTTTCGAAGCGGGTAGATATCACACTC]3'
8. iRBTA1 5'd[GAGAGGACCTCGAGCTGCAGAATTAC]3'
9. iRBRN1 5'd[CCATGTTGGGGATCTAGATGATCCG]3'
10. ECOHyg1 5'd[TCCTGCGGGTAAATAGCTGCGCCGATGG]3'
11. iRBTA2 5'd[TTACAAGGATCCGAAACTATCAGTG]3'
12. iRBRN2 5'd[TCTAGATGATCCGAAACTATCAGTG]3'
13. ECOHyg2 5'd[CGTTATGTTTATCGGCACTTTGCATCGG]3'
14. adaptorLS 5'd[GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT]3'
15. adaptorSS PO_4 -5'd[ACCAGCCC]-H_2N3'
16. adaptorP1 5'd[GTAATACGACTCACTATAGGGC]3'
17. adaptorP2 5'd[ACTATAGGGCACGCGTGGT]3'
18. lambdaH F 5'd[GCCCGGAAGTGCCGGCAC]3'
19. lambdaHADF F 5'd[GGTAGACGCAACCAC]3'
20. lambdaHADR R 5'd[CACGGCCGTGAAGGCCCG]3'
21. T7 5'd[GTAATACGACTCACTATAGGGC]3'
22. SP6 5'd[ATTTAGGTGACACTATAG]- 3'

2.1.6 Enzymes

Enzymes were purchased mainly from Boehringer Mannheim and Pharmacia with the 10 X buffer supplied. *Taq* polymerase was obtained from Gibco BRL. *Taq* polymerase for long range inverse PCR was purchased from Takara Shuzo (Takara LA PCR kit ver.2.1).

2.1.7 DNA molecular weight standards

1 kb ladder was purchased from Gibco BRL. 1 Kb ladder consists of DNA fragments of the following sizes: 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2, 1.6, 1, 0.5, 0.4, 0.3, 0.2, 0.15 and 0.075 kb.

2.1.8 Bacterial strains

organism	**host strain**	**genotype**	**reference**
E. coli	DH5-α	supE44, ΔlacU169 (ø80lacZ, ΔM15), hsdr17, recA1, endA1, gyrA96, thi-1, relA1.	Hanahan, 1983
	DH10-B	F⁻*mcr*A δ(*mrr- hsd*RMS *mcr*BC) ø80d*lacZ*, ΔM15 Δ*lacX*74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara,leu*)7697, *gal*U, *gal*K, λ⁻*rps*L, *nup*G	Calvin and Hanawalt, 1988
	XL-1 blue	supE44, hsdr17, recA1, endA1, gyrA96, thi-1, relA1, lac, F′[proAB$^+$, lac^{q}ZΔM15, Tn10(tet^r)].	Bullock *et al.*, 1987
A. tumefaciens	GV3101 (pMP90RK)	Gm^R, Rif^R, Km^R	Koncz and Schell, 1986

2.1.9 Cloning vectors

Bacteria

vector	**reference**
pBluescript II KS (+/-) (Stratagene Cloning System	Alting-Mees *et al.*, 1992
pGEM-T Easy (Promega)	Mezei *et al.*, 1994
PCR-Script SK(+) (Stratagene Cloning kit)	Simcox *et al.*, 1991
pUC19	Yanish-Perron *et al.,* 1985

Plant cloning vectors (PCVs)

Vector	Size (kb)	Resistance markers	References
pPCVTAc1	10	Amp^R, Cb^R, Hn^R	Koncz *et al.*, 1994
pPCVRN4	9	Amp^R, Cb^R, Hn^R	Koncz *et al.*, 1994

Vectors pPCVRN4 and pPCVTAc1 were provided by Czaba Koncz from the Max-Planck-Institute Köln and consist of a plasmid backbone based on PCV vectors (Koncz and Schell, 1986). Each PCV contains a conditional mini-RK2 replicon and the T-DNA border sequences. The T-DNA contains: a plant selectable marker (hygromycin phosphotransferase gene [HPT^R]); a segment of pBR322 with a ColE1 replication origin (ori_{pBR}); the β-lactamase gene providing ampicillin and carbenicillin resistance (Ap^R/Cb^R) for selection in *E. coli* and *A. tumefaciens* respectively; plasmid maintenance functions specific for *A. tumefaciens*; and the transcriptional enhancer sequence of the CaMV 35S promoter (-90 to –427) cloned as a tetramer at the right border sequence. In addition to this, pPCVTAc1 also contains the CaMV 35S promoter sequence, cloned between the right border sequence and the CaMv35S promoter enhancer tetramer.

2.1.10 Plant material

Craterostigma plantagineum Hochst. (*Scrophulariaceae*) initially obtained from Professor Volk, Universtity of Würzburg (Germany) were grown from seed or vegetatively propagated (Bartels *et al.,* 1990). Plants were grown in a Conviron growth chamber at aproximately 60% humidity, photoperiod of 14 h light at 60000 lux and a day/night temperature of 24/20°C.

In vitro growing plants or callus were maintained and propagated in MS basal medium in a growth chamber at 24± 1°C with 16 h/day fluorescent light providing 200 $\mu E/m^{-2}*s^{-1}$ in sterile conditions.

2.1.10.1 Dehydration treatment of *C. plantagineum*

Plants cultivated in pots where carefully taken out of the granules, their roots rinsed with tap water and briefly dried with paper towels, their fresh weight was determined and finally the complete plants were placed on the surface of 3 MM filter paper under the same chamber growth

conditions. Once the material was dehydrated for the desired period, the dry weight was determined and the leaves were separated from the roots and immediately frozen in liquid N_2.

The relative water content was determined by: RWC= $\frac{Wt - Wd}{Fw - Wd} * 100$

Where: *Wt* = weight at time "t" of dehydration; *Wd*= weight at complete dehydration state and *Fw* = fresh weight.

2.1.10.2 NaCl treatment of *C. plantagineum*

Pot grown plants, were carefully taken out of the granules and their roots rinsed with tap water. Three different plants were then placed into MS medium (no sucrose added) supplemented with vitamins and with different salt concentrations: 0, 50, 100 and 150 mM. Plants were maintained for 6, 12 and 24 h under the same growth conditions (see 2.1.10).

2.2 Methods

2.2.1 RNA analysis

2.2.1.1 Extraction of poly A^+ enriched mRNA extraction

Poly A^+ enriched RNA was isolated using the procedure by Bartels and Thompson (1983). Frozen plant material (2-4 g) was ground to a fine powder in liquid nitrogen with a pre-cooled pestle and mortar. The powder was shaken in buffer I under warm water for 15-30 min. Equal volume of phenol-chloroform was added to the homogenate and shake thoroughly for 10 min. The mix was centrifuged at 12,000 X g for 5 min at 10ºC. The supernatant was transferred to clean tubes and the phenol-chloroform extraction repeated. Then, a chloroform extraction was repeated and aqueous phase was collected. 0.1 grams of Oligo dT-cellulose was added for each 10 grams of tissue. The mix was agitated slowly for 15 min at room temperature. Then, the cellulose was spun down and washed three times in buffer II and subsequently three times in buffer III until the elute A_{260}=0.5.

The poly A^+ enriched RNA was eluted at 55°C with 10 mL of prewarmed buffer IV. The nucleic acids precipitated by the addition of 1/20 volumes of 4 M NaCl and 2.5 volumes of absolute ethanol. The precipitated nucleic acids were pelleted by centrifugation at 12,000 X g for 15 min at

4ºC and then washed three times in 70% ethanol. Then, the pellet was dried and resuspended in DEPC treated water and stored at –70ºC.

2.2.1.2 RNA electrophoresis

For RNA work, an electrophoresis tank was designated as being for RNA work only and kept free from RNase contamination. 4 µL of 10 X RNA loading buffer (see section 2.1.1) were added to 3-5 µg of poly A^+ RNA and incubated at 55ºC for 15 min. Following the incubation, the denatured RNA samples were separated in a 1% (w/v) denaturing agarose gel containing 1 X MOPS buffer and 2.2 M formaldehyde, using 1 X MOPS running buffer.

2.2.1.3 Northern blot transfer

The samples were blotted onto Hybond N nylon membranes (Amersham). A wick of Whatman 3MM paper was placed on a support over a reservoir of 20 X SSC. After complete saturation of the wick, the gel was placed carefully on top to ensure that no air bubbles were present between the gel and the wick. A sheet of Hybond N membrane cut to the size of the gel was wetted with 20 X SSC and placed on top of the gel. Saran Wrap was placed around the edges of the gel and membrane to prevent 'short-circuiting' of the blotting procedure. Six sheets of 3MM paper (the same size as the gel and with the first one wetted with 20 X SSC) and a stack of paper towels were placed on top of the membrane. Following overnight transfer of the RNA, the filter was rinsed with 2 X SSC and placed on two sheets of Whatman 3MM wetted with 2 X SSC. The RNA was fixed to the membrane by UV irradiation at 120,000 µJoules cm^{-2} for 30 sec using a UV Stratalinker (Stratagene).

2.2.1.4 Pre-hybridisation and hybridisation of RNA filters

Pre-hybridisation was for 0.5 to 4 h in 20 mL hybridisation solution at 42ºC. Hybridisation was carried out overnight at 42ºC using the same solution with the addition of heat denatured [α-^{32}P]dCTP-labelled probe. Filters were washed at half stringency, 2 X SSC, 0.1% SDS, at 65ºC. Briefly

rinsed at room temperature and 2 X 20 min at 65°C. Filters were sealed in a plastic bag and exposed to autoradiography at –80°C using KODAK RX film and Trimax X intensifying screens or subjected to PhosphorImager analysis.

2.2.1.5 Labelling of [α-^{32}P] and [α-^{33}P]-labelled probes

2.2.1.5.1 Random primed labelled probes

Probes were prepared from agarose gel electrophoresis-separated DNA fragments using the Random Primed DNA Labelling method. The labelling was carried out in 20 µL of the following reaction mix: 25 ng (up to 12 µL) denatured DNA, 5 µL of OLB (see section 2.1.1), 1 µL (2 U/µL) Klenow enzyme and 2 µL (10 µCi/µL) [α-^{32}P]dCTP.

The reaction was incubated at room temperature for 1 hour. The unincorporated nucleotides were removed using a Sepharose G50 column. The labelling reaction was increased in volume to 100 µL with 50 mM Tris-HCl pH8. Column effluents were collected in a single Eppendorf tube. The probe was denatured at 100ºC for 5 min and immediately chilled on ice prior to use.

2.2.1.5.2 First strand cDNA synthesis with ^{33}P labelling (from poly A$^+$ enriched RNA in 30 µL vol.)

Oligo hybridization	poly A$^+$ enriched RNA (500 ng)	X µL
	polyA$^+$ RNA GUS gene (0.25 ng)	X µL
	DEPC water	X µL
	Oligo dT$_{10}$ (500 ng)	1µL
	Total reaction volume	11µL

The mix was incubated at 70°C for 10 min. The reaction was first equilibrated at 43°C for 5 min before the Reverse Transcription (RT) reaction was started. A 1st strand cDNA synthesis reaction was carried out on the RNA by adding the following:

Reverse transcription Buffer 5 X	6 µL
DTT 0.1 M (fresh prepared)	3 µL
10 mM [dATP, dGTP, dTTP]	3 µL
dCTP 50 µM	3 µL
[α-^{33}P]dCTP 30 µCi	3 µL

Superscript RT (200 U)(Gibco BRL)	1 μL
Total reaction volume	30 μL

and incubated for 1 hour at 43°C. After this, the RNA hydrolysis was performed by adding 1 μL of 1% SDS, 1 μL EDTA 0.5 M and 3 μL NaOH 3 M, this mix was incubated for 30 min at 65°C and 15 min at room temperature. In order to neutralise the reaction, 10 μL of Tris-HCl 1 M, pH8 and 3 μL of HCl 2 N were added. At this point 4 μL of the reaction were kept for DNA yield quantification. The precipitation of the nucleic acid was performed by adding 1/10 volumes of 3 M $Na[CH_3COO]$, 1/10 volumes of yeast t-RNA carrier (10 mg/mL) and 2 volumes of ethanol. The pellet was dried and resuspended in water.

2.2.1.6 Determination of incorporated label

2 μl of the labelling reaction was spotted in duplicate onto 2 X 1 cm^2 Glass-Whatman filter paper and dried for 5 min at 80ºC. One set of samples was designated "total" radioactivity and the second set was washed two times in ice-cold 10% TCA. These samples were subsequently washed in ice cold 95% ethanol twice. Then they were dried and designated "incorporated" radioactivity. Both sets of samples were quantified by liquid scintillation counting in 4 mL of scintillation fluid (Packard). The percentage incorporation was calculated as "incorporated"/"total" x 100. The amount of first strand DNA synthesised was calculated as follows:

$$\text{yield} = \frac{A(\text{CPM}) * B\left(\frac{\mu L}{\mu L}\right) * C\left(\frac{\text{pmoldNTP}}{\text{pmoldCTP}}\right)}{\left(\frac{D(\text{CPM})/E(\mu L)}{F(\text{pmoldCTP})/E(\mu L)}\right) 3030 \left(\frac{\text{pmoldNTP}}{\mu\text{gDNA}}\right)} = \mu\text{g of first strand DNA} \qquad (1)$$

Where: A= measure of incorporated radiolabeled nucleotide (CPM); B= total RT reaction volume divided by the aliquot taken for the TCA pp. (30 μl/2 μl); C= correction. Constant value that results from the average of the four nucleotides that will be incorporated into the cDNA for every dCTP scored in the assay (4 pmol dNTP/pmol dCTP); D= total CPM of the reaction (total CPM); E= volume measured (2 μl); F= total amount of dCTP in the reaction (unlabelled+radiolabelled); 3030= constant factor, represents the amount of nucleotide that corresonds to 1 μg of single stranded DNA (pmol dNTP/μg DNA);

F results form substitution on equations 2 and 3. If isotopes were purchased from Amersham then: C=10 mCi/mL and SA=2500 Ci/mmol, when freshly used. Therefore F was considered a constant value.

The amount of dCTP in the reaction was calculated with equation 2 and 3:

dCTP (nmol) = (nmol dCTP cold)+(nmol 33P-dCTP in reaction) (2)

Where: nmol 33P-UTP=(mL 33P-dCTP)*(isotope concentration(mCi/mL)*[S.A.(mCi/nmol)] (3)

2.2.2 DNA analysis

2.2.2.1 Bacterial DNA

2.2.2.1.1 Preparation of competent cells

1 mL of an overnight culture of an *E. coli* strain (see section 2.1.8) was added to 100 mL LB medium and shaken at 37ºC until growth had reached OD_{600}=0.3 - 0.4. 50 mL of the culture were pelleted by centrifugation at 3,000 X g for 10 min at 4ºC. Pellet was resuspended in 0.5 volumes of ice-cold 50 mM $CaCl_2$ and left on ice for 30 min. The cells were pelleted as before and then gently resuspended in 10 mL ice-cold 10 mM $CaCl_2$. Cells were kept at 4ºC until required (Sambrook *et al.,* 1989).

2.2.2.1.2 Bacterial transformation

1 µL of the DNA was mixed with 100 µL competent cells and left on ice for 1 h. The mix was heat shocked at 42ºC for 5 min and immediately cooled on ice for 5 min. 250 µL LB medium was added to the cells, mixed gently and then incubated at 37ºC for 2 h. The transformation was plated out on LB agar plates containing 100 µg/mL ampicillin, 40 µg/mL X-Gal, 40 µg/mL IPTG and incubated overnight at 37ºC. Colonies were selected on the basis of their colour, blue colonies indicating non-recombinant plasmids and white colonies indicating the presence of recombinant plasmids.

2.2.2.1.3 Large-scale preparation of plasmid DNA

Single recombinant colonies were used to inoculate 3 mL LB medium containing 100 µg/mL ampicillin and then shaken overnight at 37ºC. 1000 µL of the overnight cultures were diluted in 500 mL LB medium, 100 µg/mL ampicillin and shaken at 37ºC until OD_{600}=0.5. The cells were pelleted (4 x 250 mL) at 400 X g for 20 min and the Qiagen Protocol for large-scale plasmid preparation was followed.

2.2.2.1.4 Small-scale preparation of plasmid DNA

1.5 mL of an overnight culture containing the required plasmid was pelleted in an Eppendorf tube at 15,000 X g for 5 min. The protocol described by Sambrook (1989) was followed. In brief, the bacterial pellet was resuspended in 100 µL of ice-cold solution I by vortexing. Then, 200 µL of freshly prepared solution II was added and mixed softly. 150 µL of solution III was added and the mix was softly vortexed for 10 sec. The tube was stored on ice for 5 min and then centrifuged at 15,000 X g for 5 min at 4°C. The double stranded DNA was precipitated with 200 µL 4 M ammonium acetate and 1 mL cold 2-propanol was added, mixed by inversion and incubated at –80ºC for 15 min. The DNA was pelleted at 15,000 X g for 30 min, washed with cold 70% ethanol, dried and then resuspended in 20 µL – 50 µL TE buffer (Sambrook *et al.,* 1989).

2.2.2.2 Plant DNA

2.2.2.2.1 Preparation of genomic DNA

Genomic DNA was extracted from 15 g of frozen leaf tissue according to the method of Qiagen. Frozen plant material was ground to a fine powder in liquid nitrogen with a pre-cooled pestle and mortar. The powder was added to 5 volumes of lysis buffer and mixed at 74°C. Then, 1/10 volumes of β–mercaptoethanol was added to the mix and incubated at 74°C for 20 min. The mix was cooled down to room temperature and 1 volume of chloroform/isoamylalcohol (24:1) was added, then vortexed. The lysate was centrifuged for 10 min at 5,500 X g, 4ºC. 1 volume of isopropanol was added to the aqueous phase, mixed and incubated at room temperature for 30 min.

The nucleic acid was pelleted by centrifugation for 20 min at 5000 X g, 4 ºC and the supernatant discarded. The pellet was resuspended in 0.5 mL of 1 M NaCl at 37°C; then RNase A (10 mg/mL) was added and incubated for 30 min at 37°C. Afterwards, the Qiagen protocol for genomic DNA purification from leaves was followed.

2.2.2.2.2 Southern blotting

About 30 μg of DNA from each plant sample and 100 ng of plasmid DNA were digested with the selected restriction enzymes, separated by electrophoresis in a 0.8% agarose gel. The gel was then treated by washing in 0.25 M HCl for 10 min to depurinate the DNA, followed by two 15 min washes in 0.5 M NaOH, 1.5 M NaCl to denature the DNA. This was then neutralised with two 15 min washes in 1 M Tris-HCl pH7.5, 1.5 M NaCl. The denatured DNA was then transferred and bound to Hybond N membrane (Amersham) as previously described for Northern blotting of RNA (see section 2.2.1.3). Hybridisations were performed according to Sambrook *et al*. (1989). Filters were washed two times for 20 min at 65°C.

2.2.2.2.3 Nylon filter arrays

2.2.2.2.3.1 PCR amplification of cDNA inserts from the *C. plantagineum* gene collection

The *C. plantagineum* DNA collection was amplified by PCR with universal primers in 96-microwell plates (Advanced biotechnologies) in a Peltier Thermal Cycler The PTC-225 DNA Engine Tetrad (MJ Research, Inc.). Since most of the cDNAs were cloned into either pUC19 or pBluescript derived vectors (see section 2.1.9), Universal and Reverse primers (see section 2.1.5) were used. cDNAs that were cloned into non-pBluescript derived vectors were amplified using gene specific primers. 1 µL of each clone (taken from a plasmid stock), was loaded into 96 microwell plates. A PCR master mix was prepared and 99 µL was dispensed into each well.

PCR mix	Reaction mix (µL)
H_2O	78.5
PCR 10 X buffer	10

$MgCl_2$ 50 mM	3
dNTP 10 mM (each)	2
Primer mix 10 μM (each)	5
Taq polymerase	0.5
DNA (100 ng)	1
Total volume	100 μL

The 96-microwell plate was sealed and placed onto a MJ Research thermo cycler. Amplification was carried out under the following conditions:

Cycles	Denaturation		Annealing		Extension	
	time	°C	time	°C	time	°C
1	45″	95	45″	48	1.5′	72
35	1′	95°C	45″	55ºC	1.5′	72

After the cycling, the reaction was incubated for 10 min at 72ºC and kept at 4°C.

2.2.2.2.3.2 Spotting DNA onto nylon filters

60 μL of each PCR reaction was loaded onto a 384-microtiter plate (NUNC), sized 12.5 X 8 cm^2. A 60 μL aliquot of 1% bromophenol blue was loaded at positions A-1 and P-22 to P-24, which served as a marker for the final division of the fields on each 22 cm^2 membrane. The microtiter plate was placed onto a biogridder robot (BioGrid/MicroGrid with cooling, BioRobotics) together with a pre-wetted 22 cm^2 Hybond N^+ nylon filter in denaturing conditions (2 X SSC).

The biogridder was programmed to produce DNA spots in a pattern of 2 X 2 so that a repetition of each PCR product was present in each nylon filter. The spotted volume being approximately 1 μL (ten stamps when a pin head of 0.4 mm was used). Six identical 22 cm^2 nylon membranes, each containing 6 field areas of 8 X 12 cm^2, were obtained from 60 μL of PCR product. The time of operation was approximately 2 h.

After spotting, nylon filters were placed on top of 3MM Whatman filter paper pre-wetted in neutralising solution two times for 4 min. The DNA was fixed to the membrane by UV irradiation at 120,000 μJoules cm^{-2} for 30 sec using a Stratalinker (Stratagene).

2.2.2.2.4 Pre-hybridisation, hybridisation and washing conditions of DNA filters

2.2.2.2.4.1 DNA filters

Filters were prehybridised and hybridised in 50 - 100 μL/cm^2 hybridisation solution (see section 2.1.1) at 65ºC using a hybridisation oven. Probes were prepared as previously described, the filters were washed at low stringency as follows: 2 X 20 min in (2 X SSC, 0.1% (w/v) SDS) at room temperature; 1 X 20 min in (1 X SSC, 0.1% (w/v) SDS) at 65ºC. For a high stringency wash, the following step was included: 2 X 20 min in (0.1 X SSC, 0.1% (w/v) SDS) at 65ºC. Filters were sealed in Saran Wrap (Genetic Research Instrumentation), oriented and exposed to autoradiography for the required period of time at –80ºC or subjected to PhosphorImager analysis.

2.2.2.2.4.2 Nylon array filters

Pre-hybridisation and hybridisation were carried according to Hoheisel (1993). Prehybridisation was done in 10 mL of DNA hybridisation buffer (see section 2.1) at 65ºC for 0.5 to 2 h. After that, 10 mL of hybridisation buffer was added together with 50 ng of probe, this was carried out for at least 10 h at 65ºC. The filters were then briefly rinsed twice at room temperature in washing buffer, 40 mM Na-Phosphate pH7.2, 0.1% SDS, then incubated with at 65ºC for 30 min, twice. Subsequently, the filters were briefly blotted dry, covered in Saran Wrap (Genetic Research Instrumentation) and subjected to PhosphorImager analysis by exposing to a phosphor screen overnight.

2.2.2.2.5 *In vitro* transcription

2.5 μg of DNA were used for the *in vitro* transcription reaction. The reaction mix included: 1 M DTT, 100 mM of A, G, C and U ribonucleotides, 0.25 μL of fresh label ^{35}S-UTP and 10 X T7 polymerase buffer. The components were mixed and 2 μL (25 U/μL) of RNAse inhibitor and 2.5 μL (10 U/μL) of T7 RNA polymerase were added in a total volume of 50 μL. The reaction was carried out at 37°C for 1-2 h and was stopped by adding 0.5 μL of DNAse I. An aliquote of 2 μL was TCA

precipitated and the incorporated nucleic acid quantified with the use of a scintillation counter. The amount of RNA yield was calculated as follows:

ng nucleic acid=[Σ M.W. 4 ribonucleotides (ng/nmol)] X [incorporated ribonucleotide (nmol)] (1)

the amount of incorporated ribonucleotide (IR) was calculated with equation 2:

IR (nmol) = [UTP in the reaction (nmol)] X [% incorporation/100] (2)

UTP (nmol) = (nmol UTP cold)+(nmol ^{35}S-UTP in reaction) (3)

Where: nmol ^{35}S-UTP=(mL ^{35}S-UTP)*(isotope concentration(mCi/mL)*[S.A.(mCi/nmol)] (4)

2.2.2.2.6 Cloning of T-DNA flanking regions

2.2.2.2.6.1 Long Range inverse PCR(LR-iPCR)

To rescue plant DNA sequences flanking the right arm of the T-DNA 1 μg of DNA was digested by *Eco*RI or *Xba*I restriction enzymes and religated. After purification, the ligated DNA was digested by *Sph*I or *Sma*I restriction enzymes in order to linearise the circular DNA structures. Long range PCR was performed using 0.5 μg of linearized plant DNA as template and the EcoHyg1 and iRBTA1 or iRBRN1 primers. PCR reactions were performed in 50 μL volume, using LA PCR (TAKARA ShuzoCo.) long range PCR kit as recommended by the supplier. The DNA sample was heated to 95°C for 2 min and 35 cycles were performed as follows: 94°C for 30 sec, 65°C for 30 sec and 68°C for 8 min, followed by 68°C elongation for 10 min. PCR products were analysed by agarose gel electrophoresis. When no amplified fragment was found, a second PCR reaction was performed using 1 μL of 500 X diluted PCR product as a template and EcoHyg2 and iRBTA2 or iRBRN2 primers (or nested primers). Amplified DNA fragments were purified from 0.8% agarose gels and purified PCR products were sequenced using EcoHyg2 and iRBTA2/iRBRN2 primers.

2.2.2.2.6.2 Adaptor mediated PCR

2.5 μg of plant genomic DNA was digested in 100 μL reaction volume with 10 U of restriction enzyme at 37°C. After purification, 4 μL of the DNA was then ligated to an excess of adaptor (25 μM) with 1 U T4 DNA ligase overnight at 16°C according to the manufactures conditions

(Life Technologies), in a total volume of 8 μL. After stopping the reaction, it was diluted 10 X by addition of 72 μL of 10 mM Tris-HCl pH 7.5, 1 mM EDTA and stored at –20°C.

PCR amplifications were performed according to the manufacturer's instructions (*Taq Polymerase* Gibco Co). ADP1 and iRBTA1/iRBRN1 primers were used (see section 2.1.5). The PCR reaction mix was heated to 96°C for 5 min prior addition of the polymerase. The cycle parameters were: 7 cycles of denaturation at 94°C for 25 sec and annealing/extension at 72°C for 3 min, 32 cycles of denaturation at 94°C for 25 sec and annealing/extension at 67°C for 3 min, with an additional 67°C for 7 min after the final cycle. 8 μL of the primary PCR products were analysed on a 1.5% agarose gel.

The second PCR was performed by using 1 μL of 50 X dilution of the primary PCR products and the nested primers, ADP2 and iRBTA2 or iRBRN2. The cycles were performed as follows: 5 cycles of denaturation at 94°C for 25 sec and annealing/extension at 72°C for 3 min, 20 cycles of denaturation at 94°C for 25 sec and annealing/extension at 67°C for 3 min, with an additional 67°C for 7 min after the final cycle. 5 μL of the secondary PCR products were analysed on a 1.5% agarose gel.

2.2.2.2.7 Cloning of PCR products

The cloning of PCR products was performed using the p-GEM-T-Easy vector system, according to the manufacturer instructions (Promega Co.).

Cloning of the *uid*A gene was performed by PCR with specific primers for the *uidA* gene with additional flanking sequences (see section 3.2.2.1).

2.2.2.2.8 Lambda-cDNA-library screen

The screening of a lambda Zap library (Uni Zap XR library kit, Stratagene) prepared from poly A^+ enriched RNA extracted from 2 h dehydrated *C. plantagineum* leaves (provided by Dr. Jonathan Phillips), was performed following the instruction manual (Stratagene). The plaque hybridisation was performed following Benton and Davis (1977). The HybriZap lambda library was subsequently converted, by *in vivo* excision, to a pAD-GAL4 plasmid library.

2.2.2.2.9 DNA sequencing

DNA sequences were determined by the MPIZ DNA core facility using a sequencing kit (Prism, Applied Biosystems). The reactions were resolved on a DNA sequencer (Abi Prism 377 and 3700 sequencers, Weiterstadt-Germany) using BigDye-terminator chemistry.

2.2.3 *C. plantagineum* transformation with *A. tumefaciens*

2.2.3.1 Transformation procedure

The *A. tumefaciens* strain GV3101 carrying the helper plasmid pMP90RK was used as the host strain for the constructs carrying the plant cloning vectors pPCVTAc1 and pPCVRN4 independently. The plasmid bearing *A. tumefaciens* were grown overnight at 28°C on a shaker at 150 rpm in 50 mL YEB containing the appropriate antibiotics (rifampicin for *A. tumefaciens*, kanamycin for helper plasmid and carbenicillin for the T-DNA carrying plasmid selection). The cells were then pelleted by centrifugation at 4°C, 3000 rpm and resuspended for plant infection in liquid MSAR, supplemented with 0.25 mg/L antioxidant mixture, to a final optical density of OD_{550}=0.5.

Young leaves 5 to 10 mm in length were aseptically removed from *C. plantagineum* plantlets (maintained in a proliferating state) and lightly pressed on sterile folder of fine sandpaper to increase the wounded area. The explants were immersed in the *A. tumefaciens* suspension in MSAR for 20 min in darkness and co-cultivated for 2 days on solid callus medium without antibiotics (Furini *et al.,* 1994).

2.2.3.2 Plant culture conditions

After co-cultivation, the infected explants were subcultured for 10 days on MSAR1 containing 500 mg/L cefotaxime and 15 mg/L hygromycin. Growing calli were subcultured at 3-week intervals on selective MSAR1. At each subculture step, cefotaxime was reduced by 100 mg/L until it was no longer necessary. The plant selectable marker, hygromycin, was maintained at a constant concentration until the plants were transferred to soil. To induce shoot differentiation embryogenic calli were subcultured on MSAR1a. Shoots were rooted on MS medium.

2.2.3.3 Identification of desiccation-tolerant calli

Calli were selected on hygromycin containing medium and maintained for about 3-4 months. Calli were then transferred to sterile filter paper and air-dried for 24 h at room temperature under the clean bench. During this time calli lost about 94 to 96% of their initial fresh weight. As controls, untransformed wild type callus was used as negative controls and pre-ABA treated calli was used as a positive control. Dried calli were rehydrated for two days on callus medium in the presence of hygromycin. The screening for survivors was performed with the help of a microscope.

2.2.4 Computer software

2.2.4.1 DNA sequence analysis

DNA similarities were determined using the BLAST network service (Altschul *et al.*, 1990) and the Genetics Computer Group (Madison, WI) (Devereux *et al.*,1984) version 9.0 software package.

2.2.4.2 RNA blot quantifications: PhosphorImager technology

Image Quant software version 1.0 for Macintosh (1995), Molecular Dynamics was used for basic analysis and data reporting/graphing functions. The PhosphorImager (Molecular Dynamics) (Johnston *et al.*, 1990) was used to quantify the activity of radioactive bands on the nylon membranes. The sample was exposed to a phosphor screen (KODAK). Screens are sensitive to any source of ionising radiation if exposed for an appropriate time.

The phosphor screen was loaded onto the STROM 860 (PhosphorImager) and laser scanned. Once the exposure of the storage phosphor screen to ionising radiation induced a latent image formation, the laser scanning excites the BaFBR:EU+2 crystals in the screen which releases energy as blue light and return to ground state. Blue light is collected and measured to form a quantitative representation of the sample. The units used to quantify the signal are given in PhosphorImager Counts (or Molecular Dynamics Counts [MDC]), which is an arbitrary unit that describes the intensity of photon emissions released from the storage phosphor screen during scanning.

2.2.4.3 Macroarray quantifications

Imaging of the array was done with the software supplied by the scanner manufacturer (see 2.2.11.2). The Array vision (ARV), Imaging Research Inc., 5.0 software version was used for the quantification and basic analysis of the DNA array data. This software gave a final output of spot volume quantifications (in MDC) which were already normalised to a control and that included background corrections. When an MD image is loaded into ARV, ARV converts the raw grey level values to "MDC/mm^2" which is a "darkness" scale that estimates an isotope concentration. Thus, MDC is the average MDC/mm^2 value of all the pixels within the spot. "MDC" is MDC/mm^2 multiplied by the area of the spot (mm^2) and is thus an estimate of total signal.

2.2.4.4 Cluster and Tree View

Cluster analysis for genome-wide expression data from DNA microarray hybridisation is described that uses standard statistical algorithms to arrange genes according to similarity in pattern of gene expression (developed by Eisen, 1998). The output was displayed graphically by using of the Tree View software. Both Cluster and Tree View software are public available at http://www.rana.standford.edu/software.

III Expression profile analysis of desiccation tolerance related genes from *Craterostigma plantagineum*

Common molecular responses to both dehydration and salt stress suggests overlap and/or crosstalk between the two signal transduction networks (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). Tolerance to dehydration and salinity conditions involves similar mechanisms of osmotic adjustment (Hsiao, 1973; Ashraf and O'Leary, 1996). In contrast to dehydration, salt stress has two main components: a) a hydric component, due to the decrease in the external osmotic potential, and b) an ionic component associated with the toxicity of some ions, mainly sodium and high doses of chloride, and to the salt induced decrease in other elements such as potassium (Serrano and Gaxiola, 1994; Liu *et al.,* 2000).

In an attempt to detect specific responses to water deficit, dehydration and salt treatments were compared using the plant *C plantagineum*. This plant is desiccation tolerant (Gaff *et al.*, 1971), but sensitive to relatively low concentrations of sodium chloride. In this study an expression monitoring method was followed using a collection of desiccation related cDNA arrayed on a nylon support. The technique offered the possibility to measure the expression levels of a large number of dehydration responsive genes simultaneously. Moreover, genes that were unaffected, as well as those that showed reduced transcript accumulation were identified. Transcript accumulation patterns or profiles were compared and, in some cases, confirmed individually by RNA gel blot analysis.

3.1 Responses of *C. plantagineum* to sodium chloride and dehydration treatments

3.1.1 *C. plantagineum* is desiccation tolerant, but sensitive to sodium chloride

The desiccation tolerance capacity of *C. plantagineum* is depicted in figure 3.1.1.1a. Plants that have lost 98% of their relative water content will recover full physiological activity within 24 hours of rehydration. Given that some resurrection grasses are salt tolerant (Gaff *et al.*, 1988), the hypothesis that *C. plantagineum* could be salt tolerant was tested. Initially, concentrations of sodium chloride that are tolerated by salt tolerant species (e.g. the halophytic species *Suaeda maritima*, *Atriplex nummularia* and *Mesembryanthemum crystallinum*) (Greenaway and Munns, 1980; Braun *et al.,* 1986; Löw *et al.*, 1996) were applied to *C. plantagineum* plants. Sodium chloride at concentrations of 0, 150 and 300 mM was added to MS medium in hydroponic culture. For each concentration, five plants were treated for a period of 24 hours (see section 2.1.10.2). In both cases plants wilted within the first 3-4 hours and after completion of the treatment the plants became fully

flaccid whereas control plants remained turgid. To test whether or not sodium chloride treated plants were able to recover, sodium chloride treated the plants were transferred to sodium chloride-free media for 1 to 7 days. During this time vegetative tissues showed no sign of recovery (Figure 3.1.1.1 shows the state of recovery at the fifth day). Although, in plants pre-treated with 150 mM newly grown leaves were observed, this was not a resurrection process since the original vegetative tissues did not recover.

Figure 3.1.1.1 *C. plantagineum* is tolerant to desiccation but sensitive to sodium chloride

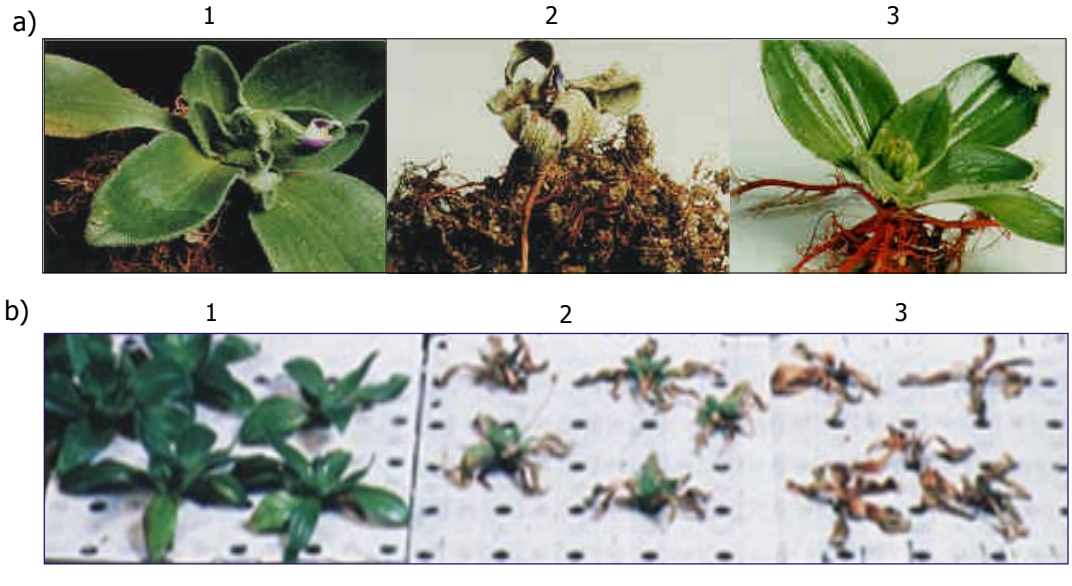


Figure 3.1.1.1 Panel a) fully hydrated *C. plantagineum* plant (1) was submited to 24 h of desiccation (2) and left to recover for 24 h on hydrated conditions (3). Panel b) *C. plantagineum* fully hydrated plants were previously treated for 24 h in hydroponic culture which medium was supplemented with 0 mM (1), 150 mM (2) and 300 mM (3) of sodium chloride. Media was replaced for sodium chloride-free media and plants were left to recover for a period of 5 d.

The effect of sodium chloride on *C. plantagineum* leaves water availability was assayed by measuring the relative water content (RWC) (see section 2.1.10.1) of leaves after dehydration and sodium chloride treatments. Five plants were dehydrated and samples were taken at different time points (6, 12 and 24 hours). Simultaneously, five plants were placed in hydroponic culture supplemented with 0, 150 and 300 mM of sodium chloride and sampled after 6, 12 and 24 hours. The relative water content measurements revealed that leaf tissues lost up to 30% RWC when treated with 150 or 300 mM sodium chloride for 24 hours (see figure 3.1.1.2). This, according to the plot, would be the equivalent to the RWC of leaves that would have been dehydrated for

approximately 4 hours. After 24 hours of drying a loss of approximately 66% of the RWC was observed in leaves.

Figure 3.1.1.2 Relative water content of *C. plantagineum* leaves after 24 h of dehydration and sodium chloride treatments

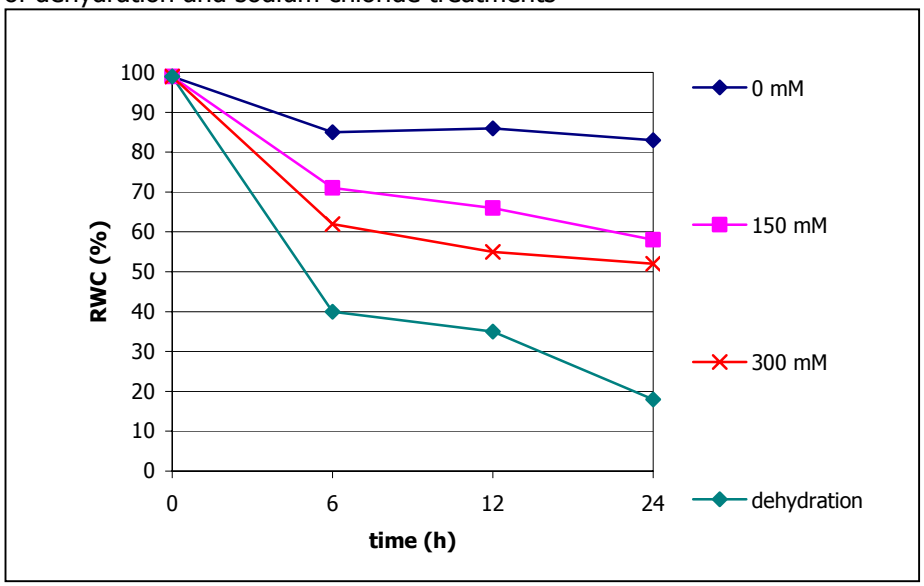


Figure 3.1.1.2 Physiological characterisation of dehydration and sodium chloride treatment. Determination of the relative water content after 24 h of dehydration and sodium chloride treatment. Leaves were separated from roots and their RWC determined. The average of five independent samples were taken per time point.

Data from the initial experiments were used to determine the conditions for the dehydration and sodium chloride treatments used in subsequent experiments. Sodium chloride treatments were applied that, rather than excessively damaging the plants, would induce a mild stress response. The final conditions for the sodium chloride treatment were: 0, 50, 100 or 150 mM, for a period of 6, 12 and 24 hours. Under these conditions, plants treated for 24 hours with 50 mM of sodium chloride showed less wilting, plants treated with 100 mM of sodium chloride showed mild wilting and plants treated with 150 mM showed complete wilting.

The dehydration treatment was applied for the following periods: 0, 2, 4, 8, 12, 24, 48 and 72 hours (ranging from 0 to 87% of water loss respectively). At each time point, plants were removed, blotted dry, dissected, frozen in liquid nitrogen and stored at -80°C until further analysis.

3.2 Comparison between the transcript profiles observed in response to sodium chloride and dehydration treatments

Recently, high-density DNA micro and macroarrays have become a useful tool for the analysis of gene expression (Schena et al., 1995; Eisen and Brown, 1999). This technology allows the expression of many genes, over multiple developmental stages and in response to environmental cues, to be simultaneously evaluated. In this study, the macroarray technique was used to compare the responses of 259 pre-selected C. plantagineum genes to either a sodium chloride or a dehydration treatment. By comparing the transcript profiles, common molecular responses to environmental stimuli were observed and dehydration specific responses were identified.

3.2.1 Nylon array filters: methodology

The nylon array filter or macroarray analysis can be divided in four main steps (see figure 3.2.1.1): 1. PCR amplification of the gene collection organised in a 384-microwell-plate (see section 2.2.2.2.3.1); 2. transfer of PCR products (gene collection), in duplicates, to nylon membranes using a "gridding" robot (see section 2.2.2.2.3.2). In this step replica filters were obtained; 3. hybridisation of independent filters to complex probes prepared from cDNA reverse transcribed from poly A^+ RNA extracted from tissue treated under specific conditions (see section 2.1.10/2.2.1.5.2); 4. filters exposed to a phosphor screen. After imaging, quantification and analysis of the signals was performed with specialised computer software (see sections 2.2.4.2/3/4).

Figure 3.2.1.1 Diagram of the DNA macroarray technology

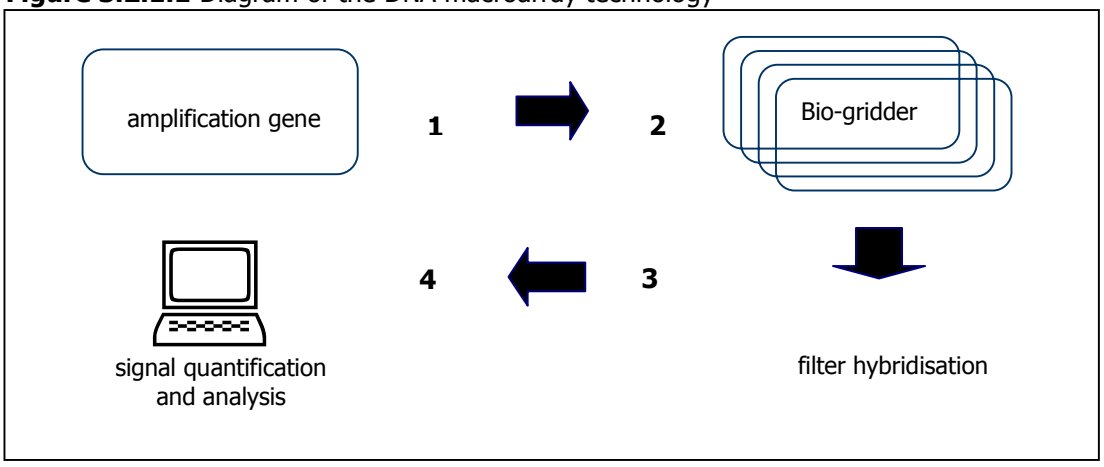


Figure 3.2.1.1 The DNA macroarray technology can be summarised in four main steps.

Each cDNA that formed the gene collection was individually amplified, and the resulting products were loaded onto agarose gels in order to visualise the presence of single products and to ensure a concentration of at least 50 ng/μL. The filter sets were prepared so that each individual PCR fragment was represented in two positions (see figure 3.2.1.2). The settings used consisted of 10 stamps per spot with a stamp time of 1 second. Each pin tool was 0.7 mm in diameter and delivered 90 nL per stamp. In this way approximately 1 μL of PCR product represented approximately 50 ng of spotted DNA.

Figure 3.2.1.1 Organisation of the gene collection in a 384-microwell-plate

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	EST 002	EST 003	EST 004	EST 005	EST 006	EST 007	EST 008	EST 0011	EST 0012	EST 0013	EST 0014	EST 0015	EST 0016	EST 0018	EST 0019	EST 0021	EST 0023	EST 0024	EST 0026	EST 0027	EST 0028	EST 0029	EST 0030	EST 0031
B	EST 0032	EST 0034	EST 0035	EST 0037	EST 0038	EST 0040	EST 0042	EST 0045	EST 0047	EST 0048	EST 0049	EST 0050-OB	EST 0050-UN	EST 0051	EST 0054	EST 0055	EST 0056	EST 0058	EST 0059	EST 0060	EST 0061	EST 0062	EST 0063	EST 0064
C																								
D	EST 0067	EST 0068	EST 0069	EST 0070	EST 0071	EST 0072	EST 0073	EST 0074	EST 0075	EST 0076	EST 0094	EST 0095	EST 0096	EST 0097	EST 0098	EST 0099	EST 0100	EST 0101	EST 0102	EST 0103	EST 0104	EST 0105	EST 0106	EST 0110
E	EST 0111	EST 0112	EST 0113	EST 0114	EST 0115	EST 0116	EST 0117	EST 0118	EST 0119	EST 0120	EST 0121	EST 0122	EST 0124	EST 0125	EST 0126	EST 0127	EST 0128	Cp-PIP a1	Cp-PIP a2	Cp-PIP a3	Cp-PIP a4	Cp-PIP a5	Cp-PIP a6	Cp-PIP a7
F																								
G	Cp-PIP a8	Cp-PIP b	Cp-PIP c	HDZIP	DD 6/7	DD 20/5	DD 22/5	DD 22/6	DD 28/4	DD 28/5	DD 48/6	DD 65/1	DD 65/4	DD 98/6	DD 98/11	DD 104/5	pcC 2	pcC 6-19	Cp-M7	pcC 11-24	pcC 11-24	pcC 13-62	pcC 16-19	pcC 16-41
H	pcC 16-81	pcC 25-52	pcC 27-45	pcC 39-62	pcC 37-31	pcC 86	pBSS18	SPSII/5a	Aldolase	Galactinosyl	C8 msr	pcC 6-19	pcC 6-1	SPSIst	HSF 4/7	HSF 7a/5	tc 17	CDT-1	pcC119	clone 4-1	clone 6-1	clone 10-1	clone 13-1	clone 14-1
I																								
J	clone 18-1	clone 1-2	clone 2-2	clone 4-2	clone 5-2	clone 6-2	clone 7-2	clone 11-2	clone 12-2	clone 13-2	clone 15-2	clone 16-2	clone 17-2	clone 18-2	clone 10-3	clone 12-3	clone 13-3	clone 14-3	clone 15-3	clone 17-3	clone 18-3	clone 19-3	clone 20-3	clone 21-3
K	clone 23-3	clone 25-3	clone 26-3	clone 28-3	clone A 48-1	clone A49-1	clone A 21-3	clone A 22-3	clone A 29-3	clone A 31-3	clone A 36-3	clone A 37-3	clone A 39-3	clone A 43-3	clone A 44-3	clone A 7-3	clone A 19-3	clone A 27-3	clone A 30-1	clone A 32-1	clone A 33-1	clone A 37-1	clone A 39-1	clone A 40-1
L		PLD pcr-p		PLD 50ng		PLD 10ng				Ubiq. 50ng		Ubiq. 10ng						PTA 25ng		PTA 10ng				
M	clone A 41-3	clone A 45-1	clone B6 (10,2)	clone B8 (10,2)	clone B9 (10,2)	clone B13 (10,2)	clone B17 (10,2)	clone B19 (10,2)	clone B39 (10,2)	clone C12 (10,2)	clone D3 (10,2)	clone D7 (10,2)	clone D11 (10,2)	clone D35 (10,2)	TH 1/1	TH 5/1	TH 7/9	TH 7/10	TH 7/15	TH 9	TH 11/1	TH 12/6	TH 16/2	TH 20/1
N	TH 22/3	TH 23	TH 25	TH 34	TH 36	TH 37/1	TH 38/1	TH 39/1	TH 40/1	BP-1	small heat shock	TH 21	TH 23	TH 44	TH 62	TH 65	TH 81	TH 123	EST 0020	EST 0039	EST 0044	EST 0046	EST 0052₁	EST 0052₂
O		GUS 100ng		GUS 50ng		GUS 10ng		GUS 5ng		GUS 1ng		GUS 100ng		GUS 0.1ng		GUS 0.05ng		Pblue 50ng		Pblue 10ng				
P	EST 0053	EST 0066	EST 0065	THR1	THR2	THR3	THR4	THR5	THR6	THR7	THR8	THR9	THR10	THR11	THR12	THR13	THR14	THR15	THR17					

Figure 3.2.1.1 Gene collection in a 384-microwell-plate format of an area of 8 x 12 cm^2. PCR products were loaded on rows from A:1-24 to P:1-24. An empty row was left every two rows, unless specified.

Probes from independent samples were labelled with ^{33}P-dCTP and hybridised to the nylon array. After washing, the radioactive signal associated with each element of the array was read with a scanning device and images were captured (imaging). Quantitative values for each gene, between independent probes, were calculated using the ArrayVision (AV) software. Highly radioactive spots represented abundant mRNA species, whereas low abundant mRNA species exhibited a relatively

weak radioactive signal. An example of the signal obtained from a nylon array filter that has been hybridised, washed and scanned, is shown in figure 3.2.1.2. In this example, the probe was a labelled cDNA reverse transcribed from mRNA corresponding to fully hydrated *C. plantagineum* leaves. From this figure it can also be observed that no background was obtained in the spaces left in blank.

Figure 3.2.1.2 Gene collection organisation within the nylon filter arrays

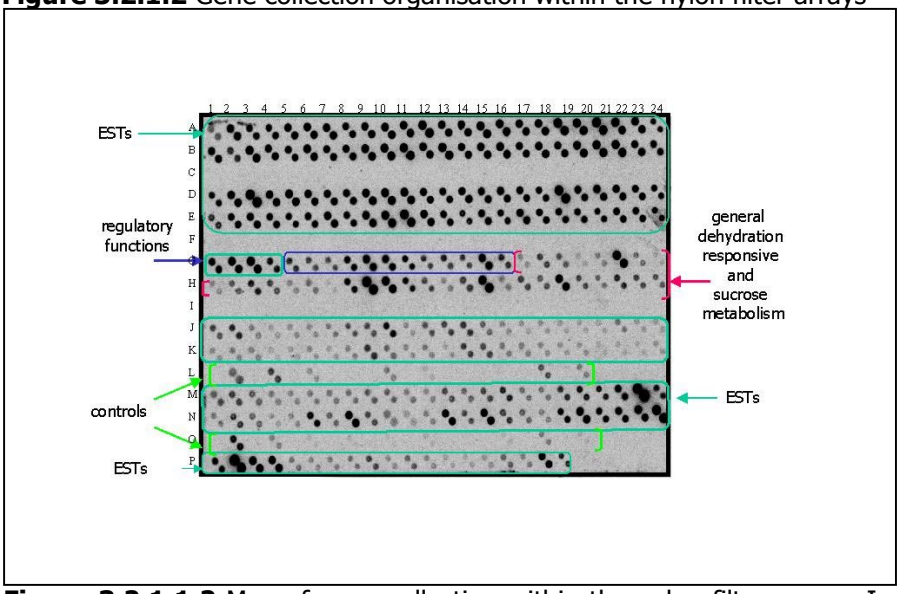


Figure 3.2.1.1.2 Map of gene collection within the nylon filter arrays. In this example the filter was hybridised to a cDNA that corresponded to fully hydrated leaf poly A^+ enriched RNA. Each gene was spotted twice, 2 x 2 pattern. No clones were loaded on the blank spaces.

3.2.1.1 Characteristics of the *C. plantagineum* gene collection

Transcript profiling of dehydration inducible genes under different abiotic conditions was performed using the macroarray technique. Over 260 desiccation-related genes have been isolated and, in some cases, fully characterised over the past decade (Phillips and Bartels, 2000). These clones fall into the following four categories:

1. Sugar metabolism genes
2. General drought stress related genes
3. Genes with a regulatory function
4. Genes of unknown function

The genes were isolated using a variety of molecular techniques such as differential, subtractive and cold plaque cDNA library screening (Bartels *et al*., 1990; Bockel *et al*., 1998; Kleines *et al*., 1999); genomic library screening (Iturriaga *et al.,* 1996); differential display reverse transcriptase-PCR (DDRT-PCR) (Frank *et al*., 1998; 2000; Kirch and Bartels, unpublished); two-hybrid library screening (Frank *et al*., 1998; Phillips and Bartels, unplublished) and one-hybrid screening (Hilbricht and Bartels, unpublished). In this study, a collection of 259 desiccation related genes was established (see table 3.2.1.1.1).

Most of the genes within the collection have been partially or fully sequenced, and their homologies with reported sequences determined. 68.6% of the clones have shown significant homologies to reported genes, from which only about 6% have been characterised in detail. The remaining 31.4% had no overall homology to other reported genes.

Table 3.2.1.1.1 *C. plantagineum* gene collection and homologies

EST	Plasmid DNA	Homology
1	EST 002	ACC synthase
2	EST 003	Alpha-tubulin
3	EST 004	Anthranilate synthase alpha subunit
4	EST 005	ATP synthase, chloroplastic
5	EST 006	β- amylase
6	EST 007	Chaperonin 10 kd
7	EST 008	Cherry-allergen protein
8	EST 0011	Cystein protease
9	EST 0012	Cytochrome P-450
10	EST 0013	DOPA decarboxylase
11	EST 0014	Ethylene-inducible protein
12	EST 0015	Ferredoxin
13	EST 0016	Fructose-bisp-aldolase
14	EST 0018	GAPDH
15	EST 0019	Geranyl geranyl hydrogenase
16	EST 0021	GTP-binding FTP1/OBG protein
17	EST 0023	Heat shock transcription factor
18	EST 0024	Histone H4
19	EST 0026	Light-inducible tissue-specific ST-LS1 gene
20	EST 0027	Major latex protein type 2
21	EST 0028	Metallothionein
22	EST 0029	Monodehydroascorbate reductase
23	EST 0030	Mrna
24	EST 0031	NADH dehydrogenase, mitochondrial
25	EST 0032	Nuclear RNP protein GAR1
26	EST 0034	Peptidyl-prolyl cis-trans isomerase
27	EST 0035	Phosphatase 2A
28	EST 0037	Polyphenol oxidase
29	EST 0038	Polyubiquitin
30	EST 0040	PSI-F subunit precursor
31	EST 0042	PSII 23kda oxygen evolving Protein
32	EST 0045	PSI-L protein
33	EST 0047	Recombination protein DRT100
34	EST 0048	Ribosomal protein L41
35	EST 0049-1	Ribosomal protein S11
36	EST 0050-OB	Ribosomal protein S18
37	EST 0050-UN	Ribosomal protein S18
38	EST 0051	Ribosomal protein S14A, S14B
39	EST 0054	S-phase specific protein
40	EST 0055	Stress-related pvsrp protein
41	EST 0056	Sucrose synthase
42	EST 0058	Transcetolase
43	EST 0059	Translation elongation factor 1 alpha
44	EST 0060	Ubiquitin carrier protein
45	EST 0061	Water stress-induced protein
46	EST 0062	β-phosphoglucomutase
47	EST 0063	Extensin
48	EST 0064	Heat shock transcription factor homolog
49	EST 0067	
50	EST 0068	
51	EST 0069	
52	EST 0070	
53	EST 0071	
54	EST 0072	
55	EST 0073	
56	EST 0074	
57	EST 0075	
58	EST 0076	
59	EST 0094	
60	EST 0095	
61	EST 0096	
62	EST 0097	
63	EST 0098	

EST	Plasmid DNA	Homology
64	EST 0099	
65	EST 0100	
66	EST 0101	
67	EST 0102	
68	EST 0103	
69	EST 0104	
70	EST 0105	
71	EST 0106	
72	EST 0110	
73	EST 0111	
74	EST 0112	
75	EST 0113	
76	EST 0114	
77	EST 0115	
78	EST 0116	
79	EST 0117	
80	EST 0118	
81	EST 0119	
82	EST 0120	
83	EST 0121	
84	EST 0122	
85	EST 0124	
86	EST 0125	
87	EST 0126	
88	EST 0127	
89	EST 0128	
90	H2-1	Cp-PIP a1
91	H2-5	Cp-PIP a2
92	H2-9	Cp-PIP a3
93	H2-11	Cp-PIP a4
94	H2-15	Cp-PIP a5
95	H2-17	Cp-PIP a6
96	H2-21	Cp-PIP a7
97	H2-23	Cp-PIP a8
98	H2-27	Cp-PIP b
99	H2-C	Cp-PIP c
100	HDZIP	HDZIP
101	DD 6/7	Kinase
102	DD 20/5	Kinase
103	DD 22/5	Kinase (?)
104	DD 22/6	
105	DD 28/4	14-3-3 homologue
106	DD 28/5	PLD
107	DD 48/6	Malic enzyme (?)
108	DD 65/1	Malic enzyme
109	DD 65/4	JP3 receptor
110	DD 98/6	PLD
111	DD 98/11	Transelongation factor
112	DD 104/5	
113	pcC 2	LEA
114	pcC 6 19	LEA (G2 or dehydrin)
115	cp m7	MYB
116	pcC 11-24	LEA
117	pcC 11-24	LEA
118	pcC 13-62	LEA
119	pcC 16-19	LEA
120	pcC 16-41	LEA
121	pcC 16-81	LEA
122	pcC 25-52	LEA
123	pcC 27-45	LEA

EST	Plasmid DNA	Homology
124	pcC 34-62	LEA
125	pcC 37-31	ELIP
126	pcC 86	LEA
127	PBSS 18	Sucrose synthase
128	SPS II /5a	Sucrose phosphate synthase II
129	Aldolase	Aldolase
130	Galactinol synt.	Galactinol synthase
131	C8 msr	Methyl sulfoxyde reductase
132	pcC 6-19	LEA (G2 or dehydrin)
133	C 6-1	
134	SPSI	Sucrose phosphate synthase I
135	HSF 4/7	Heat shock transcription factor homologue
136	HSF 7a/5	Heat shock transcription factor homologue
137	Tc 17	
138	CDT-1	
139	pcC 119	
140	Clone 4-1	NADP dependent oxidoreductase
141	Clone 6-1	LEA-protein (DC8, G3)
142	Clone 10-1	Tyrosine phosphoprotein
143	Clone 13-1	VP4- viral capsid protein (?)
144	Clone 14-1	
145	Clone 18-1	
146	Clone 1-2	
147	Clone 2-2	
148	Clone 4-2	Aldehyde dehydrogenase
149	Clone 5-2	N-acetylglucosaminyltransferase III
150	Clone 6-2	Ca^{2+}-channel/Dihydropyridine receptor
151	Clone 7-2	
152	Clone 11-2	Ribonucleoprotein (?)
153	Clone 12-2	Hypothetical membrane protein
154	Clone 13-2	Rrna-synthetase
155	Clone 15-2	Hypothetical *A. Thaliana* protein
156	Clone 16-2	Hypothetical *A. Thaliana* protein
157	Clone 17-2	
158	Clone 18-2	
159	Clone 10-3	Polycomb group (CLF, MEDEA,A-Z)
160	Clone 12-3	Isocitrate dehydrogenase ($NADP^+$)
161	Clone 13-3	
162	Clone 14-3	
163	Clone 15-3	Ca^+ dependent protein kinase *A. Thaliana*
164	Clone17-3	
165	Clone 18-3	
166	Clone 19-3	
167	Clone 20-3	
168	Clone 21-3	Protein kinase *A. Thaliana*
169	Clone 23-3	
170	Clone 25-3	Receptor protein kinase (Cf-2/LRR)
171	Clone 26-3	
172	Clone 28-3	Protein kinase *A. Thaliana*
173	Clone A48-1	Aldehyde dehydrogenase
174	Clone A49-1	rRNA-methylase
175	Clone A21-3	DNA binding (SPF, ABF, WRKY)
176	Clone A22-3	Flavonol 3-O-glucosyltransferase
177	Clone A29-3	Metal ion transporter
178	Clone A31-3	
179	Clone A36-3	Flavonol 3-O-glucosyltransferase

EST	Plasmid DNA	Homology
180	Clone A37-3	Mt phosphate transporter
181	Clone A39-3	Legumin/d-hand (?)
182	Clone A43-3	Transmembrane transporter
183	Clone A44-3	
184	Clone A7-3	Bacterial protein, Hox-A10
185	Clone A19-3	
186	Clone A27-3	Tyrosine protein kinase (?)
187	Clone A30-1	Pattern formation gene
188	Clone A32-1	Splicing factor Prp 8
189	Clone A33-1	DNA repair protein RAD 16
190	Clone A37-1	DNA repair protein RAD 16
191	Clone A39-1	MAP3-like protein kinase
192	Clone A40-1	Unkown protein, Cl^-. Channel
193	Clone A41-3	
194	Clone A45-1	Galactose kinase
195	Clone B6(10,2)	Seed imbibition protein
196	Clone B8(10,2)	Beta-D-glucosidase
197	Clone B9 (10,2)	CDPK-related kinase
198	Clone 13(10,2)	Sulfide-quinone reductase
199	Clone B17 (10,2)	Putative adenylate kinase
200	Clone B19 (10,2)	NADP-dependent oxido-reductase
201	Clone B39 (10,2)	Integral membrane protein (?)
202	Clone C12 (10,2)	Hypothetical protein
203	Clone D3 (10,2)	
204	Clone D7 (10,2)	
205	Clone D11 (10,2)	Seed imbibition protein
206	Clone D35 (10,2)	Pollen alergen/beta-expansin
207	TH 1/1	Serine threonin kinase (sorgum) (?)
208	TH 5/1	
209	TH 7/9	
210	TH 7/10	Gluthatione transferase (soybean)
211	TH 7/15	
212	TH 9	
213	TH 11/1	
214	TH 12/6	LEA, pcC 3-06 (*Cp)* (?)
215	TH 16/2	
216	TH 20/1	
217	TH 22/3	Putative zinc finger motive (?)
218	TH 23	
219	TH 25	S-adenosyl-methionine-sterol-C-methyltransferase
220	TH 34	Cyclophilin 40
221	TH 36	Wheat mytocondria 26S-rRNA
222	TH 37/1	
223	TH 38/1	Chaperonine, T-complex protein TCP
224	TH 39/1	Cotyledon CAP shape *A.th.* pos. transcription factor NAM
225	TH 40/1	Ribosomal protein *A.th.*
226	PTP	DNA binding protein
227	sHSP	Small Heat Shock Protein
228	TH 21	Hypothetical protein *A.th.*
229	TH 23	
230	TH 44	
231	TH 62	
232	TH 65	Hypothetical protein *A.th.*
233	TH 81	LEA type/embryogenic potential marker
234	TH 123	Low molecular heat shock protein 17 kDa (small)
235	EST 0020	GTP-binding beta subunit-like protein
236	EST 0039	PSI reaction centre protein subunit VI
237	EST 0044	PSII 33 kDa protein of the water oxidizing complex
238	EST 0046	Ras-related GTP-binding protein
239	EST 0052-1	Rubisco activase
240	EST 0052-2	Rubisco activase
241	EST 0053	Rubisco small subunit
242	EST 0066	
243	EST 0065	Sodium/dicarboxylate transporter
244	TH R1	Poly A binding protein
245	TH R2	
246	TH R3	Epoxide hydrolase
247	TH R4	Epoxide hydrolase
248	TH R5	
249	TH R6	
250	TH R7	
251	TH R8	LEA
252	TH R9	Dehydrin
253	TH R10	Putative protein
254	TH R11	
255	TH R12	
256	TH R13	Putative mine protein
257	TH R14	
258	TH R15	LEA (seeds rice and wheat)
259	TH R17	Ubiquitin

Table 3.2.1.1.1 *C. plantagineum* gene collection and homologies. Blank boxes represent clones to which no significant homologies were found. (?)- clones which showed low homology.

3.2.1.2 Construction of control DNA samples

The selection of the appropriate gene controls was a key step for the establishment of the array experiments. In order to identify differentially expressed genes, filters cannot usually be compared directly. Instead they need to be normalised to compensate for differences due to varying efficiencies of reverse transcription, probe purification, hybridisation, filter quality, etc. These errors should be detected and corrected by including certain controls.

In this study, the selected controls were: a) pBluescript: background control in order to assess non-specific hybridisation to the cloning vectors in which most of the ESTs were cloned. An excess of plasmid, was spotted onto the nylon membranes at two different concentrations, at positions O-18 and 0-20 (see figure 3.2.1.1). b) *pTA 71* cDNA: internal control. A ribosomal DNA fragment from a barley clone (Gerlach and Bedbrook, 1979) was spotted on the nylon membranes at two different concentrations, at positions L-18 and L-20, in order include an internal non-varying reference. A second internal control, a barley ubiquitin cDNA clone (Gausing and Barkardottir, 1986) was spotted at two different concentrations, positions L-9 and L-11. c) PLD-1 cDNA: internal control. The *C. plantagineum* PLD-1 cDNA was spotted at different concentrations, at positions L-2 and L-4 and L-6, in order include a third non-varying reference. The PLD-1 gene is constitutively expressed in fresh leaves and during the dehydration response in *C. plantagineum* (Frank *et al.*, 2000). d) *uidA* gene: internal control non-coded by plants. A fragment of the *uidA* gene (Blanco, 1987) was spotted at eight different concentrations, positions 0-2, 4, 6, 8, 10, 12, 14 and 16, in order to provide an internal quantification standard that will not vary between probes and permits a comparison between independent hybridisations (see section 3.2.2.1).

Other genes that served as controls for the dehydration treatment were included within the gene collection. These genes have been extensively characterised and their transcript accumulation pattern in response to dehydration has been well described, for example *pcC 6-19, pcC 27-45* or *pcC 11-24* (Bartels *et al.,* 1990; Piatkowski *et al.,* 1990; Velasco *et al.,* 1998). They belong to different *LEA* gene families and serve as an important control for induced genes.

3.2.2 mRNA Expression profiling

3.2.2.1 Development of a non-varying reference: poly A^+ *uidA* RNA

For normalisation of signals between independent filters, "housekeeping" genes are frequently used as internal references. However, comparing unknown states of gene expression, constancy of housekeeping gene expression cannot be assumed a priori. Furthermore, there are several reports in the literature describing housekeeping genes to be regulated (Savonet *et al.*, 1997; Bhatia *et al.*, 1994).

In order to circumvent these drawbacks of housekeeping genes as internal standards, a synthetic *uidA* RNA was synthesised as external reference to be added to the RNA pull prior to cDNA reverse transcription. Important criteria to be met by the normalisation standard was: a) being an RNA molecule, b) presence of an oligo(A) tail for selection with oligo-dT cellulose as primer binding site for reverse transcription, c) a sequence not related to plant sequences, d) the presence of hybridisation targets for the standards on the arrays, and e) an easy way of synthesis.

Consecuently, the corresponding *uidA* gene was included in the gene collection as a reference. In order to obtain a constant signal in the entire array, a known concentration of *uidA* RNA was added to each independent reverse transcription labelling reaction, spiked together with the plant poly A^+ enriched RNA used for each probe (see section 2.2.1.5.2).

The *uidA* poly$(A)^+$ RNA was generated from a *uidA* DNA fragment with an engineered poly$(A)^+$ stretch. The *uidA* DNA fragment that was constructed via the PCR, using the primers GUST7pgaf (forward) and GUSpolyAgpar (reverse). The T7 promoter was appended to the forward primer, and incorporated into the PCR product. This PCR primer contained an additional six bases upstream of the core T7 promoter sequence that were necessary for maximal transcription efficiency (Milligan *et al.*, 1987). The remainder of the primer corresponded to position 550-571 of the *uidA* sequence (Acc No.S69414) (see figure 3.2.2.1.1). The reverse primer, GUSpolyAgpar, contained a 12mer T tail and 23mer corresponding to position 781-803 of the *uidA* sequence. A PCR reaction with the following conditions was performed: 35 cycles, each cycle consisted of a denaturation step at 95°C for 1 minute, an annealing step at 58°C for 1 minute and an elongation step at 72°C for 45 seconds. Amplification of the target DNA yielded a PCR product of the expected fragment size (283 bp) (see figure 3.2.2.1.2).

The PCR product was composed of (i) the T7 DNA polymerase promoter sequence (ii) a 253 bp fragment of the *uidA* gene and (iii) a poly$(A)^+$ tail , as shown in the following figure:

Figure 3.2.2.1.1 Engineered *uidA* DNA gene fragment

T7 promoter

a) GUST7gpaf F 5'd[**TAATACGACTCACTATAG**GGAGGTGGACGATATCACC]3' 37 mer

GUSpolyAgpar R 5'd[TTTTTTTTTTTTCGAAGCGGGTAGATATCACACTC]3' 35 mer

b) PCR product:

5' coreT7 _____ *uidA* _____ AAAAAAAAAAAA 3'

Figure 3.2.2.1.1 Engineered *uidA* gene fragment. Panel a) a set of primers were designed: GUST7gpaf (F), which contained the T7 promoter sequence; and GUSpolyAgpar (R), which contained a poly T tail. Panel b) final product, the sense strand of the engineered *uidA* gene including the T7 promoter sequence and a poly(A)$^+$ tail.

In order to obtain the poly(A)$^+$ *uidA* RNA molecule, the 283 bp PCR product was used as a template in an *in vitro* transcription reaction (see section 2.2.2.2.5). The reaction occurred in the presence of ^{35}S-UTP, and after completion a 3 µL aliquot was directly loaded on an RNA agarose gel for RNA gel blot analysis to check the quality and integrity of the sample. To be able to determine the size of the reaction product, a transcript of a known size, 385 bp, was *in vitro* transcribed in parallel and included in the gel. The blot was exposed to a phosphor screen and, after imaging, a band with the predicted size, for the synthesised poly(A)$^+$ *uidA* RNA, was detected (see figure 3.2.2.1.2). A total of 3 µg of transcript were synthesised, this was determined after an aliquot of the reaction was TCA precipitated (see section 2.2.1.6).

Figure 3.2.2.1.2 *uidA* poly(A)$^+$ RNA *in vitro* product

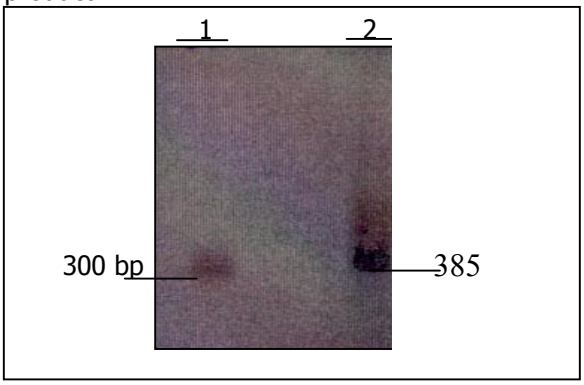


Figure 3.2.2.1.2 RNA gel blot analysis. The synthetic *uidA* transcript of approximately 283 bp was loaded on lane (1), and a transcript of 385 bp was included as a control in lane (2).

After this quality test, the sample was used for hybridisation of a nylon filter. To test whether the synthesised *uidA* poly(A) RNA could cross hybridise with the plant DNA present in the filters, a nylon array filter hybridisation was performed using 0.5 ng of the *uidA* cDNA as a probe. As a result, the *uidA* cDNA did not show cross hybridisation to any of the spotted plant DNAs or to the pBluescript background control.

Figure 3.2.2.1.3 Synthetic *uidA* cDNA, cross-hybridisation test

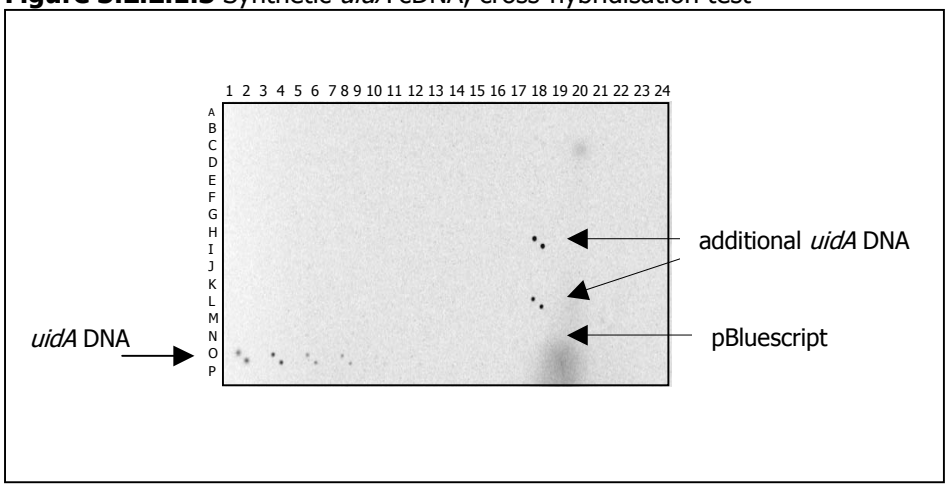


Figure 3.2.2.1.3 Cross-hybridisation test. A nylon array filter was hybridised to the synthetic *uidA* cDNA probe. The *uidA* gene was included in the filters at positions: O-2 to O-12, H-18 and L-18. The pBluescript was included on positions O-18 and O-20.

3.2.2.2 Linearity and sensitivity of the macroarray system

Preliminary hybridisations were performed in an attempt to determine the linearity and sensitivity of the nylon array system. An assessment was made by comparing the signals obtained from a series of hybridisations, performed with probes containing the synthetic *uidA* cDNA in increasing amounts, to the amount of *uidA* gene present in the filters. That is, four independent filters were hybridised with probes in a range from: 0.001, 0.01, 0.1 and 1% of *uidA* cDNA (in relation to the 500 ng of plant poly A^+ enriched RNA used in subsequent experiments). The corresponding *uidA* DNA clone was represented on the filters at known concentrations, ranging from 0.1 to 100 ng.

The linearity, amount of probe proportional to the amount of DNA in the filter, was assessed by plotting the amount of spiked *uidA* cDNA against the amount of DNA present in the

corresponding *uidA* spoted, (see figure 3.2.2.2.1). When the percentage of *uidA* cDNA was 1% the plotted values presented a polynomial behaviour with a correlation coefficient of R=0.99. That is, when the amount of *uidA* cDNAs in the probe was of 5 ng, inaccurate measurements were obtained for *uidA* DNAs spotted at concentrations ranging from 10-100 ng. Moreover, a levelling of the signal was observed for *uidA* DNA spots with concentrations higher than 50 ng. In those cases, the signal intensities may be underestimated.

When the percentage of *uidA* cDNA in the probe was 0.1%, the relation -amount of cDNA in the probe:amount of DNA in the spot- was not linear for those *uidA* DNA spotted at amounts higher than 50 ng. The plotted values presented a polynomial behaviour with a correlation coefficient R=0.98. That is, the detection system was more accurate when the amount of cDNA was of 0.5 ng only for those DNA spots within a range of 10 ng to 50 ng.

When the percentage of *uidA* cDNA in the probe was 0.01%, the resulting signal intensities were proportional to the amount of spotted *uidA* DNA and a linear relation was obtained with a correlation coefficient R=0.98. That is, the detection system was more accurate when the amount of *uidA* cDNA in the probe was of 0.05 ng and for DNA spots with a concentration range between 10 to 100 ng. When the percentage of *uidA* cDNA in the probe was 0.001%, no signal could be detected (not shown).

Figure 3.2.2.2.1 Array filter sensitivity and linearity determination

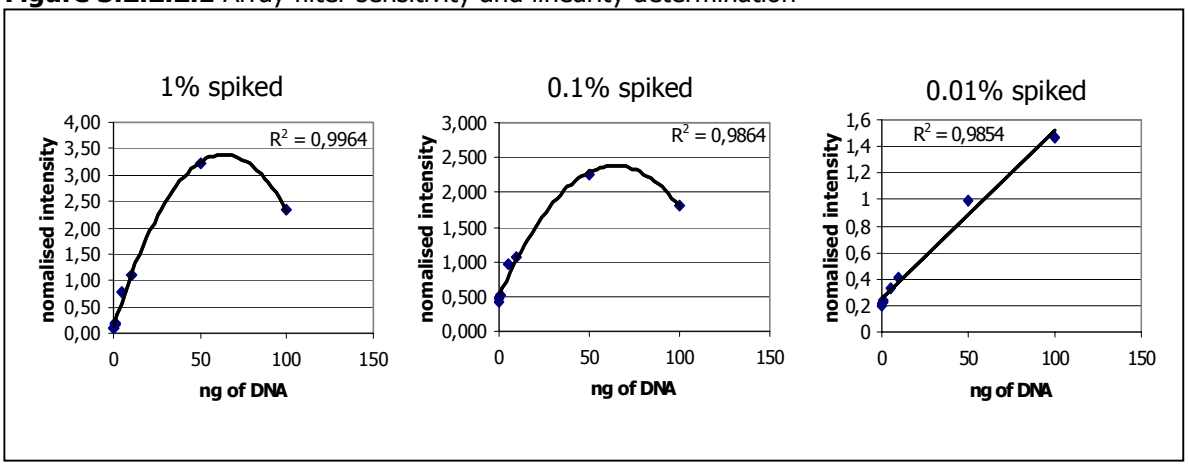


Figure 3.2.2.2.1 Determination of the system linearity and sensitivity. Different percentages of reverse transcribed *uidA* cDNA were used in filter array hybridisations. The amount (ng) of spotted *uidA* DNA were plotted against the normalised intensity of each spot after hybridisation with synthetic *uidA* cDNA.

The linearity range was found when 0.05 ng of cDNA were probed. Considering that the average efficiency of a reverse transcription reaction is of approximately 20-15% (previously determined), then 0.25 ng of *uidA* RNA should yield 0.05 ng of single stranded cDNA. This was the

amount of spiked *uidA* RNA spiked together with the plant RNA samples in each of the following reverse transcription reactions.

Based on estimates that the total number of average-sized transcripts ranges from 100,000 to 500,000 per cell in higher plants (Kamalay and Goldberg, 1980; Kiper *et al.,* 1979) and, given that the ratio (w/w) of control *uidA* cDNA probes to sample (plant) DNA probe were of 1000:100,000, 100:100,000, 10:100,000 and 1:100,000 [1000 X (1:100; 0.1:100; 0.01:100 and 0.001:100)]; then, this macroarray system will detect a range of 10 to 50 RNA copies per cell in an efficient manner, assuming that a similar number of RNA copies per cell are present in *C. plantagineum* cells.

In order to assess the minimal range of sensitivity, the signals obtained from a probe prepared from 72 hours dehydrated (totally dehydrated) material, were compared with the signal intensity obtained from the background control. The probe, 72 hours dehydrated leaves, was chosen so that it would induce a clear gene response and a combination of highly and low abundant transcripts would be present in the mRNA population. The confidence intervals were calculated and the normal distribution of the data plotted as shown in figure 3.2.2.2.2. Approximately 90% of the clones gave a significant hybridisation signal and 10% of the cDNAs showed signals below background values, which corresponded to low abundant mRNAs.

Figure 3.2.2.2.2 Normal distribution of the intensities after hybridisation with a labelled cDNA probe corresponding to the polyA$^+$ RNA population in 72 hours dehydrated leaves

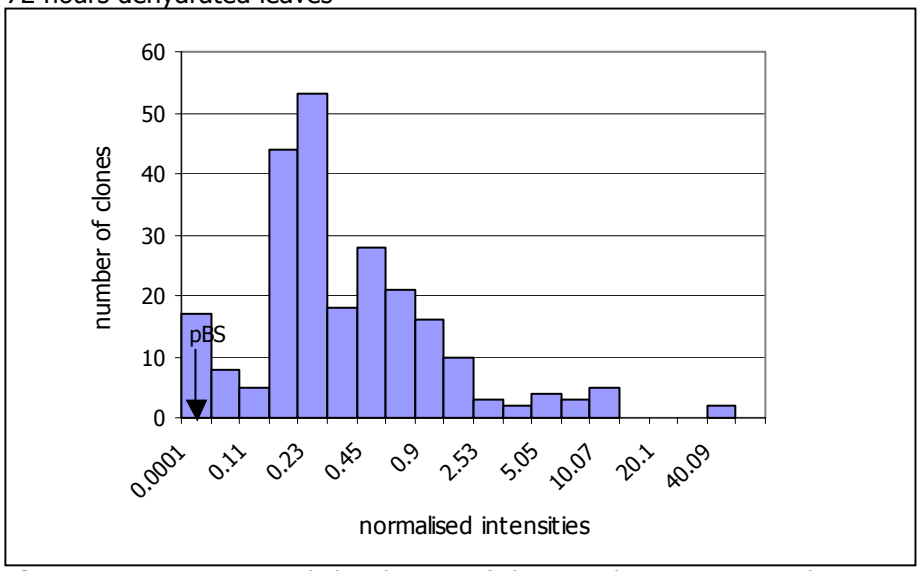


Figure 3.2.2.2.2 Normal distribution of the signal intensities. Probes were prepared from 72 hours dehydrated leaves. Filters contained 259 duplicated cDNA fragments. Average normalised hybridisation signals were plotted and the distribution of the average signals for the 259 clones shown. The arrow points to the average signal obtained for the pBluescript control clone.

3.2.2.3 Quantification of the dehydration and salt treatment transcript profiles

Expression data obtained from the different dehydration and sodium chloride treatments was compared and analysed quantitatively. Twenty-one independent filters were hybridised to twenty one independent probes in each set of experiments. Probes were prepared by reverse transcription in the presence of ^{33}P-dCTP and poly A$^+$ enriched RNA extracted from dehydrated and sodium chloride treated plants:

a) dehydration kinetic*:

1- fully hydrated
2- 2 h dehydration
3- 4 h dehydration
4- 6 h dehydration
5- 8 h dehydration
6- 12 h dehydration
7- 24 h dehydration
8- 48 h dehydration
9- 72 h dehydration

*(fully hydrated leaves correspond to 100% RWC and 72 h dehydrated leaves, or completely dehydrated leaves, to 5% RWC).

b) sodium chloride treatment kinetic:

1- 6 h. 0 mM NaCl
2- 6 h: 50 mM NaCl
3- 6 h: 100 mM NaCl
4- 6 h: 150 mM NaCl
5- 12 h: 0 mM NaCl
6- 12 h: 50 mM NaCl
7- 12 h: 100 mM NaCl
8- 12 h: 150 mM NaCl
9- 24 h: 0 mM NaCl
10- 24 h: 50 mM NaCl
11- 24 h: 100 mM NaCl
12- 24 h: 150 mM NaCl

Total, 21 cDNA probes.

A total of three repetitions of the macroarray experiments were performed. Set number 1 consisted on: 21 filters hybridised to cDNA reverse transcribed from RNA extracted from leaves treated as described before. Set number 2 consisted on: an identical repetition of set number 1, where independent filters (obtained from the same spotting experiment –see section 3.2.1- as the filters used in set number 1), and freshly labelled probes reverse transcribed from the same RNA stock. Set number 3 consisted on: 21 filters that were prepared from and independent spotting experiment, hybridised to cDNA reverse transcribed from RNAs extracted from an independent repetition of the dehydration and sodium chloride experiments.

These repetitions were performed in order to assess the reproducibility of the profiles, moreover, each filter contained duplicates of the 259 PCR fragments in order to have an indication of the reproducibility within the same experiment (see section 3.2.1.2). 0.25 ng of the synthetic *uidA* RNA control was included in each cDNA probe synthesis reaction (see section 3.2.2.2.2). The

temperature and duration of hybridisation, and washing conditions were broadly constant in all experiments. The amount of probe added (50 ng of first strand cDNA) was determined in each case by measuring the cDNA yield of each reaction (see section 2.2.1.6).

Figure 3.2.2.3.1a/b shows the hybridisation profiles of a set of 21 filters corresponding to the dehydration (9 filters) and salt treatment (12 filters) kinetics. The signals were detected using the PhosphorImager technology and the quantification of the signal intensity, corresponding to each cDNA, normalisation and the subtraction of the local background was carried out using ArrayVision (ARV) commercial software (see section 2.2.4.3). The quantification procedures were rapid, the analysis of a set of filters generally took no more than 60 minutes including the manual verification steps. Each signal was automatically corrected for variations in the amount of probe hybridised to the filters, by normalising the measured value with the average intensity of the non-varying reference control (the *uidA* gene fragment). The ARV quantification involved the following procedure:

1. A set of circles that defined where data would be gathered from the image were produced and called a data template. The data template definition (X,Y spacing) allowed all the circles to align with the spots. The same data template was then aligned with the spots that were present on the other filters.
2. In order to remove background signals, the option "user-defined regions" was taken from a set of defined spots corresponding to pBluescript. Background was reported for each spot, and then atomatically subtracted from the spot intensity value.
3. Each filter was normalised to a reference value calculated from the array, in this case the *uidA* gene fragment. To do this, these data points were programmed as a "reference" with the "user defined regions" option of the software. An average reference value was calculated for all of the spots in the reference regions. Data from every spot in the array was referenced to this single value.

Data was reported in a tabular form. Each column contained the characteristic data (e.g. EST names). The entire column set was exported directly to Microsoft Excel. The data obtained for each filter using ARV was expressed as Molecular Dynamics Counts (MDC) (see section 2.2.4.2), which are arbitrary units reflecting the amount of pixels located in each spot. These values represented the total amount of hybridisation signal being emitted from an element, minus the non-specific hybridisation signal. The substracted volume, SV was given by: SV = volume – background, where volume = density X area. Therefore the volume value reflects the total amount of hybridisation signal being emitted from a spot or element. Finally, normalised Volumes (nVolume), to the reference clone, were directly obtained from the quantification of the signals.

Figure 3.2.2.3.1a Nylon filter arrays after hybridisation with cDNAs corresponding to the dehydration treatment kinetic

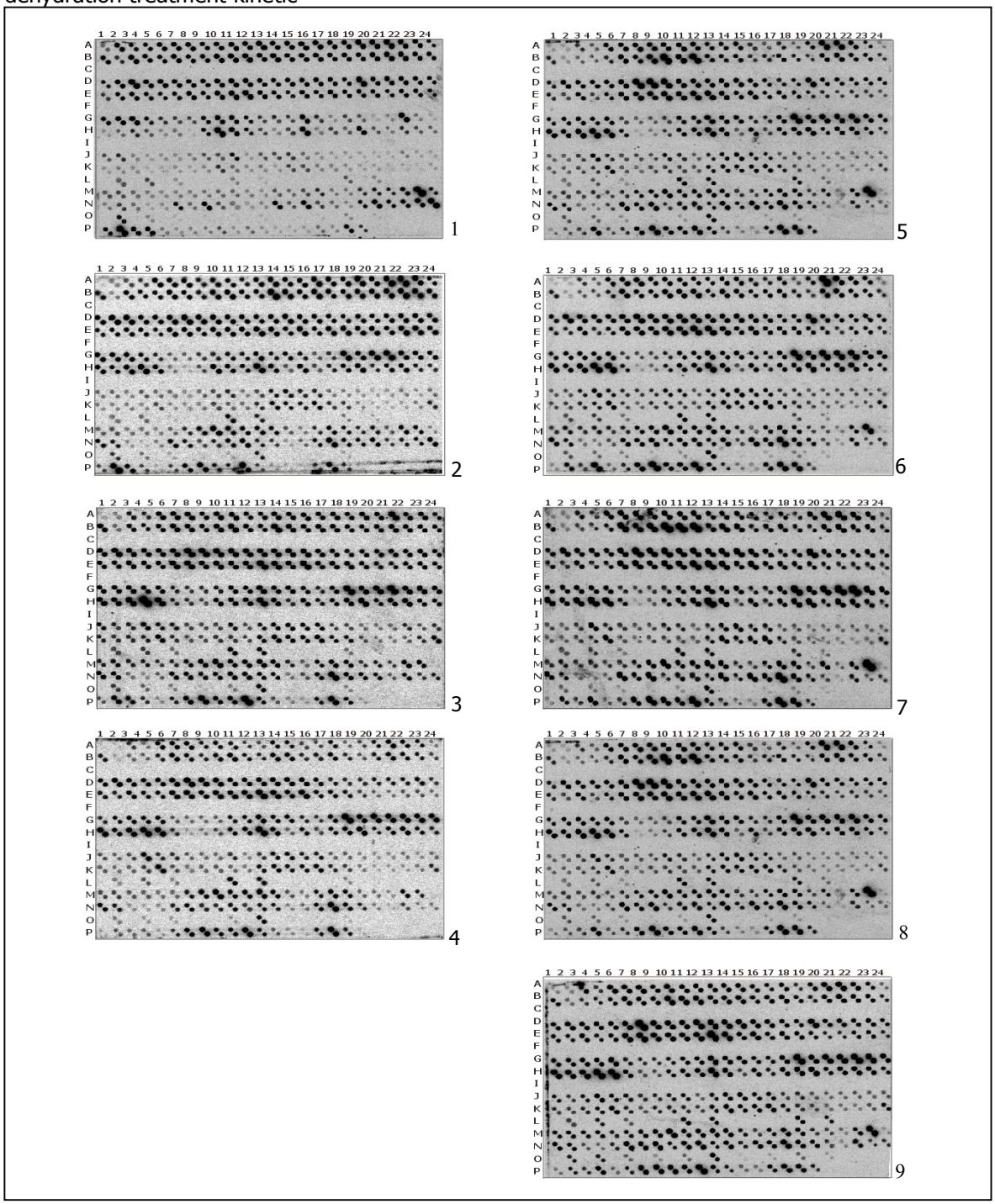


Figure 3.2.2.3.1a Nylon filter arrays hybridised with radiolabelled first strand cDNA reverse transcribed from poly A^+ enriched RNA extracted from fully hydrated leaves (1), and leaves dehydrated for: 2 h (2), 4 h (3), 6 h (4), 8 h (5), 12 h (6), 24 h (7), 48 h (8) and 72 h (9).

Figure 3.2.2.3.1b Nylon filter arrays after hybridisation with cDNAs corresponding to the sodium chloride treatment kinetic

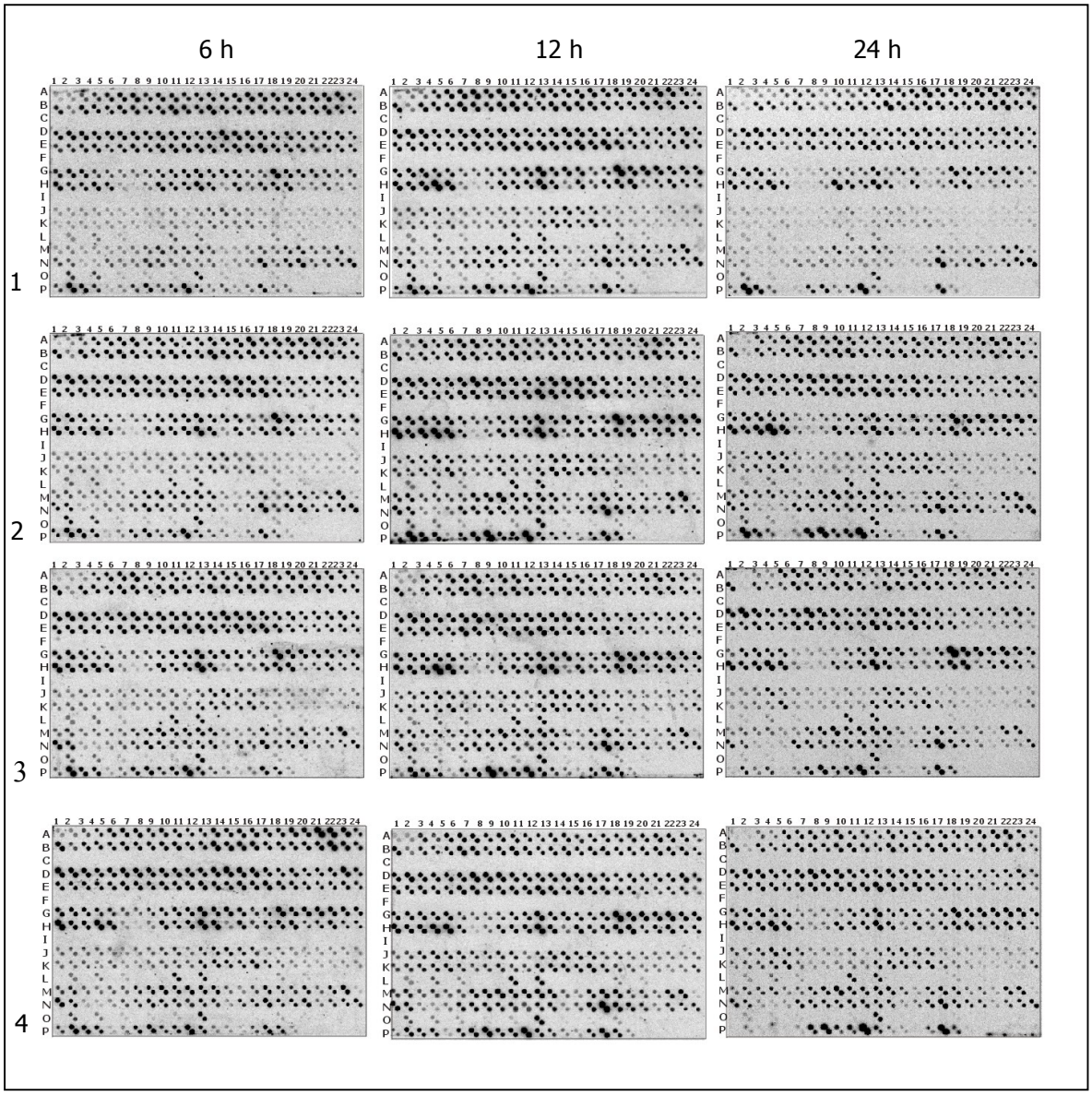


Figure 3.2.2.3.1b Nylon filter arrays hybridised with radiolabelled first strand cDNA reverse transcribed from poly A^+ enriched RNA extracted from sodium chloride treated leaves with increasing concentrations: 0 mM (1), 50 mM (2), 100 mM (3) and 150 mM (4), for 6, 12 and 24 hours.

To assess the reproducibility of the method, hybridisation signals obtained for the repeats of each double spot on the same filter were compared by plotting the normalised intensity of the spot repetition 1 against the normalised intensity of the spot repetition 2 in a log / log plot (an

example is shown in figure 3.2.2.3.2). The signal intensities obtained for the three sets of experiments, were analysed with log/log plots. The signals were reproducible, except for a small number of clones (between two and five clones per experiment, representing between the 1 to 1.6% of the ESTs present on the filter) that corresponded to low abundant mRNA.

Figure 3.2.2.3.2 Reproducibility of the system assed by comparing double spots

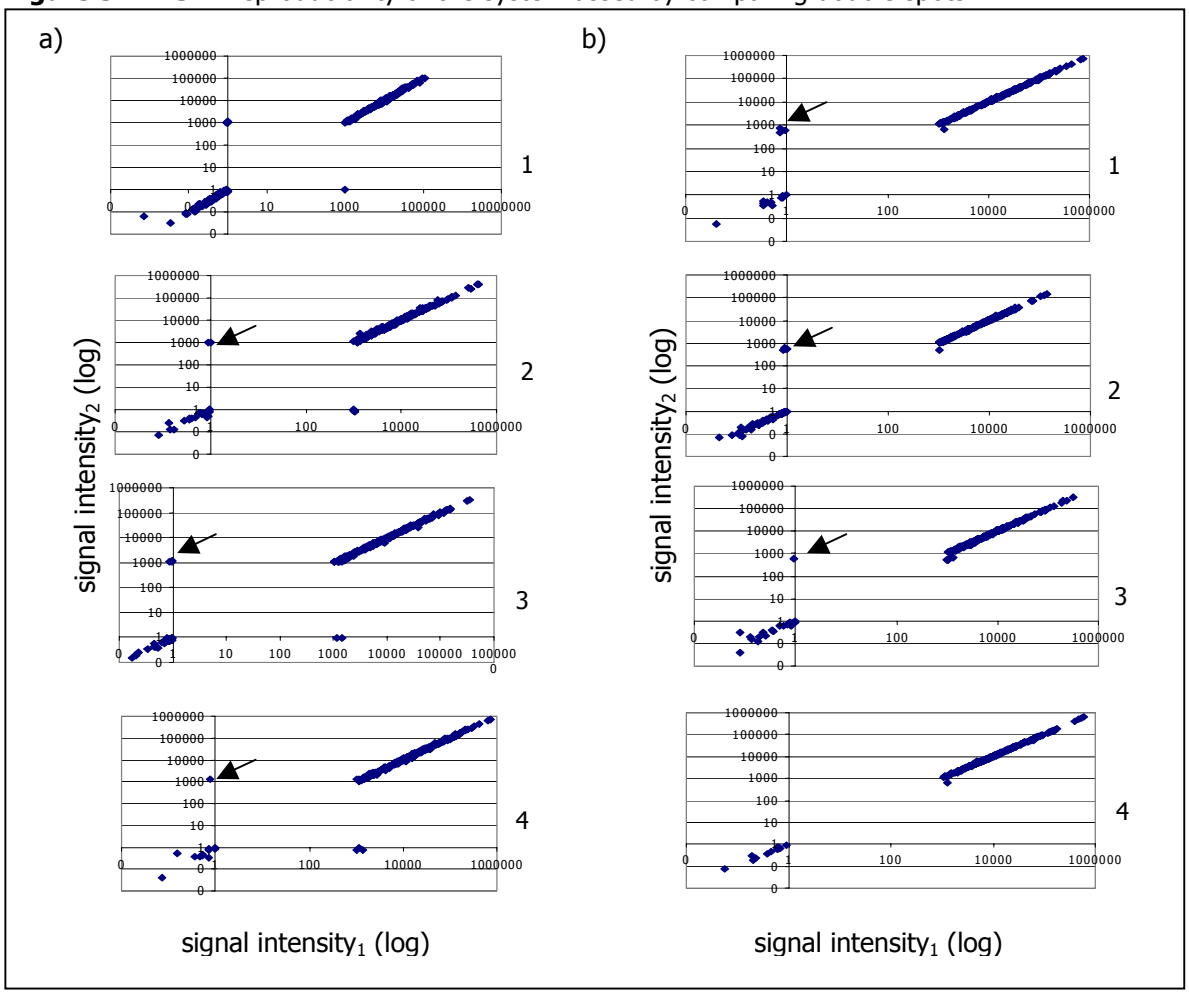


Figure 3.2.2.3.2 Reproducibility of the nylon array system. A set of filters were evaluated which are representative of one set of experiments. Double spots were compared in log / log plots of the obtained signal intensities corresponding to each DNA present in each of the filters. Panel a): filters hybridised to cDNAs corresponding to, fully hydrated (1), 6 h (2), 12 h (3) and 24 h (4) dehydrated leaves. Panel b): filters hybridised to cDNA probes corresponding to 12 h sodium chloride treated leaves with increasing concentrations: 0 mM (1), 50 mM (2), 100 mM (3) and 150 mM (4). For a small number of DNAs a variation was observed between the repeat values (double spots) as a result of hybridisation artefacts, one example per experiment was highlighted by arrows.

3.2.2.4 Analyses of transcript profiles in response to dehydration and sodium chloride treatment

In order to compare the steady state transcript levels following the different treatments, basic statistics were applied to the data obtained from the macroarray experiments which were organised in a database using Microsoft Excel (commercial software). The analysis used to compare induction or repression of a certain transcript included the ratio and the difference scores.

Taken by themselves, ratio and difference scores can be misleading. The basic problem with ratio scores (i.e. ratio = signal intensity of sample / signal intensity of the control), is that they do not take the magnitude of the differences into account and the basic problem with difference scores is that they do not take relative differences into account (i.e. difference = signal intensity of the sample – signal intensity of the control). For example, in the case of a sample that exhibits a hybridisation signal of 5 in the control image and 10 in the data image, and a second sample whose respective signals are 5,000 and 10,000. Both samples exhibit a two-fold increase in expression over control values. However, more confidence may be attributed to the ratio score from the second sample, because the difference between the control and data values is much greater than that observed in the first. Again, in the case of a sample that gives a hybridisation signal of 1,000 in the control image and 2000 in the data image, and a second sample whose respective signals are 9,000 and 10000. Both samples exhibit a 1,000-unit increase in signal over control values. However, more confidence is attributed to the difference score of the first sample, because the ratio of the control and data values is higher than that observed in the second. One way to overcome this problem was to organise the hybridisation signals based on a combination of ratio scores and difference or *z* scores (Bulyk *et al.,* 1999). An analysis report was created in the Microsoft Excel worksheet by the application of basic statistical formulations.

The following relations were loaded on an Excel worksheet:

a) up-regulation:

Ratio: $$\text{Ratio}_k = \frac{\text{signal intensity sample } \kappa}{\text{signal intensity control}}$$

z score: $$z_k = \frac{\left(\Delta_k - \Delta mean\right)}{SD_\Delta}$$

where:

Δ_k=(signal intensity sample *k*) – (signal intensity control)

$$\Delta mean = \frac{\sum_{k=1}^{n} \Delta_k}{n}$$

$$SD_\Delta = \sqrt{\frac{\sum_{k=1}^{n} (\Delta_k - \Delta mean)^2}{n}}$$

with k=1 …..n corresponding to each of the features(data) on the array

b) down-regulation:

Ratio: $\text{Ratio}_k = \frac{\text{signal intensity control}}{\text{signal intensity sample } \kappa}$

z score (as above), substituting the difference value with:

Δ_k=(signal intensity control) – (signal intensity sample k)

The average of each double value (stored in the database) was loaded in a worksheet and the statistical relations a) and b) applied to the data. The output was given automatically in a separate column in the worksheet that contained the name of the corresponding gene and its location in the filter. If the R_k >2 and Z_k score>0 in relation a), the transcripts were considered to be up-regulated. Transcripts were considered down-regulated if R_k >2 and Zscore<-0.15 in the relation b). The range of values used to discriminate between up-regulation or down-regulation, proved to be suitable for these system, the output obtained for transcripts whose expression profiles are known and well characterised were as expected. For example, transcripts corresponding to the *pcC 6-19* or *pcC 27-45* genes were classified as up-regulated by dehydration and transcripts corresponding to the *PIPa.2* gene were classified as down-regulated by sodium chloride after the application of relations a) and b) respectively. Overall statistical analysis of the treatments permitted the identification of a number of gene expression patterns: 1. transcripts up-regulated, 2. down-regulated, or 3. responsive to both dehydration and salt treatments. The percentage of transcripts in one set of experiments, that were either up-regulated or down-regulated are summarised in table 3.2.2.4.1.

Table 3.2.2.4.1 Percentage of transcripts regulated by the different treatments

transcript accumulation*	dehydration	sodium chloride	dehydration and sodium chloride	up-dehydration down-salt	up-salt down-dehydration
up-regulated	5.6%	16.3%	23.8%		
down-regulated	5.4%	8.4%	11.2%		
				19.1%	10.3%

*Transcript profiles obtained from the statistical analysis of one experiment set.

A graphical view represented in the following Venn diagram:

Figure 3.2.2.4.1 Transcripts were organised according to their expression profile in response to dehydration and sodium chloride treatments

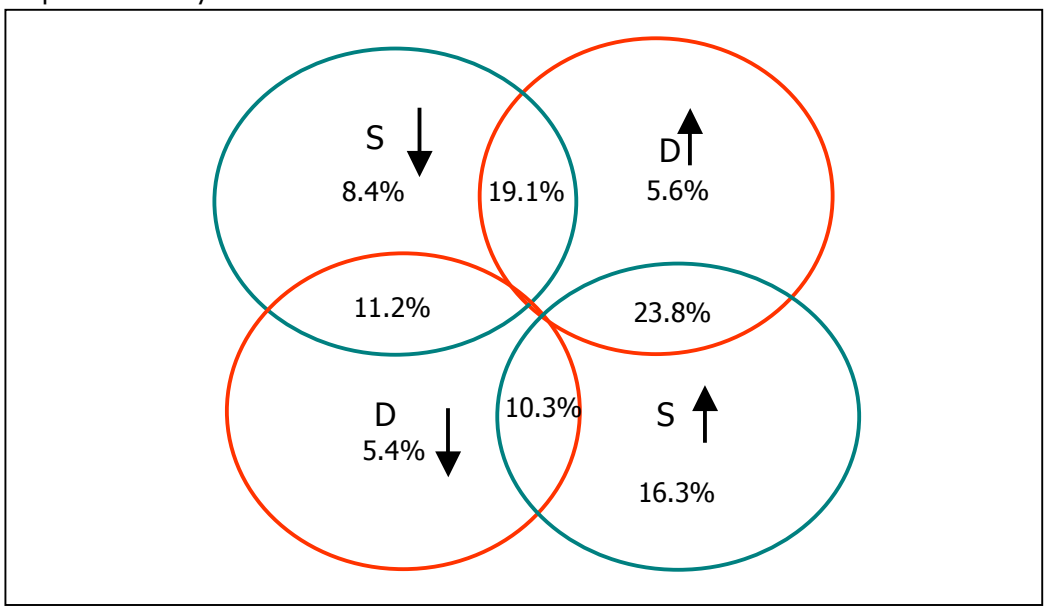


Figure 3.2.2.4.1 A Venn diagram of shared and specifically differentially expressed cDNA fragments for all treatments analysed. The percentage of transcripts that were up-regulated ↑, down-regulated ↓, or both in response to dehydration and sodium chloride are presented.

LEA genes were distributed among the group of transcripts that were up-regulated by dehydration and sodium chloride. Macroarray analysis confirmed that transcripts encoding proteins with a photosynthetic function are down-regulated by dehydration. Genes whose transcript profiles showed specific up-regulation in response to dehydration were also identified and examples of candidate genes are listed in the following tables:

Table 3.2.2.4.2 Candidate genes that are up-regulated by dehydration only

Gene identification*	**Homology**
EST 0011	Cystein protease
EST 0061	Water stress induced protein
EST 0103	Unknown
Clone 2-2	Unknown
Clone 23-3	Unknown
Clone 13-2	rRna synthethase
TH 7/10	Gluthatione transferase (soybean)

*These clones were selected from three independent macroarray repetitions.

Table 3.2.2.4.3 Candidate genes up-regulated by dehydration, down-regulated by sodium chloride

Gene identification*	**Homology**
EST 0095	Unknown
EST 0096	Unknown
EST 0120	Unknown
DD 104/5	Unknown
CpPIP-a1	Water channel protein (PIP)
CpPIP-a2	Water channel protein (PIP)
CpPIP-a3	Water channel protein (PIP)
CpPIP-a7	Water channel protein (PIP)
CpPIP-a8	Water channel protein (PIP)
CpPIP-b	Water channel protein (PIP)
CpPIP-c	Water channel protein (PIP)
Clone 4-1	NADP dependent oxidoreductase
Clone 4-2	Aldehyde dehydrogenase
Clone 5-2	N-Acetylglucosaminiyltransferase III
Clone 6-2	Ca^{2+}-channel/dihydropyridine receptor
Clone 12-2	Ribonucleoprotein (?)

Gene identification	**Homology**
Clone 10-3	Polycomb group (CLF, MEDEA, A-Z)
Clone 12-3	Isocitrate dehydrogenase ($NADP^+$)
Clone 13-3	Unknown
Clone 14-3	Unknown
Clone 15-3	Ca^+ dependent protein kinase *A. Thaliana*
Clone 18-3	unknown
Clone 21-3	Protein kinase *A. thaliana*
Clone 31-3	unknown
Clone 36-3	Flavonol 3-O-glucosyltransferase
Clone 37-1	DNA repair protein RAD 16
Clone B6	Seed imbibition protein
Clone B9	CDPK-related kinase
Clone B39	Integral membrane protein (?)
Clone A39-1	MAP3-like protein kinase
TH44	Unknown

*These clones were selected from three independent macroarray repetitions.

Two previously characterised cDNAs were chosen to test the reliability of these results. RNA gel blot experiments were performed with the same poly A^+ enriched RNA material as the one used in the macroarray hybridisations. One class of transcripts were up-regulated by sodium chloride and dehydration treatment (e.g. the *LEA* gene *pcC 27-45*). Another class of transcripts were down regulated by sodium chloride but early responsive to dehydration (e.g. the aquaporin gene *PIPa.2*). An example of both classes of gene expression is presented in figure 3.2.2.4.2.

Figure 3.2.2.4.2 RNA gel blot analysis to confirm the nylon array profiles

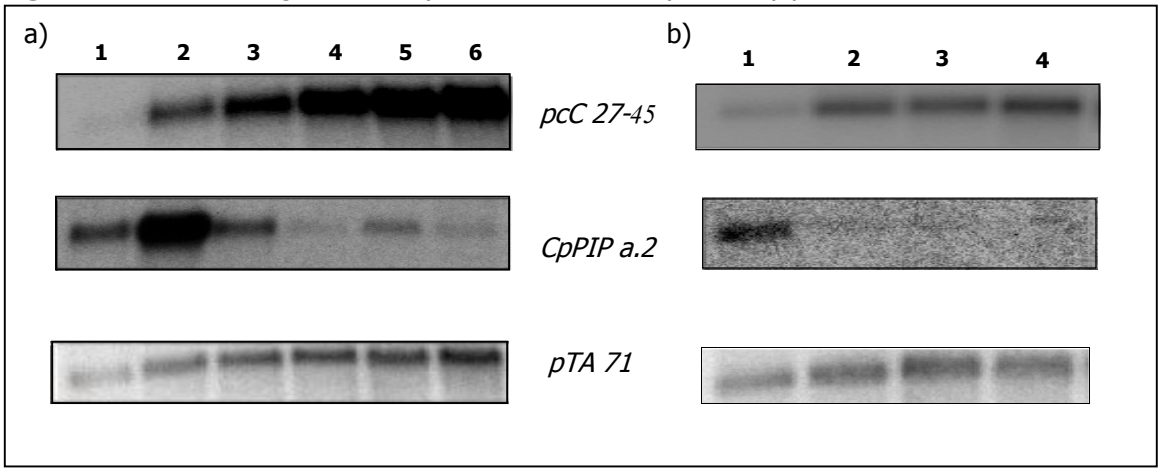


Figure 3.2.2.4.2 RNA gel blot analysis to confirm the array profiles.
3 μg of poly A^+ enriched RNA extracted from *C. plantagineum* leaves corresponding to different treatments, were separated on a denaturing agarose gel. Panel a) fully hydrated leaves (1), 2 h (2), 4 h (3), 8 h (4), 12 h (5) and 24 h (6) dehydrated leaves, and panel b) 24 hours of sodium chloride treatment at four different concentrations: 0 mM (1), 50 mM (2), 100 mM (3) and 150 mM (4). The resulting RNA blots were hybridised with a *LEA* cDNA clone insert *pcC 27-45* and a cDNA clone insert corresponding to a member of an aquaporin gene family, *PIP a.2* gene. The ribosomal *pTA 71* insert was used to asses the loading of the RNA.

In order to compare the expression data in more detail, the similarities between transcript profiles were measured using the clustering, and visualised using the tree view software (see section 2.2.11.3/4; Eisen *et al.*, 1998). The basis of this program for organising gene expression data is to group together genes with similar patterns of expression by using standard statistical algorithms. The first step to this end is to adopt a mathematical description of similarity (based on the Pearson's correlation; Eisen *et al.,* 1998). For any series of measurements, a number of measures of similarity in the behaviour of two genes can be used. In this case, as there was prior knowledge of some of the transcripts, the unsupervised method (cluster software) was chosen. A learning method is considered unsupervised if it learns in the absence of a teacher signal. Unsupervised gene

expression analysis methods begin with a definition of similarity (or a measure of distance, based on the Pearson's correlation) between expression patterns, but with no prior knowledge of the true functional classes of the genes (Brown *et al.,* 2000). Genes are then grouped by using a clustering algorithm such as hierarchical clustering. The average-linkage cluster analysis was applied to the gene expression data recorded. This method is a form of hierarchical clustering, similar to the methods used for sequence and phylogenetic analysis. Relationships among genes are represented by a tree whose branch lengths (node values) reflect the degree of similarity between the objects as assessed by a pairwise similarity function (based on the Pearson's correlation). Node values closer to 1 being the most similar and equal to 1 identical. The graphical representation of the primary data is represented with a colour that quantitatively and qualitatively reflects the original experimental observations. A dendogram is appended to the coloured table to indicate the nature of the computed relationship among genes in the table.

After independently loading the normalised volume obtained from the sodium chloride and dehydration treatments into the clustering software, a graphical output was obtained that showed the clustering and the underlying expression data. The same was done for the data obtained from the three repetitions of the macroarray experiments. Figure 3.2.2.4.3, a and b, shows the tree view output of the 259 transcript profiles in response to dehydration or sodium chloride treatment of *C. plantagineum* leaves for one of the three repetitions.

Using any ordering, the primary data table (which contain the quantified volumes of each transcript) is represented graphically by colouring each cell on the basis of the measured value. No changes were coloured black, increasingly positive log values with reds of increasing intensity, and increasing negative log values with greens of increasing intensity. The application of the hierarchical clustering organised each cluster by placing the most different patterns at the most distal proximity of each group.

In the cases where duplicate genes were included, similar values were obtained. For example, the cDNA clone corresponding to the *LEA pcC 6-19* was spotted at two different locations within the filters and the resulting transcript profiles found to cluster next to, or in the immediate vicinity, of each other. This same pattern was observed for both the dehydration and the sodium chloride treatment and for the three sets of experiments. In addition, hybridisation of different macroarrays with the same mRNA samples gave broadly the same results, although variations were found when mRNA samples from independent sets of treatments were used, specially for low abundant transcripts (i.e. transcription factors).

Figure 3.2.2.4.3a Cluster analysis of the dehydration and sodium chloride treatment data set

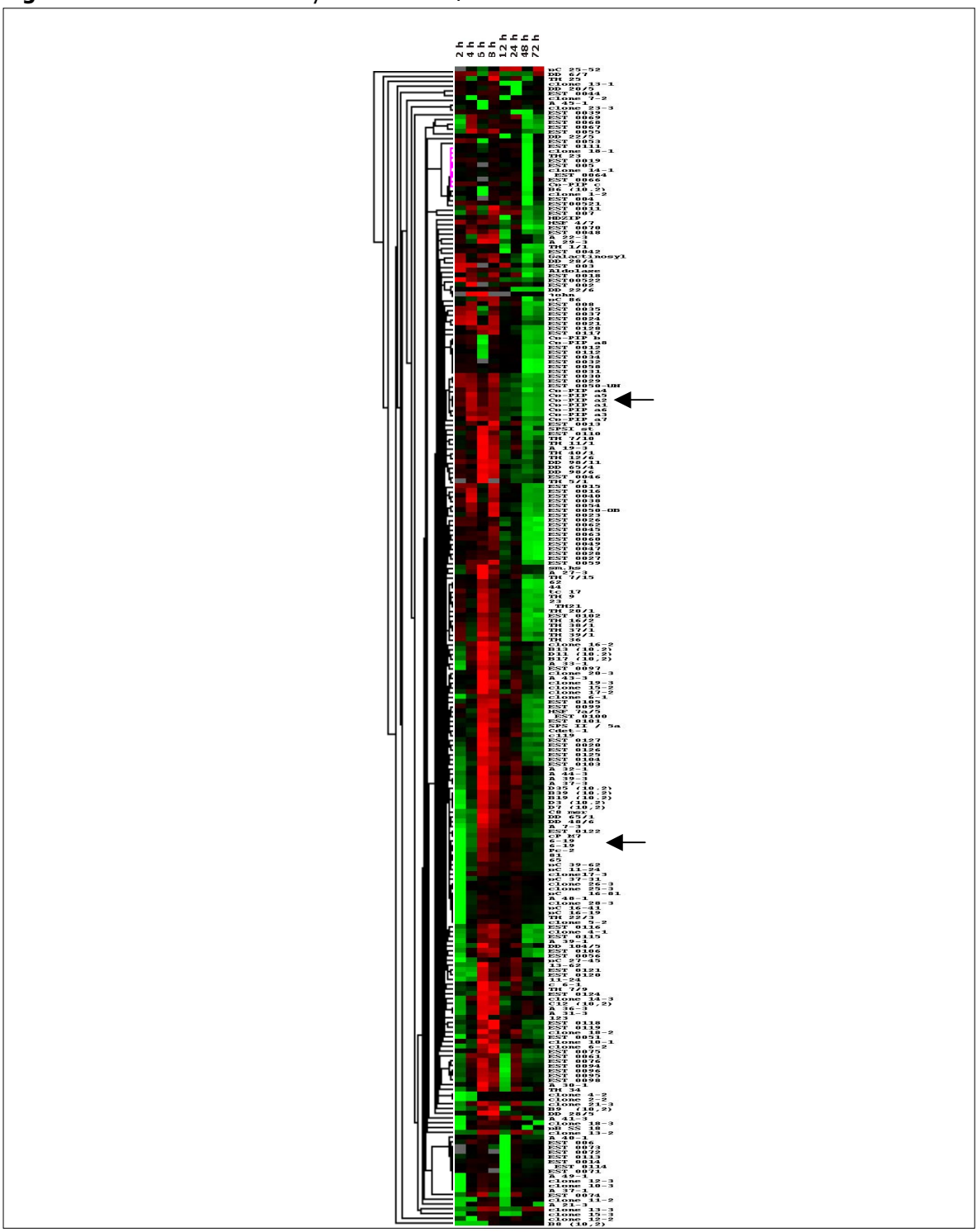


Figure 3.2.2.4.3a Transcript profiles of dehydrated leaves, were compared and clustered according to their degree of similarity using the cluster and tree view software (Eisen *et al.,* 1998). Color code: black - no change, reds of increasing intensities, increasingly positive values; greens of increasing intensity, increasingly

negative values. The arrow points to the clustering of the two repetitions of the *pcC 6-19* and to the *CpPIPa.2* clone.

Figure 3.2.2.4.3b Cluster analysis of the sodium chloride treatment data set

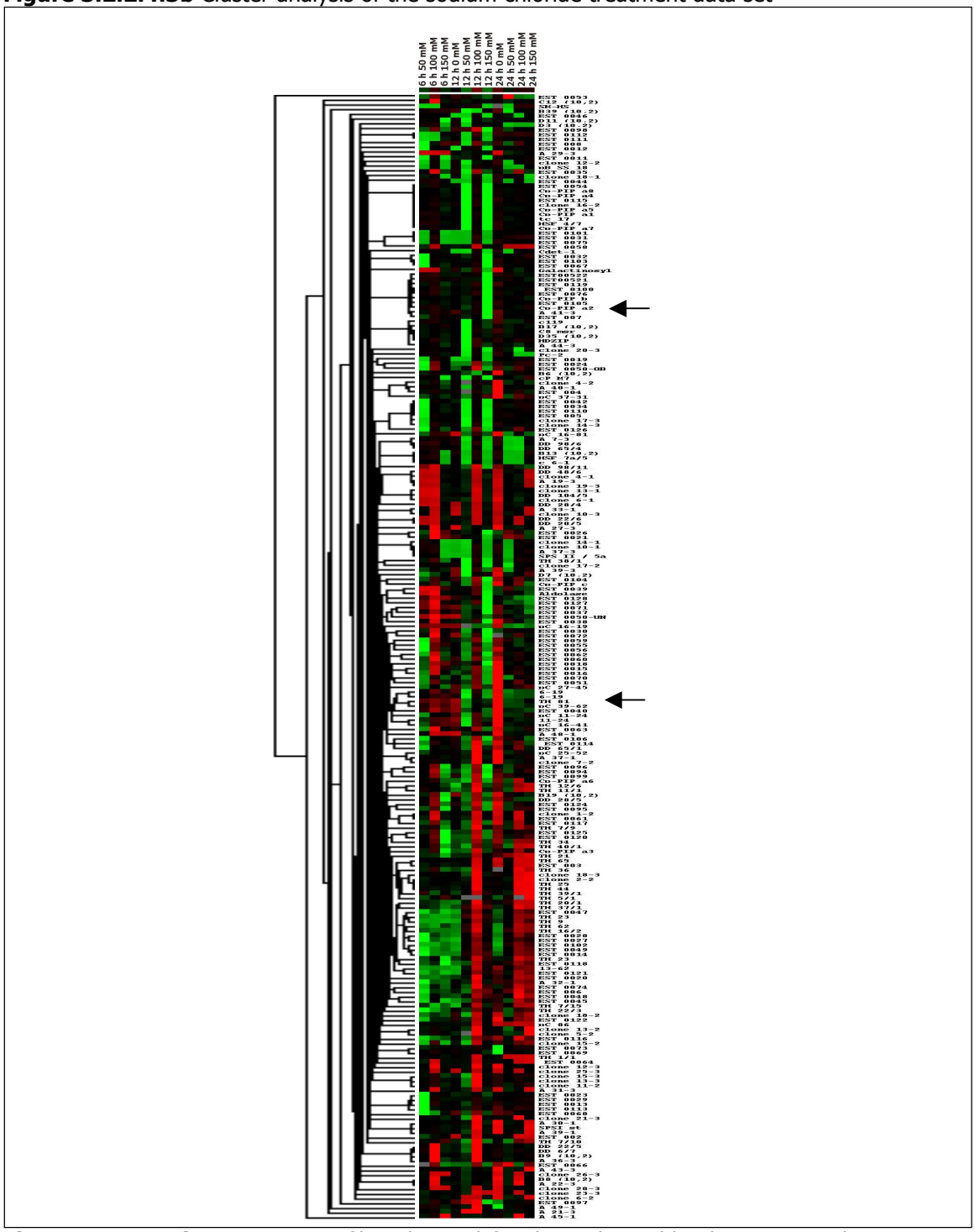


Figure 3.2.2.4.3b Transcript profiles obtained for the sodium chloride treatment kinetic, were compared and clustered according to their degree of similarity using the cluster and tree view software (Eisen *et al.,* 1998). Color code: blak - no change, reds of increasing intensities,

increasingly positive values; greens of increasing intensity, increasingly negative values. The arrow points to the clustering of the two repetitions of the *pcC 6-19* and to the *CpPIPa.2* clone.

A natural way of viewing the complex data set displayed in figure 3.2.2.4.3 is first to scan and survey the large-scale features and then to focus on the interesting details. The time-course analysis of gene expression revealed groups of genes with similar behaviour. A group of transcripts that showed an interesting behaviour were those corresponding to the *LEA* genes. In this case, the normalised intensities of all *LEA* transcripts included in the macroarrays were documented and loaded into the cluster program in an independent run (see figure 3.2.2.4.4).

From figure 3.2.2.4.4 it can be observed that the *LEA* genes tend to share a similar pattern of transcript accumulation, high accumulation at the early stages of dehydration and maintaining the levels for the duration of the treatment. The clone *TH81,* showed homology to *LEA* genes and its transcript was accumulated from the early stages of dehydration and maintained until complete dryness. The *clone 6-1* showed sequence homology to *LEA* genes and it was found to accumulate at the early stages of dehydration but accumulated at low levels at the point of complete dryness. A similar situation was true for the *LEA* genes *pcC 13-62* and the *pcC 86,* whose transcripts were accumulated at high levels at the early stages of dehydration but were not maintained until complete dryness. However, these genes clustered with a different pattern in response to sodium chloride treatment.

Figure 3.2.2.4.4 Cluster analysis of *LEA* gene transcripts in response to dehydration and sodium chloride treatments

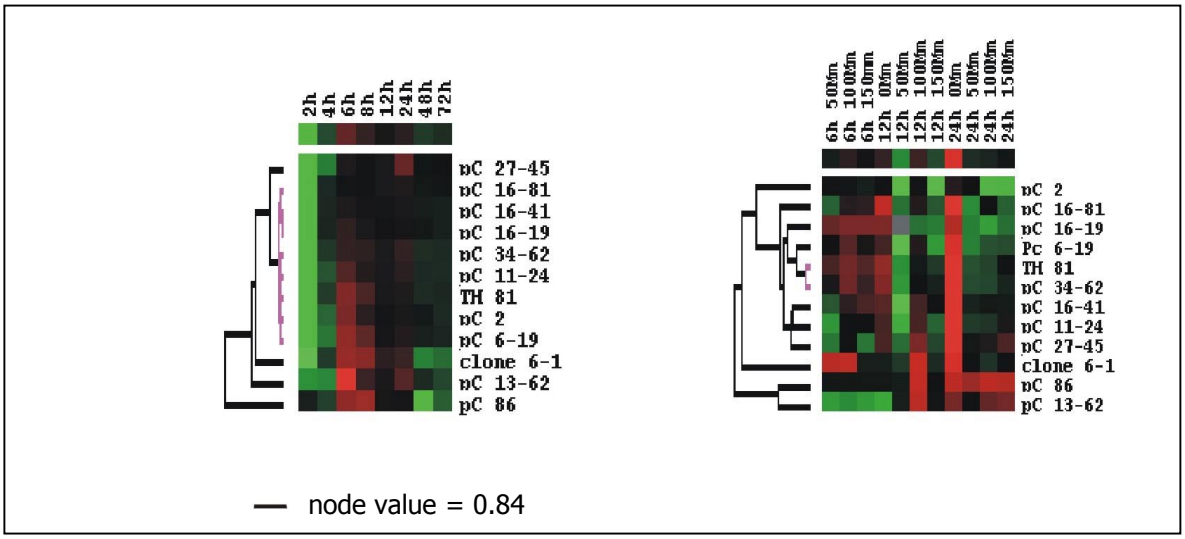


Figure 3.2.2.4.4 Cluster analysis of *LEA* gene transcripts in response to dehydration and sodium chloride treatments. The tree branches reflect the degree of similarity between transcript profiles. The bar shows the similarity value (node value).

Although it was observed that most of the transcripts accumulated in response to the sodium chloride treatment (see Venn diagram), only *LEAs pcC 34-62* and *TH81* clustered with values comparable to the highest similarity value found in response to dehydration. A second most-similar-cluster comprised *LEAs pcC 16-41, pcC 11-24* and *pcC 27-45*, although their profiles were less similar than the one they presented in response to dehydration. The remaining *LEA*s did not present significant similarities with each other.

3.2.2.5 Nylon array transcript profiles of *LEA* related genes were confirmed by RNA gel blot analysis

Transcript profiles were confirmed by RNA get blot assays. Based on the cluster results ten *LEA* genes were chosen that showed similar accumulation pattern in response to dehydration. In table 3.2.2.5.1, the different *LEA* genes, their homologies and in some cases their corresponding gene family (in brackets), are listed.

Table 3.2.2.5.1. LEA genes included in the *C. plantagineum* nylon filter arrays

Name	Homology	Reference
pcC 6-19	*rab 16* (rice) [G2]	Mundy and Chua, 1988
pcC 86	*rab 17* (maize) [G2]	Vilardell *et al.*, 1990
pcC 27-45	LEA 14 (cotton) [G4]	Galau *et al.,* 1993
pcC 11-24	LTI 65 *rd29* (*A. thaliana*)	Nordin *et al.,* 1993 Yamaguchi-Shinozaki and Shinozaki, 1993
pcC 34-62	LEA D7 (cotton) [G3] pLEA 76 (brassica)	Baker *et al.,* 1988 Harada *et al.*, 1989
pcC 13-62	*A. thaliana*	Bartels, unpublished
pcC 16-19	LEA	Bartels, unpublished
pcC 16-41	LEA	Bartels, unpublished
pcC 16-81	LEA	Bartels, unpublished
pcC 2	LEA	Bartels, unpublished
TH81	LEA type	Hilbricht, unpublished
clone c6-1	LEA type [G3]	Kirch, unpublished

Poly A^+ enriched RNA samples were extracted from fully hydrated, dehydrated and sodium chloride treated leaves - identical to those used in the nylon array experiments (see section 3.2.2.3). RNA blots comprised a complete set of dehydrated and sodium chloride treated samples. Blots were

consecutively hybridised with the *LEA* cDNA inserts. One set of filters was obtained that contained the complete dehydration kinetic and one set of filters corresponding to 6, 12 and 24 hours of sodium chloride treatment (figure 3.2.2.5.1a/b) (only the *LEA pcC 27-45* was hybridised to three independent filters, each containing a sodium chloride treatment for 6, 12 or 24 hours, respectively). A final hybridisation with the ribosomal DNA clone, *pTA 71*, was used to monitor equal loading of the RNA. The resulting hybridisations were analysed using the PhosphorImager technology and the volume quantification of the signals determined (see section 2.2.5.2). The transcript accumulation observed for each treatment was normalised to the value obtained from the corresponding loading control. After this, the sample/control ratio was calculated for each transcript and plotted.

Most of the *LEA* genes probed gave rise to at least 12 and up to several 1,000 fold increase in transcript accumulation in the dehydrated samples in comparison to the fully hydrated control. RNA levels were broadly constant during the later stages of dehydration (see figure 3.2.2.5.1a). The same genes were hybridised to filters containing the sodium chloride kinetic (see figure 3.2.2.2.5.1b). Three main patterns of accumulation were observed in response to increasing concentrations of sodium chloride, 1.- transcripts that were up-regulated in response to increases in sodium chloride concentrations, (i.e. *pcC 11-24, pcC 27-45 and pcC 16-41)*; 2.-transcripts that show minor ratios of accumulation upon increasing concentrations of sodium chloride in comparison to their corresponding non-treated control (i.e. *pcC 16-81*). And 3.- transcripts that showed higher accumulation ratios in response to higher sodium chloride concentrations, for the longest treatment periods (i.e. *pcC 13-62, pcC 2*). The ratios of induction varied between clones for most of the treatments, although the ratios of induction were not comparable to those found in response to dehydration. The most similar profiles under the sodium chloride treatment were found for the LEA genes *pcC 34-62* and *TH81,* that is, at early stages of the treatment, their transcripts accumulated in a ratio of 1.5 fold times upon increasing concentrations of sodium chloride. At later stages of the treatment, the accumulation ratio was of approximately 2 fold times and maintained in a similar ratio for higher concentrations of sodium chloride.

Figure 3.2.2.5.1a RNA gel blot assay for dehydrated *C. plantagineum* leaves

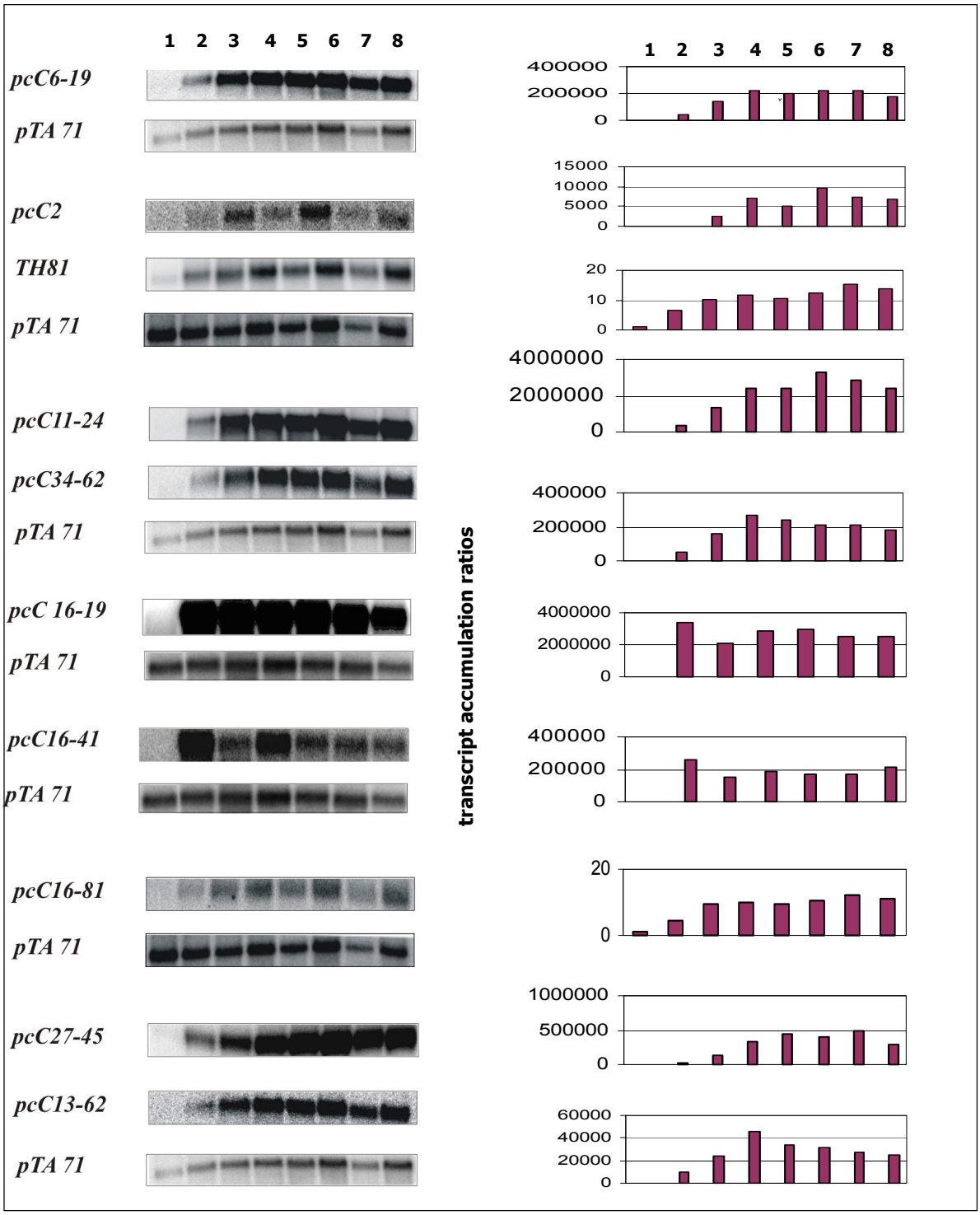


Figure 3.2.2.5.1a RNA gel blot assay using different LEA transcripts. 3µg of poly A^+ enriched RNA were extracted from *C. plantagineum* leaves submitted to dehydration. Fully hydrated leaves (1), and dehydrated leaves for a period of: 2 h (2), 4 h (3), 8 h (4), 12 h (5), 24 h (6), 48 h (7) and 72 h (8). The plots represent the accumulation ratio for each clone, normalised to the hybridisation performed with the ribosomal *pTA 71* fragment.

Figure 3.2.2.5.1b RNA gel blot assay for sodium chloride treated *C. plantagineum* leaves

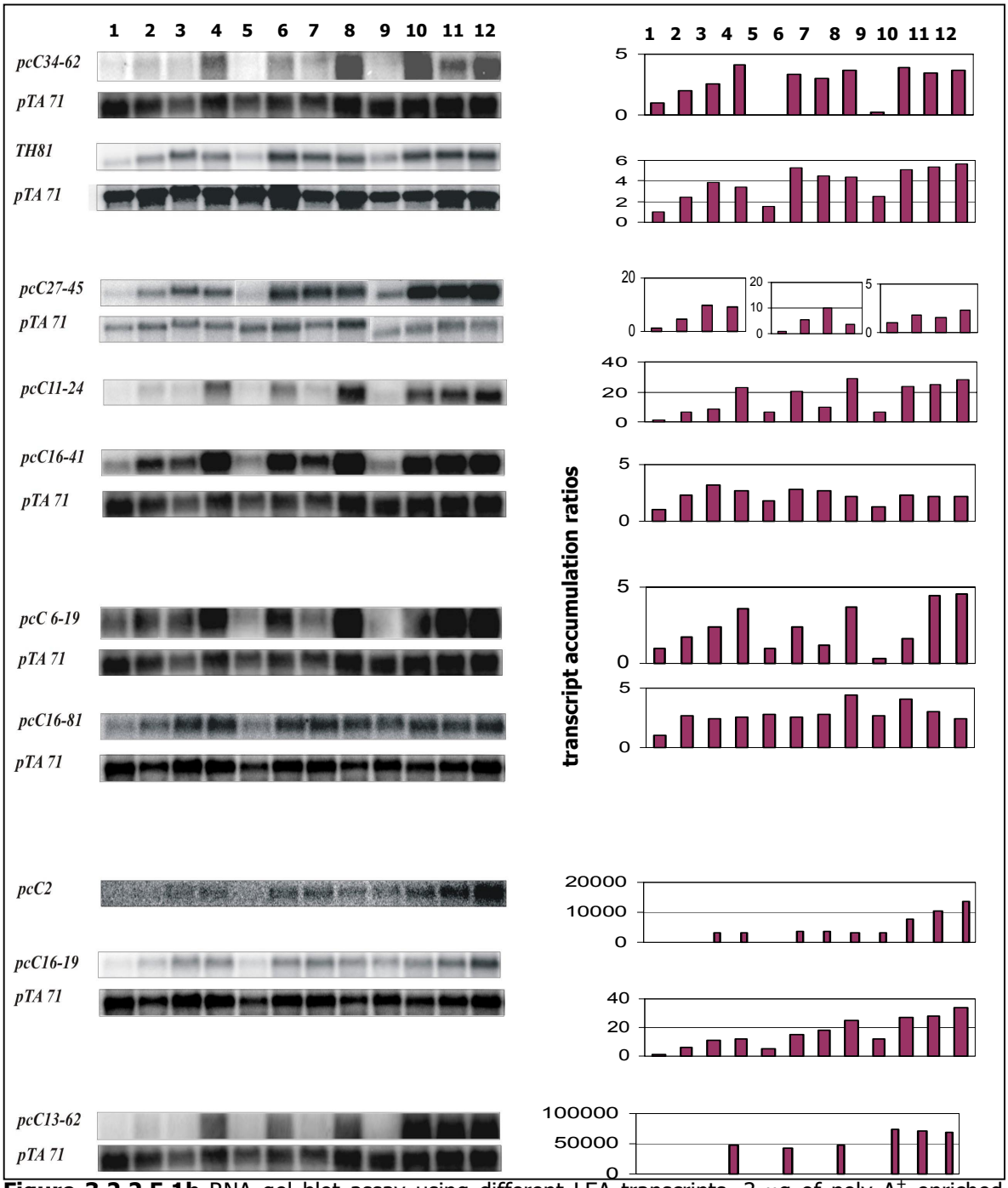


Figure 3.2.2.5.1b RNA gel blot assay using different LEA transcripts. 3 µg of poly A^+ enriched RNA was extracted from *C. plantagineum* leaves submitted to sodium chloride treatment for a period of 6 h: 0 mM (1), 50 mM (2), 100 mM (3), 150 mM (4); 12 h : 0 mM (5), 50 mM (6), 100 mM (7), 150 mM (8); and 24 h : 0 mM (9), 50 mM (10), 100 mM (11), 150 mM (12). The plots represent the accumulation ratio for each clone, normalised to the hybridisation performed with the ribosomal *pTA 71* fragment.

IV Identification and analysis of *C. plantagineum* dominant mutants affected in ABA mediated desiccation tolerance

The acquisition of desiccation tolerance in *C. plantagineum* is mediated by ABA. This theory is supported by two observations: firstly, a comparison of dried and fully hydrated tissues showed that many new transcripts and proteins accumulated during drying, and many of these molecules also appeared when ABA was applied to non-stressed tissues. Secondly, ABA is required at the callus stage in order to acquire desiccation tolerance. Dried, non-ABA treated callus is not desiccation tolerant (Bartels *et al,* 1990). This observation suggests that a number of ABA mediated pathways that lead to the acquisition of desiccation tolerance are not solely activated by dehydration at the callus level. The aim of the present study was to dissect dehydration induced signalling pathways by ectopically activating genes downstream of the ABA signal in order to generate desiccation tolerant callus.

The objective of the study was to create dominant mutants of *C. plantagineum* by gene activation tagging. For this a T-DNA mediated stable transformation of *C. plantagineum* was used (Furini *et al.,* 1994) in combination with activation tagging vectors (Koncz *et al.,* 1994). T-DNA activation tagging is based on the insertion of a strong promoter upstream of a gene, such that its transcriptional activity will be affected resulting in a gain of function of the tagged gene. By implementing a rigorous screening strategy, in this case the ability of callus tissue to survive desiccation without prior ABA treatment, informative mutants can be isolated. Rescuing and sequencing of the flanking DNA will subsequently identify genes that may play a role in ABA signalling. Molecular characterisation of the mutants will then provide more information about the function of the tagged locus.

4.1 Wild type *C. plantagineum* callus require ABA pre-treatment in order to acquire desiccation tolerance

C. plantagineum callus tissue does not survive desiccation and, in order to obtain tolerance, a four day ABA treatment prior to desiccation is necessary (see figure 4.1.1 and table 4.1.1). This phenomena was used as a positive control on the screening for dominant mutants involved in the ABA signalling.

Desiccation tolerance in callus was assed by comparing the survival rate of pre-ABA treated to non-treated callus. After four days of pre-treatment ABA induced the production of flavonoids, this gave rise to a reddish colour observed on the callus (see figure 4.1.1b) (Furini *et al.*, 1997). After desiccation of both, non- and pre-ABA treated callus (see section 2.2.3.3), the clusters were transferred to fresh MS media and checked for their ability to recover after one to two weeks. None of the desiccated non pre-ABA treated wild**-**type callus was able to recover and after 20 days they appeared completely necrotic (see figure 4.1.1c).

Figure 4.1.1 Desiccation tolerance screening in callus tissue

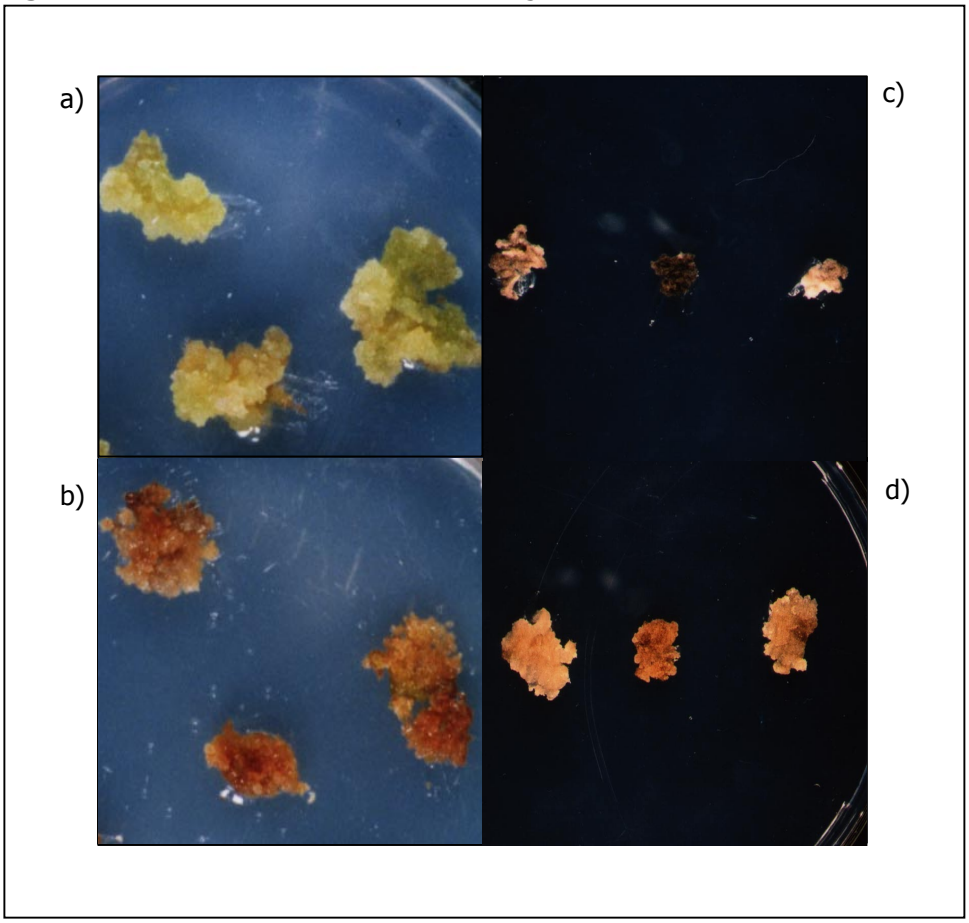


Figure 4.1.1 4 d ABA pre-treatment of wild type *C. plantagineum* callus tissue results in desiccation tolerance. Panel a) callus maintained under standard growth conditions, b) callus after 4 d pre-treatment with 1 E-4 M of ABA. After 24 hours of dehydration, the clusters were transferred to fresh MS plates and left to recover for two weeks, panel c) callus after dehydration-rehydration and d) 4 d ABA pre-treated calli after dehydration-rehydration treatment.

In contrast, the pre-ABA treated callus showed signs of recovery after 24 hours of rehydration (see figure 4.1.1d). Full recovery was observed within one week following rehydration, that is, regained volume and re-started cell division. Two weeks after rehydration the callus began to proliferate at a normal rate.

A summary of the callus survival data is presented in the table 4.1.1:

Table 4.1.1 Number of survivors upon 24 hours of dehydration treatment

tissue	**number survivors***	**% of survivors** (20 days after of recovery)
wt callus	0 out of 60	0%
wt pre-ABA treated callus	29 out of 50	58%

*This results represent the average of four independent experiments.

4.2 T-DNA activation tagging /Creation of dominant mutants

4.2.1 Description of the Plant Cloning Vectors (PCVs)

Two different PCVs were chosen in order to create a T-DNA activation tagged *C. plantagineum* population. The vectors are named pPCVTAc1 and pPCVRN4 (see figure 4.2.1.1; Koncz *et al*, 1994) and were selected on the basis of their potential to activate the expression of genes once the T-DNA has been inserted into the plant genome. In both cases the T-DNA carries between the left and right borders a plant selectable marker (*HPT*-hygromycin resistance gene), multiple cloning sites, a segment of the plasmid pBR322 containing the ColE1 origin of replication (ori_{pBR}), and a β-lactamase gene providing ampicillin and carbenicillin resistance (Ap^R/Cb^R) for selection in *E. coli* and *A. tumefaciens*. The pPCVTAc1 vector contains the Cauliflower Mosaic Virus (CaMV) 35S promoter (Odell *et al.,* 1985) carrying 4 repeats of the -90 to –440 enhancer domain positioned at the right border of the T-DNA. pPCVRN4 is identical to pPCVTAc1, but does not contain the +1 to -90 CaMV 35S promoter region. Following T-DNA insertion it is expected that genes near the insertion site can be transcriptionally activated resulting in a dominant mutation, this allows the direct selection of specific phenotypes from a population of primary transformants. The reason for using both binary PCVs is that transcriptional activation resulting from the insertion of the pPCVTAc1

vector may result in improved gene activation since it contains four copies of the CaMV 35S transcriptional enhancer and the CaMV35S promoter

Figure 4.2.1.1 Plant cloning vectors (PCVs)for activation tagging

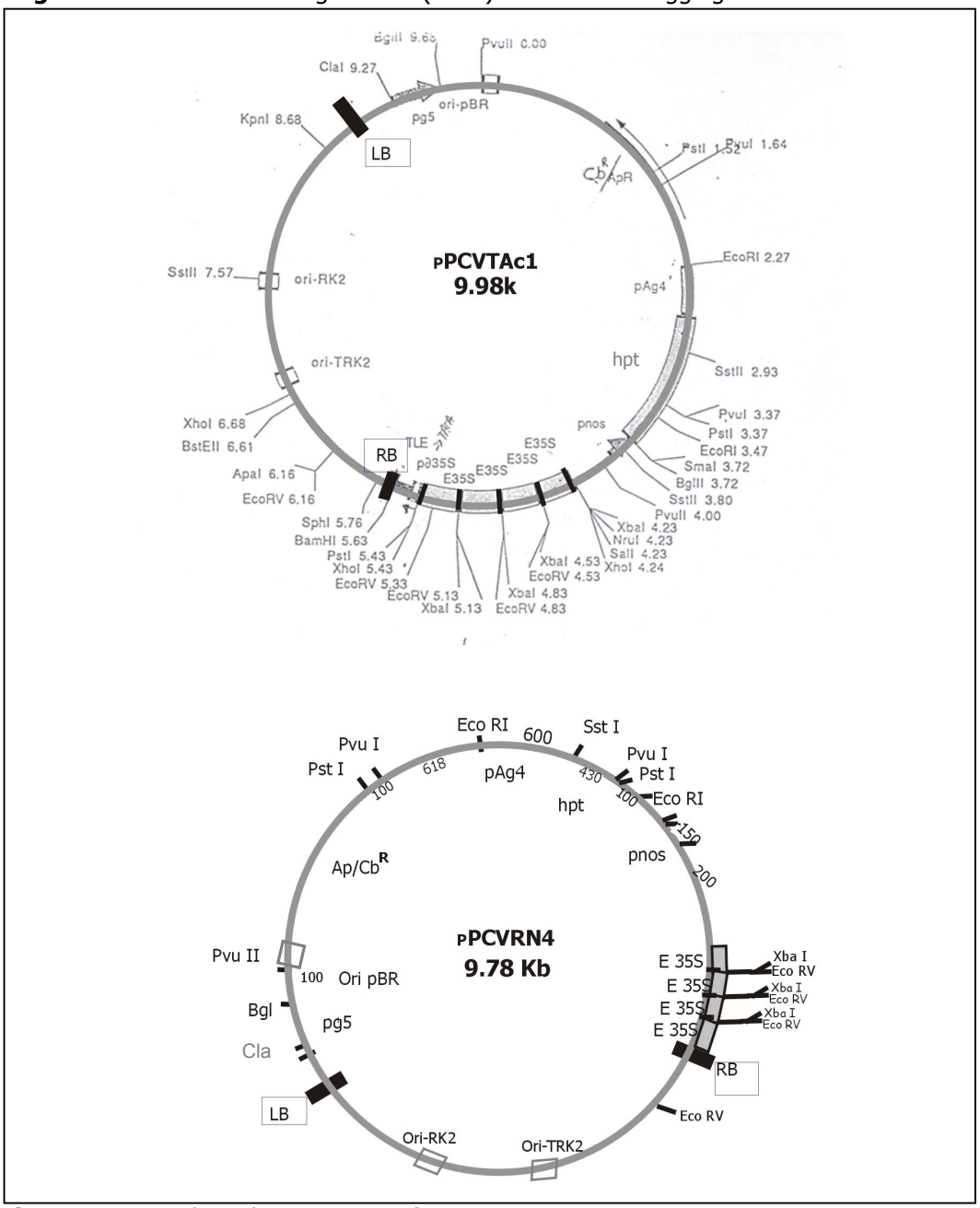


Figure 4.2.1.1 Plant cloning vectors for activation tagging.
Abbreviations: (E 35S)- CaMV 35S transcriptional enhancer; (*hpt*)- hygromycin resistance gene marker; (Ap/Cb^R) – ampicillin and carbenicillin resistance gene; (LB)-left border; (RB)-right border; (p35S) – CaMV 35S promoter.

4.2.2 Transformation of *C. plantagineum*

A prerequisite for creating the T-DNA activation tagged mutant population was the establishment of an efficient transformation system for *C. plantagineum*. Following a strategy based on that described by Furini *et al.*, (1994) (see table 4.2.2.1) a series of *C. plantagineum A. tumefaciens* -mediated transformations were performed. In this way, engineered T-DNA with activation tagging properties (see section 4.2.1) was inserted into the plant genome.

The transformation protocol initially involved wounding young leaves and co-cultivating the wounded explants with the appropriate *A. tumefaciens* strain. After co-cultivation, the infected explants were subcultured in media that stops bacterial growth and promotes the growth of transformed plant tissue. Approximately nine weeks after following leaf infection, a proliferation of hygromycin resistant callus was observed from at least three different locations in each leaf explant. The tissue exhibited a light green appearance, and was compact with a slightly nodular surface. The individual callus clusters were subcultured at 3-week intervals after culture initiation in order to maintain each potential transformation event.

Although most of the callus clusters turned brown at the contact point with the medium, these calli could be subcultured and maintained viable. Three months after transformation the callus clusters became friable and could not be distinguished from wild type callus. It was evident, however, that hygromycin inhibited formation of non-transformed callus tissue. However, identification of transformants on the basis of antibiotic resistance was performed during the plant regeneration process, since "escapes" from the callus stage failed to form roots in the presence of hygromycin.

The frequency of callus formation was influenced largely by the age of the explant, the degree of wounding and the presence of the antioxidant mixture. Leaf explants longer than 1 cm developed callus only at the base of the petiole and usually this callus became necrotic within 3-4 weeks. Younger leaf explants gave a higher frequency of callus initiation. Callus development was observed on more than 50% of the cultured leaf explants. *C. plantagineum* transformation required extensive wounding of the leaf surface to enhance infection. When explants were not damaged by scalpel or by treatment with sand paper, they responded poorly to the tissue culture conditions. Because of the high amount of phenolic compounds produced from wounded *C. plantagineum* tissue, it was necessary to add the antioxidant mixture to the callus induction and maintenance media as well as to the *A. tumefaciens* suspension culture used for leaf inoculation. In the absence of the antioxidant, the explants released phenolic substances whose oxidation products darkened both tissue and medium. In such conditions the explants died within a few days.

Table 4.2.2.1 Media used and time scale of the experiment

Procedure	Medium	time	Light/Dark
1. leaf explant infection	MSAR	20'	dark
2. co-cultivation	MSAR1	2 d	dark
3. callus initiation	MSAR1 + 15 mg/L hygromycin[1] + 500 mg/L cefotaxime[2]	2-9 weeks	Light[3]
4. callus maintenance	as above	3 week intervals	light
5. shoot differentiation	MSAR1a	4-5 weeks	light
6. root formation	MS	2-3 weeks	light
7. plant development	MS	2-6 months	light
8. plant maturation	granules soaked with water or soil	2-3 months	light

After approximately six months of continuous subculture, a total of approximately 6400 independent hygromycin resistant callus clusters were obtained. The calli were proliferated for a period of one to two months before the first desiccation screen was performed. In order to confirm whether the calli were indeed transformed, 8 random samples were selected and subjected to a PCR test using specific primers that were designed to amplify a 1.5 kb hygromycin resistance gene and a 0.5 kb fragment of the carbenicillin resistance gene (see section 2.1.5). The result of this experiment was that PCR products of the predicted size (2 kb) were amplified in 7 out of the 8 callus clusters (see figure 4.2.2.1).

[1] hygromycin was maintained at constant concentration during the culture *in vitro*.
[2] at each subsequent subculture step the cefotaxime was reduced by 100 mg/L
[3] light intensity was maintained at 200 $\mu E/m^2*s$

Figure 4.2.2.1 PCR test to confirm the frequency of callus transformation.

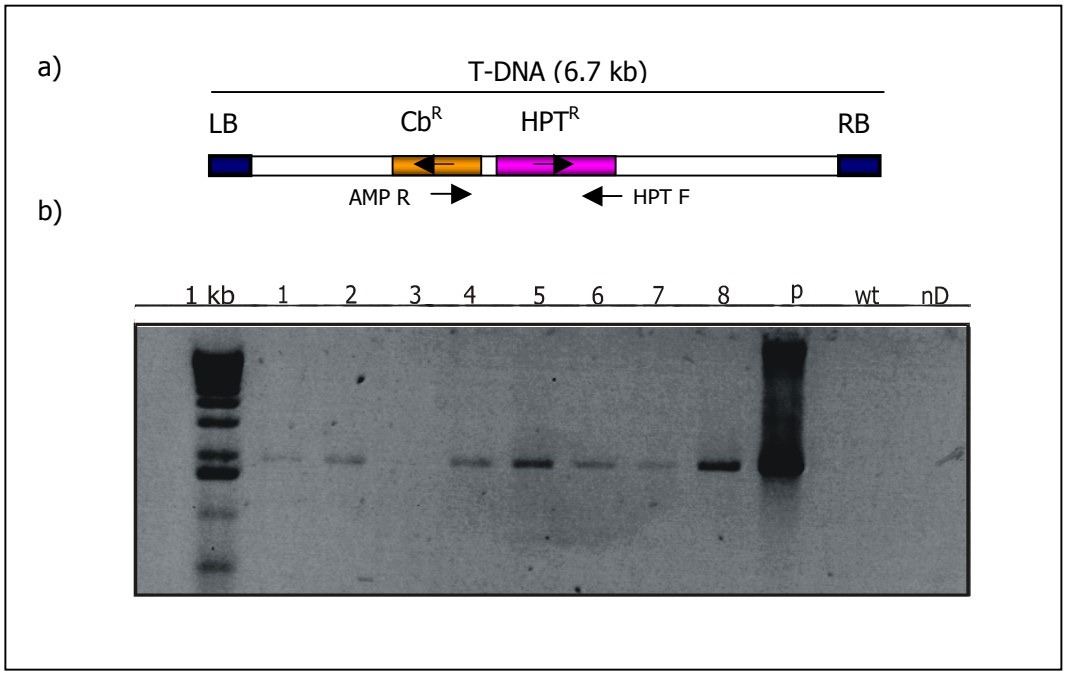
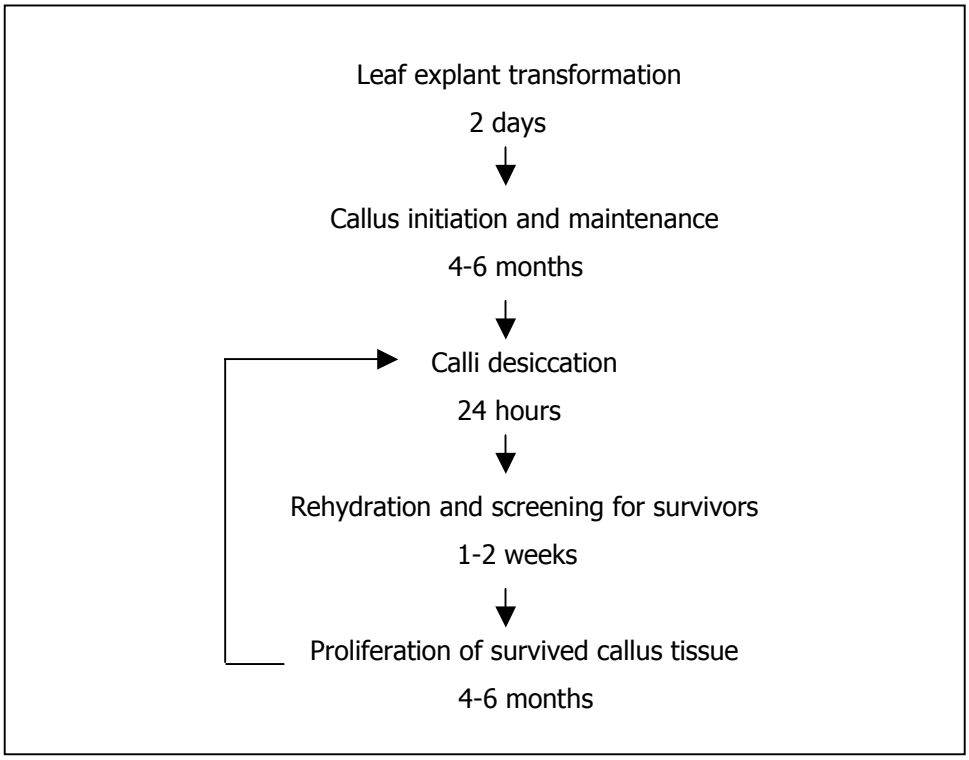


Figure 4.2.2.1 PCR test to confirm the frequency of callus transformation.
Panel a) schematic representation of the inserted T-DNA to which specific primers were designed for amplification of a 1.5 kb fragment of the hygromycin and a 0.5 kb fragment of the carbenicillin resistance gene. Panel b) PCR products from 9 callus tissue samples. Independently transformed *C. plantagineum* callus clusters lanes (1) to (8); pPCVTAc1 DNA (positive control) (P), wild type callus (negative control) (wt) and no DNA added (PCR control) (nD).

4.2.3 Isolation of desiccation tolerant mutants

Desiccation tolerant transgenic calli were selected on the basis of their ability to survive a 24 hour dehydration treatment without prior application of ABA. The screening was performed as follows: transformed calli were dehydrated overnight under sterile conditions -without ABA pre-treatment- and callus clusters that were able to survive dehydration were selected following one to seven days of recovery on MSAR1 media (see scheme 4.2.3.1).

Scheme 4.2.3.1 Selection of desiccation tolerant callus clusters



The mutant screen resulted in the survival of approximately 0.62% of the callus population (see table 4.2.3.1) following the first round of screening. An example of phenotypes observed is depicted in figure 4.2.3.1. The selected callus lines obtained from the first screening were kept for proliferation. In order to screen for single transformation events, each single cluster was subsequently divided into three independent clusters.

In order to confirm that each of the selected callus clusters were desiccation tolerant, the desiccation/rehydration cycle was repeated. After six months of continuous subculture they were submitted to a second dehydration-rehydration experiment (see table 4.2.3.1). From this second screening, approximately 16% of the dehydrated calli survived (from transformation events resulting from pPCVTAc1 and pPCVRN4 T-DNA insertions). The desiccation tolerant callus clusters were then divided into several sub-clusters and kept for proliferation.

Figure 4.2.3.1 First screen for T-DNA activation tagged callus

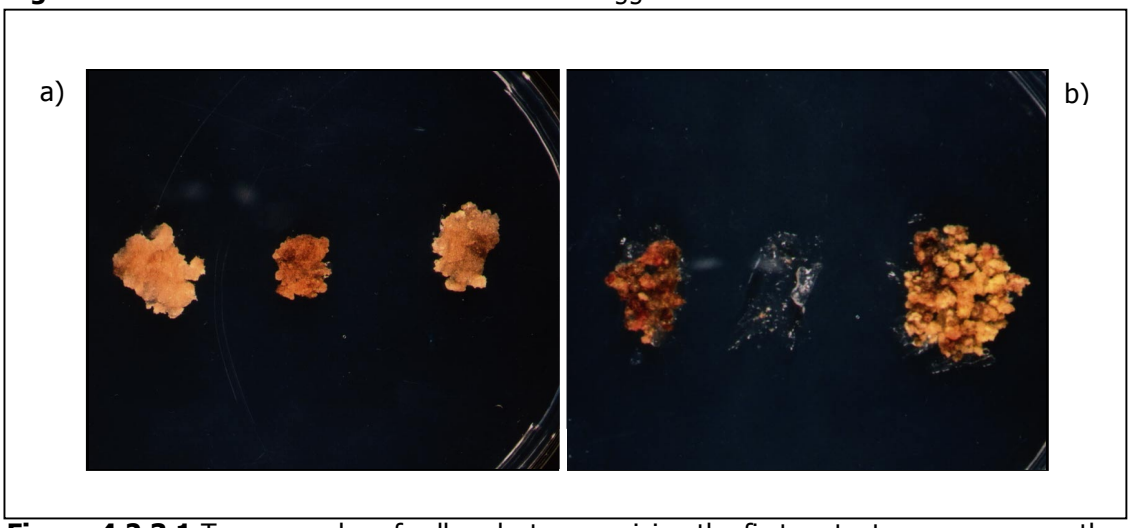


Figure 4.2.3.1 Two examples of callus clusters surviving the first mutant screen one month after callus rehydration: transgenic callus harbouring T-DNA originated from (a) pPCVTAc1 and (b) pPCVRN4.

Table 4.2.3.1 Mutant screening summary

tissue	**survivors dehydration screen No.1**		**survivors dehydration screen No.2**	
	number	**%**	**number**	**%**
wt callus	0	0	0	0
wt-pre-ABA treated callus	30 out of 50	61	27 out of 50	54
callus transformed with pPCVTAc1	12 out of 3200	0.37	3 out of 36	8.3
callus transformed with pPCVRN4	8 out of 3200	0.25	2 out of 24	8.3

4.2.4 Regeneration of plants carrying T-DNA insertions

In order to fully characterise the transgenic *C. plantagineum* lines, plants were regenerated from the selected calli. Dehydration resistant callus clusters were subcultured for approximately three months, when roughly half of the callus per line was transferred to either shoot induction media (see section 2.1.3) or proliferated in an undifferentiated manner in the presence of hygromycin. On shoot induction media the subcultured calli became greener, with a semi-friable

consistency. During the second subculture callus tissue became more friable and a large number of embryogenic structures proliferated on the surface of the cluster (see figure 4.2.4.1a). After three to four weeks of growth on the same medium, individual embryoids developed (see figure 4.2.4.1b) that gave rise to the formation of multiple green shoots (see figure 4.2.4.1c). During further subculturing, the shoots continuously proliferated and did not display any differences to that observed for wild type shoots regenerated on the same medium in the absence of hygromycin. On the other hand, non-transformed explants did not form any shoots and became necrotic within 3 weeks of subculture on MSAR1a containing hygromycin.

Figure 4.2.4.1 The selected transgenic callus were regenerated into plants

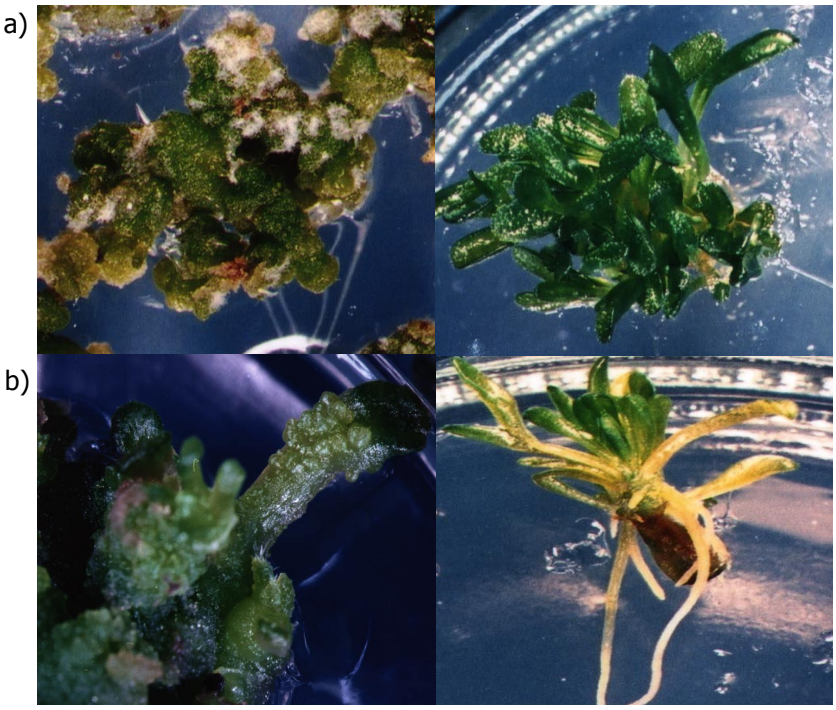


Figure 4.2.4.1 Shoot formation. Panel: a) transgenic callus formed embryogenic structures approximately three months after continuous proliferation, b) one month after transfer to MSAR supplemented with BAP and NAA, leaflets started to form from individual embryoids, c) 1-2 months after the formation of the first leaflets, a dense leaflet structure is formed and d) 2 months after transfer to MSAR with no hormones, roots started to form.

Approximately one month after the first leaflet was initiated, the shoots started to form a dense structure in most of the mutant lines (see figure 4.2.4.1c). Green shoots were transferred to

MS medium without hormones and supplemented with hygromycin for rooting and further development. At this stage of *C. plantagineum* plant regeneration, the hygromycin selection is extremely strict, such that non-transformed lines are unable to produce roots in the presence of hygromycin (Furini *et al.,* 1994). In the present study, more than 50% of the transferred shoots gave rise to plantlets with roots (see figure 4.2.4.1d). Transgenic lines obtained from the transformation experiments performed with the pPCVRN4 vector developed into plants within two to three months after subculture on plant formation media.

For the lines corresponding to the transformation experiments performed with the pPCVTAc1, plant formation occurred after five to six months (or more) of continuous subculture. In both cases none of the regenerated plants showed visible phenotypic differences when compared to wild type (wt) *in vitro* cultured *C. plantagineum* plants grown on medium without hygromycin (see figure 4.2.4.2). Transgenic lines were labelled with letters, if they were transformed with pPCVRN4, and with numbers if they were transformed with pPCVTAc1.

Figure 4.2.4.2 *In vitro* cultured wild type and transgenic plants

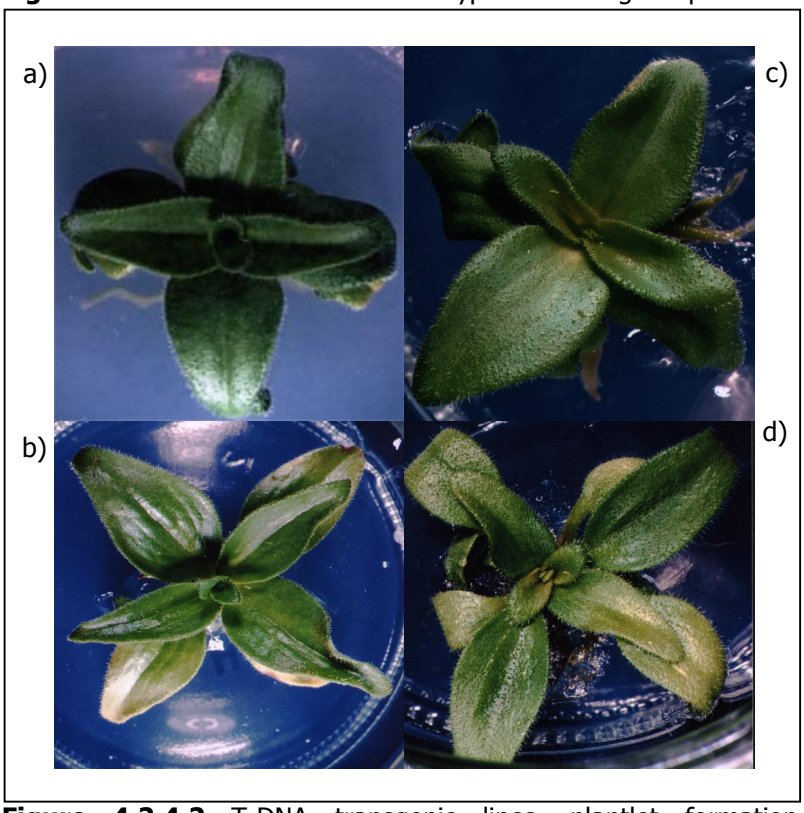


Figure 4.2.4.2 T-DNA transgenic lines, plantlet formation. Examples of *in vitro* grown plants are shown in panels: a) wild type (wt), b) transgenic line E, c) transgenic line H and d) transgenic line 10.

A final number of 28 and 35 transgenic lines harbouring T-DNA originating from the pPCVRN4 and pPCVTAc1 vectors respectively were selected as putative mutant lines. From these plants, seed formation was induced in order to allow segregation analysis. Rooted plants with six to eight leaves and roots that were 2-3 cm long, were soaked with tap water for one day to induce hardening, and then transferred to a growth chamber (Conviron growth chamber) (see 2.1.10), until each plant matured. After two weeks of growth under controlled conditions, only lines H and L displayed an altered phenotype in comparison to wild type plants (see figure 4.2.4.3 b and c), which was not further observed when the plants were fully developed in green house conditions (see figure 4.2.4.3 e and f).

Figure 4.2.4.3 Green house grown wild type and transgenic plants

Figure 4.2.4.3 Examples of regenerated plants one month after transfer from *in vitro* conditions to a growth chamber. Panel a) wild type (wt), b) transgenic line H and c) transgenic line L. Fully developed plants were obtained two months after transfer to green house conditions. Panel d) wt, e) transgenic line H and f) transgenic line L. All plants were grown under the same conditions and were approximately of the same age

The T-DNA insertion did not affect the process of organ differentiation because the plants regenerated from the transgenic callus were morphologically similar to wild type plants. In all cases, plants derived from the transgenic callus clusters displayed reduced fertility, in that none of the tested plants produced seeds, even after pollination with wild type pollen. Furini *et al*. (1997) reported a similar phenomenon.

4.2.5 Tertiary screening of putative desiccation tolerant non-ABA treated callus lines

In order to confirm that the regenerated transgenic plants originated from dehydration tolerant callus clusters, another round of screening was performed on callus derived from the leaves of each regenerated plant. 10 to 20 leaflets were taken from the shoots, replaced onto MSAR1 and subcultured for approximately three months until callus clusters had formed. Callus was successfully regenerated from approximately 40% of the transgenic plants, since this step is prone to contamination most of the leaflets did not regenerated into calli.

A desiccation screen was performed on the regenerated calli (see section 2.2.3.3). As a result, 5 out of 10 independent lines tested, gave rise to desiccation tolerant non-ABA treated callus (see table 4.2.5.1). This corresponded to transgenic lines: 8, 10 and 15, (transformed with pPCVTAc1) and E and H (transformed with pPCVRN4). A summary of the third screen is presented on table 4.2.5.1.

Table 4.2.5.1 Summary of the survival rate of non-ABA treated callus lines

Plant material	Transgenic lines	Number of survivors	% survival
Wt negative control	-		0%
Wt pre-ABA positive control	-	36 out of 58	61.5%
Regenerated calli (pPCVTAc1)	line 8 line 10 line 15	1 out of 10 4 out of 21 3 out of 43	10% 19% 6.9%
Regenerated calli (pPCVRN4)	line E line H	4 out of 38 2 out of 20	10.5% 10%

Other lines tested were P, V, W and Z from transformation events with pPCVRN4 (V, W and Z were shown to be the same line –see section 4.3.1), and line 13 from transformation events with pPCVTAc1. They did not show a desiccation tolerant phenotype. The plants that, at the callus level

were able to survive complete dehydration without ABA pre-treatment, were subjected to further analysis.

4.3 Molecular characterisation of transgenic lines

4.3.1 Characterisation of the T-DNA insertions

To confirm the presence and number of T-DNA insertions, genomic DNA from untransformed and putatively transformed plants was extracted from leaves, digested and hybridised with either the 300 bp CaMV 35S transcriptional enhancer region or a 1.5 kb DNA fragment corresponding to the *HPT* gene, both obtained from the pPCVs used. 30 µg of DNA was digested with three different restriction enzymes: *Eco*RI, *Bam*HI and *Hind*III. The enzyme *Eco*RI cleaves at two sites within the T-DNA in both of the PCVs, releasing a fragment of 1.5 kb that corresponds to *HPT* gene. *Bam*HI cleaves at one restriction site in the pPCVTAc1 T-DNA, whereas no restriction sites for *Hind*III are present in either of the PCVs used. The digested DNA was separated on a 0.6% agarose gel, transferred to a nitrocellulose membrane and hybridised at 65ºC overnight. Following a half stringency wash, the filters were exposed over night to a phosphor screen, scanned and analysed using the Image Quant software. Figure 4.3.1.1 shows the results for the mutant lines tested.

A band of 1.5 kb was observed in all of the selected mutant lines when the *HPT* fragment was used as a probe. This confirmed that the mutant lines were indeed transformed. In control lines no specific signals were detected when hybridised with the PCV-derived probes. Bands of different sizes were observed in all of the selected mutant lines when the CaMV 35S promoter transcriptional enhancer was used as a probe. In general, the number of T-DNA insertions per line was between 1 and 3. One exception, line 44, showed the presence of 6 bands. As expected, fragments larger than 6.6 (T-DNA size) were observed in the samples digested with *Hind*III and *Bam*HI. This observation suggests that the T-DNA was not rearranged after insertion into the *C. plantagineum* genome. In the case of mutant line N, however, the presence of a 2.2 kb *Hind*III fragment suggested that a T-DNA rearrangement might have occurred. These results are summarised in table 4.3.1.1.

A total of 18 transgenic lines were analysed, from which ten independent lines could be detected (see figure 4.3.1.1). The complete T-DNA tagged population could not be characterised, the reason being the lack of plant material. Some plants regenerated at lower rates and the required amount of plant material could not be collected.

Figure 4.3.1.1 T-DNA insertions in the transgenic lines obtained by activation tagging

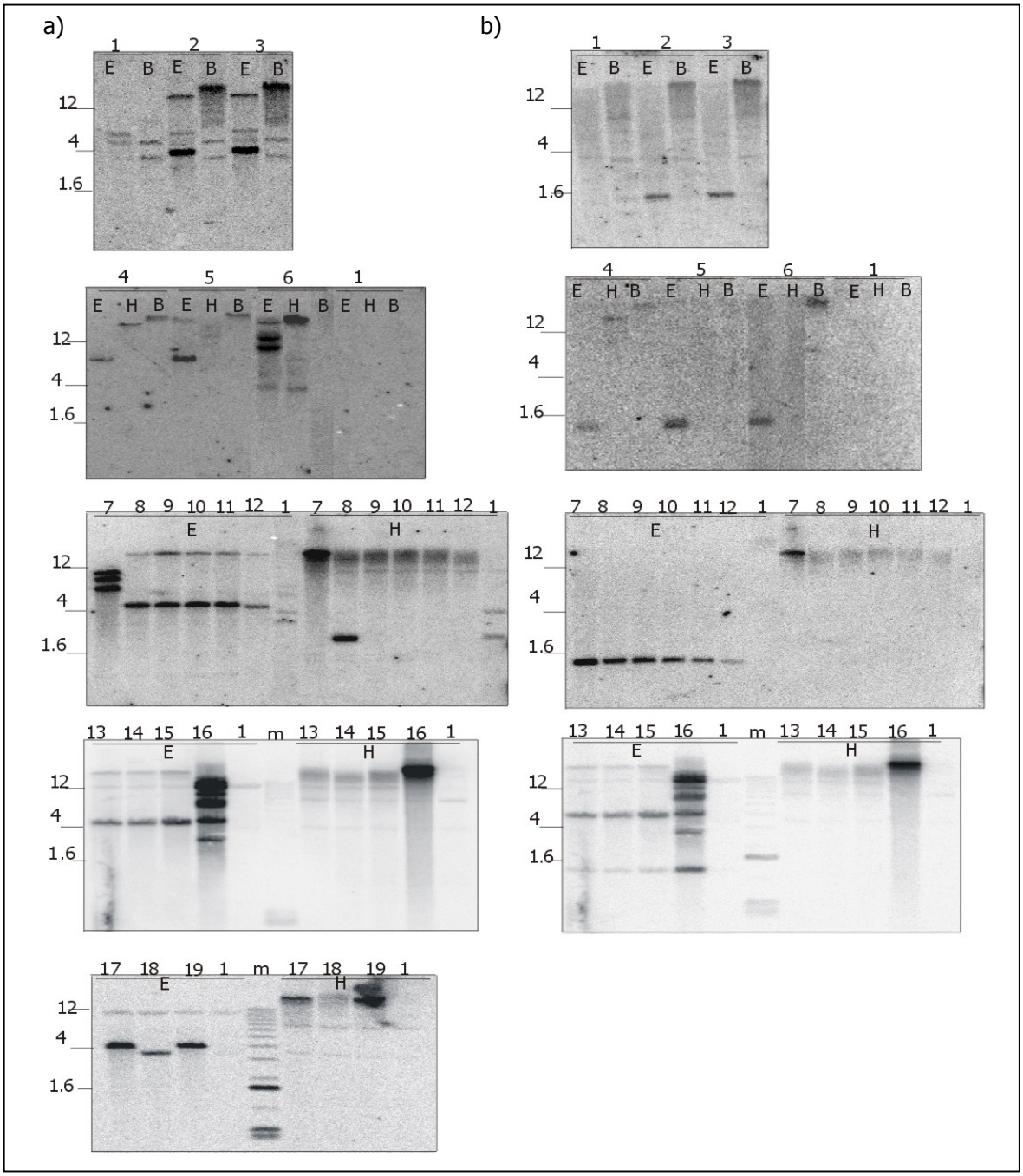


Figure 4.3.1.1 DNA gel blot analysis of the T-DNA insertions in the transgenic lines obtained. Panel a) blots were hybridised to the CaMV 35S enhancer and panel b) blots were hybridised to the *HPT* fragment. 30 mg of genomic DNA were digested with *EcoR*I (E) and *BamH*I (B), or E, B and *Hind*III (H). Lanes: wild type (1); transgenic line C (2), D (3), 10 (4), H (5), E (6), L (7), N (8), S (9), V (10), W (11), Z (12), J (13), K (14), M (15), 44 (16), 8 (17), 18 (18) and 9 (19). The corresponding hybridisation with the *HPT* fragment of transgenic lines 44, 8, 18 and 9 is not shown.

The results obtained from the Southern blot analysis are summarised in the following table:

Table 4.3.1.1 Number of T-DNA insertions in the transgenic lines

Transgenic lines	**No. of insertions**	**Inserts size (kb)**		
		***Eco*RI**	***Hind*III**	***Bam*HI**
lines 8 and 9	1	4	15	
line 10	1	8	14	16
line 18	1	3.6		
line 44	6	13 12 10 8 4 3		
lines C and D	2	13 3.5		14
line E	3	14 13 9	14 6	
line H	2	13 6	13	15
lines J, K and M	2	15 4	12 6	
line L	3	10 8.5 7	14	
line N	2	14 4.2	14 2.2	
lines S, V, W and Z	2	14 4.2	14 13	

Note: *Bam*HI restriction enzyme was only used in DNA blots that included transgenic lines: 10, C, D and H.

4.3.2 Structure of the insertion sites

To determine whether the mutant phenotypes were caused by mutation of genes adjacent to the T-DNA insertions, isolation of the T-DNA flanking regions was carried out in lines that gave rise to desiccation tolerant calli in the third screen. Two PCR based methods were used: Long Range inverse PCR (LR-iPCR) and an adaptor mediated PCR.

4.3.2.1 Long Range Inverse PCR (LR-iPCR)

This method allows the isolation of long T-DNA flanking regions. It requires specific primers designed to amplify outside the borders of the inserted T-DNA (see figure 4.3.2.1.1) and the use of a DNA polymerase I enzyme with enhanced thermostablity. Genomic DNA, isolated from the T-DNA tagged lines, was digested with *Eco*RI. This enzyme contains two restriction sites within the T-DNA, which, after digestion, releases a 1.5 kb fragment corresponding to the HPT gene. The resulting RB-plant genomic DNA and LB-plant genomic DNA fragments were re-circularised and purified (see section 2.2.2.2.6.1). The purified DNA was PCR amplified using a programme that is designed for amplification of relatively long DNA stretches. In total, three independent T-DNA tagged lines were selected and their flanking regions isolated by LR-iPCR, transgenic lines E, H and 10.

Figure 4.3.2.1.1 LR-iPCR approach for the isolation of T-DNA flanking regions from *C. plantagineum* transgenic lines

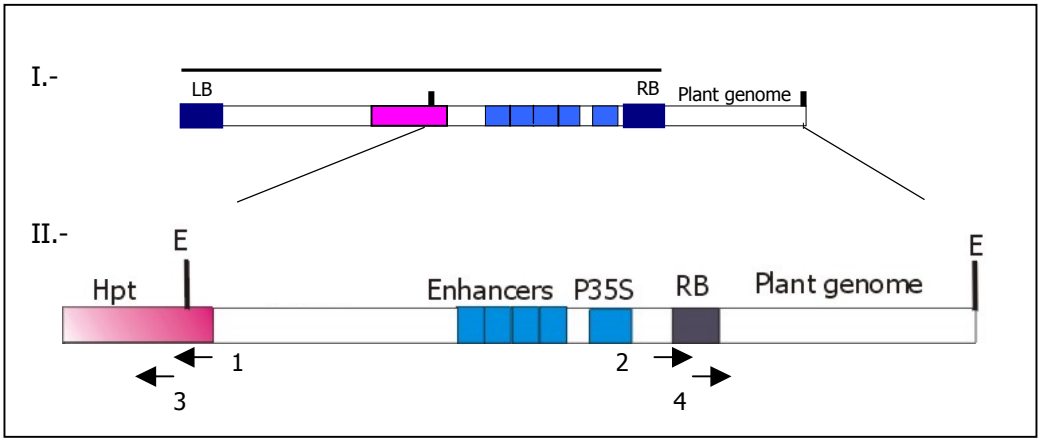


Figure 4.3.2.1.1 LR-iPCR consists of two basic steps. Step I: restriction digestion of genomic DNA isolated from the mutated plant, in this case with EcoRI (E) enzyme and step II: after religation, LR-iPCR with the use of specific primers. Arrows: 1 and 2 primers for the primary PCR reaction, 3 and 4 primers for the secondary PCR reaction

For T-DNA tagged line E three different bands were detected on an agarose DNA gel, each corresponding to 6, 5.5 and 2.6 kb respectively (see figure 4.3.2.1.2). Each of the bands was purified and cloned into the pGEM-T Easy vector (see section 2.2.7.3). Ends of the DNA fragments were sequenced using the Universal and Reverse, or T7 and SP6 primers. Analysis of the sequences revealed that two of the fragments were homologous to one another and to a 500 bp fragment corresponding to the *HPT* gene. The sequence corresponding to the larger clone, 6 kb, showed homology to a 60 bp region corresponding to the *HPT* gene. This was expected since a 200 bp fragment of the 3' end of the *HPT* gene was predicted to be included in the recircularised plant

genomic DNA fragments (as a result of the digestion with *Eco*RI). After searching the database with the obtained sequence of the 6 kb rescued fragment, no homology was found to any reported sequence.

Figure 4.3.2.1.2 T-DNA flanking regions rescued from *C. plantagineum* T-DNA tagged lines using LR-iPCR

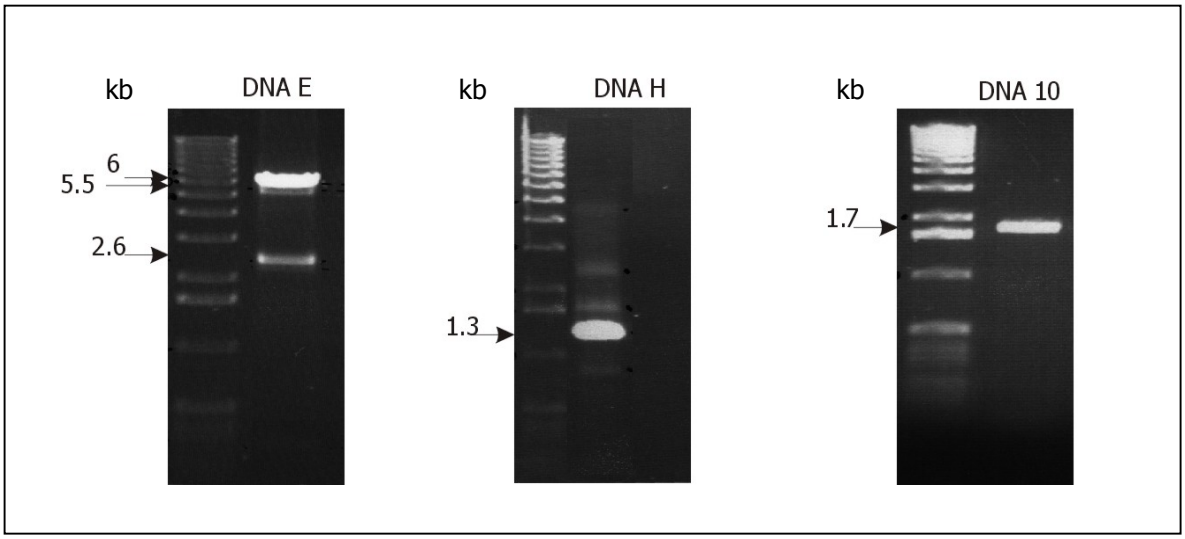


Figure 4.3.2.1.2 Rescue of T-DNA flanking regions from T-DNA tagged transgenic lines using LR-iPCR. Agarose gel of the fragments amplified from the independent LR-iPCR reactions using genomic DNA isolated from transgenic lines E (DNA E), H (DNA H) and 10 (DNA 10).

The partial sequence corresponding to the 6 kb fragment, rescued from transgenic line E (DNA E) was obtained and analysed. The highlighted areas in the sequence correspond to the homologies found after searching the databases: in red - cloning vector, pink - hygromycin resistance gene sequence, bold - *EcoR*I restriction site and no colour - no significant homology.

DNA E: (Univ)

```
  1  GAATTGTAAT  ACGACTCACT  ATAGGGCGAA  TTGGGCCCGA  CGTCGCATGC
 51  TCCCGGCCGC  CATGGCGGCC  GCGGGAATTC  GATTCTATCG  GCACTTTGCA
101  TCGGCCGCGC  TCCCGATTCC  GGAAGTGCTT  GACATTGGGG  AATTCCTTGA
151  ATATAAAAAG  CAGAAAGCAA  AGTCTGCTGA  AGGAACATCT  TACGCAAAAA
201  TAATTGCGGA  AGTAGAATAT  GAGGTTGATT  ATGAGAAAAT  TTCTCATACT
251  GAGGTAATTA  TATTACTTGA  ATTTAAAGAT  TTAAAATGGT  CTGATGAACC
301  TTGGACTTTA  ATGTCAAGGT  ATTTAGACAG  AGCATCATAT  GCTGCTACTT
351  TTTATAAAAA  CCGTGACACT  TATGAAAATA  TTCTAAGGTC  TACGGGAACA
401  TGTGACTTTG  AGCACTTTAA  TGCGGGAAAT  ACTCCTTATG  CTTTGAAAAA
451  GGGCTATACC  TTTAGCAAAA  TAATTTTTAA  GCAAATTATT  TCGCCTGAAA
501  AATGGGCTAT  ACACCCATTA  AAGGATAGAG  AATATCTCCA  TCCTGAGTTA
551  AAAACTCTTG  TTAAATTTAA  TTATTACGAT  TATATCGATG  CTTTTTATAA
601  AGTATTTCTA  TACCAGAATG  AAAGTAAAAA  ACATTCTTGG  TGGATAAAAA
651  TTTGTGCGGA  TGTATATAAG  TCCGATATAC  CAAATTGGTT  TTATCAATGG
```

```
 701  TGGCTTTCTT  ATGGAGCTAC  AATCGAAATT  CTTAAAGAAC  CCTTCTTTGG
 751  ATTATATCAA  GAATGGGTTA  AAGTTTCTCC  GACTCTTATA  GAAGCGGAAC
 801  ATAAAAAAAG  AATGTATTGG  AAGGATGGCT  TTCTTTACAT  TTTTATATTG
 851  GAATTANTAT  TCCCTTGGAT  TTGGAGATNG  GACTNCANAA  AGGGAGTTTT
 901  TACTTCAACA  AAAAATTTCC
```

For T-DNA tagged line H one specific product of 1.3 kb was detected on an agarose DNA gel (see figure 4.3.2.1.2). The DNA band was purified, cloned and after sequence analysis the structure of the fragment and homology to other known sequences determined. Immediately after the Universal primer a 150 bp fragment corresponding to the HPT gene was found, which was adjacent to a 1.1 kb *C. plantagineum* genomic DNA sequence. The T-DNA right border fragment was found between the genomic DNA sequence and the T7 primer sequence. The sequence corresponding to the plant DNA did not show significant homology to any gene of known function. The following sequence shows in the highlighted areas the corresponding homologies found: in red - cloning vector, blue - right border, pink - hygromycin resistance gene sequence, bold- *EcoR*I restriction site and no colour - no homology.

DNA H: (SP6-Universal)

```
   1  CCTATGGTTA  AAGTGTGTCC  TTTGGTCGAT  ACGGGTACTA  ATGCGGTTCG
  51  ATAAATCCAC  TGTGATATCT  TATGAGTTCG  ATACGTAGGT  TGCGCAACCC
 101  TCGAGAGGGT  ATACCAGCTG  GACGTCCGCC  GGCGCTTAAG  TGATCACTAA
 151  GGTACAACCC  CTAGATCTAC  TAGGCAGGAC  GCCCATTTAT  CGACGCGGTA
 201  CCAAAGATGT  TTCTAGCAAT  ACAAATAGCC  GTGAAACGTA  GCCGGCGCGA
 251  GGGCTAAGGC  CTTCACGAAC  TGTAACCCCT  TAAGGTGTAG  TAAGGTAGTA
 301  AAGCTCTGAA  CCTTCTTCTT  TCCGAATCAC  CTTGCGTCGT  CTTTATTCTC
 351  TTATACTTCT  GCCGCATTAG  TTTTCCCAAC  TAGGACCGCG  CGAAATCTGT
 401  TACTGTACTT  CTGGCTGTGC  TGACGATGAG  TATACACTAG  GGTGATGGTG
 451  GAGTAATGAT  AGACTTGGGT  AAATTGGGCT  TTTCTGGAAG  ACTAAGTACG
 501  ATTGACGAAA  TTCAACATCT  GCACGCTAAC  TGATCGTTCG  TATACCATCT
 551  CTGGTCTAAT  AACGTACTTA  ACTCGCTAAG  TGTCTAAATA  ATCGGATATG
 601  TGACTCACTA  AACTCACTTA  GGGACACTCC  ACACCTAGAG  CTAATCATCA
 651  CGATTACTTT  AAGCACAGCT  ACTCACGACT  AATAACAACG  TCACAGATAT
 701  TAACGAACGA  ATTAAGGATA  AACACAAAAG  GTAGACGCAA  CCACTTAACA
 751  CTTACCAGCA  CGGCAATAGA  ACTATTGGAA  AAGAACGAAA  ATAAAACGAA
 801  CTCCTGTTCG  TTTTCGAATT  CACACCCCTC  CAAACTATTT  GCATAAAGTA
 851  AGATACGTAA  ATAACCGAGA  TAAATGTGGG  TAAATTTATA  ACATCCTGGA
 901  AGCTTACTAA  TAGCTCGCTT  TTCCTTGCTT  TTGCTCCGCC  TTTGCTGCGC
 951  TTTAACCGGT  TCATACTTCT  ACAGCCTGTT  CCTCAGTGCC  GGCACTCGAA
1001  ATGGAGTTAT  CCGGCACTTA  GAGCCGTCTT  TTCACCCTAT  AGCTGTTACA
1051  AGTATAAGTG  CCGGCACTTG  TGGAAACGTT  GCTCGGCACT  TTTCGCCGTC
1101  TCTCTTTTCA  TTCACTGGAA  GTGCCGGCAC  TATGTAGTAC  GCTACCCGGC
1151  ACTTGAACCC  GTCATATACT  TCTAGACCTT  GTACATGTGT  AAGTGCCGGC
1201  ACTTCGCAGT  TCGTTCCTCG  GCACTTAGTG  CCGTCATCGA  GTTCCTGAAT
1251  TGCAGTGCCG  GCACTTGAAA  ATACATGCTC  CGGCACTTGG  ACCTGTCTCT
1301  CGAGTTGCTT  CCTTTTAAGG  TAAGTAAGTG  CCGGCACTGA  AGTTTGTGAC
1351  TATCAAAGCC  TAGTAGATCT  AGGGGTTGTA  CCTTTAGCTT  AAGGGCGCCG
1401  GCGGTACCGC  CGGCCCTCGT  ACGCTGCAGC  CCGGG
```

For T-DNA tagged line 10, the LR-iPCR reaction amplified a single band of 1.7 kb (see figure 4.3.2.1.2). Sequence analysis showed that the SP6 primer was followed by a 136 bp fragment corresponding to the *HPT* gene, which was next to a 1.45 kb sequence corresponding to plant genomic DNA. Immediately adjacent to the T7 primer sequence was the T-DNA right border DNA sequence. Database searches revealed that the rescued genomic T-DNA flanking sequence shared high homology to two short stretches of 27 and 22 bases long (see DNA 10 sequence), with the *CDT-1* gene sequence of *C. plantagineum*. Highlighted areas in the following sequence corresponds to: red - cloning vector, green - sequence homology, blue - right border, pink - hygromycin resistance gene sequence, bold - *EcoR*I restriction site and no colour - no homology.

DNA 10: (SP6-T7)

```
   1  ACTCAAGCTA  TGCATCCAAC  GCGTTGGGAG  CTCTCCCATA  TGGTCGACCT
  51  GCAGGCGGCC  GCGAATTCAC  TAGTGATTTC  CTGCGGGTAA  ATAGCTGCGC
 101  CGATGGTTTC  TACAAAGATC  GTTATGTTTA  TCGGCACTTT  GCATCGGCCG
 151  CGCTCCCGAT  TCCGGAAGTG  CTTGACATTG  GGGAATTCTA  TAATTTTTTG
 201  TCCTTTGCAT  ATTACGGCGA  TTATAAAATT  TCTCAAGTAA  GACTAAACAG
 251  ACCTGATGTC  AAGTTGAAAA  ACAGGTACAT  GAAATTGACA  TGTTTGGCAG
 301  TTATCAATAA  TAAGTAGCAT  AAATTGAATT  AGTACAATTT  CTAGATTGTA
 351  GAAGCTCTCT  CTCCCATTTA  CTTTTAATTT  GCATCGTCAA  AATATGGACG
 401  GAGATCATGT  TATATATTAT  TTTTCTCTAA  GTGGGAATAT  AAAATTAAAA
 451  GAAAAGTTTT  AAAGTAGGCA  CACACACACA  CAACCACTCA  CCTCTTTTTA
 501  TTTTCATGCA  TTTCCTCTAA  ATGTATTTAA  GAGAATAGCA  TTGGTTACAT
 551  TTAGAAATAC  TCAATTATAT  AAAAAATTGC  AAGTCAGTAA  TCTGTCAGTA
 601  TTTTTTTATA  TGTAGATACC  TATTAGAATA  AATTTTTGCT  TATTGACACC
 651  CATCCCGCCA  CAAGGTACGC  CATATAACAC  ATTTACGTAC  CATAGAGAAA
 701  CGTATTATGG  TACATAACAC  AGTGAGAAAT  GGTACATCAT  ATAACACATT
 751  AAAGTAGCAT  ATAGAAACGA  TATAGGTACC  ATATAAACGC  AGGACGTGGT
 801  ACGTAGCACA  CATATAAAAA  GGTACGTCGA  GACTAAAAAA  AATGGTACAC
 851  ACAGTCAAAT  GGGTACTTAC  ATAAAAAATT  TTCTGACATT  AGTTGTGACT
 901  TGCAAATCTG  TTATACAAAT  GGATATTTTT  CTATAGATTC  ACCTACATTT
 951  TATATATTAT  CACAGCACTG  GATGACTTGA  ACAAAACCTG  AAAAAAAAAG
1001  TCATAATCAT  AGTGGGAAAA  GAAAGTTGAA  TGATAACACC  ATTTATCAAA
1051  TTCGTTAAGG  TTGCGTTTGG  ATTGGCGTAT  TCGAACGAAA  TCGCGTGATT
1101  TGAAATCATG  AATTTCAAAA  CCTTAATTTC  AAATCAAAAT  CCATTGTTTG
1151  GCAAACGTTA  GGATTTCGAA  ATTCAGATTT  CAAAATGAAG  TTCGACCTCA
1201  AGCGCTCGAG  TAGCTCGTCG  AACTCGACTT  CTAGCTTTTT  GGGNTTTTTT
1251  TTTTTTTTTT  TTTTTTTTTC  CTTTNNTTTT  TTTTTTNTTT  TTTTTTTTNN
1301  CTTTTCTCCC  GGAAACGATA  GCTTGCATTA  ATAAACAAAG  ATACAGGGGC
1351  TAAATAGCCC  AAAGGGTCTC  TCCGGTAATA  CAGAATCCGG  TAGAGAAACA
1401  AAGGCTCATA  AGGAGCAATG  CAACACGAAA  ACAAAAACAA  GCCGACAGAA
1451  AACGAAATAA  ACCTCGAACT  TGGACCAAAA  ATCTATAGAA  CATACGACAG
1501  AAAAGGTTAT  TAATCCGAAC  CTACGGTACT  CTACATTATT  TTTGTCCAAC
1551  GACTCGAGGT  TATCGTCTTC  ATCGGAAGAG  GCTCCACAAG  GGAGAAAAGG
1601  CTCCACAACG  GGAGAAAAGG  CTCCCGACCT  AATGGCCGGG  AGAAACGAGC
1651  TCCCCACGGG  AGACTCGCGG  GTGGACGATA  GTGAGGTGTG  GCGCGGCGGT
1701  CAGCGGTCGG  TGGTCGGCGG  TCGGCAGTCG  GCGGTCGGCG  GTCGGCGGTC
1751  GGCGGTCAGC  GAGTTGGCGG  CGAGGTGGTG  AGATGGTGGA  GTGGCGGTGG
1801  ATGCCGGCCT  CAACTTCAGC  GAGACGCCGG  CAAGACGGGG  CGGGCGACGA
1851  CTCTCAGCAG  TGATGAACGG  ATTACTTGTA  ATTGTAAATA  GTAATTCTGC
```

```
1901 AGCTCGAGGT  CCTCTCAATC  GAATTCCCGC  GGCCGCCATG  GCGGCCGGGA
1951 GCATGCGACG  TCGGGCCCA
```

These results are summarised in the table 4.3.2.1.3 and shown schematically in figure 4.3.2.1.3.

Table 4.3.2.1.3 Sequence homologies of T-DNA flanking regions rescued by LR-iPCR

cloned fragment	size (kb)	sequenced	homology
DNA E	6	920 bp	no homology
DNA H	1.3	all	no homology
DNA 10	1.7	all	no homology

Figure 4.3.2.1.3 Structure of the T-DNA flanking regions rescued from *C. plantagineum* T-DNA tagged lines using the LR-iPCR approach

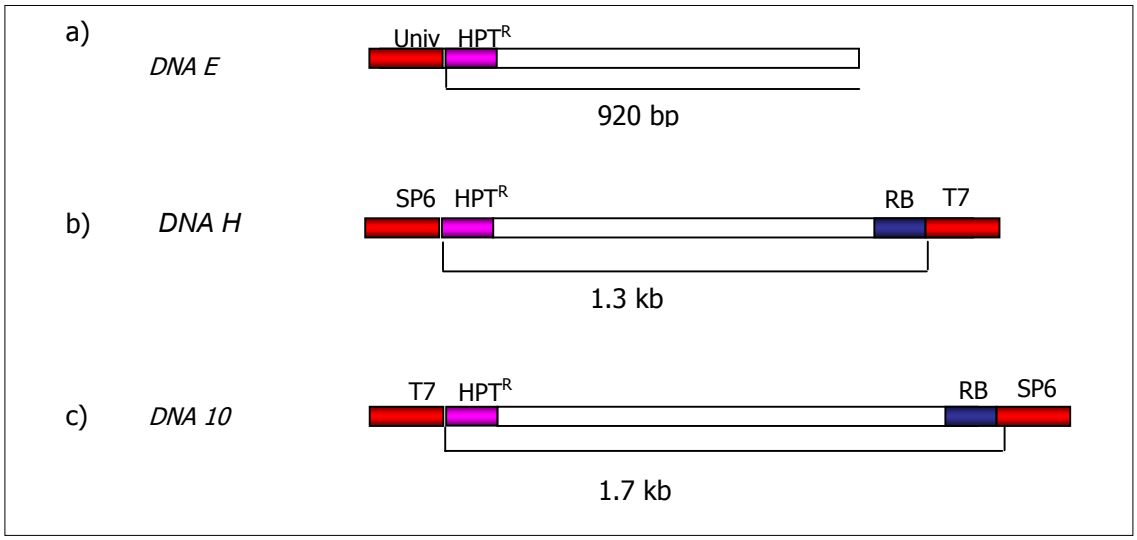


Figure 4.3.2.1.3 T-DNA flanking regions rescued from *C. plantagineum* T-DNA tagged lines. Panel a) transgenic line E (DNA E), b) transgenic line H (DNA H) and c) transgenic line 10 (DNA 10). Colours correspond to: red- cloning vector, blue- right border (RB), pink- hygromycin resistance gene (HPT^R), and no colour- plant genomic DNA.

4.3.2.2 Adaptor mediated PCR

In parallel with LR-iPCR, adaptor ligation PCR was used in order to isolate T-DNA flanking regions (Siebert *et al.,* 1995). This method was selected because it is suitable for handling larger

number of samples (in comparison to the LR-iPCR approach). It is based on the addition of blunt-end adaptors to previously digested genomic DNA. After this, specific adaptor and T-DNA right border primers were used in a PCR programme (see section 2.2.2.2.6.2). The sequences of the adaptor and primers are shown in section 2.1.5. One end of the adaptor is designed to ligate to both ends of blunt-ended DNA fragments. The unique feature of the adaptor is the presence of an amine group at the 3' end of the lower strand. This blocks DNA polymerase catalysed extension of the lower adaptor strand, thus preventing the generation of the primer binding site unless a defined, distal, gene specific primer extends a DNA strand opposite to the upper strand of the adaptor. This "suppression" technology uses an adaptor primer that is shorter in length than the adaptor and is capable of hybridising to the outer primer-binding site. If any PCR products are generated which contain double-stranded adaptor sequences at both ends (due to non-specific DNA synthesis), the ends of the individual DNA strands will form "panhandle" structures following every denaturation step, due to the presence of inverted terminal repeats (see figure 4.3.2.2.1B). These structures are more stable than the primer-template hybrid and therefore will suppress exponential amplification. However, when a distal gene-specific primer extends a DNA strand through the adaptor, the extension product will contain the adaptor sequence only on one end and this cannot form the "panhandle" structure. PCR amplification can then proceed.

Figure 4.3.2.2.1 Strategy of adaptor mediated PCR for cloning T-DNA flanking regions

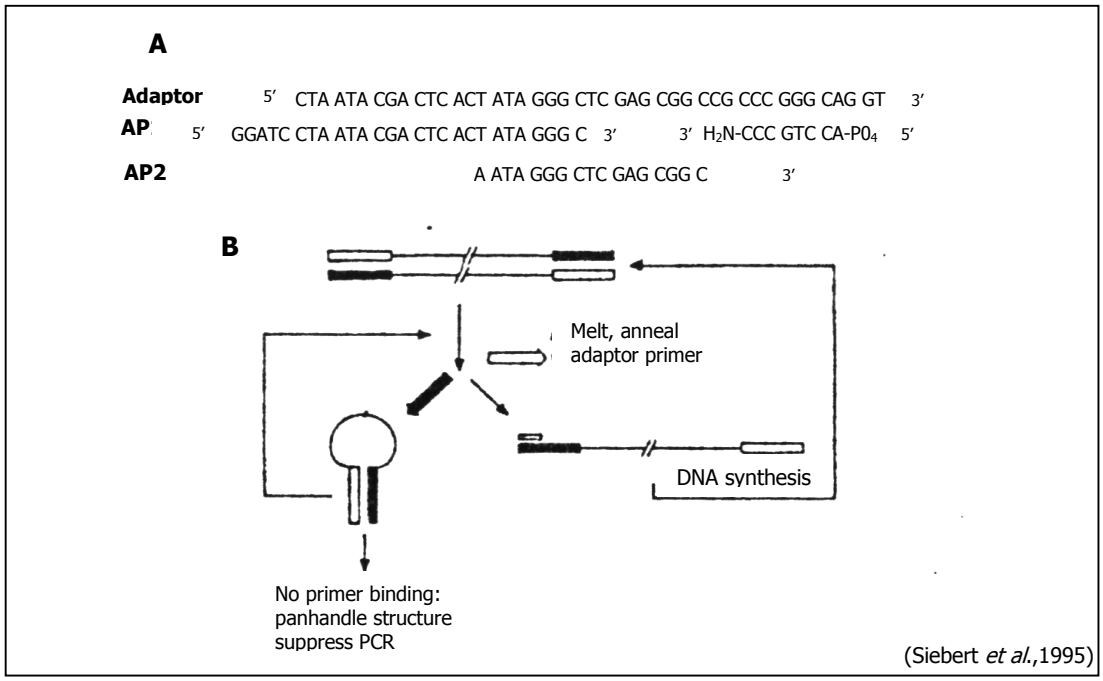


Figure 4.3.2.2.1 Panel A) sequences of the adaptor and adaptor primers (AP1 and AP2). Panel B) illustration of the suppression PCR effect. Structure of PCR products formed by non-specific amplification. The sequences on the ends of the products contain inverted terminal repeats and will form "panhandle" structures that suppress PCR.

This strategy applied to *C. plantagineum* transgenic lines is depicted in figure 4.3.2.2.2. Specific primers, right border and adaptor primers, were used for the rescue of T-DNA flanking regions.

Figure 4.3.2.2.2 Adaptor mediated PCR approach for rescuing T-DNA flanking regions

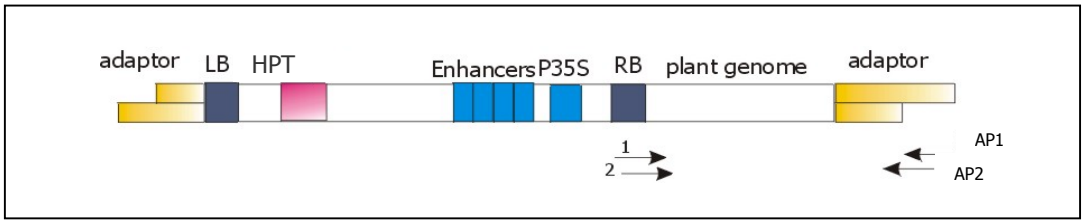


Figure 4.3.2.2.2 Rescued T-DNA flanking regions from T-DNA tagged lines by adaptor mediated PCR. Scheme of the T-DNA insertion in the plant genome and the primers used for amplification of the T-DNA with the help of an adaptor region ligated to the ends of the restriction-digested genomic DNA. Arrows: adaptor primers AP1 and AP2, and T-DNA specific primers (1) and (2) for the primary and second PCR reaction respectively.

Genomic DNAs extracted from 5 independent mutant lines were subjected to this analysis. A number of PCR products were amplified. After gel analysis of the primary AD mediated PCR, a smear was detected in most of the reactions. Only after the secondary AD-mediated PCR specific bands could be detected (an example is shown in figure 4.3.2.2.3).

Figure 4.3.2.2.3 Rescued T-DNA flanking region of transgenic line 18 following the AD-PCR approach

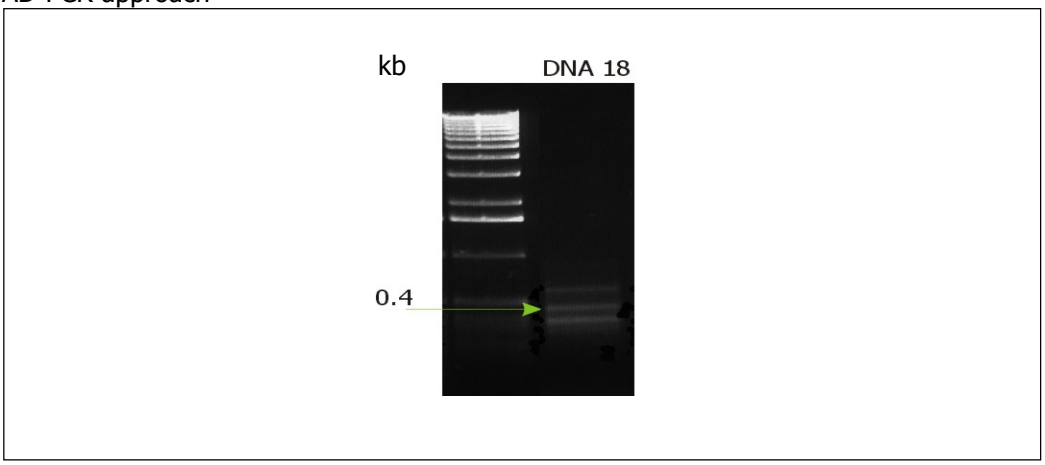


Figure 4.3.2.2.3 Rescue of T-DNA flanking region from T-DNA tagged transgenic line 18 with AD mediated PCR. Agarose gel of the fragments amplified on the second PCR reaction. Three bands were isolated and further analysed.

In all the cases, the specificity of the bands was determined by DNA blots using the T-DNA sequence as a probe. The DNA fragments that showed to be specific were purified and cloned into the pGEM-T Easy vector. A total of 14 fragments, with sizes ranging from 200 to 600 bp, were isolated and their sequences determined with the use of the Universal and Reverse primers. The sequences of the clones revealed that most of the flanking regions were homologous to regions of plant cloning vectors such as the hygromycin resistance gene. Only one clone showed homology to a non-plasmid borne sequence, this corresponded to the T-DNA flanking region isolated from transgenic line 18. Analysis of the sequence showed homology to a retrovirus sequence. In the following sequence, the highlighted areas correspond to: in red - cloning vector, yellow - adaptor primer sequence and no color - plant genomic DNA.

DNA 18 (Universal)

```
  1  CTATGCATCC AACGCGTTGG GAGCTCTCCC ATATGGTCGA CCTGCAGGCG
 51  GCCGCGAATT CACTAGTGAT TACTATAGGG CACGCGTGGT CGACGGCCGG
101  GCTGGTCTGA ATTCCTCCTT GGTTGTCACA GAAAACATCC ACATACGCTT
151  GAGTCATGCC CAGCTCATTC ATTAATCCTC TCAGCCACAG AGCCTCCTTA
201  ACAACTTCAG TAAGAGCAAT ATATTCTGCC TCTGTTGAAG ATAATGCCAC
251  GAGCTTATGC AGGCTGGCTT TCCAACTAAT AGCAGTCCCA TATGAGGTGA
301  ATACATAGCC GGTCAGAGAT TTCCTAGAGT CCAAACAGCC GGCGAAATAT
351  GAGTCCACAA AACCACTCAC TTGACCACTG ATAGTTTCGG ATCCTTGTAA
401  AATCGAATTC CCGCGGCCGC CATGGCGGCC GGGAGCATGC GACGTCGGGC
451  CCAATTCGCC CTATAGTGAG TCGTATTACA ATTCACTGGC CGTCGTTTTA
501  CAACGTCGTG ACTGGGAAAA CCCTGGCGTT ACCCAACTTA ATCGCCTTGC
551  AGCNCATCCC CNTTTGNGCC AGTTTGGGGG TAAATAGGCG AAAGAGGCCC
601  GCACGGGATT GGCCCTTTCC
```

These results are summarised in the table 4.3.2.2.1 and shown schematically in figure 4.3.2.2.4.

Table 4.3.2.2.1 Sequence homology of the T-DNA flanking region rescued by AD mediated PCR

cloned fragment	size (kb)	sequenced	homology
DNA 18	0.4	all	retrovirus related sequence

Figure 4.3.2.2.4 Structure of the T-DNA flanking region of transgenic line 18 isolated by the adaptor PRC approach

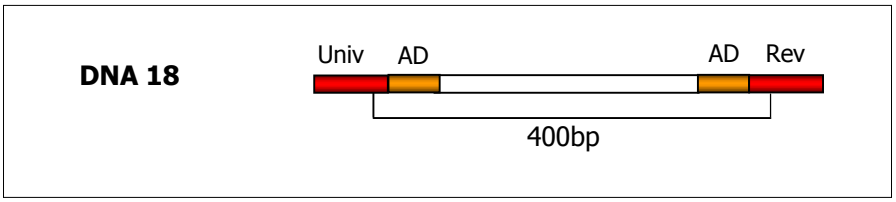


Figure 4.3.2.2.4 T-DNA flanking region rescued from *C. plantagineum* T-DNA tagged line 18 (DNA 18) following the adaptor mediated PCR approach. Colours correspond to: red- cloning vector, yellow – adaptor sequences, and no colour- plant genomic DNA.

A summary of the cloned T-DNA flanking regions, their size and homologies to other reported sequences are listed in table 4.3.2.3.2.

Table 4.3.2.3.2 Summary of rescued T-DNA flanking regions

Transgenic line	Clone name	Rescued insert size (kb)	Method	Homology
10	DNA 10	1.7	LR-iPCR	no homology
18	DNA 18	0.4	AD-PCR	retrovirus related sequences
E	DNA E	6	LR-iPCR	no homology
H	DNA H	1.3	LR-iPCR	no homology

4.3.3 Gene expression analysis of T-DNA tagged plants using rescued genomic flanking regions as probes

To investigate whether or not the T-DNA insertion lead to altered expression of the gene or genes present in the isolated flanking regions, RNA gel blot analysis were performed. The rescued genomic T-DNA flanking regions were digested with *Eco*RI, purified, radioactively labelled and hybridised with a filter containing poly A^+ enriched RNA from wild type and mutant *C. plantagineum* tissues. This analysis was performed with DNA isolated from mutant lines 10 and H, since both passed a tertiary desiccation tolerance screen (see section 4.2.5).

In RNA blots that included wild type callus and leaf tissues, and transgenic callus tissue, the 1.1 kb rescued genomic DNA from mutant H (DNA H), accumulated at lower quantities in the transgenic line in comparison to the transcript in wild type tissue. The transcript showed accumulation in both, ABA pre-treated and non-treated callus tissue, and in fully hydrated roots (see figure 4.3.3.1). No transcript was present in wild type hydrated or dehydrated leaves.

Figure 4.3.3.1 The LR-iPCR rescued DNA from transgenic line H corresponds to a transcript present in transgenic and wild type callus, and in wild type fully hydrated roots

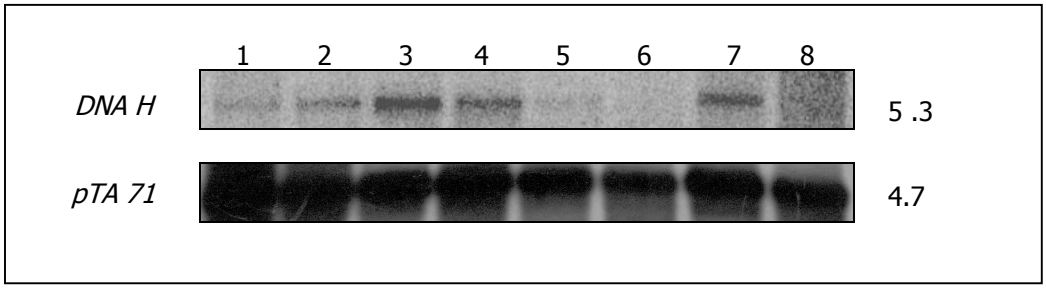


Figure 4.3.3.1 LR-iPCR fragment rescued from transgenic line H corresponds to a transcript of approximately 5.3 kb. 3 μg of poly A^+ enriched RNA were loaded from: callus tissue from transgenic line H (1), transgenic line 10 (2), wild type: fully hydrated callus tissue (3), 4 d ABA pre-treated callus tissue (4), fully hydrated leaves (5), 72 h dehydrated leaves (6), fully hydrated roots (7) and 72 h dehydrated roots (8). The same blot was sequentially hybridised to the 1.1 kb T-DNA flanking region rescued from mutant line H (*DNA H*) and to the ribosomal cDNA fragment, *pTA 71*, which was used to monitor the loading of the RNA.

The transcript accumulation pattern found in response to dehydration in wild type leaves was compared to the transcript accumulation that occurred in the T-DNA tagged transgenic lines. Poly A^+ enriched RNA was extracted from leaves of fully hydrated and completely desiccated wild type plants and from a selection of in vitro grown transgenic plants. DNA H hybridised to a low accumulated transcript of approximately 4 kb in leaves of transgenic lines E, H, L and 10, but not in wild type leaves (see figure 4.3.3.2).

Figure 4.3.3.2 The LR-iPCR rescued DNA from transgenic line H shows constitutive accumulation a transcript present in transgenic leaf tissue

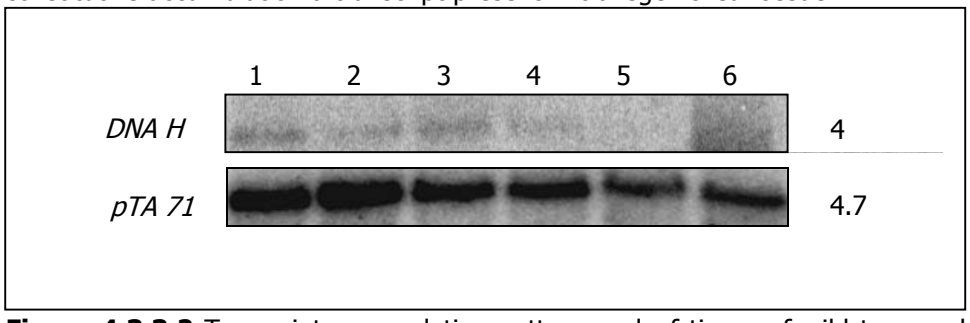


Figure 4.3.3.2 Transcript accumulation pattern on leaf tissue of wild type and transgenic lines using the LR-iPCR rescued DNA from transgenic line H as a probe. 3 μg of poly A^+ enriched RNA were loaded on each lane: transgenic lines (1) E, H (2), 10 (3) and L (4), and wild type leaves: fully hydrated (5) and 72 h dehydrated (6). The ribosomal cDNA fragment *pTA 71* was used to monitor the loading of the RNA.

In RNA blots that included wild type callus and leaf tissues and transgenic callus tissue, the 1.45 kb genomic DNA fragment originating from mutant line 10 hybridised to transcripts of sizes ranging from 5, to 1 kb, present in all tissues although at different intensities, except on completely dehydrated leaves. Signals were found in transgenic callus tissue of line H and 10, of transcripts corresponding to approximately 1.9 and 1.2 kb. A unique transcript of approximately 1 kb was found in the transgenic callus line 10. In fully hydrated wild type leaves and roots, transcripts of 5, 1.9 and 1.2 kb were highly abundant in comparison to the other tissues. One transcript of 1.2 kb was highly abundant in wild type callus, and wild type ABA-treated callus. None of the transcripts were detected in wild type completely dehydrated leaves (see figure 4.3.3.3).

Figure 4.3.3.3 LR-iPCR fragment rescued from transgenic line 10 corresponds to transcripts of different sizes

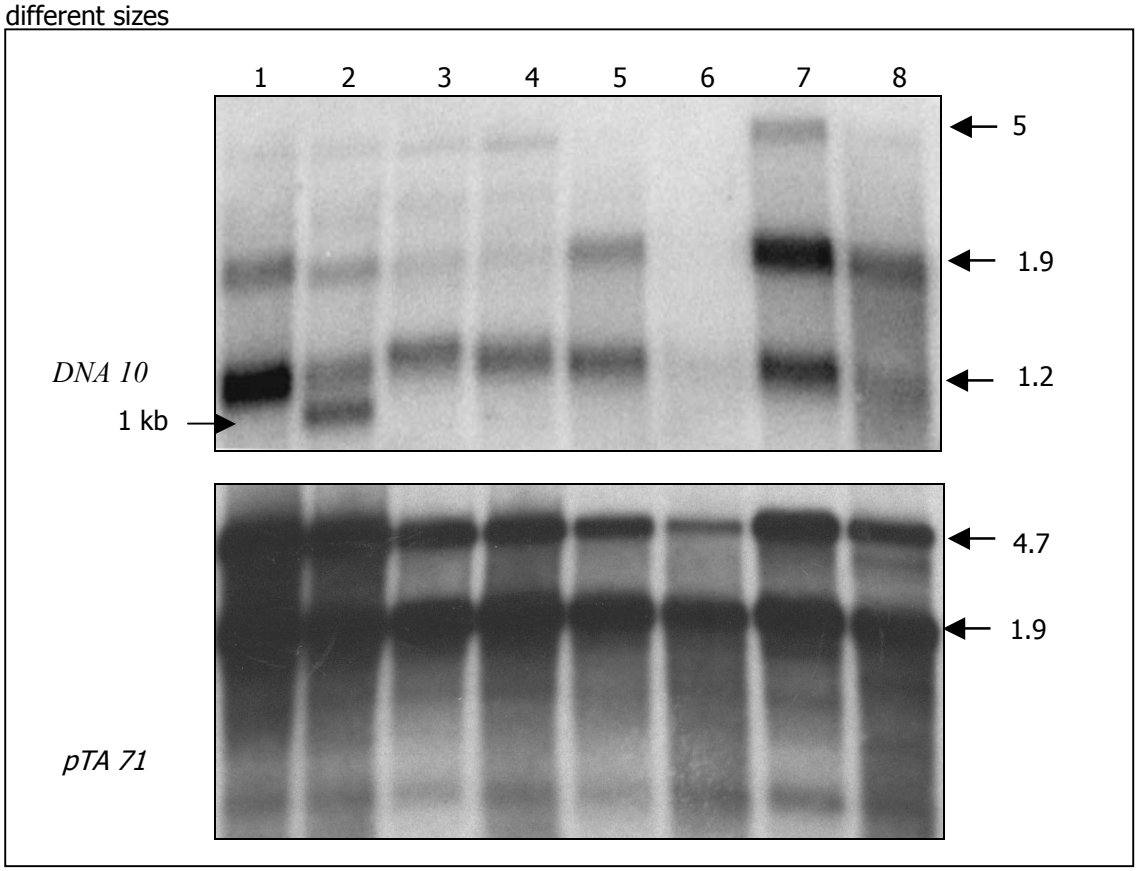


Figure 4.3.3.3 LR-iPCR fragment rescued from transgenic line 10 corresponds to transcripts of different sizes, which are not present in fully hydrated leaf tissue from wild type plants. 3 μg of poly A^+ enriched RNA were loaded on each lane: callus tissue from transgenic line H (1), transgenic line 10 (2); wild type fully hydrated callus tissue (3), 4 d ABA pre-treated callus tissue (4), fully hydrated leaves (5), 72 h dehydrated leaves (6), fully hydrated roots (7) and 72 h dehydrated roots (8). The ribosomal cDNA fragment *pTA 71* was used to monitor the loading of the RNA.

The possibility that the putatively tagged gene might be involved in the ABA/drought mediated signalling network was tested by comparing the transcript profiles of different known desiccation responsive genes in wild type and transgenic backgrounds. To do this RNA gel blots containing wild type and transgenic leaf and callus tissue were hybridised with two previously characterised dehydration responsive genes *pcC27-45* and *pcC6-19* (Bartels *et al.,* 1990). The corresponding transcripts are normally highly accumulated during the desiccation process in wild type *C. plantagineum* leaves and in ABA treated callus.

The transcript corresponding to the *pcC6-19* gene was detected in transgenic callus tissue corresponding to lines H and 10. This transcript accumulated in callus from transgenic line 10 at levels comparable to those found in ABA treated callus. *pcC6-19* transcript levels were low in callus originated from transgenic line H in comparison with wild type callus pre-treated for 4 d with ABA (see figure 4.3.3.4). *pcC27-45* gene expression was detected in callus originated from transgenic line 10 but not in callus originating from transgenic line H. Normal accumulation levels were found in fully hydrated and dehydrated tissue.

Figure 4.3.3.4 Two desiccation responsive transcripts differentially accumulated in callus originating from transgenic lines H and 10

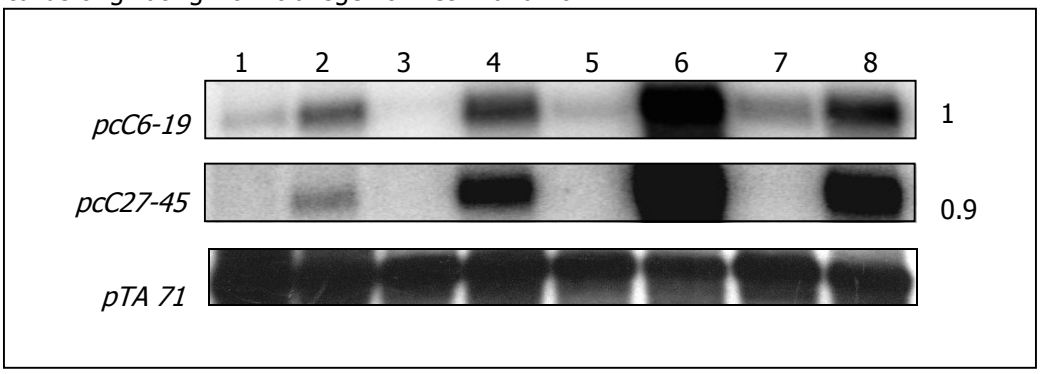


Figure 4.3.3.4 RNA gel blot. 3 μg of poly A^+ enriched RNA were loaded from: transgenic callus lines H (1), 10 (2); wild type: fully hydrated callus (3) and 4 d ABA pre-treated callus (4); fully hydrated leaves (5) and 72 h dehydrated leaves (6), fully hydrated roots (7) and 72 h dehydrated roots (8). Two different *LEA* gene fragments, *pcC27-45* and *pcC6-19* were used as probes. The ribosomal cDNA fragment *pTA 71* was used to monitor the loading of the RNA.

Interestingly, *pcC6-19* transcript was detected in leaves originated from transgenic line 10, but not in leaves originated from transgenic line H. Moreover, *pcC27-45* was not present in leaves from either of the transgenic lines (see figure 4.3.3.5).

Figure 4.3.3.5 Two desiccation responsive transcripts differentially accumulated in leaves of transgenic lines H and 10

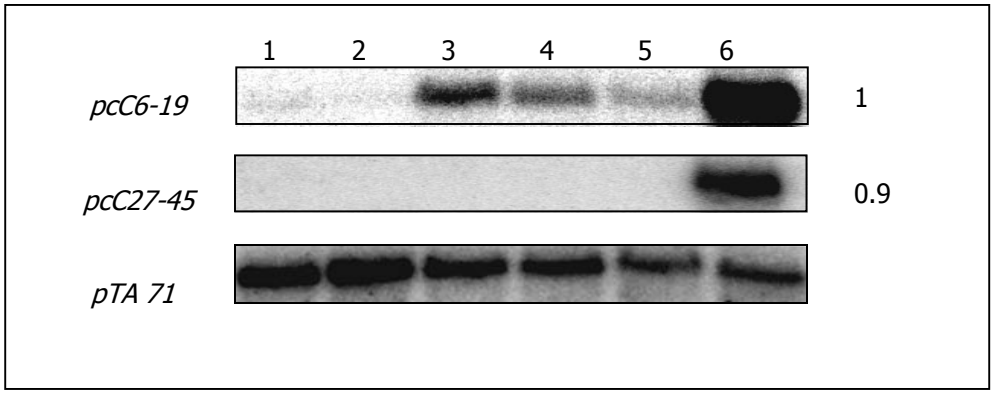


Figure 4.3.3.5 RNA gel blot analysis of transgenic and wild type leaves. 3 μg of poly A^+ enriched RNA from leaves originated from transgenic lines were loaded on: E (1), H (2), 10 (3) and L (4), and wild type: fully hydrated (5) and 72 h dehydrated (6). Two different *LEA* gene fragments, *pcC27-45* and *pcC6-19,* were used as probes. The ribosomal cDNA fragment *pTA 71* was used to monitor the loading of the RNA.

4.3.4 cDNA isolation of the rescued T-DNA flanking regions

In order to obtain information about the corresponding locus where the T-DNA had inserted in transgenic lines H and 10, attempts to isolate the corresponding cDNAs were made. A cDNA library prepared from 2 h dehydrated *C. plantagineum* leaves was screened using the LR-iPCR rescued fragments as a probe. As a result, a 1.6 kb cDNA clone was isolated when the 1.1 kb fragment corresponding to transgenic line H was used as a probe. A 0.8 kb cDNA was isolated when the 1.45 kb LR-iPCR product from line 10 was used as a probe. Sequence analysis showed homology to the corresponding sequence used as a probe in the library screen for both of the obtained cDNAs.

The sequence of the cDNA clone corresponding to the T-DNA flanking region of transgenic line H (*cDNA H*) was adenine-rich and reliable sequence data proved difficult to obtain. Only 600 bp were accurately sequenced and used in a homology search of the public databases, NCBI and EMBL. No significant homology to any reported sequence was found and, furthermore, no clear open reading frame was observed (see figure 4.3.4.1). The sequence of *cDNA H* is shown, highlighted areas correspond to: red – cloning vector, blue – homologue region to the probe (the sequence is 100% homologous to the region of 1118-547 of the LR-iPCR fragment) and bold – *EcoR*I restriction site.

cDNA H (ADF)

```
   1  CCCCACCAAA  CCCAAAAAAA  GAGATCGAAT  TCGGCACGAG  AAAAAAAAAA
  51  AAAAAAAAAA  AAAAAAGAAC  CTTTCCTTTG  ATAATCTTCC  GGTCTCGTGA
 101  ATTCCACATC  ATTCCATCAT  TTCGAGACTT  GGAAGAAGAA  AGGCTTANTG
 151  GAACGCAGCA  GAAATAAGAG  AATATGAAGA  CGGCGTAATC  AAAAGGGTTG
 201  ATCCTGGCGC  GCTTTANACA  ATGACATGAA  GACCGACACG  ACTGCTACTC
 251  ATATGTGATC  CCACTACCAC  CTCATTACTA  TCTGAACCCA  TTTAACCCGA
 301  AAAGACCTTC  TGATTCATGC  TAACTGCTTT  AAGTTGTANA  CGTGCGATTG
 351  ACTAGCAAGC  ATATGGTANA  GACTAGATTA  TTGCATGAAT  TGAGCGATTC
 401  ACAGATTTAT  TAGCCTATAC  ACTGAGTGAT  TTGAGTGAAT  CCCTGTGAGG
 451  TGTGGATCTC  GATTAGTAGT  GCTAATGAAA  TTCGTGTCCG  ATGAGTGCTG
 501  ATTATTGNTG  CAGNGGCTAT  AATTGCTTGC  TTAATTCCTA  TTTGNGTTTT
 551  CCATCTGCGT  TGGTGAATTG  TGAATGGGCC  GTGCCCGTTA  TCTTTGGANA
 601  ACCCNTTTTN  CTTGCCTTTT  TAATTTTTGG  CTTNGNGGGA  ACAANGCAAA
 651  AAAAGCNTTA  AAAAGGNGGN  NGGGGGGGAG  GGNTTTTGGA  AANAAAAACC
 701  GTTTATTTTT  TTAAAAANAA  CCCCCCTCTC  AAATTTNGGG  GTAAAAAGGG
 751  GGNCNAANTT  TTTGGGGGGG  GGGTTTTTTT  TTTTTTTNTT  TANGGGGGGN
 801  AAAAAANAGG  TTTTTTTTGG  GGGAAAACCC  AATTTTTNCT  TTATTGGNCC
 851  CCAATTTTTT  AAATTNGGGG  GGCTNTTTTT  TTTTTTTCCC  NCCCCCCCCC
 901  TTTTTNTAAA  AAANTTTTTG  GGGNNNGGGN  CCCTTTTCNA  AANTNGGATT
 951  TTTTTNCNNN  NCCNNAAAAA  NGGGAACCCA  AAAACCGGGG  GGGGGAAACC
```

The full 0.8 kb sequence of the *cDNA 10* clone corresponding to the T-DNA flanking region of transgenic line 10, was obtained. Sequence analysis showed a stretch of 28 bases homology to the *C. plantagineum* gene *CDT-1.* It does not contain a clear open reading frame, although a putative peptide of 159 aa was predicted with the Gene Finder software (see figure 4.3.4.1). A stretch of 110 aa showed 94 to 96% homology to a mouse EST (not annotated). The sequence of the *cDNA 10* clone is further shown with highlighted areas corresponding to: red – cloning vector, blue - homologue region to the probe (LR-iPCR rescued fragment), green – homology to *C. plantagineum* mRNA hypothetical protein CDT-1 (100% homologue to the sequence [tgggctatttagcccctgtatctttgtt]); bold red/blue – *EcoRI* and *Xho*I restriction sites respectively, (*EcoR*I-*Xho*I, 0.5 kb fragment) and bold black – start and end of the hypothetical protein.

cDNA 10 (ADF-ADR):

```
   1  ATAACTATCT  ATTCGATGAT  GAAGATACCC  CACCAAACCC  AAAAAAAGAG
  51  ATCGAATTCG  GCACGAGGTT  TAAAAAAAAA  AAAAAAAATT  CGTCCACCCC
 101  GCCGCTGCCA  CCACCAAGCC  GCCAACGCTG  CCACCACCAA  GCCGCCTCTG
 151  CCTTCGCCTC  CTCTGCAGCC  GCCTGCGCCT  CCGCCTCCTC  CGTTGCCATC
 201  GCCGCCGCCT  CCGTCTTCTT  TGGCCCCGTC  TTTCCCGCCA  GATCTGCCAC
 251  AGATCCTGCC  TCTGCCGGCC  GCCACCACCT  GGCCGCTGCC  TCTGCTGTCC
 301  GCCGCCCCTG  CCGCCACCTC  CATCGCTACA  GTCTGCCATA  ACTACAGTCC
 351  GCCACATCGC  ACTATCGTCC  ACCCGCGAGT  CTCCCGTGGG  GAGCTCGTTT
 401  CTCCCGGCCA  TTAGGTCGGG  AGCTTTTTCT  CCCGTTGTGG  AGCCAATTCT
 451  CCCTTGTGGA  GCCTCTTCCG  ATGAAGACGA  TAACCTCGAG  GCGTTGGACA
 501  AAAATAATGT  AGAGTACTGT  AGATTTGGAT  TAATAACCTT  CTCTGTCGTA
 551  TGTTCTATAG  ATTTTTGGTC  CAAGTTCGAG  GTTTATTTCG  TTTTCTGTCG
```

```
601  GCTTGTTATT  TTGTTTTCGT  GATGCATTGC  TCCTTATGAG  CCTTTGTTTC
651  TCTACCGGAT  TCTGTGTTAC  CGGAGAGACC  CTTTGGGCTA  TTTAGCCCCT
701  GTATCTTTGT  TTATTAATGA  AAGCTATCGT  TTACGGAAAA  AAAAAAAAAA
751  AAAAAAAAAA  AAAAAAAAAA  AACTCGAGCC  CGGGTCGACT  CTAGTGCCCT
801  ATAGTGAGTC  GTATTACTGC  A
```

Figure 4.3.4.1 *DNA 10* hypothetical protein shows high homology to a mouse EST

a) *DNA 10* hypothetical protein: Frame 5-3'

RLRLRLLRCHPRRRLRLLWPRLSRQICHPRSCLCRPPPPGRCLCCPPPPLPPPP**SLQSAITTVPRHIALS STRESPVGSSFPLPAIRSGAFSPVVEPILPPCGASSDEDDNLEALDTQNNVEYCRFGLITFSV VPCSIDF WSKFEVYFVF CRPLVILFS**

159 aa

b) *DNA 10* hypothetical protein, significant homologies:

```
mus_musculus1     1 ------------------------------------SPVVEPILPPCGASSDEDDNLEA
DNA10             1 SLQSAITTVPRHIALSSTRESPVGSSFPLPAIRSGAFSPVVEPILPPCGASSDEDDNLEA
mus_musculus2     1 ------------------------------------SPPADPCLVS-GLSSDEDDGLEC
homo_sapiens      1 ---------------------------------------SSLSHLIIHITLSSSRHS----
oryza_sativa      1 ------------------------------------PSLAALACSVDTPAPSSESCST

mus_musculus1    24 LDTQNNVEYCRFGLITFSVVPCSIDFWSKFEVYFVFCTKVVVLFS---
DNA10            61 LDTQNNVEYCRFGLITFSVVPCSIDFWSKFEVYFVFCRPLVILFS---
mus_musculus2    23 LDVANN----RLSAGCDSLSDCLASFSITFSLDFVT--KVVVLFS---
homo_sapiens     19 ---PVN-----LTLPSPSITPFAPTFSPLLRP----------------
oryza_sativa     23 IRAGRP-----PPPPSPDASPPAQARKAEDDEEEALPRRHRPRRCRGG
```

Figure 4.3.4.1 *DNA 10* hypothetical protein, corresponding to the T-DNA flanking region rescued from transgenic line 10. Panel a) *DNA 10* translated sequence (Gene Finder); highlighted in bold the region that showed significant homology. Panel b) 110 aa of the hypothetical protein showed significant homology to reported ESTs.

The 0.5 kb fragment, corresponding to the *cDNA 10 Eco*RI-*Xho*I restriction fragment, was used as a probe in RNA gel blot experiments. It contained the fragment corresponding to the hypothetical protein. As a result transcripts ranging form 5 to 1.2 kb detected in all tissues although at different levels of accumulation (see figure 4.3.4.2). The RNA blot included transgenic and wild type callus, wild type leaves and roots both fully hydrated and dehydrated. One transcript of 1.2 kb was accumulated in callus tissue from transgenic line H. Three transcripts were detected in callus tissue from transgenic line 10 and wild type, corresponding to approximately 4.5, 1.3 and 1.2 kb. In ABA treated wild type callus tissue only one transcript of 1.2 kb accumulated. In fully hydrated wild type leaves two transcripts were detected, corresponding to approximately 1.9 and 1.2 kb. Only one transcript of 1.2 kb was detected in all samples with the exception of totally dehydrated leaf tissue.

Three transcripts of different sizes, 5, 1.9 and 1.2 kb, were detected in fully hydrated roots, which did no longer accumulate when the roots were totally dehydrated (instead, a smear was present).

Figure 4.3.4.2 *cDNA 10,* 0.5 kb fragment, corresponds to transcripts of different sizes, differentially accumulated in dehydrated and fully hydrated tissues.

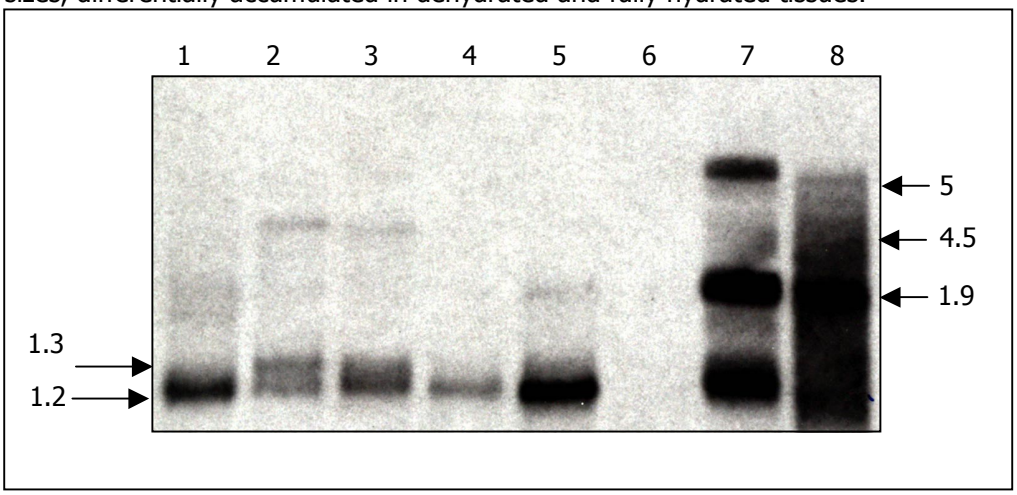


Figure 4.3.4.2 RNA gel blot. 3 μg of poly A^+ enriched RNA were loaded from plant material originated from: callus tissue from transgenic line H (1) and line 10 (2); wild type: fully hydrated callus (3), 4 d ABA pre-treated callus (4), fully hydrated leaves (5), 72 h dehydrated leaves (6), fully hydrated roots (7) and 72 h dehydrated roots (8). The ribosomal cDNA fragment *pTA 71* was used to monitor the loading of the RNA (not shown).

The transcript sizes and accumulation pattern are summarised in table 4.3.4.2.

Table 4.3.4.2 Summary of the transcripts homologous to *cDNA 10* in *C. plantagineum* tissues

Transcripts size	1.2 kb	1.3 kb	1.9 kb	4.5 kb	5 kb
wild type					
fully hydrated leaves	+		+		
fully hydrated roots	+		+		+
fully hydrated callus		+		+	
wild type					
totally dehydrated leaves					
totally dehydrated roots			+		
4d ABA treated callus	+				
transgenic callus					
H	+				
10	+	+		+	

(+) denote presence of the transcript of the corresponding size and empty spaces denote no transcript.

V Discussion

5.1 Transcript profiling of C. plantagineum genes

Abiotic stresses such as drought, high salinity or extreme temperature result in water loss. In order to get a better understanding of the molecular mechanisms that regulate dehydration tolerance, one strategy is to dissect the signalling networks that activate gene expression. Previous reports have identified distinct pathways that control gene expression in response to either dehydration or low temperature, however prior to this study dehydration and salt signalling pathways remained closely linked.

Here, transcript profiling was performed with dehydration responsive genes isolated from *C. plantagineum*. It was observed that many transcripts accumulated in response to both types of stress. Detailed analysis revealed that *LEA* genes, which have been well characterised and related to osmotic stress responses (Dure, 1989; Dure *et al.,* 1993a/b; Garay-Arroyo *et al*, 2000), showed a synchronised pattern of transcript accumulation in response to dehydration that differed from that observed in response to sodium chloride treatment. Thus, changes at the molecular level can be associated with dehydration tolerance and distinguished from salt induced responses.

5.1.1 *C. plantagineum* is a suitable model system for the identification of dehydration specific responses

In order to define dehydration specific responses at the mRNA level *C. plantagineum* was chosen as an experimental system because it tolerates extreme dehydration and is sensitive to sodium chloride: relatively low concentrations of salt for short periods of time caused a wilting phenotype (100 mM sodium chloride for 24 hours). In contrast, the non-halophytic plant *A. thaliana* can grow in media supplemented with 100 mM sodium chloride for up to four days (Liu *et al.,* 1997) and the halophyte *A. nummularia* withstands up to two months of 400 mM sodium chloride treatment (Braun *et al*., 1986). Although both dehydration and salt stress lead to osmotic stress, salt stress is also comprised of an ionic component, therefore, it is proposed that this component is toxic to *C. plantagineum*.

Genes are induced by both dehydration and high salinity, among them are *A. thaliana* genes described as being responsive to dehydration (*RD),* early responsive to dehydration *(ERD)* and members of the *LEA* gene family (Yamaguchi-Shinozaki *et al.;* 1992; Kiyosue *et al.,* 1994). Other genes are thought to regulate dehydration and salt stress tolerance such as the DREB2A transcription factor (Liu *et al.,* 1998). In the case of salt stress, the *SOS2* and *SOS3* genes have been identified and are thought to be involved in the tolerance of the ionic but not the osmotic component of salt stress. They play specific roles in plant adaptation to high Na^+ stress (Liu and Zhu, 1998; Liu *et al*., 2000). These observations suggest the existence of specific cellular signal transduction pathways that lead to abiotic stress tolerance in plants.

5.1.2 Macroarrays to study gene responses

The ability to investigate global patterns of gene expression is revolutionising biology and the understanding of plant processes by providing a general perspective of responses to environmental and developmental changes. The use of high-density DNA arrays attached to nylon membranes or glass slides as a tool to study gene expression has been demonstrated in a number of studies. For example, expression of the yeast genome (over 6000 yeast genes) was monitored (DeRisi *et al*., 1997; Wodicka *et al*., 1997) and in plants a comparison of gene expression between plants grown in dark or light conditions has been made (Desprez *et al.,* 1998). These studies allowed subtle changes in gene expression to be detected. Recently, the expression pattern of 1300 *A. thaliana* genes were monitored under drought and cold stress conditions (Seki *et al.,* 2001), which helped to identify target genes of stress related transcription factors.

In this study a nylon filter array technique was used. Nylon filters that included 259 *C. plantagineum* dehydration responsive genes were hybridised to various probes. The reliability of the procedure was demonstrated by the following observations. Firstly, a comparison of the duplicate spot values revealed a high reproducibility for the majority of clones. Only in 1-1.6% of the cases was significant variation in the hybridisation signals observed. Such a comparison has been used in other reports and shown to be a reliable method for the detection of aberrant signals. In the work of Desprez (1998), 816 cDNA genes were studied and the variation between spot repetitions was estimated to be 0.3%.

Secondly, for previously characterised dehydration responsive genes the expected differential expression was observed. For example, *LEA* transcripts were seen to accumulate to high levels in response to dehydration. Moreover, two repetitions of the *pcC 6-19 LEA* gene, included on

the filters at multiple positions, clustered at the same position in response to both dehydration and sodium chloride treatments.

Thirdly, differential gene expression patterns identified via the macroarray approach were confirmed by RNA gel blot analysis. Although the absolute values determined by RNA blot analysis and DNA array hybridisations were different, the patterns of gene expression were comparable. The results obtained from RNA gel blot hybridisations proved to be more reproducible and sensitive than those obtained from macroarray hybridisations. A possible explanation could be that the hybridisation kinetic, the nature of the probe, and the general conditions of both techniques differ from each other.

Although the macroarray results were on the whole reproducible, some variation in gene expression was observed, particularly in the case of low abundant transcripts. One example concerns a member of the homeobox transcription factor family, *CpHB-1* (Frank *et al.,* 1998), whose transcript accumulation observed in the macroarrays was not reproducible when compared to the results obtained from RNA blot experiments. A similar discrepancy has been described elsewhere: macroarray systems are unable to detect transcripts accumulated at low levels (Mochii *et al.,* 1999).

5.1.2.1 The applied statistical analyses were reliable

In order to be able to quantify the signals and compare them between independent filters, the *uidA* gene, a non-plant control was included. This reference allowed elimination of the error resulting from the different hybridisation or labelling reaction efficiencies. Direct comparison between transcript accumulation values could therefore be performed for each gene. This synthetic control proved to be reliable in that it did not cross-hybridise with plant DNAs. Other types of genes have been reported as internal quantification standards, such as human *desmin* and *nebulin* cDNAs (Desprez *et al.,* 1998), or the synthetic clones derived from antibiotic resistance genes (Eickhoff *et al.,* 1999).

Most approaches to global transcription analysis rely on generating ratios of signal intensities between the control and experimental samples. The ratio between signals for a particular spot provides a measure of change in expression level. However, experimental conditions often result in only a small number of genes with altered levels of expression. Induction and repression ratios identify dramatic changes in transcript abundance but ignore variations in signal intensities between spots that do not significantly change. Multiple replicates of an experiment are

necessary before one can be confident that the signal corresponds to the true steady state mRNA level. Therefore, the macroarray experiments presented here were repeated three times and the data analysis included not only the ratio of induction between the control and the experimental samples (transcripts whose ratio of induction or down-regulation were <2 fold were eliminated), but also a second statistical parameter, the *z* score (which takes differences of induction into account). Candidate genes and differentially transcribed genes were classified on this basis. The results obtained for the known transcripts proved the reliability of the statistic analysis applied.

It should, however, be noted that variations in signal between two different genes in an array cannot be compared. Since the variations detected may not reflect the absolute abundance of different transcripts in the cell but might be due to differences in size, base composition, or concentration of DNA in each spot.

5.1.3 Differences in the transcriptomes obtained from dehydrated and sodium chloride treated *C. plantagineum* plants showed molecular fingerprints that may be specifically related to dehydration tolerance

5.1.3.1 Organisation of *C. plantagineum* genes according to transcript accumulation characteristics

Macroarray experiments not only enabled the possibility to better assess the behaviour of genes previously implicated in dehydration responses, but also to identify novel differentially regulated transcripts. Most of the genes included in the arrays were dehydration responsive, this was expected since they were pre-selected on this basis. However, 3% of the genes included could not be classified as being dehydration responsive. Because of the stringent criteria used for data acceptance, it is likely that some differentially expressed genes may have been eliminated (after application of the ratio and *z* score parameters, only those transcripts that showed a response in more than two points along the kinetic were selected). Low abundant transcripts, which were not efficiently measured, were also eliminated after analysis of the data sets.

From the total number of differentially accumulated transcripts 23% were up-regulated by both dehydration and sodium chloride treatment. As expected, most of the *LEA* gene transcripts grouped within this class, e.g. *pcC 6-19, pcC 27-45, pcC 16-19*; several novel ESTs (e.g. EST0067, 0068), and some ESTs with homology to known genes (e.g. clone 10-1 with homology to tyrosine

phosphoprotein). A lower percentage of transcripts, 11%, were down-regulated by both treatments, e.g. ESTs with sequence homology to genes involved in photosynthesis such as EST005, EST0015 and EST0044, with homology to chloroplastic ATP synthase, ferredoxin and PSII 33KDa protein of the water oxidising complex, respectively.

A small group of transcripts were regulated specifically by dehydration: either up-regulated (e.g. EST0011 with homology to a cystein protease or EST0061 with homology to a water stress-induced protein). They, together with those transcripts that were down-regulated upon sodium chloride and up-regulated upon dehydration treatment may represent key molecules that are specific determinants of dehydration tolerance. 19% of the differentially accumulated transcripts belonged to this second group, one of these genes showed homology to a protein kinase, clone A39-1, and to an aquaporin gene, *CpPIPa.2.* In the case of transcripts that are only down-regulated (either upon dehydration or sodium chloride) one should be cautious at taking them as candidates. They might be constitutively low accumulated, therefore yielding inaccurate measurements, or they might be true down-regulated transcripts. Further analysis should confirm these results.

In the case of the gene *CpPIPa.2* the transcript profile was confirmed by RNA gel blot analysis. This gene has been previously characterised in response to dehydration in *C. plantagineum* (Mariaux *et al*., 1998), and was found in this study to possess a unique profile in response to sodium chloride. *CpPIPa.2* encodes for a plasma membrane intrinsic protein (PIP), which belongs to the aquaporin protein family. Aquaporins are thought to facilitate water transport in order to achieve osmotic adjustment between cytoplasm and vacuole and are members of the major intrinsic protein (MIP) family. MIPs are a family of channel proteins that allow translocation of small solutes across membranes. Enhanced aquaporin expression has been reported in drought and salt stressed plants, i.e. the *rd28* gene from *A. thaliana* (Yamaguchi-Shinozaki *et al.,* 1992). Others, like Mc-MIPA, B and C genes from *Mesembryanthemum crystallinum*, are transiently down-regulated by salt stress but up-regulated during the recovery of plant cell turgor (Yamada, 1995). The *CpPIPa.2* transcript profile suggests that mechanisms for water transport are induced at the early stages of dehydration and only in response to osmotic change.

The *C. plantagineum* PIP cDNA clones included in the macroarray filters belong to three different groups: *Cp-PIPa*, *Cp-PIPb* and *Cp-PIPc*, classified according to their sequence homologies (Mariaux *et al.,* 1998). *Cp-PIPa2* transcripts are both ABA and dehydration inducible. In contrast *Cp-PIPa6* and *a7* and *c* are dehydration inducible, and *PIPb* transcripts are constitutively expressed. In this study *Cp-PIP* genes showed differential transcript regulation in response to dehydration and sodium chloride treatment within the family, i.e. *CpPIPc* versus *CpPIPa.2*. Both transcripts were up-regulated upon dehydration and down-regulated upon sodium chloride, however, they did not clustered at positions close to each other. Differential expression within gene families in response

to dehydration in *C. plantagineum* has been reported for other multigene families such as Myb and homeobox transcription factors (Iturriaga *et al.,* 1996; Frank *et al.,* 1998). Complexity of gene families may indicate functional complexity in different cells or tissues, developmental specificity of expression or functional redundancy.

The ability to study individual transcript profiles within a gene family using the DNA-array hybridisation procedure was also confirmed by the *CpPIP* data. Differences can be detected in the macroarrays since the complex probe is synthesised by reverse transcription initiated from the 3' end of the mRNA, using an anchored oligo-dT primer. This procedure enriches the probes for sequences corresponding to the highly variable 3'-untranslated region of mRNA. The application of a similar transcript profile approach permitted the characterisation of *A. thaliana* gene family members involved in the wound response (Reymond *et al.,* 2000).

5.1.3.2 Transcript profiling reveals dehydration specific responses

The dehydration and sodium chloride induced transcript profiles were analysed by measuring the similarity between transcript accumulation patterns within a kinetic. The cluster and tree view software, developed by Eisen (1988), allowed the measurement of the degree of similarity between transcript profiles and gave a qualitative and quantitative output.

Although a common response to both treatments was the up-regulation or down-regulation of certain transcripts, the coordination between transcript profiles often differed. According to the similarity values, the different *LEA* transcripts gave rise to patterns of induction that were more similar with each other in response to the dehydration than in response to the sodium chloride treatment. Three main patterns were observed: (1) transcripts that were up-regulated in response to increases in sodium chloride concentrations (50 mM to 150 mM; e.g. *pcC 11-24*, *27-45*), (2) transcripts that show minor variations in comparison to their corresponding non-treated control (50 mM to 150 mM for 6, 12 and 24 h; e.g. *pcC 16-81*) or (3) transcripts that were up-regulated in response to higher sodium chloride concentrations for long periods of time (e.g. *pcC 13-62*). These patterns of induction were subsequently confirmed by RNA gel blot analysis.

One implication in the common temporal pattern of expression is that the genes might share similar or related roles in cellular processes and/or may be regulated by the same signalling molecules. It has been shown that transcripts with similar profiles share similar functions, or are involved in the same pathway. Perou (2000) reported that specific transcript accumulation patterns correspond to specific types of tumours in human cell samples. In a similar way, the specific and

coordinated transcript accumulation pattern presented by the *LEA* genes in response to dehydration can be considered as molecular "fingerprints" for desiccation tolerance. Once a distinct expression fingerprint has been correlated with a complex process, such as desiccation tolerance, this expression fingerprint can be potentially used as a tool to evaluate new genetic materials for the trait in conjunction with phenotypic tests.

By determining expression patterns of *LEA* genes across a wider range of environmental conditions it may be possible to develop a hypothesis about their specific function. So far, LEA proteins are thought to function in protecting other proteins and membranes from dehydration-induced damage by providing a surrogate water film to solvate cellular components (Ingram and Bartels, 1996). Other LEA proteins may form amphiphilic helices that sequester ions that are concentrated during seed maturation (Baker, 1988). Recently, LEA proteins were classified in a more widespread group referred as "hydrophilins" (Garay-Arroyo *et al.,* 2000). By using the genomic information of several organisms, database searching showed that this criterion selectively differentiates most known LEA proteins as well as additional proteins from different taxons. It was demonstrated that the criterion that defines hydrophilins seems to be an excellent predictor of responsiveness to high osmotic pressure since most of the genes encoding these proteins in *E. coli* and *S. cerevisiae* were induced by osmotic stress. Apparently, hydrophilins represent analogous adaptations to a common problem in such diverse taxons as prokaryotes and eukaryotes. *LEA* transcripts are, however, not only regulated in response to water stress responses but also to normal plant development (Colmenero-Flores *et al.,* 1999; Jayaprakash *et al.*, 1998), which suggests cross talk between stress and developmental signalling networks.

5.1.4 Future experiments

Further studies should use a larger *C. plantagineum* gene collection, including known and unknown EST clones. The use of the macroarray technique is a suitable approach to study plants that posses a unique physiological response and have a complex genome. Moreover, the DNA arrays can be used to screen the T-DNA tagged *C. plantagineum* population (see section 5.2) for polymorphic "expression fingerprints" which, together with other methods, would help to identify genes or groups of genes involved in specific plant processes, such as ABA-mediated dehydration tolerance.

Although DNA arrays offer significant advantages for gene expression studies, they have limitations. The current methods of producing probes rely on a significant amount of poly(A)$^+$

enriched RNA. Improvements in protocols that would permit the isolation of poly A^+ enriched RNA from small quantities of tissue or even from single cells would extend the application of the technique. Also, not all genes that participate in a process exhibit changes in transcript levels. In some signal transduction pathways, regulation occurs at the level of phosphorylation of the gene product. Developments in the complementary field of proteomics will provide the tools needed for global analyses of changes in polypeptides.

More recently microarray technology has been developed, the main advantage of which is the use of two dyes (Cy3 and Cy5) to individually label cDNA derived from samples to be compared in a single hybridisation. Current concerns include non-linearity of Cy3 and Cy5 responses, reproducibility between arrays and variation due to slight differences in hybridisation conditions. Slide attachment chemistries, hybridisation solution reagents and alternative nucleotide derivatives for improved labelling and hybridisation are all active areas of research.

In a study by Richmond (1999), both macro and microarrays were compared. Considerable variation was observed between the two methods. In conclusion, the microarray approach was found to be more reproducible than radioactive hybridisation to nylon arrays. One explanation for the higher variability with the hybridisation to separate membranes is variability in spot deposition, which can only be overcome by performing multiple, independent hybridisations.

Finally, improved statistical and computational methods for measuring and relating transcript accumulation patterns may permit biological pathways to be determined. For example, the work of Zien *et al.* (2000) suggests the use of a sample scoring function in order to generate biologically possible pathways by scoring them with respect to gene expression measurements. This method uses the available knowledge on specific biological networks for the evaluation of gene expression data (e.g. glycolysis pathway in yeast). Starting from known reaction networks, the possible pathways are extracted and examined according to how well they are supported by a given expression data. The idea is to define scores for putative pathways and scores for genes with respect to a given pathway, both based on gene expression measurements. In this way, the relevant pathways are selected based on gene expression (Zien *et al.*, 2000).

5.2 Dissection of the ABA/dehydration signalling network by the study of dominant mutants in *C. plantagineum*

Like other plant hormones, ABA has multiple roles during the life cycle of a plant. In particular it is thought to modulate the response of plants to stress conditions. The integrated

response of the whole plant to water deficit must also comprise sensing and signalling mechanisms. Recently, the use of *A. thaliana* ABA perception and sensitivity mutants have helped to assign a function to a number of molecules involved in the ABA signalling network (Leung *et al., 1994, 1997*; Gosti *et al.,* 1998; Finkelstein *et al.,* 1994, 2000).

Many homologous genes have been identified in different species, such as *A. thaliana,* maize*,* barley and *C. plantagineum*, as a consequence of what appears to be a universal response to water deficit. Nevertheless, there are clear differences in stress survival rates between these species. Differences between tolerant and non-tolerant species in gene expression and genome content should be identified which may play a role in stress tolerance.

C. plantagineum can tolerate extreme dehydration, however *in vitro* propagated callus derived from this plant has a strict requirement for exogenously applied ABA in order to survive severe dehydration. ABA treatment induces the subset of the genes in the callus that are induced by dehydration in the whole plant (Bartels *et al*., 1990). This property has been exploited for the isolation of dominant mutants, in which the ectopic expression of resident genes activated by insertion of a foreign promoter would confer desiccation tolerance to the transformed cells without prior ABA treatment.

Previous activation tagging experiments permitted the isolation of a gene (*CDT-1*), which encodes a signalling molecule in the ABA transduction pathway (Furini *et al.,* 1997). Constitutive expression of *CDT-1* led to desiccation tolerance in the absence of ABA and to the constitutive expression of a subset of dehydration and ABA responsive transcripts. Therefore, the activation tagging technique was used in this work in order to identify potential signalling intermediates in the ABA activation of gene expression in response to stress. Two transgenic callus, lines 10 and H, were identified. T-DNA tagged genes in these lines may play a role in the ABA signal transduction network leading to desiccation tolerance.

This method is especially effective in organisms with functional redundancy, such as the polyploid *C. plantagineum*. The problem of functional redundancy has become particularly apparent during the past few years, as sequencing of eukaryotic genomes has revealed the existence of many duplicated genes that are very similar both in their coding regions and their non-coding, regulatory regions (Weigel *et al.,* 2000).

5.2.1 A T-DNA tagged population was created in *C. plantagineum*

A T-DNA tagged population in *C. plantagineum* was generated by *A. tumefaciens* mediated transformation with the use of two different plant cloning vectors (Koncz *et al.,* 1994). It consists of

49 transgenic lines, which were selected at the callus level by their ability to survive two independent desiccation tolerance screenings. The regenerated plants are kept both *in vitro* and in green house conditions. 10 independent lines have been identified which contain between 1 to 6 T-DNA insertions. Since each explant was regenerated from a single cell, the possibility that the desiccation tolerant phenotype resides only in certain plants must be considered. Further studies are required to determine whether or not the callus derived from the selected plants are able to reconstitute the desiccation tolerance phenotype. Regenerated plants were indistinguishable from greenhouse grown, wild-type *C. plantagineum*. They were however sterile, even after cross-pollination with wild-type pollen. This phenomenon was also described by Furini (1997), where the use of a similar plant transformation system resulted in transgenic plants that were not able to develop seeds. Unlike *A. thaliana*, the regeneration of *C. plantagineum* plants takes longer periods (approximately 6 months). This together with the large amounts of plant material needed for molecular analysis were the limiting steps for the full characterisation of the tagged population.

Previous screenings of other T-DNA populations created with the use of activation-tagging vectors have led to the identification of a histidine kinase from *A. thaliana*, whose overexpression can bypass the requirement for cytokinin in the regeneration of shoots (Kakimoto, 1996), and recently to the identification of two *A. thaliana* transcription factors such as the LEAFY PETIOLE (leaves without a petiole) which encodes a protein with a domain with similarity to the DNA binding domain of members of the AP2/EREBP family that is thought to function in either cell division activities or leaf patterning (Van der Graaf *et al.,* 2000). Other examples include a *MYB* gene that regulates phenylpropanoid biosynthesis and enhances the accumulation of lignin, hydroxycinnamic acid, esters, and flavonoids, including various anthocyanins that gave a purple colour phenotype (Borevitz *et al.,* 2000) and the *ORCA3* gene from *Catharanthus roseus*, a jasmonate-responsive transcriptional regulator with an AP2-domain which is thought to regulate plant primary and secondary metabolism (van der Fits and Memelink, 2000).

5.2.2 Two putatively dominant mutants were identified that are involved in desiccation tolerance

Two dehydration tolerant callus lines, H and 10, were chosen by their ability to survive three independent desiccation tolerance screenings. Their T-DNA flanking regions were isolated

and used as probes for the identification of the corresponding cDNAs by screening a *C. plantagineum* cDNA library.

Sequence analysis of the isolated flanking region corresponding to transgenic line H loci, called *DNA H,* showed low sequence homology to a rice EST. Limited sequence analysis was performed with the isolated *cDNA H*. The complete sequence was not obtained, which was possibly due to the long stretches of adenines that are present in the clone. Further isolation of the corresponding genomic clone would help determining the nature of the T-DNA insertion and its homology to reported sequences.

DNA H corresponds to a transcript of approximately 5.3 kb, which is accumulated in fully hydrated and ABA treated wild type callus only and it is lowly accumulated in its corresponding transgenic callus line. DNA blot analysis showed that two T-DNA insertions occurred on transgenic line H. However, only one of the two T-DNA flanking regions was isolated, a genomic fragment of 1.1 kb. Further studies should determine the flanking regions of the second T-DNA insertion, which might contain a gene that could be responsible for the observed desiccation tolerance phenotype.

Sequence analysis of the rescued flanking region, corresponding to the single T-DNA insertion in transgenic line 10 (called *DNA 10*), with Gene Finder revealed that *DNA 10* corresponds to a multiexon gene. Its longest putative open reading frame codes for a peptide of 110aa rich in proline which showed high homology to a mouse EST and no significant homology to any other reported sequence. Moreover, the same peptide was predicted after the corresponding *cDNA 10* sequence was translated.

The *DNA 10* hybridised to a number of different transcripts distributed along differentiated and non-differentiated tissues. The accumulation of the transcripts varied, as well as the transcript size. One unique transcript was detected in the corresponding transgenic callus line. This band was not detected when the isolated *cDNA 10* was used as a probe, although the same transcript accumulation pattern and distribution was similar to the one detected with the genomic rescued fragment. This supports the hypothesis that probably more than one gene was included in the rescued DNA 10 locus.

Further studies are required in order to determine whether the complete genes have been isolated and to determine if the putatively activated tagged genes are responsible for the acquisition of desiccation tolerance in callus through retransformation experiments. It must be considered that the gene responsible for the phenotype observed could be further downstream of the rescued flanking sequences. However, it has been reported that overexpressed genes are almost always found immediately adjacent to the inserted T-DNA, at distances ranging from 380 bp to 3.6 kb (Weigel *et al.,* 2000; van der Fits and Memelink, 2000).

5.2.3 Possible role of the putatively tagged genes

The transcript accumulation pattern observed for two dehydration responsive *LEA* transcripts in transgenic line H suggested that the putatively tagged gene might act in a response pathway that leads to accumulation of the *pcC 6-19* transcript, but not *pcC 27-45*. The ABA induction of the endogenous *pcC 27-45* mRNA requires *de novo* protein synthesis, whereas *pcC 6-19* mRNA does not. Moreover, an element has been described that is present in *pcC 27-45* and is essential but not sufficient for the activation of genes in response to ABA (Nelson *et al.,* 1994). These suggest that different regulatory pathways of ABA mediated gene expression are responsible for the expression of *pcC 6-19* compared to *pcC 27-45* (see figure 5.4.1).

Moreover, the observation that the corresponding *DNA H* transcript was detected at lower levels in transgenic line H than in the wild type callus tissue suggests a possible role of the tagged gene as a negative regulator of ABA signalling. A similar mode of regulation has been shown for two *A. thaliana* protein phosphatases, ABI1 and ABI2, which act as negative regulators of the ABA signalling network (Gosti *et al.,* 1999).

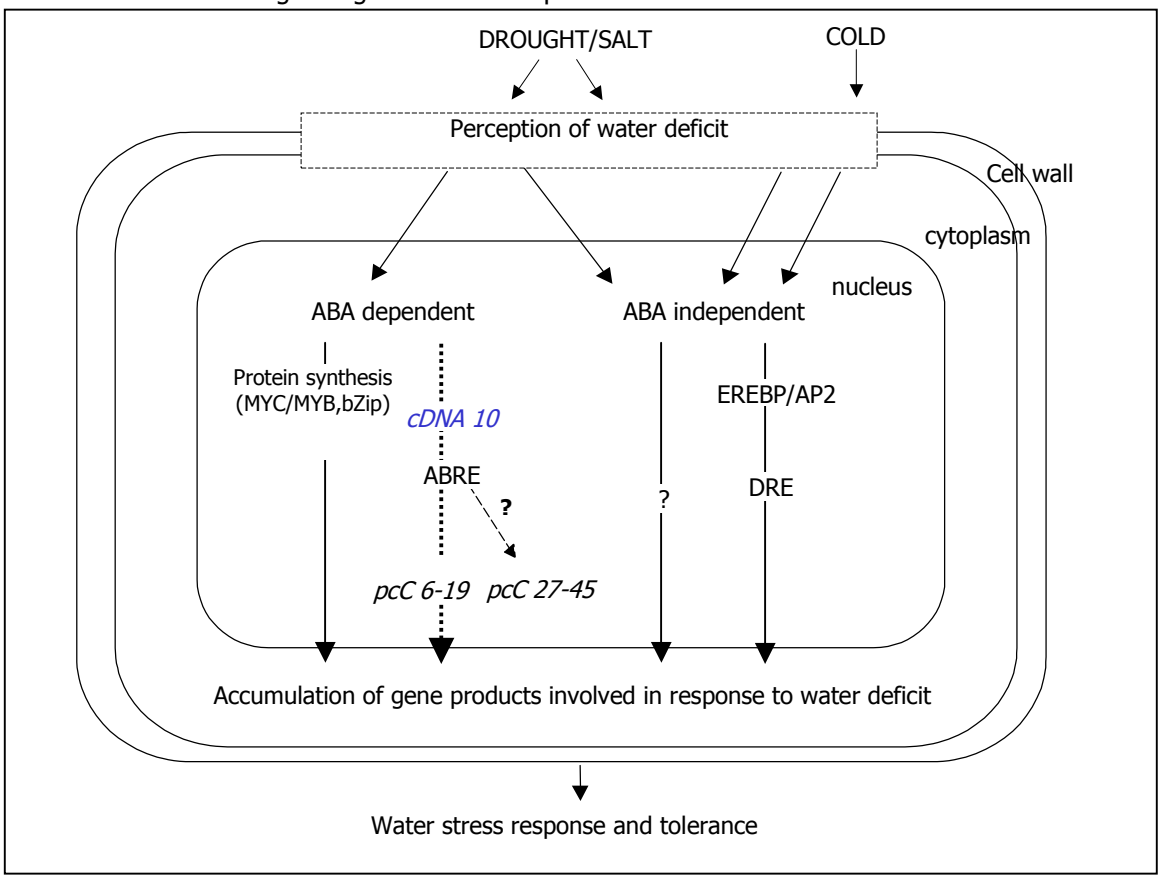
The DNA 10 locus gives rise to multiple transcripts that may be related to different stages of differentiation or perhaps to the hydration level of the tissues. No transcripts are detected in 72 hour dehydrated leaves and only one transcript is present in ABA treated callus. Moreover, *pcC 6-19* and *pcC 27-45* transcripts were accumulated in transgenic callus tissue from line 10. This suggests that the putatively tagged gene is inducing a dehydration response in callus that may be part of the ABA mediated signalling network.

The multiple transcripts observed when *cDNA 10* was used as a probe in the RNA blot experiments correlates with a possible regulatory cascade based on alternative RNA splicing. The best understood example is the mechanism that leads to sex determination in *Drosophila* (Bell *et al.,* 1988). A cascade of regulated RNA splicing events are necessary for the production of three gene products involved in transmitting the information that determines sex. A similar genetic mechanism was described for the *A. thaliana FCA* gene (MacKnight *et al.,* 1997). *FCA* controls flowering time and encodes a protein containing RNA-binding domains (RRM). The identification of the *FCA* gene product as an RNA-binding protein and the alternative processing of the *FCA* transcripts demonstrated that posttranscriptional regulation is an important mechanism in the control of flowering time in *A. thaliana*. The *FCA* transcript is alternatively spliced with only one form encoding the entire FCA protein. One speculation is that this RNA-splicing cascade is an ancient control device, left over from a stage of evolution where RNA was the predominant logical molecule and controls of gene expression had to be based almost entirely on RNA-RNA interactions. In this scheme, a number of RNAs produced under fully hydrated conditions may no

longer be transcribed when the plant is dehydrated. Under that situation specific RNA splicing might produce a functional protein involved in dehydration tolerance.

Based on the ABA signalling network proposed in recent reviews (Bray, 1997; Yamaguchi-Shinozaki and Shinozaki, 1997) (see section 1.3), the possible role of the putatively tagged gene of transgenic line 10 (*cDNA10*) in the ABA signalling network is depicted in the following scheme (see scheme 5.4.1).

Scheme 5.4.1 ABA signalling network in response to water deficit



Scheme 5.4.1 The multiple cellular pathways leading to water-deficit-induced gene expression. It is uncertain in what subcellular location the receptors are located, and how many different receptors are involved. In this model, *cDNA 10* is placed in an ABA-dependent signalling pathway upstream of two LEA genes (*pcC 6-19* and *pcC 27-45*). Abbreviations: ABRE, ABA-responsive element, DRE, dehydration-responsive-element. Dashed lines represent not known mechanisms.

Recent efforts have led to major advances in understanding the molecular mechanisms of ABA signalling, however many components within the non-linear and branched pathways of this network remain to be identified. New insights into ABA signalling will lead to future advances in this field that will be relevant to plant signal transduction, environmental ecology and agriculture.

Moreover, many genes utilised by the tolerant species should be present in all land plants. Vegetative desiccation tolerance may be based on changes in regulatory circuits and networks rather that on the biochemical hardware that accomplishes the actual tolerance. Postgenomic and proteomic approaches will be essential for future advances.

VI Summary

6.1 Summary

The aim of the present study has been to get a better understanding of the molecular mechanisms leading to desiccation tolerance using the desiccation tolerant resurrection plant *Craterostigma plantagineum* as a model. To achieve this, two methods have been pursued; (1) a transcript profiling approach to determine specific patterns of gene expression that lead to stress tolerance and (2) a mutational approach to dissect signalling networks that modulate plant responses to environmental cues.

Molecular responses to either dehydration or salt treatment were compared in *C. plantagineum*. Although desiccation tolerant, *C. plantagineum* was shown to be sensitive to sodium chloride, thus offering an ideal system for the detection of specific dehydration responsive mRNAs. The analysis consisted of comparing the temporal sequence of gene expression in response to dehydration and sodium chloride treatment. For this purpose, a collection of 258 dehydration responsive genes were studied at the RNA level using a macroarray technique. Classification of a large number of genes based on temporal transcript accumulation patterns following sodium chloride and dehydration treatments allowed insight into the genetic programmes employed by *C. plantagineum* during the acquisition of desiccation tolerance. Of the transcripts that showed differential accumulation in response to dehydration or sodium chloride treatment, 23% were up-regulated in response to both dehydration and sodium chloride. 19% of the gene collection were up-regulated by dehydration, but down-regulated by sodium chloride. This class of transcripts represent good candidates for further studies of specific gene responses to dehydration. A gene member of an aquaporin gene family was found to group in this class, and represents a good candidate for further analysis.

Closer inspection revealed specific transcript profiles for groups of genes in response to dehydration treatment. One gene family, the so-called LEA genes, was studied in detail. This family displayed a synchronised transcript accumulation pattern in response to dehydration that differed from the pattern observed in response to sodium chloride stress. Therefore, transcript profiles observed only in the dehydration response, that differ from a more general stress response such as sodium chloride treatment, can be considered as molecular finger prints for the acquisition of desiccation tolerance.

Most desiccation responses are mediated by abscisic acid (ABA). In order to dissect ABA/dehydration mediated signalling pathways, dominant *C. plantagineum* mutants were created

via a T-DNA activation tagging approach. The screening of mutants was based on the observation that wild type *C. plantagineum* callus tissues do not survive desiccation without ABA treatment. Through the insertion of transcriptional enhancers, ectopic expression of genes is induced. The application of this approach has permitted the isolation of five independent desiccation tolerant callus lines that do not require ABA pre-treatment. Two of the transgenic lines were selected for further characterisation, lines 10 and H. The T-DNA flanking regions were rescued and the corresponding cDNAs isolated. None of the cDNA sequences had significant homology to reported genes present in public databases. However, the cDNA isolated from mutant line 10 showed limited homology to a previously characterised *C. plantagineum* gene and a mouse gene encoding a hypothetical protein. Expression analysis revealed that desiccation responsive genes were highly accumulated in fully hydrated callus and leaf tissues from mutant line 10, suggesting that the tagged locus is involved in the ABA/dehydration signalling network.

6.2 Zusammenfassung

Ziel der vorliegenden Arbeit war es, mit Hilfe der austrocknungstoleranten wiederauferstehungspflanze *Craterostigma plantagineum* ein besseres Verständnis der Mechanismen zu gewinnnen, die zur Austrocknungstoleranz führen. Um dies zu erreichen, wurden zwei Methoden verwendet: (1) ein Transkript-Profilierungsansatz, um spezifische Genexpressionsmuster zu bestimmen, die zur Stresstoleranz führen, und (2) ein Mutagenese-Ansatz, um Signalnetzwerke zu analysieren, die pflanzliche Reaktionen auf Umweltreize modulieren.

Molekulare Reaktionen auf entweder Austrocknung oder Salzbehandlung hin wurden in *C. plantagineum* verglichen. Es war gezeigt worden, dass die austrocknungstolerante *C. plantagineum* salzempfindlich reagiert, so dass sie ein ideales System darstellt zur Identifizierung von spezifisch auf Austrocknung reagierenden mRNAs. Die Analyse bestand im Vergleich der zeitlichen Genexpressionsabfolge als Reaktion auf Austrocknung und Salzbehandlung. Zu diesem Zweck wurde eine Sammlung von 258 austrocknungsreaktiver Gene auf RNA-Ebene mit Hilfe der Macroarray-Technik untersucht. Die Klassifizierung einer grossen Anzahl von Genen auf zeitliche Transkriptakkumulierungsmuster nach Salz- und Austrockungsbehandlung gewährten Einsicht in die von *C. plantagineum* verwendeten genetischen Programme bei der Ausprägung der Trockentoleranz. Von den Transkripten, die eine differentielle Akkumulierung als Reaktion auf Salz oder Austrocknung hin zeigten, wurden 23 % aufreguliert sowohl auf Salz- alsauch Austrocknungsbehandlung hin. 19 % der Gensammlung wurden auf Austrocknung hin aufreguliert, aber herunterreguliert durch Salz. Diese Transkriptklasse stellt gute Kandidaten für weitere Untersuchungen genspezifischer Reaktionen auf Austrocknung. Ein Mitglied einer Aquaporin-Familie fand sich, das sich in diese Gruppe einordnete.

Nähere Untersuchung erbrachte spezifische Transkriptprofile für Gruppen von Genen als Reaktion auf Austrocknung. Eine Genfamilie, die sogenannten LEA-Gene, wurde im Detail untersucht. Diese Familie zeigte ein synchronisiertes Transkript-Akkumulierungsmuster als Reaktion auf Austrocknung, das von dem Muster auf Salzstress hin verschieden ist. Daher können die nur in der Reaktion auf Austrocknung hin beobachteten Profile als molekulare Fingerabdrücke für den Erwerb der Trockentoleranz angesehen werden.

Reaktionen auf Austrocknung werden durch ABA vermittelt. Um ABA- und Austrocknungsvermittelte Signalwege zu untersuchen, wurden dominante *C. plantagineum* Mutanten durch einen T-DNA activation tagging Ansatz geschaffen. Die Suche nach Mutanten basierte auf der Beobachtung, dass

Wildtyp-Kallusgewebe von *C. plantagineum* eine Austrocknung ohne ABA-Vorbehandlung nicht überlebt. Durch die Insertion von Transkriptionsverstärkern (enhancer) wird eine ektopische Genexpression induziert. Die Anwendung dieses Ansatzes erlaubte die Isolierung von fünf unabhängigen austrocknungstoleranten Kallus-Linien, die keine ABA-Vorbehandlung benötigen. Zwei der transgenen Linien wurden zur weiteren Charakterisierung ausgewählt, Linie 10 und H. Die T-DNA flankierenden Bereiche und die entsprechenden cDNAs wurden isoliert. Keine der cDNA-Seqüenzen hatte signifikante Homologien zu bekannten Genen in den Datenbanken. Wie auch immer, die cDNA der Mutantenlinie 10 zeigte begrenzte Homologie zu einem zuvor charakterisierten *C. plantagineum* Gen sowie zu einem Mausgen, das für ein hypothetisches Protein kodiert. Die Expressionsanalyse zeigte, dass Gene, die auf Austrocknung reagieren, stark angereichert waren in gut gewässertem Kallus und Blattgewebe von Linie 10, was nahelegt, dass der getaggte Locus am ABA/Austrocknungssignalnetzwerk beteiligt ist.

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