Stress proteins and identification of interacting partners in the resurrection plant *Craterostigma plantagineum*

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1 Introduction

1.1 The study of the drought stress tolerance in plant: economic and scientific relevance.

Plant productivity is strongly influenced by stresses induced by drought, high salt, and low temperature conditions. Most cultivated crop plant species are highly sensitive and either die or display reduced productivity after they are exposed to long periods of stress (McKersie and Leshem, 1994). It has been estimated that two-thirds of the yield potential of major crops are routinely lost due to unfavorable environments (Boyer, 1982; Bajaj et al., 1999). Increased drought-tolerance of crop plants has therefore become one of the major objectives of plant breeding programs on a worldwide scale.

The understanding of the plant responses to water stress has been advanced by the application of the molecular techniques but details of the mechanisms regulating these responses remain to be examined. Also questions of ecological and evolutionary nature need to be studied in depth. So, the knowledge gained in basic research could significantly contribute to produce agronomically useful plants with high levels of drought stress tolerance.

1.2 Environmental stresses in plants

Under both natural and agricultural conditions, plants are frequently exposed to stress. Stress is usually defined as an external factor that exerts a disadvantageous influence on the plant (Levitt, 1972). Water deficit, chilling and freezing, heat stress, salinity and oxygen deficiency are major stress factors restricting plant growth (Boyer, 1982; Salisbury and Ross, 1989). Some environmental factors (such as air temperature) can become stressful in a few minutes; others may take days to weeks (soil water) or even months (mineral nutrients) to become stressful.

During stress, a plant adapts its metabolism and responds by leading to a variety of biochemical and physiological changes. Carbohydrates, lipids, proteins and nucleic acid metabolisms are severely damaged (reviewed in Jones et al., 1989; Cushman et al., 1990; Katterman, 1990; see also Jain et al., 1997; Nguyen et al., 1997; Pareeek et al., 1997) and effects of exposure to stresses have been noted with respect to almost all major physiological processes including photosynthesis, nitrogen fixation and respiration (Dilks and Proctor, 1979; Bjoerkman et al., 1980; Jackson and Drew, 1984; Drew, 1987; Kaiser, 1987; Osmond, 1987; Smirnoff and Cumbes, 1989; Chapin III F.S., 1991; Waring, 1991; Bjoerkman and Demming-Adams, 1994; Downs and Heckathonrn, 1998). Different developmental stages of the plant growth including seed germination, seed maturation and senescence, are differentially affected in response to stress conditions (reviewed in Skriver and Mundy, 1990; see also Chiariello and Gulmon, 1991; Gyoergyey et al., 1991; Hall, 1993; Leprince et al., 1993; Gagliardi et al., 1995; Kermode et al., 1997).

A signal of abiotic stress is often injury to membranes (Senaratna et al., 1987). Chilling and freezing, as well as high temperature conditions, affect the membrane fluidity (Murata et al., 1982; Clegg, 1986; Schwab et al., 1986; Smirnoff and , 1989; Crowe and Crowe, 1992; Vernon et al., 1993; Steponkus et al., 1993; Botella et al., 1994) and perturb membrane-bound processes such as the photosynthetic apparatus (Sung and Krieg, 1979; Kaiser, 1987; Rao et al., 1987; Schwab et al, 1989; Bjoerkman and Demming-Adams, 1994; Downs and Heckathonrn, 1998). The formation of reactive oxygen intermediates, a secondary effect of stress, also damages membranes (Perl-Treves and Galun, 1991; Sgherri et al., 1993a,b; Mittler and Zilinskas, 1994).

With the exception of flooding, the major environmental stresses all result in water-deficit stress (Kramer, 1993). Cold or frozen soil can reduce water uptake and thus produce water stress (Tranquillini, 1976; Oberbauer and Billings, 1981); in the same way, salt accumulation in the soil decreases the water potential that makes soil water less available (Osmond et al., 1980; Fitter and Hay, 1987). In order to survive under water deficit conditions, plants have to maintain their water status to keep homeostasis. When a depletion of water occurs in the plant tissues, the extracellular solute concentration results altered and there is a flux of water from the cells, causing a decrease in turgor and an increase in concentrations of intracellular solutes (Crowe et al., 1984; Adams et al. 1992; Bray, 1993;

Ding and Pickard,1993; Boyer, 1995; Lichtentaler, 1995). Consequently, osmotic adjustment plays a fundamental role in cellular stress response. There are several biochemical functions

involved in the response of the plants to osmotic stress, such as ion exclusion, ion export, cell wall modification, accumulation of various organic compounds, synthesis of late-embryogenesis-abundant (LEA) proteins and activation of several detoxification enzymes (for review see Bohnert et al., 1995; Allen, 1995; Bray E.A., 1997). The concept of stress is associated with that of stress tolerance, which is the plant's fitness to cope with a unfavorable environment (Levitt ,1972, 1980). On the basis of their water requirements and adaptation to environmental stresses, plants are commonly characterized as xerophytes (drought tolerant), halophytes (salt tolerant) and glycophytes (stress-sensitive; Barbour et al., 1987; Salisbury and Ross, 1989). Very similar mechanisms in stress protection have been observed for different plant species, supporting the theory that stress tolerance mechanisms are ubiquitous and that the advantages of halophytes and xerophytes over glycophytes may result from the more efficient performance of a few basic biochemical pathways (Leopold et al., 1992; Donoghue M.J., 1994; Bohnert et al., 1995).

Concomitant to induced stress tolerance, protein metabolism of the cells undergoes changes in terms of acquiring specific stress proteins, which are either not detected or present in low amount in not induced cells. Stress proteins may be involved in avoiding stress, in repairing damage or in protecting cellular machinery from the effects of stress (Grover et al., 1993; 1998; Ingram and Bartels, 1996; Bray, 1997; Singla et al., 1997). Fig. 1 shows examples of stress proteins that are expressed in response to the major abiotic stress factors in plants.

Fig. 1

Characteristic features of plants stress proteins (after Grover, 1999)

Stress protein	*Induction agents*	*Characteristic features*
HSPs (heat-shock proteins) Vierling, 1991; Harrigton et al., 1994	High temperature; also water stress, salt stress, low temperature; in some cases, also abscisic acid.	Classified as small (low molecular weight) HSPs and high molecular weight HSPs; highly conserved amino acid sequence; stress inducibility of HS genes regulated by HSEs (heat-shock elements); some HSPs are shown to act as chaperones
Osmotic stress proteins such as WSPs (water stress proteins) and SSPs (salt stress proteins) Ingram and Bartels, 1996; Bray, 1997; Shinozaki, 1997.	Low water availability and salt stress; also induced by abscisic acid	Varied molecular weight and cellular location; mostly are enzymes involved in diverse functions such as production of osmolytes, regulation and transport. Role of some WSPs is not well defined (dehydrins, LEA proteins, etc.)
ANPs (anaerobic proteins) Sachs et al., 1996; Drew, 1997.	Anaerobic stress (caused by flooding or submergence stress)	Mostly are enzymes of the fermentative or the glycolytic pathway; most genes contains AREs (anaerobic responsive elements) in the promoter
Cold stress proteins Guy, 1990 Hughes and Dunn, 1996	Low temperature stress; also by osmotic, oxidative stress and abscisic acid	Conserved proteins of varied sizes and functions; most genes contain specific nucleotide sequence that stimulate transcription in response to low temperature

1.2.1 Common responses to different environmental stresses

Several investigations suggest that plants exhibit a phenomenon called cross-tolerance (Levitt, 1980; Crawford, 1989), whereby exposure to one stress can provide tolerance to another stress. If tolerance increases as a result of exposure to a previous stress, the plant is said to be acclimated. Acclimation can be distinguished from adaptation, which usually refers to a genetically determined level of resistance acquired over many generations (Fitter, 1987; Salisbury and Ross,

1989). Evidence has been accumulated in favor of a linkage at the cellular level between various abiotic stresses. For example, it has been well recognized that plants responses to low temperature and water stress are linked through a common dehydration mechanism. Early studies of Chen et al. (1975, 1979) showed that cold hardiness of red osier dogwood stems could be increased by water stress and dehydrins (Close, 1993, 1996; see also 1.2.3). Other dehydration-induced stress proteins have been reported to be common to cold and water stress in several plant species (Guy et al., 1982; Anisko and Lindstrom. 1995; Hughes and Dunn, 1996; Webb et al., 1996; Artlip, 1997; Stockinger et al., 1997; Danyluk et al., 1998; Ismail et al., 1999; Wisniewski, 1999; Fu et al., 2000; Richard et al., 2000). Finally, Palta et al. (1981) noted that potato species acquired higher heat tolerance during cold acclimation. Data also indicated that heat and drought stresses in plants are often correlated (Wallner et al., 1982). It has been shown that water stress increased heat tolerance in geranium plants (Arora et al., 1998) and exogenous application of abscisic acid (ABA), a growth regulator known to accumulate during cold acclimation and water stress (Quarrie, 1980; Chen and Lin, 1982; Ryu and Li, 1984), increased heat stress tolerance in grape and bromegrass cell cultures (Abass and Rajashekar, 1993; Robertons, 1994). Furthermore, ABA accumulated in grape cell cultures during heat acclimation (Abass and Rajashekar, 1993).

Differences in the expression of specific genes between stress-sensitive and stress-tolerant plants indicate that tolerance is conferred by genetically encoded mechanisms (Bray et al., 1993; Basra, 1994). Alteration in gene expression is an important part of the ability of the plant to respond to the environment and in the last years several stress-related genes have been characterized (reviewed by Grover et al., 1999). The study of the diverse genes that are induced and repressed by dehydration (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997) shows that many also respond to salt stress. In addition, there is evidence that among the genes that respond to salinity and drought, some respond also to cold stress (Shinozaki and Yamaguchi-Shinozaki, 1996). Several plant genes that are induced by salt but differ in their response to dehydration, cold and the plant hormone abscisic acid (ABA) were reported by Winicov (1998).

With regard to the heat stress, a typical stress-induced gene family is represented by the heat-shock proteins (HSPs). It is well know that HSPs are synthesized in response to high temperature (Nover et al, 1984; Schlesinger M.J., 1990; Gurley and Key, 1991. See also 1.2.3). Nevertheless, many HSPs may are up-regulated by a variety of treatments such as cold, salt, heavy metal and oxidative stress (Nagao et al., 1990; Vierling, 1991; Gething and Sambroock, 1992; Nedunchezhian et al., 1992; Berkel et al., 1994; Ferullo et al., 1994; Alamillo et al, 1995; Coca et

al., 1996; Eckey-Kaltenbach et al., 1997; Banzet et al., 1998). The common responses to different stresses may indicate similar functions of stress-responsive gene products for plants under stress conditions involving water deficit. The existence of interacting signal perception and transduction pathways, which promote the plant stress response, is suggested by studies on gene expression during dehydration. Endogenous ABA levels increase as result of water deficit and it is thought to be involved in signal transduction (Chandler and Robertson, 1994; Giraudat et al., 1994). At least four signal-transduction pathways have been reported to be involved in plants between the initial dehydration signal and gene expression: two are ABA (abscisic acid)-dependent and two ABA-independent (Shinozaki and Yamaguchi-Shinozaki, 1996, 1997). These pathways lead to activation or synthesis of transcription factors (Nakagawa et al., 1996; Abe, 1997; Hollung et al., 1997; Stockinger et al., 1997; Nakashima et al., 2000) and it has been demonstrated that this four signaling pathways cross-talk and converge to activate stress response gene expression (Shinozaki and Yamaguchi-Shinozaki, 1996, 1997).

1.2.2 Adaptations to drought stress

Drought stress occurs when water uptake from soil cannot balance water loss through transpiration. The subsequently cellular water loss is referred to as dehydration (Levitt, 1980). Response to water loss during dehydration is particularly achieved by osmotic adjustment. Most plants maintain their cytoplasm osmotic balance by production of low molecular weight compounds, which help to maintain the cellular water potential (Yancey et al., 1982; Arakawa and Timasheff, 1985; Wiggins, 1990; Adams et al, 1992). The solutes accumulated in the cytoplasm must be non-toxic (“compatible”) with respect to metabolic processes and should not interfere with protein structure or function when present at high concentration (Morgan, 1984; Somero, 1986). Quaternary ammonium compounds such as glycine betaine are among the commonest compatible solutes and occur in bacteria, cyanobacteria, algae, higher plants and animals (reviewed in Yancey et al., 1982; see also Grumet and Hanson, 1986; Rhodes et al., 1989). The osmoprotectans include tertiary solfonium compounds, amino acids without net charge like proline, various polyols, polyamines and oligosaccharides and sugars (Wyn and Gorham; 1983; Morgan, 1984; Smirnoff and Cumbes, 1989; McCue and Hanson, 1990; Hanson, 1992; Timasheff, 1992; Vernon et al., 1993; Niu et al., 1995; Ishitani et al., 1996). Trehalose and sucrose are the most widely distributed carbohydrates involved in drought tolerance (Crowe et

al., 1992; Leprince 1993; Leprince et al., 1993). Sugar is assumed to stabilize dry membranes and dry proteins: membrane stabilization is thought to occur through water replacement of the lipid bylayer, preventing dense packing of phospholipids and spurious membrane-protein interactions during dehydration (Clegg, 1986; Crowe and Crowe, 1988a,b; Leopold et al., 1992). Protein stabilization presumably occurs through formation of hydrogen bonds between hydroxyl groups of the sugar and the polar residues of the proteins (Carpenter and Crowe, 1989).

Besides sugar, the LEA (late embryogenesis abundant) proteins are proposed to protect membranes and protein structures against drought induced damage. LEA proteins accumulate in seeds during the later stages of embryogenesis (Close et al., 1993; Parcy et al., 1994) and some also accumulate in vegetative tissues in response to stress (Baker et al., 1988; Cammue et al., 1989; Houde et al., 1992). They are hypothesized to act as solubilizing agents with chaperone properties, maintaining cellular structural organization and preventing ion crystallization during desiccation (Baker et al., 1988; Close et al., 1989; Kort and Chandler, 1989; Roberts and al., 1993; Close, 1996). The presence of LEA proteins in seeds and seedlings is correlated with tissue dehydration tolerance and salt tolerance (Blackman et al., 1991; Galvez et al., 1993; Ried and Walker-Sommons, 1993; Moons et al., 1995; Backer et al., 1995; Xu et al., 1996). With regard with this observation, direct evidence of a role of LEA proteins in water stress comes from work with rice transformed with a LEA protein from barley, which showed an increased tolerance to water deficit and salinity (Xu et al., 1996). Finally, recovery of native protein conformation involves either enzymes with function in protein repair or chaperones. Drought induced proteins with chaperone function include high and low molecular weight heat-shock proteins (HSPs and LMW or small HSPs respectively). HSPs represent a large protein family that includes several subfamilies (HSP90, HSP70 and HSP60 or GroEL; Morimoto et al., 1990). In particular, HSP70s are evolutionary conserved and belong to the protein class known as chaperones (Anderson et al., 1994). Most HSPs are believed to be able to act by stabilizing and protecting proteins and by enhancing their refolding (Jinn et al., 1989, 1993, 1995; Borkird et al., 1991; Gethin and Sambrook, 1992; Ferguson et al., 1994; Kiyosue et al., 1994).

Small HSPs are evolutionarily related to the vertebrate lens protein α-crystallin (Ingolia and Craig, 1982; Wistow, 1985). The *in vivo* functions of the plant small HSPs are largely unknown, yet several studies have demonstrated their ability to act as molecular chaperones *in vitro* (Forreiter et al., 1997; Helm et al., 1997; Jinn et al., 1995; Lee et al., 1995, 1997; Yeh et al., 1995; Lee and Vierling, 2000; Löw et al., 2000; Smykal et al., 2000).

1.2.3 The role of dehydrins and heat-shock proteins (HSPs) during drought stress

In natural environments plants are affected during drought by two different physical factors: water deficit and high temperature. Water deficit-induced accumulation of dehydrin proteins is noteworthy (Close, 1993a,b; 1996). Dehydrins are an immunologically distinct family of proteins, also known as LEA D-11 family, which typically accumulate in plants during the late stage of embryogenesis and in response to low temperature or ABA application, besides to any environmental cue (drought, salinity, extracellular freezing) having cellular dehydration as a common induction factor (Close, 1993, 1996). Dehydrins are highly hydrophilic and heat-stable. Close (1996) hypothesized that dehydrins and compatible solutes act synergistically to stabilize macromolecules such as unfolded proteins and nucleic acids. This stabilization could be achieved through the hydrophilic regions of the dehydrin, resulting in the formation of an envelope of order water that, particularly in the presence of compatible solutes, drives partially unfolded proteins back to a folded state or at least inhibit further denaturation. In this regard, evidence has been presented for a protective role of dehydrins, *in vitro*, against freeze-thaw- and heat stress-induced denaturation of proteins (Carpenter and Crowe, 1988; Robertson, 1995).

Heat is the other environmental cue present during drought. Plants respond to elevated temperatures by expressing several families of evolutionarily conserved heat-shock proteins (HSPs)(Key et al., 1985; Lindquist and Craig, 1988;). The heat-shock response is widely conserved in all living cells and is a model system for studying the molecular mechanisms of stress gene expression regulation (Morimoto et al., 1990). One common features is the rapidly induced expression of HSPs. Induction of transcription of heat-shock genes at high temperatures is mediated by the activation of pre-existing transcription factors (HSFs), which bind to heat-shock elements (HSEs) in the promoter region (Sorger, 1991). The genes for HSFs have been isolated and characterized from yeast, *Drosophila*, chicken, mice, humans (Wu, 1995), tomato (Scharf et al., 1990), *Arabidopsis* (Hubel et al., 1994), maize (Gagliardi et al., 1995) and soybean (Czarnecka-Verner et al., 1995).

Dehydrins and HSPs represent two very different classes of stress proteins that accumulated upon drought conditions. The dehydrin functions *in planta* are poorly known, yet synthesis of dehydrin transcripts were observed in drought-tolerant sunflower plants (Cellier et al., 1998, 2000) and drought-tolerant wheat (Labhilili et al., 1995). On the other hand, HSPs are expressed during phases of the plant life cycle where water stress conditions occur (pollen development, seed

development and germination) and it has been suggested that they may play a role in plant desiccation tolerance (Almoguera and Jordano, 1992; DeRocher and Vierling, 1994; Magnard et al., 1996; Wehmeyer et al., 1996). Moreover, it has been reported that adaptation of cotton plants to soil drought was accompanied by enhancement of their resistance to both water deficiency and overheating (Kuznetsov et al., 1982). In that study, two polypeptides (70 and 80 kDa) similar to the corresponding HSPs were synthesized in response to drought. In addition, accumulation of HSPs have been reported in laboratory as well as in field-grown plants subjected to water stress (Prasad, 1997).

1.3 Drought tolerance studies by the yeast two-hybrid system in the resurrection plant *C.plantagineum*.

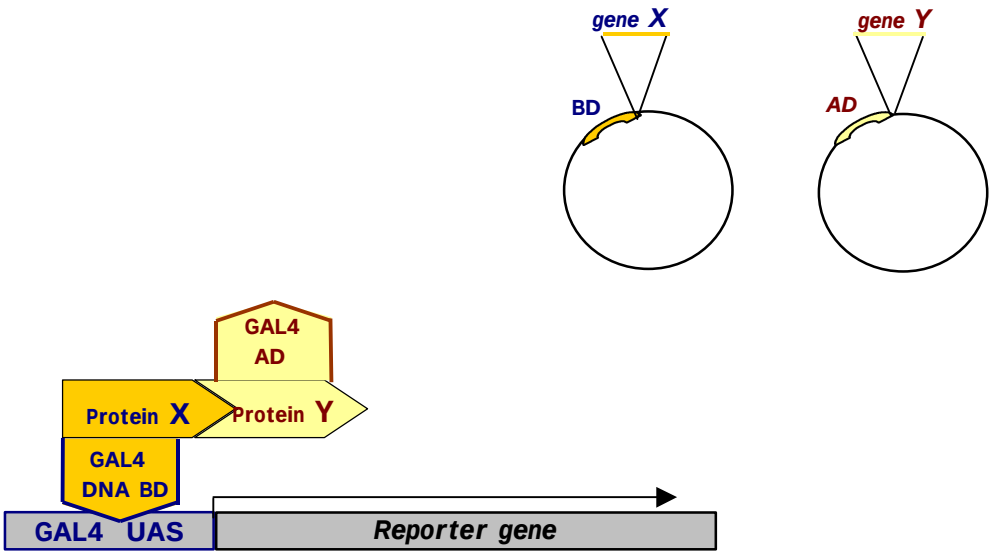
There is a group of higher plants known as resurrection plants, which posses a unique effective mechanism for coping with drought stress by being desiccation tolerant (reviewed by Hartung et al., 1998). Desiccation is defined as the severest form of water loss (Chandler and Bartels, 1999). Virtually all plant species, at some point in their life cycle, are at least partially tolerant to desiccation. For example, seed and pollen lose large quantities of water during the maturation process (Bewley, 1979). But the ability of mature tissues, such as roots and leaves, to survive severe desiccation is rare. The resurrection plant *Craterostigma plantagineum* Hochst. (Fam. Scrophulariaceae) is one example of the known desiccation-tolerant species (Gaff, 1981). The study of the molecular response to water deficit in *Craterostigma plantagineum* has been mainly directed towards the understanding of the functions of genes up-regulated during dehydration. Many of them encode hydrophilic polypeptides related to the LEA (late embryogenesis abundant) proteins, for which an osmoprotective function has been suggested (Bartels et al., 1990,1992; Piatkowski et al., 1990; Schneider et al., 1993; Alamillo et al., 1994). Several of them are involved in biochemical pathways and show homology to key enzymes as sucrose synthase and sucrose-phosphate synthase (Ingram et al., 1997; Kleines et al., 1999), transketolase (Bernacchia et al., 1995), GAPDH (Velasco et al., 1994), phospholipase D (Frank et al., 2000), or encode for water channel protein homologues (Mariaux et al., 1997). In addition, *C.plantagineum* genes with a potential regulatory function have been identified (Iturriaga et al., 1996; Furini et al., 1997; Chandler and Bartels, 1997; Frank et al., 1998; Bockel C., 2000, Ph.D. thesis).

The identification and isolation of stress-responsive proteins is a central theme in plant-stress studies. The yeast two-hybrid system represents a powerful method for detecting and analyzing protein-protein interactions. The basic concept of the strategy is to detect the interaction between two proteins via transcriptional activation of reporter genes (Fields and Song, 1989). The system is based on the modular nature of eukaryotic transcriptional activators, which have two domains: one domain that specifically binds to DNA sequences (the binding domain, BD) and a domain that promote the transcription (the activation domain, AD). The domains most commonly used are the yeast GAL4 DNA-binding and activation domains. It is known that the yeast transcriptional activator GAL4 binds specific sites on DNA to activate transcription of adjacent genes (Yocum et al., 1984; Bram and Kornberg, 1985; Keegan et al., 1986). The distinct activation regions of GAL4 are rich in acidic residues and it has been suggested that these regions interact with other protein components (such as the TATA-binding protein or RNA polymerase II), while the DNA-binding region serves to position the activating region near the gene (Patshne, 1986; Brent and Patshne, 1987; Struhl, 1987). Consequently, the interaction of two hybrid proteins, one carrying the GAL4 BD and the other expressing the GAL4 AD, could reconstitute the GAL4 activity (Fields and Song, 1989; Bartel et al., 1993). In the two-hybrid system, plasmids are constructed that encode two hybrid proteins: one consists of the DNA binding domain of GAL4 fused to one test protein (“X”) named "bait" and the other consists of the GAL4 activation domain fused to another test proteins (“Y”), representing the "target". These plasmids are transformed into the yeast host strain that contains a reporter gene (e.g. *lacZ*) whose regulatory region contains the GAL4 binding site. A schematic diagram of how the two-hybrid system works is shown in Fig.2.

The basis of the assay is that transcription will occur only if the two hybrid proteins X and Y interact together (see 4.1.3 for details).

Fig. 2

General scheme of the yeast two-hybrid system



The GAL4 DNA-binding domain hybrid protein (BD and bait protein X) binds to the GAL4 UAS present upstream of the reporter gene. The GAL4 activation domain hybrid protein (AD and target protein Y) binds transcription factors in the nucleus bur does not localize to the GAL4 UAS. If the bait (X) and target (Y) proteins interact, the GAL4 activation and DNA-binding domains are brought close to each other and act together to initiate transcription of the reporter gene.

1.4 Objectives of the thesis

To investigate the complex network of dehydration-related gene products in desiccation tolerance, protein-protein interactions were analyzed *in vivo* by the yeast “Two-Hybrid System” (Fields and Song, 1989; Chien et al., 1991; Bartel et al., 1994). A cDNA expression library derived from dehydrated *C. plantagineum* leaves was generated and screened using two dehydration-induced proteins, Dsp16 and *Cp*-HSF1, as bait. Dsp16 is a dehydrin-like protein encoded by the low copy number gene CDeT6-19 (Bartels et al,1990);

Cp-HSF1 is a heat-shock transcription factor homologue encoded by the *Cp*-Hsf1 gene (Bockel C., PhD thesis). Putative positive clones were subjected to sequence analysis to identify gene function and RNA blot analyses were performed to determine dehydration-induced expression of the interacting proteins. To further determine the role of the different regions of *Cp*-HSF1 for the transactivation of the protein, putative interactions among the different functional domains of *Cp*-HSF1 were studied.

In order to identify potential target genes of the heat-shock transcription factor *Cp*-HSF1, experiments have been extended to the investigation in *C. plantagineum* of the small heat-shock proteins (small HSPs). These proteins represent the major family of stress-responsive genes induced by elevated temperature (Water E.R., 1995) and are expressed also in specific developmental stages in seeds and flowers. It has been hypothesized that the small HSPs present during seed maturation have a role in seed desiccation tolerance (Almoguera and Jordano, 1992; Jakob et al., 1993; Coca et al., 1994, 1996; DeRocher and Vierling, 1994; Alamillo et al., 1995; Wehmeyer et al., 1996). Previously the presence of small HSPs was observed in vegetative tissues of *C. plantagineum*, where their accumulation was induced both by heat-shock and by water stress (Alamillo et al., 1995). During this study, a small heat-shock protein homologous cDNA isolated from *C. plantagineum* (*Cp*-Hsp20.6) has been characterized and its genomic organization was examined. A heat-shock responsive element (*Cp*-HSE) has been identified in the *Cp*-Hsp20.6 promoter region and the ability of the heat-shock transcription factor homologue *Cp*-HSF1 to bind to this heat-shock element-like sequence and to activate the target gene has been tested by *in vitro* binding assays and by *in vivo* tobacco protoplast transformation experiments.

2 Material and Methods

2.1 Abbreviations

aa	amino acid
ABA	abscisic acid
APS	ammoniumperoxodisulfate
3-AT	3-amino-1,2,4-triazole
β-gal	β-galactosidase
bp	base pair
BSA	bovine serum albumine
cDNA	complementary DNA
Cp	*Craterostigma plantagineum*
cpm	counts per minute
CPRG	chlorophenol red-β-D-galactopyranoside
d	day
2,4-D	2,4-dichlorophenoxyacetic acid
dATP	deoxy-adenosintriphosphate
dCTP	deoxy-cytidinsintriphosphate
ddH_2O	destilled water
EDTA	ethyllendiamintetraacetic acid
EMSA	electrophoretic gel mobility assay
g	gram
GUS	*E.coli* β-glucoronidase gene
h	hour
His^-	histidine auxotroph
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilo base pair
KCl	potassium cloride
kDa	kilodalton
l	liter

Leu^-	leucine auxotroph
m	meter
M	molar
mA	milliAmpere
MES	2-[N-morpholino]-ethanesulfonic acid
$MgCl_2$	magnesium chloride
min	minute
ml	millilietr
mM	millimolar
mRNA	messenger RNA
4-MUG	4-methylumbelliferyl glucuronide
ng	nanogram
nmol	nanomol
OD	optical density
PAGE	polyacrylamide-gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
PIPES	1,4-piperazin-N,N'-2-ethansulfonate
pmol	picomol
PMSF	phenylmethansulfonylfluorid
rpm	revolutions per minute
s	second
SD	synthetic minimal medium
SDS	sodium dodecyl sulfate
SSC	3M NaCl, 0.3 M Na-citrate
TEMED	N, N, N', N'-tetramethylethylendiamin
Tris	tris(hydromethyl)aminmetane
Trp^-	tryptophan auxotroph
U	unit
µ	micro
V	Volt
% [v/v]	volume percent

% [w/v] weight percent

2.2 Materials

2.2.1 Chemicals, enzymes, radioisotopes and kits

Chemicals were provided, unless otherwise mentioned, by the following companies: Biomol (Ilversheim), Boehringer (Mannheim), DIFCO (Detroit, USA) Merk (Darmstadt), Sigma (Deisenhofen), and Serva (Heidelberg). Enzymes were supplied by Amersham Buchler (Braunschweig), Boehringer (Mannheim), DIFCO (Detroit, USA), GIBCO BRL (Neu-Insenburg), New England Biolabs (Schwalbach), Pharmacia (Freiburg) and Stratagene (Heidelberg). P^{32}-labelled radioisotopes (α-[^{32}P] dATP, α-[^{32}P]dCTP, γ-[^{32}P]dATP) were purchased from Amersham Buchler (Braunschweig). The following kits were used: HybridZAP™ two-hybrid lambda kit (Stratagene, La Jolla, USA); Genomic DNA purification kit (Quiagen, Hilden), Plasmid DNA preparation kit (Quiagen, Hilden), Quiaquick spin PCR purification kit (Quiagen, Hilden), Quiaex gel extraction kit (Quiagen, Hilden), BioRad protein assay (BioRad, München), ECL kit (Amersham Buchler, Braunschweig) and QIAexpress System (Quiagen, Hilden).

2.2.2 Media, buffers and solutions

All media, buffers and solutions were prepared with deionised water. Thermostabile components were autoclaved for 20 min at 121°C, 2 bar. Thermolabile componets were sterile-filtered. Media, buffers and solutions were prepared as described in Sambrook et al. (1989), Murashige and Skoog (1962) and Negrutiu et al. (1987). Yeast two-hybrid media and reagents were prepared according to the instruction manual (Stratagene, La Jolla, USA, 1995).

2.2.3 Vector, bacterial and yeast strains.

HybridZAP™ vector	Stratagene (Short et al., 1988)
pBluescript II SK +/- vector	Stratagene (Alting-Mees et al., 1992)
pQE expression vector	Quiagen (Bujard et al., 1987)
pBD-GAL4(Cam) phagemid vector	Stratagene (Short and Sorge, 1992)
pAD-GAL4 phagemid vector	Stratagene (Short and Sorge, 1992)

p53 expression vector	Stratagene (Iwabuchi et al., 1993)
pSV40 expression vector	Stratagene (Li and Fiels, 1993)
pBI101 expression vector	Clontech (Jefferson et al., 1987)
pRT104 expression vector	Töpfer et al., 1987
E.coli XL1-Blue MRF'	Stratagene (Bullock et al., 1987)
E.coli DH 5α	Hanahan et al., 1983
E.coli K803	Fedoroff et al., 1983
E.coli M15(pREP4)	Quiagen (Villajero and Zabin, 1974)
S.cerevisiae YRG-2	Stratagene (Guthrie and Fink, 1991)

2.2.4 Plant material

2.2.4.1 *Craterostigma plantagineum* Hochst. Fam . Scrophulariaceae

Craterostigma plantagineum plants were originally collected in South Africa and propagated under the grown conditions described by Bartels et al (1990). *Craterostigma* plants were grown onto artificial clay (Lecaton-fein, Hydro GmBH, Koeln) in Conviron growth chambers with a light intensity of 60-200 μmol m^{-2} s^{-1} , 60% relative humidity, day/night temperature of 24°C/ 20°C and daily light period of 14 hours.

2.2.4.2 *Nicotiana tabacum* cv. Petit Havana, Fam. Solanaceae

Nicotiana tabacum plants were grown axenically in a sterile culture system (Binding, 1975) and propagated *in vitro* under long-day conditions with a light/dark period of 16 h/ 8 h and day-night temperature of 24°C/16°C. After approximately 6 weeks, the plant shoots were subcultureted on solid MS medium (Murashige and Skoog, 1962).

2.2.4.3 Collection and storage

Whole *C.plantagineum* plants were subject to dehydration or heat-shock treatments after removal of necrotic parts. At the end of the treatments, the plant material was frozen in liquid nitrogen

and stored at -70°C until further usage. Leaves and roots were stored separately. Detached leaves were used for the ABA treatment.

Shoot cultures of fully expanded tobacco (*N.tabacum* cv Havana line SR1; Maliga et al., 1973) leaves (4-6 weeks old) were used for mesophyll protoplasts preparation.

2.2.5 Protoplasts preparation

Tobacco leaves were cut into 1-2 cm squares and digested in K3 solution (Murashige and Skoog, 1962) containing 1% cellulase Onozuka R10, 0.5% macerozyme R10 (Serva), 4 g / l MS salt, 0.2% mg / l 2,4-D, 1 mg / l α-naphtalene acetic acid, 0.2 mg / l kinetin, 200 mg / l inositol, 230 mg / l xylose, 0,4 M saccharose (600 mOsm) for 16 h in the dark. Protoplasts were separated by gentle shaking for 20 min at 100 r.p.m., filtered and purified by centrifugation at room temperature. The floating protoplasts were recovered, washed in the same MS medium without enzymes and centrifuged again. Then the protoplasts were collected in W5 solution (Negrutiu et al., 1987) by centrifugation and resuspended at a final concentration of 1×10^6 protoplasts / ml in MaMg solution (0.45 M mannitol, 15 mM $MgCl_2$, 3 mM MES-KOH, pH 5.6). An average of 6×10^6 protoplasts was obtained from 10 medium size leaves.

2.2.6 Plants stress treatments

2.2.6.1 Dehydration

Dehydration experiments were performed with whole plants, which were placed on filter paper and air-dried in the growth chamber for various lengths of time.

2.2.6.2 ABA treatments

Detached *Craterostigma* leaves were floated on sterile water supplemented with 1 mM ABA. As ABA was dissolved in a 1:100 ethanol water mixture, detached leaves were incubated in sterile water supplied with an equal volume of ethanol as a control.

2.2.6.3 Heat stress

Well water *Craterostigma* plants from the growth chamber were used for heat-shock experiments. Pots were put for different lengths of time into an incubator at 42°C, 45°C or 48°C respectively. During the treatments the plants were exposed to the artificial light (a lamp was

collocated into the incubator) or at the natural light (by putting the plants into an incubator with transparent walls. The incubator was placed far from heat-sources but by well lighting conditions). The humidity was kept constant by putting a beaker with water into the incubator.

2.3 Methods

2.3.1 Nucleic acid extraction

2.3.1.1 Genomic and plasmid DNA isolation from bacteria

Genomic DNA was isolated using the Quiagen Genomic DNA purification kit; plasmid DNA was isolated using the Quiagen Plasmid kit according to the instruction of the manufacturer. Concentration and purity of the DNA were examined spectrophotometrically at an optical density (OD) of 260 nm and 280 nm. DNA size was checked by agarose gel electrophoresis. Genomic DNA was stored at 4° C, plasmid DNA was stored at -20° C.

After restriction enzyme digestion, plasmid inserts were isolated from native agarose gels via the Quiaex DNA extraction kit according to the instructions of the manufacturer.

2.3.1.2 Plasmid DNA isolation from yeast

Plasmid DNA from yeast cells was performed according to Hofmann and Winston (1987). Because the procedure yields a mixture of intact plasmid DNA and fragmented chromosomal DNA, the resultant plasmid DNA was used directly to transform *E.coli* DH *5a* strain . The further analyses were performed after the isolation of the plasmid DNA form bacterial colonies, as described in 2.3.1.1.

2.3.1.3 Total RNA isolation

Fully hydrated (8 g), dehydrated (2 to 4 g) or heat-shocked (8 g) leaves and roots from *Craterostigma* were collected and total RNA was extracted according to De Vries et al (1986). 30 µg of each RNA samples were used for Northern blot analysis (2.3.2.2).

2.3.2 Hybridization analysis

2.3.2.1 Southern blot analysis

After agarose gel electrophoresis, DNA was fixed onto membranes (Hybond N, Amersham; Sambrook et al., 1989). Prehybridization (2 h) and hybridization (overnight) was performed under high (65° C) stringency conditions (Sambrook et al., 1989). The filters were subsequently washed in 2X SSC and 0.1% SDS for 3 x 15 min under high stringency (65° C) conditions. The filters were exposed on X-ray films (Kodak X-Omat AR5) at -70° C.

2.3.2.2 Northern blot analysis

After gel electrophoresis using a formaldehyde denaturing 1.2% agarose gel, total RNA was transferred onto nylon membranes (Hybond N, Amersham) according to Sambrook et al. (1989). Prehybridization (3 h) and hybridization (overnight) was performed in Northern hybridization buffer (50% [v/v] formamide, 5X SSC, 10 mM PIPES pH 6.8, 0.1% [w/v] SDS, 1X Denhardt' s) at high hybridization temperature. Equal loading was controlled by

hybridization of the Northern filters with the ribosomal probe pTA71 (Gerlach and Bedbrock, 1979). The blots were subsequently washed for 3 x 15 min under high stringency conditions (Sambrook et al., 1989). The filters were exposed on X-ray films at -70°C.

2.3.3 PCR

Specific DNA fragments were generated by PCR (polymerase chain reaction) amplification. The following standard conditions were used:

10	ng	template DNA
5	µl	10x PCR buffer (Gibco BRL)
1.5	mM	MgCl
20	pmol/µl	forward primer
20	pmol/µl	reverse primer
100	µM	dNTPs
2.5	U	*Taq*-DNA-polymerase (GIBCO BRL)
X	µl	dH_2O to a final volume of 50 µl

The reaction mixture was covered with paraffin oil.

The PCR reactions were performed in a TRIO-thermoblock (Biometra, Goettingen). The number of cycles of DNA denaturation, primer annealing and DNA synthesis varied from 25 to 35. The primer annealing temperature Ta was determined as followed:

melting temperature :Tm = (G+C) 4°C + (A+T) 2°C

annealing temperature:Ta = Tm - 5°C

The optimal number of cycles and primers Ta was determined empirically for each primer/template combination.

2.3.4 The yeast two-hybrid system

2.3.4.1 Two-Hybrid cDNA expression library construction and activation domain (pAD-GAL4) plasmid preparation

Oligo (dT)-primed cDNA was synthesized from 5 μg poly $(A)^+$ RNA derived from 2 hours dried leaves using the Hybrid ZAP™ cDNA synthesis kit (Stratagene, LA Jolla, USA). the cDNA was directionally cloned into the Uni-ZAP XR vector, packaged using the ZAP

cDNA Gigapack II Gold packaging extract (Stratagene, La Jolla, USA) and plated on the *E.coli* XL1-Blue MRF' according to the instructions of the manufacturer. The Hybrid-ZAP™ lambda library was subsequently converted, by *in vivo* excision, to a pAD-GAL4 plasmid library, where the GAL4 activation domain sequence was fused *in frame* to the cDNA library sequence in order to expressed the hybrid protein (the target) required for the two-hybrid screening.

2.3.4.2 DNA-Binding Domain (pBD-GAL4) bait plasmids preparation

<u>BD-Dsp16 expression vector</u>

The full-length cDNA expressing the *Cp*-Dsp16 dehydrin (Bartels et al., 1990) was amplified with a 26-mer (5'-CGGGAATTCATGCGTCAGTTCGGCGG-3') and a 25-mer (5'-TGCATGCAGGCTGGCCGCCGGGAAG-3') as forward and reverse primers respectively. The 468 bp fragment obtained, was digested and cloned into the pBD-GAL4 expression vector at the *EcoRI/PstI* sites. In order to produce the hybrid protein (the bait) for the two-hybrid screening,

the cDNA sequence was fused *in frame* with the GAL4 binding domain sequence present in the vector,

Cp-HSF1 constructs

cDNA sequences representing different domains of the *Cp*-HSF1 protein were prepared either by restriction digestion or by PCR amplification of the *Cp*-Hsf1 full-length cDNA (Bockel C., 2000, PhD thesis). For the *Cp*-HSF1157 construct, a 24-mer (5'-AGAAAAAGCCCGGGAGACTGGCGT-3') and a 26-mer

(5'-TGAAAACATCCCGGGCTTTATGCTAT-3') oligos were used as forward and reverse primers respectively. The obtained 1157 bp fragment was digested and cloned into the pBD-GAL4 expression vector at the *SmaI* site.

The *Cp*-HSF960 construct was obtained by digestion with the *EcoRI* restriction endonuclease. The obtained 960 bp fragment was digested and cloned into the pBD-GAL4 vector at the corresponding restriction site.

For the *Cp*-HSF775 construct, a 26-mer (5'-CGGGAATTCATGCGTCAGTTCGGCGG-3') and a 17-mer (5'-GAATCTGCAGACGTTGC-3') oligos were used as forward and reverse primers respectively. The obtained 775 bp fragment was digested and cloned into the pBD-GAL4 vector at the *EcoRI*/*PstI* sites.

The 29-mer (5'-ACGCGTCGACTCGATCCATCGAAGCAGAG-3') and a 17-mer (5'-GAATCTGCAGACGTTGC-3') oligos were used as forward and reverse primers respectively, to prepare the *Cp*-HSF302 construct. The obtained 302 bp fragment was digested and cloned into the pBD-GAL4 expression vector at the *SalI*/*PstI* sites.

All the cDNA sequences were fused *in frame* with the GAL4 binding domain sequence present in the vector, in order to produce the hybrid protein (the bait) for the two-hybrid screening.

2.3.4.3 Two-hybrid screening

To screen the cDNA yeast expression library, YRG-2 yeast cells, containing the bait, were transformed according to the TRAFO lithium acetate method (Gietz and Schiestl, 1995). with individual target plasmids. Transformants were selected for histidine prototrophy (Leu, Trp^-, His^-). Transformed yeast clones were screened for β-galactosidase activity using a filter lift assay

(Breeden and Nasmyth, 1985). Colonies were transferred onto nitrocellulose filters, permeabilized by freezing in liquid nitrogen and thawed at room temperature. The freeze/thaw cycle was repeated for three times. Filters were then overlaid on Whatman 3MM paper saturated with lacZ-X-gal solution (Breeden and Nasmyth, 1985) and incubated at room temperature. The time required for color development ranged from 10 min to 8 hours). Colonies corresponding to positives were further analyzed. β-galactosidase activity was quantified using chlorophenol red-β-D-galactopyranoside (CPRG, Boehringer Mannheim) as substrate (Bartel and Fiels, 1995). Assays were performed in triplicate and β-galactosidase units were calculated as 1000 $(OD574)$/Vt $(OD600)$, where V = colture volume in 1 ml and t = reaction time in minutes.

2.3.5 Sequencing and sequence data analysis

Plasmid DNA preparation and dye terminator Thermosequenase (TM) cycle-sequencing reactions were performed using the DNA Vistra Labstation 625 (Molecular Dynamics,

Sunnyvale, USA). The sequence ladders were resolved on an ABI373A DNA sequenator (Applied Biosystems, Foster City, USA). Sequence were edited manually to remove vector sequences and ambiguous base calls. Most of the cloned cDNA sequences were sequenced both from the putative 5'-end and from the putative 3'-end. Sequence similarities of the DNA sequences to nucleotide sequences in GenBank, EMBL, DDJB and PDB data base were detected using FASTA and BLASTN programs (Pearson and Lipman, 1988; Altshul et al., 1990). The cDNA sequences were translated in the three reading frames in both possible orientations and the putative deduced amino acid sequences were compared with non-redundant protein sequence data bases (GenBank, CDS translation, PDB, SwissProt and PIR) by using the BLASTP program (Altshul et al., 1990; Pearson, 1991). All sequences were analyzed with the Wisconsin package version 10.0 of the Genetic Computer Group (GCG). Protein sequences alignment was performed using the PILEUP program with the GapWeight of 6 and the GapLengthWeight of 2. Phylogenetic analyses of the aligned amino acid sequences were conducted using the PAUP program (Swofford, 1993). The PSORT software (Gavel and von Haijne, 1990) was used for prediction of protein localization sites and putative pre-sequences targeting.

2.3.6 Expression and purification of recombinant proteins in *E.coli*

Expression and purification of recombinant His-tag-fusion proteins in *E.coli* was performed using the Quiaexpress System (Quiagen, Hilden). Optimal induction conditions and solubility of

the recombinant protein were determined according to the instructions of the manufacturer. Recombinant proteins were purified under native conditions (Sambrook et al., 1989).

2.3.7 Purification of recombinant proteins from yeast

Small-scale protein extract preparations were performed according to Mount et al (1996). Proteins extracts were obtained by mechanical disruption of the yeast cells, which were vortexed in the presence of glass beads.

2.3.8 Determination of protein concentration

Protein concentration was determined using the BioRad protein assay (BioRad, München; Bradford, 1976). Bovine serum albumin (BSA) was used as protein standard.

2.3.9 Gelelectrophoretic protein separation (SDS PAGE)

Proteins were separated by one-dimensional electrophoresis in 12% polyacrylamide gels (Laemmli, 1970). Protein samples were boiled for 5 min and cooled on ice before loading. Gels were run at 40 mA.

2.3.10 Protein staining

Protein detection by Poinceau staining (0.2% Poinceau S in 3% TCA; Salinovich and Montelaro, 1986) was applied to visualized the proteins before transfering them onto nitrocellulose membranes (2.3.11).

2.3.11 Western analysis

After separation by SDS PAGE proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel) by semidry-blotting technique according to Westermeier et al. (Pharmacia special edition RE-72). The transfer buffer contained 39 mM glycine and 48 mM Tris; blotting was performed at 2 mA / cm^2 for 1h. Proteins transferred onto nitrocelluose were detected by specific antiserum as described in the following. The membranes were blocked for 30 min in 5% milk powder, 1X TBS buffer (pH 9.6), incubated for 1 h with the 1: 10000 diluited antiserum, washed 3 x 10 min with 0.5% Nonited P-40, 5% milk powder, 1X TBS buffer (pH

9.6), and incubated for 45 min with the 1:5000 diluited secondary antiboby (anti-rabbit IgG conjugated horseradish peroxidase, Sigma). Subsequently, the membranes were washed 2 x 10 min with 0.5% Nonited P-40, 1X TBS buffer (pH 9.6), 2 x 10 min with 1X TBS buffer (pH 9.6) and 1 x 50mM Tris-HCl
pH 7.5. All steps were carried at room temperature. For protein detection, "ECL Western blotting system" (Amersham Buchler) was used according to the instructions of the manufacturer.

2.3.12 Electrophoretic gel mobility shift assay (EMSA)

Protein/DNA interaction was investigated in electrophoretic gel mobility shift assays (EMSA). The buffer, the protein samples and the unspecific competitor were first incubated for 5 min at room temperature. The ^{32}P-labelled DNA probe was added and the mixture was incubated for 15 min at room temperature. The reaction mixture was loaded on a 4% native
polyacrylamide gel (4% acrylamide, 0.1% N,N-methylenbisacrylamide, 0.2x TAE, 7% glycerin, 0.06% APS, 0.06% TEMED). Before loading, the gel was pre-run at 100 V for 1 h
at 4°C. After loading, the gel was run at 150 V for 4 to 5 h. After electrophoresis, the gel was dried for 1 h at 80°C under vacuum on Whatman 3MM paper and exposed to X-ray film (Kodak X-Omat AR5) overnight. The composition of the reaction mixture is described in the following:

2.4	µg	*E.coli* protein sample containing the *Cp*-HSF1 fusion protein (2.9.1)
2	µl	sonicaton buffer (50 mM Na-phosphate pH 7.8, 300 mM NaCl)
2	mM	$MgCl_2$
10	µl	2X binding buffer (14% (v/v) glycerin, 15 mM Hepes pH 7.5 (KOH), 8 mM Tris pH 7.5, 0.14 mM EDTA, 7mM β-mercaptoethanol, 0.1 mM PMSF)
1	µg	dIdC
30,000	cpm	^{32}P-labelled *Cp*-HSE DNA (5'-GTGTGATCGAACTTTTCTCGAAAAATATC-3')
x	µl	dH_2O to a final volume of 20 µl

For the competition assays, 100-fold molar excess of *Cp*-HSE sequence was added to the mixture reaction containing the 30,000 cpm ^{32}P-labelled HSE1 sequence (Bockel C., 2000, Ph.D thesis).

2.3.13 Tobacco protoplasts experiments

2.3.13.1 Construction of the *Cp*-HSp:GUS reporter plasmid

The 1 kb 5' region of the *Cp*-Hsp20.6 gene (-1027 to +69; see Appendix II) was inserted into the *HindIII/BamHI* sites of the pBI101.2 expression vector in front of the *E.coli* β-glucoronidase (GUS) gene, which was followed by the NOS termination site. In order to obtain a "translation fusion expression cassette", the following primers were used to amplify the 1096 bp *Cp*-Hsp20.6 promoter region:

forward primer (26-mer): 5'-GAATTCATCCTAAAGCTTAGAAGGTC-3'

reverse primer (27-mer): 5'-GTACGGAGCGGATCCAGGACAGTGTTG-3'

Subsequently, the 3226 bp *HindIII/BamHI* fragment was subcloned into the *HindIII/EcoRI* sites of pBluescript SK plasmid (Fig.3.19).

2.3.13.2 Construction of the CaMV35S:*Cp*-HSF1 expression plasmid

The full-length cDNA sequence (1150 bp) of the *Cp*-Hsf1 gene (Bockel 2000, Ph.D thesis), was cloned at the *NcoI/BamHI* sites into the expression vector pRT104 containing the stronger 35S promoter sequence and the polyadenilation signal from the cauliflower mosaic virus (CaMV35S). In order to create a "translational fusion cassette", the following primers were used to amplify the *Cp*-Hsf1 cDNA sequence:

forward primer (24-mer): 5'-GGCACGAGAGAAAAAGCCATGGAG-3'

reverse primer (26-mer) : 5'-CAATTCATGAAAGGATCCCTAGCTTT-3'

2.3.13.3 Protoplasts transformation

According to Negrutiu et al. (1987), 10 μg carrier DNA, 10 μg GUS reporter plasmid (2.13.1) plus 10 μg expression plasmid (2.13.2) and 330 μl PEG solution (40% PEG-4000, 0.4 M mannitol, 15 mM $MgCl_2$, 0.1% Hepes) were added to aliquots of protoplasts (330 μl of the suspension). After 20 min incubation at room temperature, the protoplast/DNA/PEG mixture was slowly diluted with 5 ml W5 solution (Negrutiu et al., 1987). After adding 6 ml of K3 solution (2.2.5), protoplasts were incubated for 16 h in the dark at 20 °C, then harvested by centrifugation and stored in GUS extraction buffer at -70°C.

2.2.13.4 Protoplasts heat stress treatments

Heat-shock of transformed protoplasts was performed by putting the samples into a waterbath at 37°C or 40°C respectively. For each transformation, one aliquot was kept in a waterbath at the control temperature (25°C). The length of the heat treatments and of the recovering times were as described by Treuter et al.(1993).

2.2.13.5 GUS activity assay

The GUS assay was performed according to Jefferson et al (1987). The GUS activity was measured after 1 h incubation at 37°C using a thermoblock. The assay was based on the enzymatic conversion of 4-methylumbelliferyl glucuronide to 4-methylumbelliferone, wich was measured in pmol 4-MUG / mg protein and quantify with a fluorometer (Perkin Elmer LS30, 365 nm excitation and 455 nm emission wavelengths). Protein concentration were determined according to Bradford with the BioRad kit (Bradford, 1976).

3 Results

3.1 Isolation of genes encoding drought-related proteins by the yeast two-hybrid system

3.1.1 Establishing the yeast two-hybrid system in *C. plantagineum*

The yeast two-hybrid system is an *in vivo* molecular-genetic test for protein interactions. To isolate proteins (the targets) that interact with a protein of interest (the bait protein), first the bait plasmid was constructed and used to screen a plasmid library for interacting fusion proteins. Secondly, after the elimination of false positives, sequence analysis and characterization of RNA transcription profiles were performed for those isolated clones showing significant interaction with the bait.

3.1.1.1 Verification of the yeast phenotypes

Using different selective media, the phenotype of the yeast host strain YRG-2 was verified prior to performing the screening assays. The YRG-2 strain contains a dual selection system with the *lacZ* and *HIS3* reporter genes: these should be activated only in the presence of specific protein interactions.

Plates of minimal SD agar medium prepared with the appropriate 10X dropout solutions were used to test the yeast strain for the following nutritional requirements: tryptophan (Trp), leucine (Leu), histidine (His) and uracil (Ura). As expected, the yeast host strain YRG-2 can grow only on SD agar plates without uracil (Table 3.A). The YRG-2 host strain was also assayed for the expression of the *lacZ* reporter gene by filter lift assays (Breeden and Nasmyth, 1985). As shown in Table 3.A, no β-galactosidase activity was detected, demonstrating that the activation of the *lacZ* reporter gene did not occur in the absence of protein interactions. The yeast host strain was transformed with the plasmids pBD-GAL4(Cam) and pAD-GAL4 separately, in order to verify the phenotype conferred

by the bait and the target vectors. Each of the two plasmids carries a different nutritional marker, tryptophan (Trp) and leucine (Leu) respectively, for the selection in yeast. The host strain was also transformed with the pGAL4 control plasmid (containing the entire coding sequence of the wild type GAL4 protein), to verify the induction of the *lacZ* reporter gene. Since the transformed colonies turned blue when permeabilized and incubated in the presence of a buffer containing X-Gal, the *lacZ* gene induction was confirmed (indicated in Table 3.A by a (+) in the β-gal assay column).

Finally, the YRG-2 host strain was transformed with the control plasmids p53 (expressing the GAL4 DNA-binding domain fused to the murine p53 protein) and pSV40 (expressing the AD-GAL4 domain fused to the large T-antigene) alone or in pairwise combinations. The clone p53/SV40 obtained after co-transformation with the two plasmids p53 and SV40, whose hybrid proteins interact *in vivo*, was used as positive control for specific protein interactions. In fact, typical of the two-hybrid system is the restoration of the yeast histidine auxotrophy through protein interactions. As shown in Table 3.A, only the yeast expressing both proteins could grow on plates lacking histidine. The verification tests here described were necessary to set up the yeast phenotype controls used during the two-hybrid screening, in order to verify: first, the detection of the reporter gene products in the assay used; second, that the expression of the reporter genes occurs only in the presence of specific protein interactions. The results of all control experiments are summarized in Table 3.A.

Table 3.A

Verification of the yeast phenotypes after transformation with different plasmid vectors

Yeast Strain	Vector (1)	SD -Trp	SD -Leu	SD -His	SD -Ura (2)	β-gal assay (3)
YRG-2		-	-	-	+	-
YRG-2	pBD-GAL4Cam	+	-	-	+	-
YRG-2	pAD-GAL4	-	+	-	+	-
YRG-2	pGAL4	-	+	-	+	+*
YRG-2	p53	+	-	-	n.d.	-
YRG-2	pSV40	-	+	-	n.d.	-
YRG-2	p53/pSV40	+	+	+**	n.d.	+**

(1) The vectors represent the control plasmids used for all two-hybrid experiments. (2) The URA3 gene confers the uracile prototrophy to the YRG-2 wild type strain, which can grow on the SD-Ura medium. (3) The β-gal assay was performed by filter lifts (see 2.3.4.3). (+): *lacZ*$^{+}$ phenotype (blue colonies); (-): *lacZ*$^{-}$ phenotype. * : positive control for specific -galactosidase gene activity (the pGAL4 plasmid carries the *leu2* gene as marker for the selection in yeast).

** : double transformation: positive control for specific protein interaction (the p53 and the pSV40 plasmids carry the *trp1* gene *leu2* respectively as markers for the selection in yeast).

n.d. : not determined

3.1.1.2 Construction of a target protein library

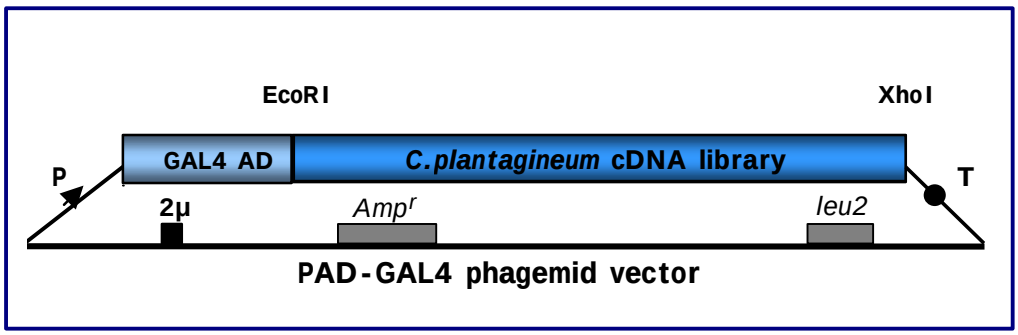
A cDNA library representing a pool of expressed proteins after 2 hours dehydration was prepared using poly(A)$^+$RNA from *C.plantagineum* leaves and cloned into the HybridZAP™ lamda vector (Stratagene, La Jolla, USA). Approximately 1,5 x 10^6 pfu (plaque forming units) were obtained. The HybridZAP™ vector was converted by *in vivo* excision to the *E.coli*-yeast shuttle vector pAD-GAL4, where the DNA insert (average size around 1 kb) representing the library of target proteins was fused to the yeast GAL4 activation domain (amino acid 768-881; Ma and Ptashne, 1987).

The plasmid library construct pAD-GAL4 (target plasmid) is shown in Fig. 3.1.

The pAD-GAL4 target plasmid was cotransformed with the pBD-GAL4(Cam) control plasmid (i.e. with an empty plasmid that does not contain any DNA insert) into the YRG-2 yeast strain and assayed for expression of the *HIS3* and *lacZ* reporter genes. Because no induced expression of both reporter genes was observed, the pAD-GAL4 plasmid library seemed a suitable target vector to be used by the two-hybrid system established for this study.

Fig.3.1

cDNA library pAD-GAL4 expression vector



The size of the pAD-GAL4 phagemid vector is 7620 bp.
The hybrid protein (GAL4 AD plus the cDNA library target proteins) is constitutively expressed by the yeast p_{ADH1} promoter (**P**) and the yeast t_{ADH1} terminator (**T**).
The cDNA insert (average size around 1 kb) was directionally inserted at the *EcoRI* and at the *XhoI* sites.

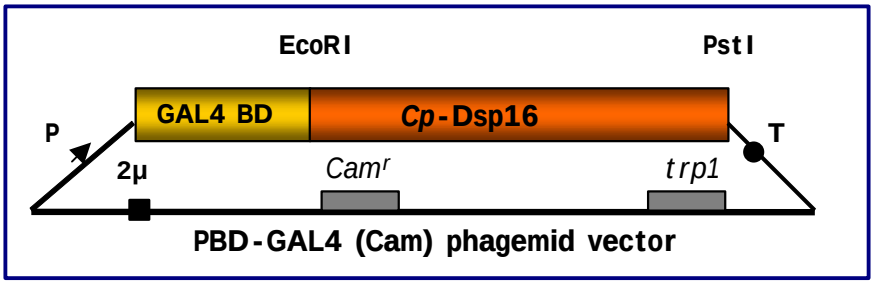
2µ : high-copy yeast origin of replication
Amp^r : ampicillin-resistance gene for growth in bacteria
leu2 : auxotrophic marker for selection in yeast

3.1.2 Identification of proteins interacting with the *Cp*-Dsp16 dehydrin

3.1.2.1 Preparation of the *Cp*-Dsp16 dehydrin as bait protein

The gene CDeT6-19 (Bartels et al., 1990) encodes for a 16 kDa protein (*Cp*-Dsp16) homologous to the Lea D-11 proteins, known as "dehydrins" and belongs to the *C.plantagineum* drought-inducible transcripts group. Dehydrins generally accumulate in response to any environmentally-induced or developmentally-programmed process that has a cellular dehydration component (Close et al., 1993; Dure L., 1993). The *Cp*-Dsp16 full-length cDNA sequence (468 bp) was cloned into the bait vector pBD-GAL4(Cam), to produce a hybrid protein between the binding domain of the yeast transcription factor GAL4 (amino acid 1-147, Keegan et al. 1986) and the dehydrin (Fig. 3.2).

Fig. 3.2
The pBD-Dsp16 expression vector



The size of the pBD-GAL4(Cam) phagemid vector is 6494 bp.
The hybrid protein (GAL4 BD plus the full-length *Cp*-Dsp16 dehydrin) is constitutively expressed by the yeast p_{ADH1} promoter (**P**) and the yeast t_{ADH1} terminator (**T**).
The *Cp*-Dsp16 cDNA insert (468 bp) was directionally inserted at the *EcoRI* and at the *PstI* restriction sites.

2µ : high-copy yeast origin of replication
Cam^r : chloramphenicol-resistance gene for growth in bacteria
trp1 : auxotrophic marker for selection in yeast

To determine whether the BD-Dsp16 fusion protein was a suitable bait for the two-hybrid system, its capability of autonomous reporter gene activation was tested. First the host yeast was

transformed with the BD-Dsp16 bait plasmid. Subsequently, the cells expressing the BD-Dsp16 fusion protein were transformed with the pAD-GAL4 plasmid, which does not contain any DNA insert.

Generally the yeast cotransformants are plated both on minimal medium lacking leucine and tryptophan (SD-LT) and on minimal medium lacking leucine, tryptophan and histidine (SD-LTH). The growth on SD-LT indicates the presence of both the proteins (bait and target) but does not prove that they interact. On the contrary, the interaction between bait and target (or between two proteins contain transcriptional domains able to activate the *HIS3* gene) is necessary for yeast colonies surviving on SD-LTH. As shown in Table 3.B, the absence of growth on the selective medium (SD-LTH) of the transformants expressing the *Cp*-Dsp16 dehydrin proved that the BD-Dsp16 fusion protein did not contain transcriptional activator regions able to activate the *HIS3* reporter gene independently from the presence of target proteins.

The inability of the BD-Dsp16 fusion protein to interact with an unrelated target (the large T-antigene; in Table 3.B indicated as AD fusion protein: SV40) was demonstrated by the absence of growth on the selective medium (SD -LTH) of the transformants expressing the *Cp*-Dsp16 dehydrin and the SV40 protein. This result proved that the BD-Dsp16 bait did not interact randomly with other protein. Consequently, the possibility to produce false positives by using the BD-Dsp16 as bait protein for the two-hybrid screening was estimated to be extremely low.

No β-galactosidase activity was detected for the yeast transformants expressing either the BD-Dsp16 bait or the bait protein together with SV40 target protein. The failed activation of both reporter genes confirmed that the BD-Dsp16 fusion protein was suitable bait for the two-hybrid screening.

Table 3.B

Verification of phenotypes for the yeast clones expressing the BD-Dsp16 protein together with unrelated targets.

bait BD fusion protein	target AD fusion protein	SD -Trp	SD -LT	SD -LTH	β-gal assay (1)
Cp-Dsp16	pAD-GAL4*	+	+	-	-
Cp-Dsp16	SV40	+	+	-	-
p53	SV40	+	+	+**	+**

(1) The β-galactosidase assay was performed by filter lifts (see 2.3.4.3).
(+): *lacZ*$^+$ phenotype (blue colonies); (-) : *lacZ*$^-$ phenotype.

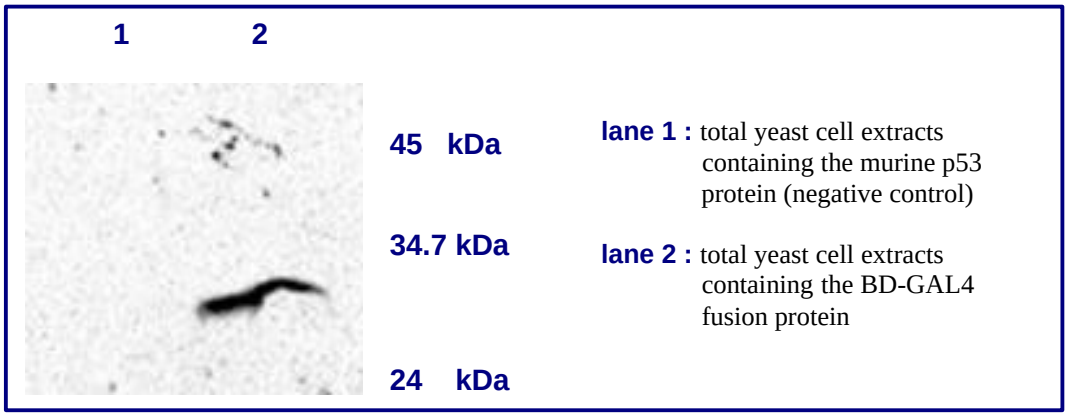
* The pAD-GAL4 vector here used was the empty shuttle vector which does not contain any additional coding DNA sequence fused to the GAL4 activation domain and therefore it is not able to produce target proteins.

** Double transformation: positive control for specific protein interaction (the p53 and the pSV40 plasmids carry the *trp1* gene and the *leu2* gene respectively as markers for the selection in yeast).

Analysis of protein extracts from the transformed yeast cultures was used to verify the expression of the BD-Dsp16 fusion protein. A band of approximately 32 kDa (Fig.3.3) was detected using polyclonal antiserum raised against the *Cp*-Dsp16 dehydrin (Schneider et al., 1993). The size of the band recognized by the antibodies is in good agreement with the expected size for the BD-Dsp16 fusion protein, where the size of the dehydrin (16 kDa, Lisse et al, 1996) has to be added to the size of the GAL4 binding domain (15 kDa, Keegan et al. 1986).

Fig. 3.3

Western analysis of the Cp-Dsp16 dehydrin expression in the yeast YRG-2



3.1.2.2 Identification of proteins interacting with the *Cp*-Dsp16 bait protein

After testing the reliability of the BD-Dsp16 fusion protein as bait (3.1.2.1), the yeast cells expressing the *Cp*-Dsp16 dehydrin were transformed with the pAD-GAL4 plasmid library (3.1.1.2), to identify dehydration-related proteins interacting with the *Cp*-Dsp16 dehydrin. Approximately 2 x 10^6 transformants were plated on synthetic medium containing glucose but lacking leucine, tryptophan and histidine (SD-LTH).

To distinguish whether the growth of the transformants on minimal medium was due to the leaky expression of the *HIS3* reporter gene or to specifically interacting proteins, the activation of the second reporter gene *lacZ* was tested by the β-galactosidase-lift assay: just the transformants which grew on the medium lacking histidine and which turned blue by the β-galactosidase assay were considered candidates for expressing interacting proteins.

The first selection round resulted in a massive activation of the *lacZ* reporter gene and most of the yeast transformants turned blue, preventing the identification of true positive clones. Two hundred blue colonies were identified after the β-galactosidase lift assay. Because most of the colonies turned blue after 4 to 7 hours (while the p53/SV40 clone used as control for protein interaction needed 40 minutes), a second β-galactosidase lift assay was performed, to eliminate those clones expressing target proteins which interacted weakly with the BD-Dsp16 bait protein. Briefly: the putative positives were restreaked on the same selective medium (SD-LTH) and tested for β-galactosidase activity. After this third round, only twenty-two putative positive yeast clones were isolated.

To test whether the phenotype observed (growth on SD-LTH and turned blue colonies) was reproducible and dependent on the presence of the *Cp*-Dsp16 dehydrin, the plasmid DNA was recovered from each positive clone and used to retransform the empty YRG-2 host strain to check for plasmid dependence. The expected result was that none of the new transformants could grow on selective medium in the absence of the BD-Dsp16 bait protein. However, 13 out of 22 clones were able to grow on selective medium in the absence of specific interaction with the BD-Dsp16 bait and therefore they were excluded from the analysis because they were clearly false positives. The other 9 plasmid DNAs were used to retransform the yeast cells expressing the *Cp*-

Dsp16 dehydrin, in order to confirm the histidine auxotrophic phenotype previously observed. All were able to grow on minimal medium: all were apparently true positive clones. The corresponding plasmid DNA was isolated from the yeast clones and subjected to sequence analysis.

Most of the analyzed DNA sequences showed the GAL4 activation domain sequence fused *in frame* to DNA fragments which were too short (size from 50 to 200 bp) to encode any protein able to interact with the bait. A database search did not reveal any homology to other proteins. The sequence analysis displayed that these clones were also false positives. The frequency of false positives by the two-hybrid system and their role by this screening will be discussed in the fourth part (Discussion).

Only three ORF (open reading frame) sequences, fused *in frame* with the GAL4 activation domain, were long enough to encode for putative target proteins (their size was on average from 700 to 800 bp).The RNA profiles of the corresponding transcripts were characterized.
In addition, β-galactosidase liquid assays using chlorophenol-red-β-D-galactopyranoside (CPRG, Iwabuchi et al., 1993) were performed to quantify the strength of the interaction between these potential candidate targets and the BD-Dsp16 bait . The results of these analyses are summarized in Table 3.C.

Table 3.C

Proteins interacting wíth the Cp-Dsp16 dehydrin.

Homology Org.(1)	Protein	AA Ident. % (2)	Growth on plates lacking leu, trp, his (3)			β-gal activity (7) (β-galactosidase units)		Expression in leaves (8)				
	Putative targets		no bait (4)	Bait (5) Dsp16	3-AT (6)	no bait	bait Dsp16	Dehydration 0h	2h	4h	24h	ABA 4h
Cp	Dsp11-24	99	-	+	+	0.95 ± 0.88	1.82 ± 1.1	-	+	+	+	+
Ath	α-subunit of the TRAP complex	65	-	+	-	0.1 ± 0.07	0.43 ± 0.16	+	+	+	+	+
Sb	Kafirin	39	-	+	-	0.58 ± 0.4	1.6 ± 0.9	-	+	+	+	+

(1) *Cp*: *Craterostigma plantagineum*; *Ath*: *Arabidopsis thaliana*: *Sb*: *Sorghum bicolor*

(2) The protein sequence comparisons were determined using the BLAST network service (Altschul et al., 1990).

(3) Growth on SD minimal medium.

(4) not bait: the yeast was transformed with the pBD-GAL4(Cam) vector. This is a plasmid which does not contain any additional coding DNA sequence fused to the GAL4 binding domain and therefore it is not able to produce bait proteins. Here it was used as negative control.

(5) Dsp16: BD-Dsp16 bait protein expressing the GAL4 binding domain fused to the full-length protein of the *Cp*-Dsp16 dehydrin.

(6) 3-AT: the competitive inhibitor 3-amino-1,2,4-triazole was added to reduce the residual *HIS3* gene expression and to increase the stringency of the selection. Assayed concentrations: 5-15-25-40-50-60 mM.

(7) Liquid cultures were assayed for β-galactosidase activity to quantify two-hybrid interactions. Transformed yeast clones expressed the putative targets in combination either with the negative control (pBD, no bait) or with the BD-Dsp16 bait protein (Dsp16, bait).
The substrated was the chlorophenol red-β-D-galactopyranoside (CPRG, Iwabuchi et al., 1993).
1 unit of β-galactosidase = amount that hydrolizes 1μmol of CPRG to chlorophenol red and D-galactose per minute per cell (Miller, 1972). Interactions were assayed for 8 hours.
Reference of specific activity GAL4 wt protein : 533 units of β-gal after 10 minutes
Reference of specific protein interaction P53/SV40 : 385 units of β-gal after 40 minutes
CPRG quantification was performed in triplicate for each transformation.

(8) The RNA analyses were performed using poly(A)$^+$ RNA from *Craterostigma* detached leaves. ABA (abscisic acid) was used at the concentration of 1 mM.

The first putative target was identical to the CDeT11-24 gene product. The gene was isolated from *C.plantagineum* and already characterized (Velasco et al., 1998). The

corresponding protein (Dsp11-24) is a hydrophilic 41 kDa polypeptide, whose expression is constitutive in roots but induced by dehydration, abscisic acid and salt in leaves.

As reported in Table 3.C, the growth on selective medium (indicated by (+) in Table 3.C) was observed only for the yeast transformed with the isolated target and the Dsp16 bait protein together. In addition, the chemical inhibitor 3-aminotriazol (3-AT) was used to test how specific the interaction with the *Cp*-Dsp16 was; the transformants expressing the Dsp11-24 protein were plated on SD-LTH plates containing different concentrations of 3-AT. Growth of the yeast colonies was observed till up 50 mM 3-AT, indicating that the Dsp11-24 protein was able to interact relatively strongly with the *Cp*-Dsp16 dehydrin.

The β-galactosidase activity was estimated for the transformants expressing the target both in the absence of the bait (BD, no bait) and in the presence of bait protein (Dsp16, bait). The activity level in the presence of the specific interaction between bait and target proteins was different from that estimated in the presence of non-specific interactions, but not significantly different. Finally, the RNA profiles observed for the transcript corresponding to the isolated target protein were consistent with the expression analysis previously performed (Velasco et al., 1998) and showed a strong induction by dehydration and ABA treatment in leaves (Table 3.C). The constitutive expression in roots was also confirmed.

The second protein sequence examined showed 65% homology to the *A.thaliana* protein TRAP-α protein, a subunit of a transmembrane protein complex located at the site where the nascent secretory proteins enter the endoplasmatic reticulum (Hartmann and Prehn, 1994). The TRAP-α is membrane spanning protein identified, besides *Arabidopsis*, in man and in trout (Accession numbers Z12830 and Z12831 respectively). Like for the first identified target, the growth on selective medium (+) was observed only for the yeast transformed with the isolated target and the bait proteins together. In this case, the addition of the chemical inhibitor 3-aminotriazol (3-AT) to the medium was not necessary for the selection. Similarly to the first clone, the β-galactosidase activity level in the presence of the specific interaction between bait and target proteins was not dramatically different from that estimated in the presence of non-specific interactions. The RNA analysis revealed a constitutive expression of this transcript in the leaves. Because the aim of this work was the

study of the dehydration-stress responses and the identification of protein induced by

dehydration in *C.plantagineum*, the characterization of this clone was not continued.

The third candidate for target protein showed 39% homology to *S.bicolor* kafirin protein, a major seed storage prolamin of *Sorghum* (DeRose et al., 1989. Accession number P14692). Also for this putative target protein, the interaction with the bait seemed to be specific (growth on selective medium only in the presence of the BD-Dsp16 bait, Table 3.C) and the addition of the histidine inhibitor 3-aminotriazol (3-AT) was not necessary. The β-galactosidase activity observed was roughly the same in the presence as in the absence of the bait protein. The corresponding transcript was induced by dehydration and ABA in leaves. The low homology to plant protein (39% in *S.bicolor*, just 18% in *A.thaliana*), and the low β-galactosidase activity level observed were consistent with the hypothesis that the protein interactions taken place between this target and the bait were weak. For this reason the characterization of this clone was not continued.

3.1.2.3 Analysis of the self-interaction ability of the *Cp*-Dsp16 dehydrin

Dehydrins are characterized by a highly conserved Lys-rich region of 15 amino acids (named K segment) present near the carboxy terminus of the protein and which has been proposed to form an amphipathic α-helix domain (Dure L.III, 1993). Theoretical studies have speculated on the dimerization ability of the D-7 Lea protein family (containing the same α-helical arrangement) via binding of their hydrophobic faces (Dure L.III, 1993). In addition, the existence as a dimer of the 17.7 kDa maize dehydrin has been experimentally demonstrated (Close et al., 1993). Based on this, the two-hybrid system was used to check the dimerization ability of the *Cp*-Dsp16 dehydrin. The bait used was the same BD-Dsp16 fusion protein previously prepared for the library screening and containing the *Cp*-Dsp16 full-length cDNA sequence (3.1.2.1). The target protein to test for interactions with the Dsp16 dehydrin was a novel hybrid protein, where the GAL4 activation domain (amino acid 768-881; Ma and Ptashne, 1987) was fused to the same *Cp*-Dsp16 full-length cDNA sequence into the target vector pAD-GAL4. The bait and the target vectors were introduced
into the yeast host by sequential transformation and plated both on selective medium

lacking leucine and tryptophan (SD-LT), to check for the presence of both the plasmids. On

medium lacking leucine, tryptophan and histidine (SD-LTH) no growth was observed for the transformants expressing the bait-target dehydrins (Table 3.D). On the contrary, the yeast colonies could grow on plates lacking only leucine and tryptophan (SD-LT), where the interaction between the two proteins was not necessary for surviving. The use of the *Cp*-Dsp16 dehydrin as a bait by the two-hybrid system showed that this protein was not able to create strong interactions with other proteins, not even to interact with itself to form a homodimer.

Table 3.D

Analysis of Cp-Dsp16 self-interaction.

bait (1) BD fusion protein	target (1) AD fusion protein	SD -LT	SD -LTH
Cp-Dsp16	*Cp*-Dsp16	+	-
P53	SV40	+	+*

(1) The BD fusion protein (bait) and the AD fusion protein (target) express both the full-length *Cp*-Dsp16 dehydrin protein.

* Double transformation: positive control for specific protein interaction (the p53 and the pSV40 plasmids carry the *trp1* gene and the *leu2* gene respectively as markers for the selection in yeast).

3.1.3 Identification of proteins interacting with *Cp*-HSF1

3.1.3.1 Preparation of the *Cp*-HSF1 bait protein

The fragment introduced into the pBD-GAL4(Cam) vector had a length of 1157 bp and represented the full-length *Cp*-Hsf1 cDNA (Bockel C., 2000, Ph.D thesis). To create a hybrid protein suitable for the two-hybrid screening, the cDNA sequence was fused downstream from the GAL4 binding domain (amino acid 1-147, Keegan et al., 1986) in the bait vector pBD-GAL4(Cam), as previously was done for the BD-Dsp16 fusion protein (3.1.2.1). The translated hybrid protein used as bait was termed *Cp*-HSF1157. Before

screening the *Craterostigma* cDNA library for interacting targets, the *Cp*-HSF1157 bait was assayed for transcription of the reporter genes in the absence of specific protein interactions. The results are reported in Table 3.E.

Table.3.E

Verification of the yeast phenotypes expressing the Cp-HSF1 protein together with unrelated targets.

bait BD fusion protein	target AD fusion protein	SD -LT	SD -LTH	3-AT(1) SD -LTH	β-gal assay (2)
Cp-HSF1157	pAD-GAL4*	+	+	+	+*
Cp-HSF1157	SV40	+	+	+	+*
p53	SV40	+	+**	n.d.	+**

* The pAD-GAL4 vector here used was the empty shuttle vector that does not contain any additional coding DNA sequence fused to the GAL4 activation domain and therefore it is not able to produce target proteins.

** Double transformation: positive control for specific protein interaction (the p53 and the pSV40 plasmids carry the *trp1* gene and the *leu2* gene respectively as markers for the selection in yeast).

n.d.: not determined

(1) Assayed concentrations: 10-25-50-75 mM. The yeast colonies expressing the *Cp*-HSF1157 protein in combination with the control plasmid pAD-GAL4 could grow in the presence of all 3-AT concentrations.

(2) The β-galactosidase assay was performed by filter lifts (see 2.3.4.3). (+): $lacZ^+$ phenotype (blue colonies); (-) : $lacZ^-$ phenotype.

The host yeast strain YRG-2 was transformed first with the bait plasmid expressing the *Cp*-HSF1157 protein, and subsequently with the empty pAD-GAL4 plasmid, which does not contain any protein encoding DNA insert. Although no potential interacting proteins were present, nevertheless the activation of the *HIS3* and the *lacZ* reporter genes was observed, i.e the yeast colonies grew on medium lacking leucine, tryptophan and histidine (SD-LTH) and turned blue when permeabilized and incubated in presence of the X-Gal buffer. The control target protein SV40 was expected to not interact with the bait protein *Cp*-HSF1157. However, when they were expressed together in the yeast host, the transcription of the *HIS3* and the *lacZ* reporter genes was activated. These results together indicated that the full-length *Cp*-HSF1157 protein was an unsuitable bait for the screening.

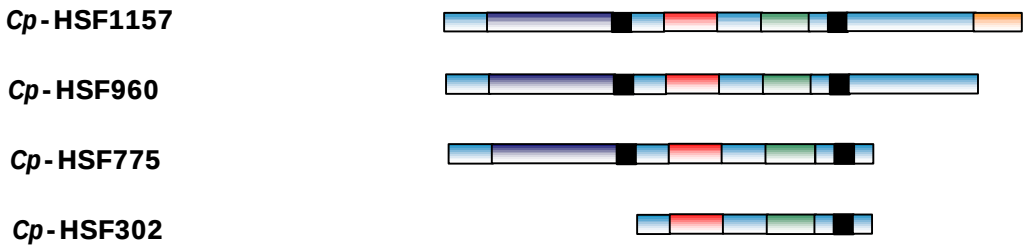
Because the minimal medium (SD-LTH) was not sufficient to abolish the transcription of the *HIS3*reporter gene in the absence of specific protein interactions, the chemical inhibitor 3-aminotriazol (3-AT), which restore histidine auxotrophy, was added to the selective medium, in order to reduce the residual *HIS3* gene expression and to increase the stringency of the selection. As shown in Table 3.E, in the presence of the histidine inhibitor 3-AT, the *Cp*-HSF1157 protein could still activate the reporter genes transcription. This result confirmed the difficulty to utilize the full-length sequence as a reliable bait protein for the two-hybrid screening.

3.1.3.2 Analysis of different *Cp*-HSF1 deletion constructs

Since the full-length *Cp*-HSF1157 protein seemed to be not a suitable bait for the two-hybrid screening (3.1.3.1), three different deletion constructs were prepared (Fig.3.4). The corresponding translated proteins were expressed as hybrids between the GAL4 binding domain and the deleted *Cp*-HSF1157 protein. The first construct, named *Cp*-HSF960, was prepared by omitting the C-terminal hydrophobic region (HR-C) of the activation domain. The second (*Cp*-HSF775) represented the DNA binding domain (DBD), the central oligomerization region (HR-A/B) and the nuclear localization sequence (NLS). The third (*Cp*-HSF302) was the shorter fusion protein prepared for this screening. In this construct neither the DNA binding domain nor the activation domain of *Cp*-HSF1 were present, but only the oligomerization region (HR-A/B) plus the nuclear localization sequence (NLS) were conserved.

Fig. 3.4

Representation of the different Cp-HSF1 deletion constructs used as bait proteins in the yeast two-hybrid screen



For all constructs, the GAL4 DNA-binding domain sequence was fused *in frame* with the cDNAs encoding for the different deleted proteins. The terminology used to distinguish the different constructs refers to the length of the different DNA fragments obtained after the amplification reactions or the restriction digestions of the full-length *Cp*-Hsf1 gene. The cDNA fragments were directionally subcloned in the pBD-GAL4 expression vector.

Cp-HSF1157 :this construct encodes for the full-length Cp-HSF1protein.

The basic structure of the protein is indicated above the draw: DBD = DNA binding domain; OD = oligomerization domain; AD = activation domain; HR-A/B, HR-C =heptad hydrophobic repeats; L1 = linker region between DBD and HR-A; L2 = linker region between HR-A and HR-B; NLS = nuclear localization signal (for details see Bockel C., 2000, PhD. thesis).

Cp-HSF960 :the 960 bp fragment was obtained after digestion with the endonuclease *EcoRI* and subcloned in the pBDGAL4 expression vector.

Cp-HSF775 :by amplification with specific primers, a 775 bp *EcoRI-PstI* fragment was obtained.

Cp-HSF302 :by amplification with specific primers, the internal hydrophobic region (HR-A/B) and the nuclear localization sequence (NLS) were obtained as a 302 bp *SalI-PstI* fragment.

Each plasmid was introduced in the YRG-2 host strain in pairwise with the empty pAD-GAL4 vector, to check whether the corresponding bait protein was capable of autonomous reporter gene activation. In addition, following the same scheme previously applied to the BD-Dsp16 and the *Cp*-HSF1157 bait proteins, each bait was tested for its ability to interact with the control SV40 target protein. The resulting phenotypes were individually assayed by colony lift assays for the *lacZ* reporter gene activation (Table 3.F).

Table 3.F

Verification of the yeast phenotypes expressing the Cp-HSF1 deletion constructs together with unrelated targets.

Bait BD fusion protein	Target AD fusion Protein	SD -LT	SD -LTH	3-AT(1) SD -LTH	β-gal assay (2)
Cp-HSF960	pAD-GAL4*	+	+	+	+
Cp-HSF960	SV40	+	+	+	+
Cp-HSF775	pAD-GAL4*	+	+	-	+
Cp-HSF775	SV40	+	+	-	+
Cp-HSF302	pAD-GAL4*	+	-	-	-
Cp-HSF302	SV40	+	-	n.d.	-
P53	SV40	+	+**	n.d.	+**

The transformants able to grow on medium lacking leucine, tryptophan and histine and showing blue colonies because of the activation of the β-galactosidase reporter gene (indicated by (+) in the SD-LTH and β-gal assay columns respectively) suggested that the deletion construct in examined was unsuitable bait for the system.

(1) 3-AT assayed concentrations: 10-25-50-75-90 mM

(2) The β-galactosidase assay was performed by filter lifts (see XX). (+): *lacZ*$^{+}$ phenotype (blue colonies); (-) : *lacZ*$^{-}$ phenotype.

* The pAD-GAL4 vector here used was the empty shuttle vector that does not contain any additional coding DNA sequence fused to the GAL4 activation domain and therefore it is not able to produce target proteins.

** : double transformation: positive control for specific protein interaction (the p53 and the pSV40 plasmids carry the *trp1* geneand the *leu2* gene respectively as markers for the selection in yeast).

n.d. : not determined

As already observed for *Cp*-HSF1157, the yeast colonies transformed with the *Cp*-HSF960 construct could induce autonomously the expression of the reporter genes

(Table 3.F). When plated on medium containing the 3-AT histidine inhibitor, the transformants could grow up to a concentration of 90 mM, which is three times higher than that routinely used for these assays. The *Cp*-HSF960 construct was therefore not used to screen the library.

The transformants expressing the *Cp*-HSF775 protein could not survive in the presence of the competitive inhibitor 3-AT, but still they were able to grow on minimal medium lacking leucine, tryptophan and histidine (Table 3.F). Surprisingly, even though in this protein no activation domain was present, gene transcription activation was observed for the *HIS3* and *lacZ* reporters. Differently to the *Cp*-HSF1157 and *Cp*-HSF960 proteins, in the presence of the unrelated SV40 control target protein, *Cp*-HSF775 did not activate the expression of the *lacZ* reporter gene. Nevertheless, because the observed growth on selective plates (SD-LTH) in the absence of any potential target protein, this deletion construct was not chosen as bait for the library screening.

Cp-HSF302 was the shorter fusion protein prepared for this screening. In this construct neither the DNA-binding domain nor the activation domain of *Cp*-HSF1 were present (Fig.3.4). After testing the protein alone and in combination with the control plasmid encoding for the SV40 target protein, no reporter gene activation was observed. The *Cp*-HSF302 protein was chosen as bait for the screening of the *C.plantigineum* cDNA library.

3.1.3.3 Identification of proteins interacting with the Cp-HSF302 bait protein

Using the *Cp*-HSF302 protein as bait, the *Craterostigma* cDNA library was screened. To avoid the identification of weak interactions, 5 x 10^4 transformants were selected for their swiftness of growth: the yeast colonies appearing after 5 days from the plating on SD-LTH medium were excluded from the screening. After several rounds of selection, 87 cotransformats grown on minimal medium lacking leucine, tryptophan and histidine were isolated. This method of selection based on the hypothesis that the transformants

expressing stable protein interactions could grow faster than those expressing proteins interacting weakly or unspecifically. The 87 clones were tested for the *HIS3* and *lacZ* reporter gene activation. The assays were performed on plates lacking leucine, tryptophan and histidine with

the addition to the medium of the competitive inhibitor 3-AT (15 mM) in order to eliminate false positive colonies growing because of the cellular endogenous histidine level.

Despite the screening on SD-LTH plates resulted positive (showing the correct activation of the *HIS3* reporter gene), no β-galactosidase activity could be detected by colony lift assay. By the same work conditions, the activation of the *lacZ* gene was observed both in the control colonies expressing the *in vivo* interacting proteins p53 and SV40 and in the colonies expressing the wild type GAL4 protein (normally used as control because their strong *lacZ* gene activation). So the failed detection of the β-galactosidase activity for the 87 isolated clones could not depend of the failed detection on the reporter gene expression.

Yeast liquid cultures were established for 40 out of the 87 clones to determine the β-galactosidase activity using CPRG (chlorophenol red-β-D-galactopyranoside) as substrate. After the assays only a weak β-galactosidase activity was observed.

This result indicated that the protein interactions taking place in the presence of the *Cp*-HSF302 bait were weak, if ever not specific. The only DNA sequence cloned and homologous to a plant protein also at the amino acid level showed 71% of identity to an *A.thaliana* glutamate/aspartate binding protein (sequence database entry AC002330).

3.1.3.4 Study of the interactions among different functional domains of the Cp-HSF1 protein

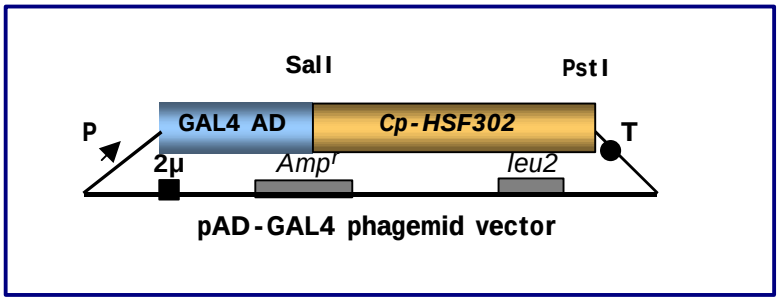
Analysis of heat-shock transcription factors from many species has defined structural and regulatory regions responsible for transcriptional activation. All studied HSFs have a common core structure containing the DNA binding domain and a trimerization domain (Sorger and Nelson, 1989; Wu C., 1995). The amino acid sequence of *Cp*-HSF1 shows the characteristic domains of the eukaryotic heat-shock transcription factors (Bockel C., 2000, Ph.D thesis).

In this study, the analysis has been focused on the short internal hydrophobic region (the oligomerization domain, HR-A/B, Fig.3.4) which is thought to be an essential element of the heat-induced activation of the protein (Sorger P.K., 1991; Morimoto R.I., 1993).

3.1.3.4.1 Preparation of a new *Cp*-HSF1 deletion construct

A deletion construct was prepared in a way that the corresponding translated protein was able to interact with the bait proteins containing the different functional domains of the *Cp*-HSF1. The new protein (*Cp*-HSF302AD) was encoded by the *Cp*-HSF302AD plasmid (Fig. 3.5) and it was a hybrid between the GAL4 activation domain (amino acid 768-881; Ma and Ptashne, 1987) and the 302 bp region of the *Cp*-HSF1 protein. This fragment, encoding the oligomerization domain of *Cp*-HSF1, was cloned into the target vector pAD-GAL4 to create a protein able to interact with all baits so far tested by the two-hybrid system. The standard controls indicated that the novel protein could be a suitable partner for the different *Cp*-HSF1 proteins.

Fig. 3.5
The Cp-HSF302AD expression vector.



The size of the pAD-GAL4 phagemid vector is 7620 bp.
The hybrid protein (GAL4 AD plus the cDNA library target proteins) is constitutively expressed by the yeast p_{ADH1} promoter (**P**) and the yeast t_{ADH1} terminator (**T**).
The 302 bp DNA fragment was the same used for the preparation of the *Cp*-HSF302 bait protein (Fig. 3.4).

2µ : high-copy yeast origin of replication
Amp^r : ampicillin-resistance gene for growth in bacteria
leu2 : auxotrophic marker for selection in yeast

3.1.3.4.2 Analysis of the interactions among the different *Cp*-HSF1 domains

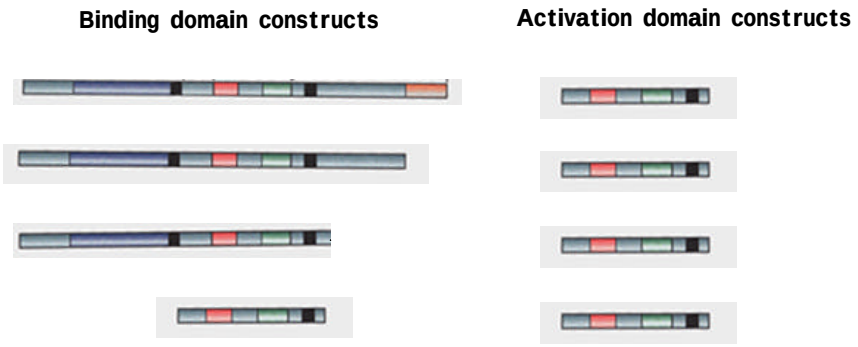
A scheme of the different combinations used in the two-hybrid system to study the interactions among the conserved domains of the *Cp*-HSF1 protein is given in Fig.3.6. The plasmid vectors expressing the BD-fusion and the AD-fusion proteins were introduced into the yeast host YRG-2 by sequential transformation and the selection was performed on SD-LTH plates. The β-

galactosidase activity was tested both by colony-lift filter screening and by liquid assays using CPRG as substrate.

No variations in growth rate were observed by the plate selection: none of the interactions among the different domain of *Cp*-HSF1 proved to be more advantageous than the others to the yeast transformants. Apparently the strength of the interaction was equivalent both when the HR-A/B oligomerization domain was in the presence of the full-length protein (*Cp*-HSF1157 x *Cp*-HSF302AD) and when the HR-A/B domain expressing protein was in combination with the homologous fusion protein (*Cp*-HSF302 x *Cp*-HSF302AD). The activity of the *lacZ* reporter gene was weak for all detected interactions, with the exception of the yeast clones transformed with the full-length protein (*Cp*-HSF1157 x *Cp*-HSF302AD) and of the *Cp*-HSF302 x *Cp*-HSF302AD combination.

Fig. 3.6

Analysis of interactions among the different Cp-HSF1 domains



B D		A D	β-gal assay
Cp-HSF1157	X	*Cp*-HSF302AD	480 units
Cp-HSF960	X	*Cp*-HSF302AD	130 units
Cp-HSF775	X	*Cp*-HSF302AD	110 units
Cp-HSF302	X	*Cp*-HSF302AD	280 units

GAL4 wild type	control for specific activity	1670 units
p53/SV40	control for specific interaction	1540 units

Control reaction

pBD-GAL4(Cam)	X	*Cp*-HSF302AD	no growth

In the upper part of the figure are represented the different plasmid combinations used for yeast transformation. The binding domain constructs are described in Fig. 3.4. All are fused *in frame* with the GAL4 binding domain. The activation domain construct is the *Cp*-HSF302AD protein shown in Fig.3.5. This protein contains the GAL4 activation domain. Below the picture, the fusion proteins expressed by the yeast clones are indicated by name. The β-galactosidase values are reported (β-gal assay column). The β-galactosidase units refer to the liquid assays performed using chlorophenol red-β-D-galactopyranoside (CPRG) as substrated. **1 unit of β-galactosidase** = amount that hydrolizes 1μmol of CPRG to chlorophenol red and D-galactose per min per cell (Miller, 1972). Interaction were assayed for 8 hours. CPRG quantification was performed in triplicate for each assay. The control reaction was performed to test the ability of the *Cp*-HSF302AD protein to activate by itself the *HIS3* reporter gene.

3.2 Characterization of the small heat-shock protein *Cp*-HSP20.6

3.2.1 Sequence analysis of the *Cp*-Hsp20.6 cDNA

3.2.1.1 Sequence of the *Cp*-Hsp20.6 cDNA and the deduced *Cp*HSP20.6 protein

The clone used for this study was kindly provided by Dr. J.Phillips. The cDNA sequence showed high homology to the plant small heat-shock proteins (small HSPs) and was chosen as potential target gene of the heat-shock transcription factor *Cp*-HSF1. All small HSPs exhibit high level of induction on heat stress (Chen et al., 1990; DeRocher et al., 1991; Hsieh et al., 1992).

The cDNA has a length of 772 bp with an open reading frame (ORF) of 612 bp (Fig 3.7). The ORF beginns with a putative translation start codon ATG in position 1 and ends with the stop codon TAG in position 612. The methionine coded by the ATG is considered to be the translation start point based on sequence similarity with other small HSP genes. The *Cp*-Hsp20.6 sequence contains a 3'-untranslated region of 146 bp and a poly(A)-tail of 14 residues.

The corresponding cDNA transcript is a 203 amino acid protein with a predicted molecular mass of 20.6 kDa and an isoelectric point of 9.64. The deduced protein, named *Cp*HSP20.6, contains two large conserved regions (consensus I and II, Fig. 3.9) found in all plant small HSP sequences so far analyzed. In addition, a 5 amino acid consensus sequence at the N-terminus identifies the maturation site typical of the plant mitochondrial small heat-shock proteins. The *Cp*HSP20.6 protein sequence is analyzed in 3.2.1.2.

Fig.3.7

Sequence of the Cp-Hsp20.6 cDNA and the deduced CpHSP20.6 protein

```
TCGTTTGCAATGGCTTCATCGATAGCTCTGAGGAGGCTCCTGTCGTCGAGCAACGCTCTCTTCGGTCC   60
       M  A  S  S  I  A  L  R  R  L  L  S  S  S  N  A  L  F  G  P

CTCCGTACGGCGCGAGCGGCGGCTCAGCCAGCCTCCGTTCGTCTGTTCAACAGCAACGCG  120
 L  R  T  A  R  A  A  A  Q  P  A  S  V  R  L  F  N  S  N  A

CTGACGGAGTACGACAGTGACGACCGAGACGTCGACGTCGCTGATCGACGGGGCGAGCGA  180
 L  T  E  Y  D  S  D  D  R  D  V  D  V  A  D  R  R  G  E  R

CGCCTATTCTCTCCATTTGCAGATATGATGTTTGGTCCCTTCTCAACGAGGAGCCGCCTC  240
 R  L  F  S  P  F  A  D  M  M  F  G  P  F  S  T  R  S  R  L

AGCCAAATCCTTAACATGATGGACCAAATCGTTGACTCACCAATGTCCTCCGCCGTTCGC  300
 S  Q  I  L  N  M  M  D  Q  I  V  D  S  P  M  S  S  A  V  R

CGGAATTGGTTTGCTAGAGAAGCTGCCGAGGGTCTCCATCTGAGGATGGACATGCCAGGC  360
 R  N  W  F  A  R  E  A  A  E  G  L  H  L  R  M  D  M  P  G

CTGGGCAAGGGGGACGTTAAAATCTCGGTGGAGCAGAACACTTTGATCATCAGAGGTGAG  420
 L  G  K  G  D  V  K  I  S  V  E  Q  N  T  L  I  I  R  G  E

GGCAAAAAGGAGGAGTTTGAAAACGATGAAGAGGTTGGTGGCCGAAGATTCTCGGGGAGG  480
 G  K  K  E  E  F  E  N  D  E  E  V  G  G  R  R  F  S  G  R

ATCGATCTGCCTGAGAAGCTGTACAAGGTGAATGACATTAAAGCTGAGATGAAGAATGGC  540
 I  D  L  P  E  K  L  Y  K  V  N  D  I  K  A  E  M  K  N  G

GTCTTGAAGGTGTTTGTTCCCAAGACAAAGGATGAGGAGAGGAGTGATGTCTTCCATGTT  600
 V  L  K  V  F  V  P  K  T  K  D  E  E  R  S  D  V  F  H  V

AATGTTGAGTAG                                                   612
 N  V  E

TATAGTCGGTAATGATGAGTGCTACCGTTGGTTGGTAGACTACGGTGAAAATAATGTGAT
GTCCCTACTTTGGTAGATGAGTTGGGAATCTTCCTGCGTCGTAAAATTCGTTACTTTCTT
GGTTAAGCGATATCTACAGATCTGTTAAAAAAAAAAAAAA                       772
```

Numbers indicate the nucleotide positions starting at the first nucleotide. The predicted translation start site ATG is underlined and marked in *blue*. The predicted termination site TAG is marked in *bold*.The methionine coded by the ATG is considered to be the translation start point based on sequence similarity to other small HSP genes. The putative mitochondrial transit peptide is underlined.

3.2.1.2 Sequence comparison of the *Cp*HSP20.6 protein with the plant small HSP superfamily

According to the sequence similarity and to their cellular localization (cytosol or organelles) the plant small HSPs are divided in different classes. The diversity of the small HSPs in plants is unique among eukaryotes and includes proteins found in the endomembrane system, chloroplasts and mitochondria (Vierling E., 1991; Lenne and Douce, 1994, Waters E.R., 1995). All plant small HSPs are nuclear encoded (Vierling E., 1991).

Phylogenetic analysis using the program PILEUP in the Wisconsin Genetic Computer Group package (GCG version 10.0) was performed to determine the relationship of the *Cp*HSP20.6 to other plant small heat-shock proteins.

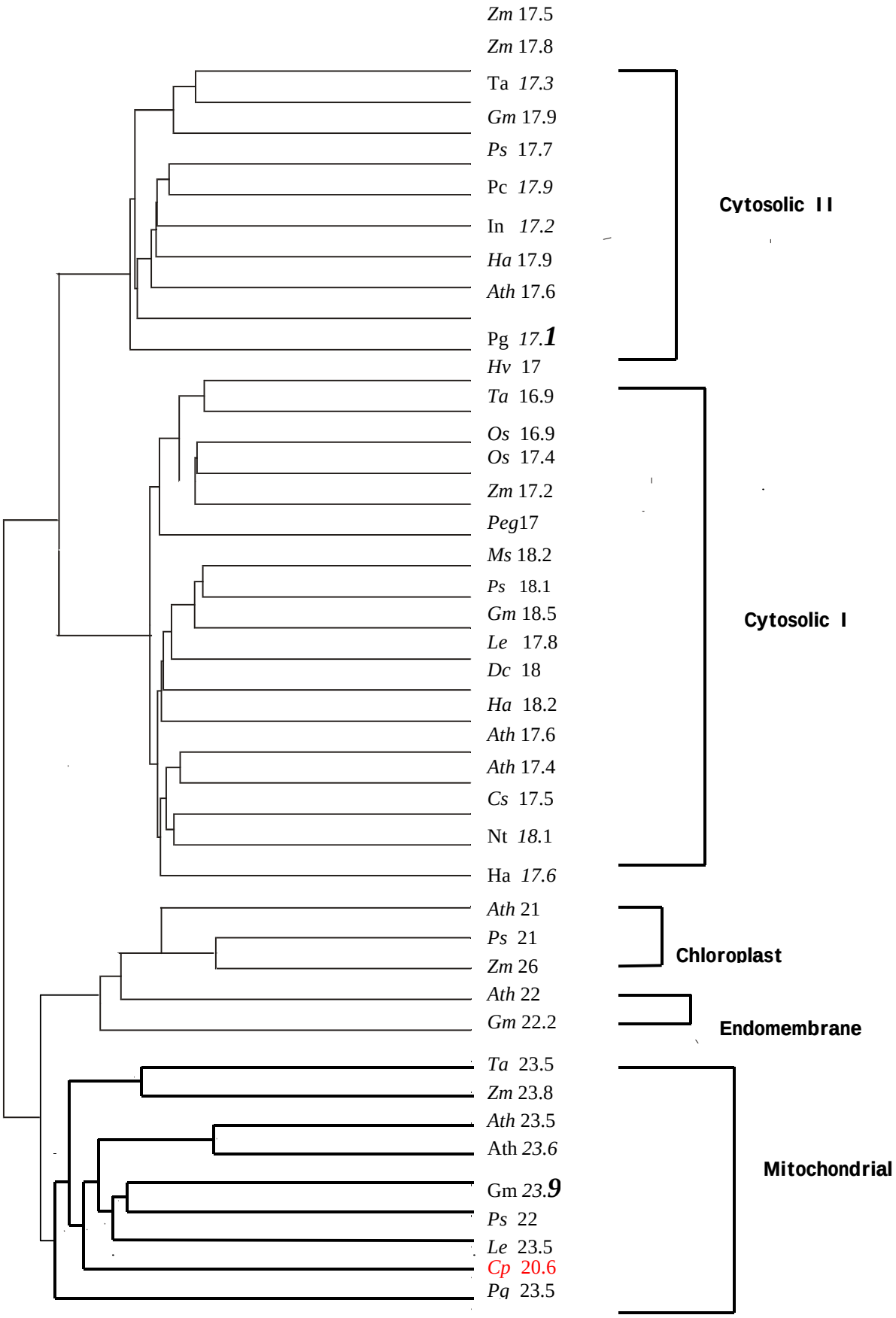
As shown in Fig. 3.8, *Cp*HSP20.6 protein is assigned to the mitochondrial small HSP family.

Fig 3.8

Phylogenetic tree showing the relationship of the CpHSP20.6 protein to other plant small HSPs.

Using the PILEUP program from the GCG package a consensus tree was generated. The five different small HSP classes are indicated. The mitochondrial small HSP region of the tree is highlighted. *Craterostigma plantagineum Cp*HSP20.6 protein is marked in *red*. The different small HSPs are indicated by their molecular weight.
Plant species are identified by their acronyms. The complete scientific names are here indicated in alphabetic order: *Ath: Arabidopsis thaliana;, Cs: Castanea sativa; Dc: Daucus carota; Gm: Glycine max; Ha: Helianthus annuus; Hv: Hordeum vulgare; In: Ipomea nil Le: Lycopersicon esculentum; Ms: Medicago sativa; Nt: Nicotiana tabacum; Os: Oryza sativa; Peg: Pennisetum glaucum; Pc: Petroslinum crispum; Pg: Picea glauca; Ps: Pisum sativum Ta: triticum aestivum; Zm: Zea mays.*
Accession numbers for all sequences are listed in Appendix I.

Fig. 3.8


Zm 17.5
Zm 17.8
Ta 17.3
Gm 17.9
Ps 17.7
Pc 17.9
In 17.2
Ha 17.9
Ath 17.6
Pg 17.1
Hv 17
Ta 16.9
Os 16.9
Os 17.4
Zm 17.2
Peg17
Ms 18.2
Ps 18.1
Gm 18.5
Le 17.8
Dc 18
Ha 18.2
Ath 17.6
Ath 17.4
Cs 17.5
Nt 18.1
Ha 17.6
Ath 21
Ps 21
Zm 26
Ath 22
Gm 22.2
Ta 23.5
Zm 23.8
Ath 23.5
Ath 23.6
Gm 23.9
Ps 22
Le 23.5
Cp 20.6
Pq 23.5
Cytosolic II
Cytosolic I
Chloroplast
Endomembrane
Mitochondrial

DNA and protein similarities to plant small HSPs were determinate using the BLAST network service (Altschul et al., 1990). Seven of the protein sequences that were more similar to the *Cp*HSP20.6 have been characterized as mitochondrial small HSPs (Table 3.G).

Table 3.G

Comparison of the C.plantagineum mitochondrial CpHSF20.6 protein sequence with other plant mitochondrial small HSPs.

Sequence are listed in descending order of percent of amino acid identity with respect to *Cp*HSP20.6

Plant	Accession No	Protein	Localization	Identity	Similarity
P.sativum	X86222	HSP22	mitochondria	65	78
G.max	U21722	HSP23.9	mitochondria	59	68
A.thaliana	U72958	HSP23.6	mitochondria	45	65
L.esculentum	AB017134	HSP23.8	mitochondria	42	63
Z.mays	AF035460	HSP23.8	mitochondria	41	63
P.glauca	L47741	HSP23.5	mitochondria	42	59
T.aestivum	AF104107	HSP23.5	mitochondria	38	52
A.thaliana	X54102	HSP21	chloroplasts	34	48
G.max	X07188	HSP22	endomembranes	32	49

The amino acid sequence alignment (Fig. 3.9) further supports the inclusion of the *C.plantagineum* protein in the mitochondrial class of small HSPs. In fact the amino acid sequence of *Cp*HSP20.6, besides the two conserved regions present in the C-terminus of all plant small HSPs ((I) and (II), underlined in *green*), shows the N-terminus -FNSNA- amino acid sequence (marked in *blue*), very similar to the -FNTNA- sequence identified in several plant small HSPs (Lenne et al., 1995). This 5 amino acid motif represents the consensus sequence around the maturation site of the protein to enter the mitochondrion (a serine (S) residue in the *Craterostigma* sequence replaces the threonine (T) in the conserved motif. Both are neutral, hydrophilic amino acids). On the basis of protein sequencing a putative transit peptide long 35 residues could be identified in the *Cp*HSP20.6 protein sequence, according to the length of the plant mitochondrial targeting peptides, which has been

estimated between 18 and 77 amino acid (Schneider et al., 1998). In addition, the *Cp*HSP20.6 amino acid composition of the mitochondrial targeting sequence shows two arginine residues in position -10 and -2 from the putative cleavage site, as described by Schneider (Schneider et al., 1998) for the plant mitochondria protein.

Fig. 3.9

Comparison of the predicted amino acid of the CpHSp20.6 protein with mitochondrial small HSPs from diverse plant species.

Conserved domains typical of small HSPs (I and II) are boxed in different colors.

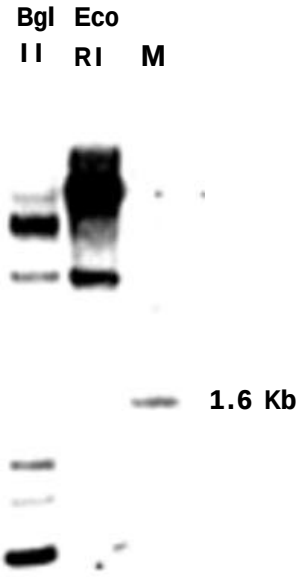
The consensus sequence at the N-terminus around the maturation site is indicated in bold.

3.2.2 Characterization of the genomic sequence of *Cp*-Hsp20.6 gene

3.2.2.1 *Cp*-Hsp20.6 gene organization

To analyze the genomic organization of the *Cp*-Hsp20.6 gene, genomic DNA was digested with the restriction endonucleases *BglII* or *EcoRI*, whose restriction sites are absent in the *Cp*-Hsp20.6 cDNA sequence. The digested DNA was blotted and the filter was hybridized with the full-length cDNA (772 bp) as a probe. As shown in Fig. 3.10, several hybridizing bands were detected. This result suggest the presence of a "small Hsp" gene family in *C.plantagineum*, as already observed for most, if not all, plant small heat-shock proteins so far studied (Waters et al., 1996; Vierling E., 1997).

Fig. 3.10
Southern analysis using the cDNA Cp-Hsp20.6 probe.



Ten µg of total genomic DNA from *C.plantagineum* were digested with the indicated enzymes, electrophoretically separeted and blotted. Hybridization with the full-lenght cDNA sequence was performed overnight at high stringency (see Material and Methods).

M = molecular weight marker

3.2.2.2 *Cp*-Hsp20.6 gene structure

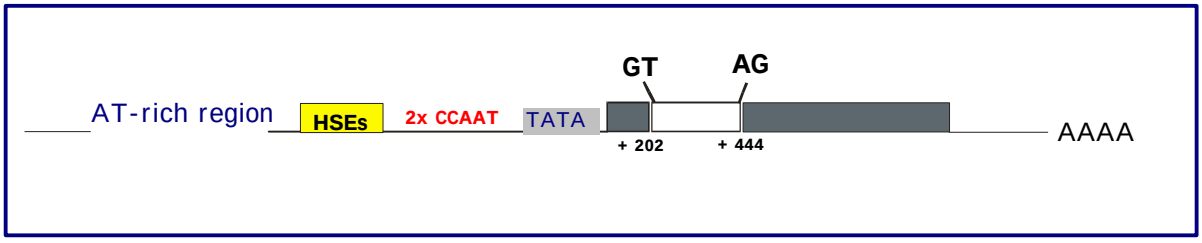
To isolate a genomic clone corresponding to *Cp*-Hsp20.6, a genomic library (kindly provided by Dr. A. Furini) was screened using the full-length *Cp*-Hsp20.6 cDNA as probe. Approximately 2,5 x 10^7 pfu were generated by plaque hybridization (Sambrook. et al., 1989); three positive phage pools were further purified. Phage DNA was *BamHI* digested and hybridized with the full-length *Cp*-Hsp20.6 cDNA probe in order to identify the exon containing fragments.

The sequence analysis confirmed that the complete nucleotide sequence is a 612 bp ORF (open reading frame), with the 3' untranslated region that extends 146 bp up to the polyadenylation site and the putative promoter region containing a TATA motif, several transcriptional regulatory regions (two CCAAT sequences, the AT-rich region) and at least two heat-shock responsive elements that represent potential binding sites for the *Cp*-HSF1 protein.

A scheme of the genomic structure is given in Fig.3.11 (see also 3.2.2.3 for the analysis of the promoter region). The identity between the cDNA sequence used as probe and the genomic sequences was 98.8%. A single intron of 242 bp splits the coding region between codons +202 and +203. The presence of the intron was deduced from the consensus 5' splicing site GT and the 3' splincing site AG characteristic for the eukaryotic introns (Padgett et al., 1986). Moreover, the intron sequence present in the *Cp*-Hsp20.6 gene is rich in A-T residues typical of dicot introns (Goodall and Filipowicz., 1989; Luehrsen et al., 1994). The complete sequence of the *Cp*-Hsp20.6 gene is shown in Appendix II.

Fig. 3.11

Cp-Hsp20.6 gene structure



3.2.2.3 Analysis of the *Cp*-Hsp20.6 promoter region

The 5' untranslated region of *Cp*-Hsp20.6 contains several putative cis-regulatory elements (Fig. 3.12), most of them characteristic for heat stress-induced genes: a modified TATA box represented by the TATAT motif (marked in *grey*), two CCAAT boxes (marked in *red*) found in several heat-shock gene promoters, numerous blocks of contiguous AT-rich sequences (marked in *blue*). These sequences exist as repeats of ATTA or TAAT motifs (Gurley et al., 1992) in the promoters of many heat-shock responsive genes and are thought to act as transcriptional activator elements (Baumann et al., 1987; Czarnecka et al., 1989).

The thermoinducible expression of heat-shock genes is attributed to the presence of controlling DNA sequences, the heat-shock elements (HSEs), located in the promoter. The essential component of the HSE is a trinucleotide repeat 5'-nGAAn-3', or its complement 5'-nTTCn-3', arranged in alternating orientations (Pelham, 1982; Amin et al., 1988). At least two nGAAn units are needed for high affinity binding of heat-shock factors *in vitro* and these may be arranged either head-to-head (nGAAnnTTCn) or tail-to-tail (nTTCnnGAAn; Perisic et al, 1989; Sorger P.K., 1991).

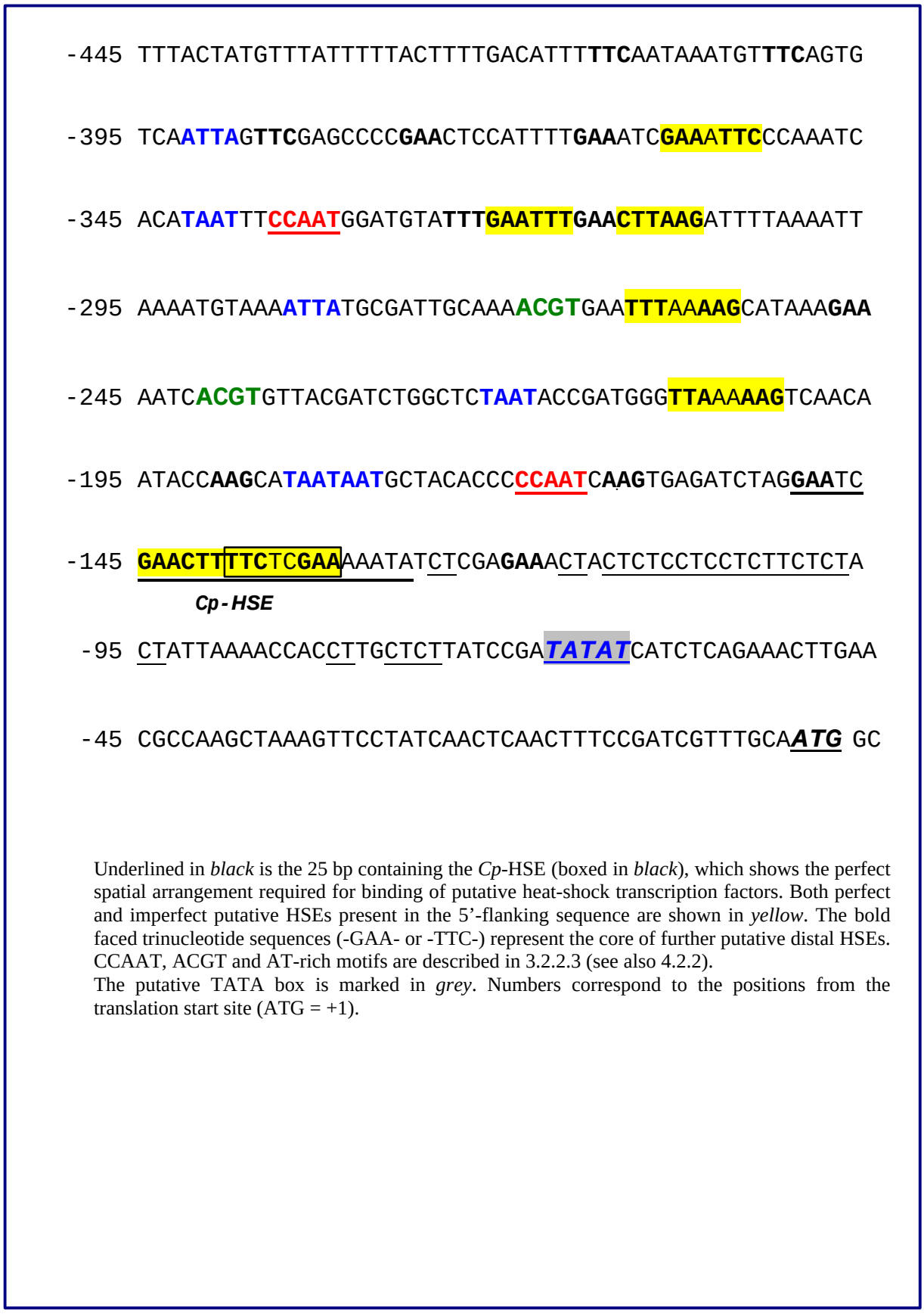
In the *Cp*-Hsp20.6 promoter, a 25 bp region (underlined in *black* and indicated as *Cp*-HSE, Fig. 3.12) shows adjacent GAA/TTC blocks that represent a potential functional sequence for the binding of the *Cp*-HSF1 protein. Particularly, the **TTC**TC**GAA** sequence (edged in *black*) provides the perfect module for the binding activity.

Upstream the *Cp*-HSE sequence, several nGAAn and nTTCn motifs form HSE-like sequences (marked in *yellow*). In addition, two ACGT elements (marked in *green*) are present in the *Cp*-Hsp20.6 promoter region. The ACGT elements represent the core of the ABA-responsive element (ABRE), a cis-acting DNA sequence involved in ABA-regulated gene expression (Giuliano et al., 1988; Giraudat at al., 1994; Ingram et Bartels, 1996; Izawa et al, 1993), but also found in genes that are expressed during seed development (Pla et al., 1991, 1993; McCarty, 1995).

Finally, CT-rich regions characteristic of many heat-shock gene promoters (Gilmour et al., 1989; Lu et al., 1992; Voellmy, 1994) have been identified; alternating C and T residue regions (underlined) were shown to function as binding sites of the GAGA transcription factor (Gilmour et al., 1989; Giardina et al., 1992).

Fig. 3.12

Nucleotide sequence of the proximal promoter region of Cp-Hsp20.6.



Underlined in *black* is the 25 bp containing the *Cp*-HSE (boxed in *black*), which shows the perfect spatial arrangement required for binding of putative heat-shock transcription factors. Both perfect and imperfect putative HSEs present in the 5'-flanking sequence are shown in *yellow*. The bold faced trinucleotide sequences (-GAA- or -TTC-) represent the core of further putative distal HSEs. CCAAT, ACGT and AT-rich motifs are described in 3.2.2.3 (see also 4.2.2).
The putative TATA box is marked in *grey*. Numbers correspond to the positions from the translation start site (ATG = +1).

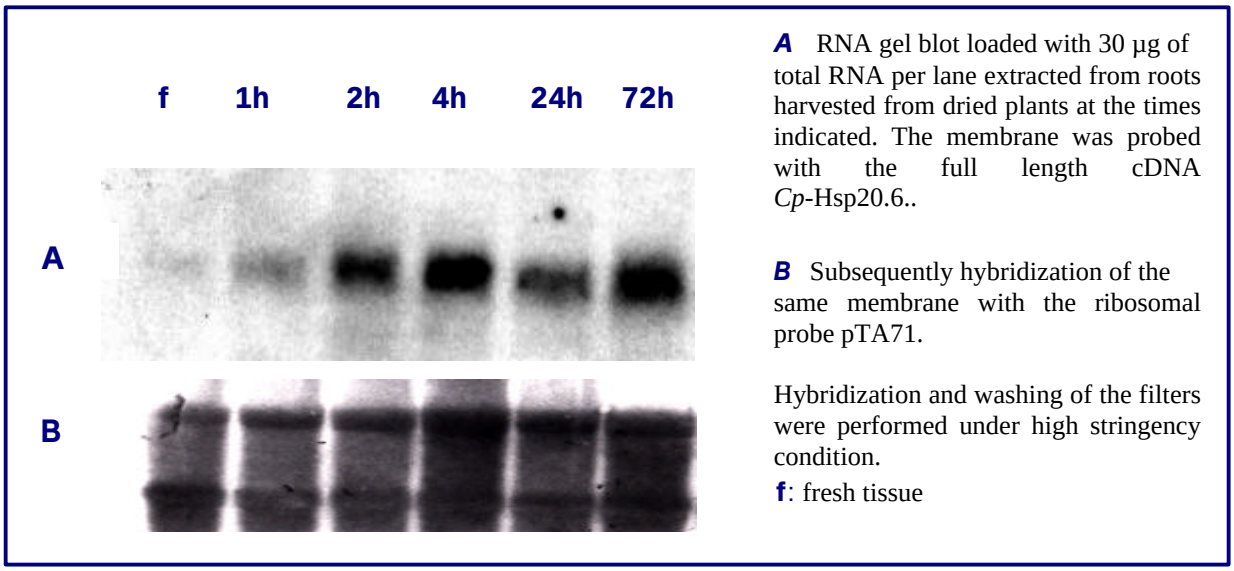
3.2.3 *Cp*-Hsp20.6 transcript expression

Northern analyses with the full-length *Cp*-Hsp20.6 cDNA probe were performed to study the heat induction of the protein and to confirm that the *Cp*-Hsp20.6 gene is expressed upon drought. The expression of the *Cp*-Hsp20.6 transcript was analyzed using total RNA isolated from fresh, dehydrated and heat stressed *Craterostigma* leaves and roots. The drought and stress treatments were performed on whole plants, whose roots and leaves were analyzed separately. The filters carrying the mRNA were hybridized subsequently with the ribosomal probe pTA71 to monitor equal loading (Gerlach and Bedbrock, 1979).

3.2.3.1 *Cp*-Hsp20.6 transcript expression in dehydrated roots

During the dehydration treatment whole plants were placed on filter paper and air dried for various lengths of time. Not detectable in fresh roots, *Cp*-Hsp20.6 is rapidly induced within two hours but its expression increases appreciably after longer times dehydration (Fig.3.13). *Cp*-Hsp20.6 expression remains high upon prolonged dehydration: after 72 hours, the signal was still significantly strong, indicating a prolonged transcription activity. Dehydration treatment over 72 hours was not applied in this study.

Fig. 3. 13
Cp-Hsp20.6 expression pattern during dehydration in roots



A RNA gel blot loaded with 30 µg of total RNA per lane extracted from roots harvested from dried plants at the times indicated. The membrane was probed with the full length cDNA *Cp*-Hsp20.6..

B Subsequently hybridization of the same membrane with the ribosomal probe pTA71.

Hybridization and washing of the filters were performed under high stringency condition.

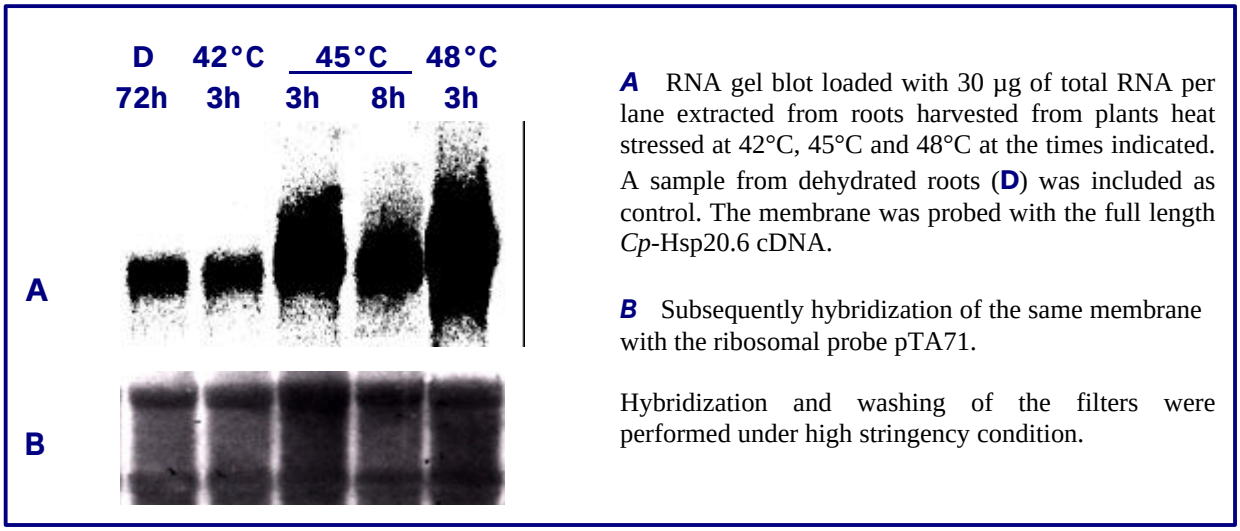
f: fresh tissue

3.2.3.2 *Cp*-Hsp20.6 transcript expression in heat stressed roots

Heat stress was performed with plants in their pots in the light for 3 hours at 42°C, 45°C or 48°C, keeping the humidity constant. A strong induction of the *Cp*-Hsp20.6 transcript was observed both upon the heat treatment (3 hours at 42°C) already applied for heat stress studies on sunflower (Almoguera et al., 1993; Coca et al., 1996) and *C.plantagineum* plants (Alamillo et al, 1995), and upon extreme heat stress conditions (45°C and 48°C). As shown in Fig. 3.14, in comparison to the dehydration treatment (lane D), the heat stress exposure induces a higher expression of *Cp*-Hsp20.6. This suggests that the *Cp*-Hsp20.6 transcript. is induced by heat-shock, besides to dehydration.

Fig. 3.14

Cp-Hsp20.6 expression pattern upon heat stress in roots



A RNA gel blot loaded with 30 µg of total RNA per lane extracted from roots harvested from plants heat stressed at 42°C, 45°C and 48°C at the times indicated. A sample from dehydrated roots (**D**) was included as control. The membrane was probed with the full length *Cp*-Hsp20.6 cDNA.

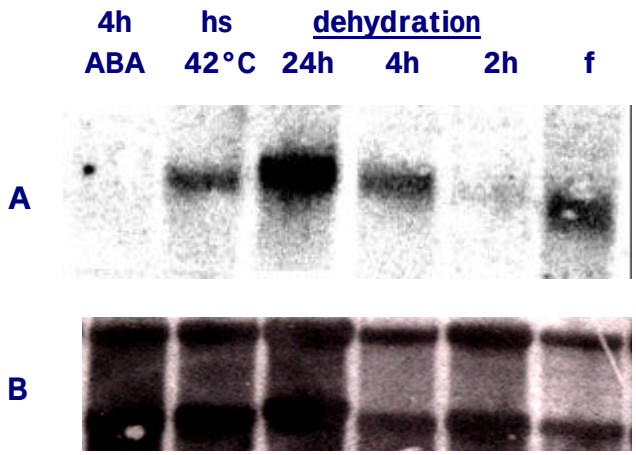
B Subsequently hybridization of the same membrane with the ribosomal probe pTA71.

Hybridization and washing of the filters were performed under high stringency condition.

3.2.3.3 *Cp*-Hsp20.6 transcript expression in dehydrated and heat stressed leaves

Both in fresh and in dehydrated leaves the expression of the *Cp*-Hsp20.6 transcript was rather low, increasing in the extremely dehydrated (24 hours) and in heat-shocked tissues. A sample of ABA treated leaves (4 hours treatment) was included to test if the gene is ABA-responsive. As shown in Fig 3.15, the *Cp*-Hsp20.6 transcript was not induced by ABA in leaves.

Fig. 3.15
Cp-Hsp20.6 expression pattern in leaves



A RNA gel blot loaded with 30 µg of total RNA per lane extracted from leaves haverested from plants treated as indicated. For the ABA treatmet 1 mM ABA solution was used.

hs : heat stress treatment

f : fresh tissue

B Subsequently hybridization of the same membrane with the ribosomal probe pTA71.

Hybridization and washing of the filters were performed under high stringency condition.

3.3 Study of the binding activity of the *Cp*-HSF1 protein to the *Cp*-Hsp20.6 gene promoter region

To investigate the ability of the *C.plantagineum* heat-shock element *Cp*-HSE to act as a functional heat-shock regulatory sequence, the 25 bp fragment containing the **TTC**TC**GAA** motif was used in gel retardation and competition assays (3.3.1) in the presence of the heat-shock transcription factor homologue (*Cp*-HSF1) isolated from *C. plantagineum* (Bockel C., 2000, Ph.D thesis). In addition, 1096 bp from the *Cp*-Hsp20.6 promoter region were cloned in front of the GUS-reporter gene and its expression was tested in tobacco protoplasts (3.3.2).

3.3.1 *In vitro* binding assays of *Cp*-HSF1 to the heat-shock responsive element *Cp*-HSE

Previous investigations have shown that this transcription factor homologue binds to a synthetic heat-shock element (HSE1) providing the optimal binding site for HSF proteins (Bockel C., 2000, Ph.D thesis). Starting from this observation, the *Cp*-Hsp20.6 promoter region containing a perfect heat-shock responsive element motif (Fig. 3.16) was used as target sequence for *Cp*-HSF1 in gel retardation and competition assays. Interestingly, this sequence is very similar to the synthetic oligomer HSE1 used successfully in binding assays with the soybean heat-shock transcription factor *Gm*HSF34 (Czarnecka-Verner *et al*., 1995).

Fig. 3.16

Comparison of the-5'-oligomer sequences used for Electrophoretic gel Mobility Shift Assay (EMSA) in previous studies and the Cp-HSE 5' oligomer sequence.

Bockel (HSE1)
5´ *gtgt*agggatcga**GAA**gc**TTC**ta**GAA**cg**TTC**tc**GAA**tggaggat 3'

Czarnecka-Verner (HSE1)
5' *tcgac*gttaggattt**TTC**tg**GAA**catacaa 3´

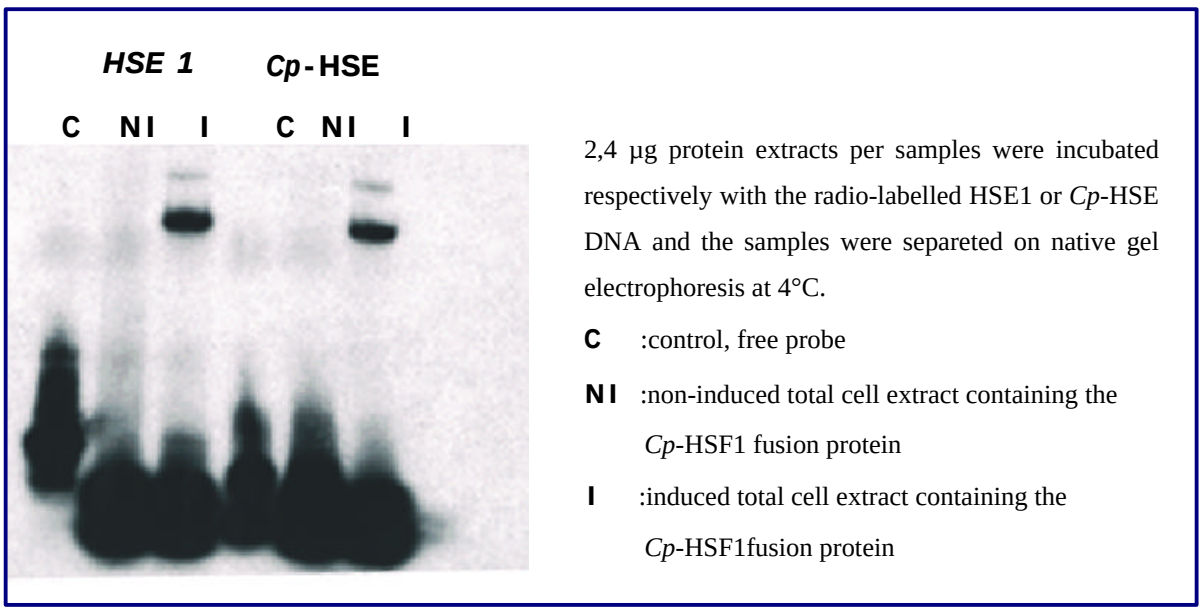
this study (*Cp*-HSE)
5' *gtgt*gatcgaactt**TTC**tc**GAA**aaatatc 3'

HSE sequences are underlined. References: Czarnecka-Verner *et al*., 1995; C.Bockel 2000, Ph.D thesis

The cloning, overexpression and purification of the recombinant full-length *Cp*-HSF1 protein in *E.coli* was originally performed by Bockel (Bockel C., 2000, Ph.D thesis). Here, the crude protein extract was prepared after induction of the bacterial clone (see Material and Methods) and directly used in the Electrophoretic gel Mobility Shift Assays (EMSA). The presence of the recombinant *Cp*-HSF1 in the total protein extract was checked by Western analysis using monoclonal anti-His tag antibodies.

The gel retardation assay was performed using as target sequence both the synthetic oligonucleotide HSE1 and the *Cp*-HSE sequence isolated from the *Cp*-Hsp20.6 promoter. The recombinant *Cp*-HSF1 was added to different reactions, containing the radio-labelled HSE1 sequence and the radio-labelled *Cp*-HSE sequence respectively. The reactions were loaded, together with the free probes and the non-induced bacterial crude protein extracts as negative controls, in the same polyacrylamide gel (Fig.3.17). The binding of *Cp*-HSF1 to the synthetic HSE1 sequence should provide the positive control for the reaction conditions. In fact, the *Cp*-HSF1:HSE1 complex was observed (Fig.3.17, left). The *Cp*-HSF1:*Cp*-HSE complex was also present and showed the same electrophoretic gel mobility (Fig.3.17, right). This result confirms the putative nature of heat-responsive cis-regulatory element for the *C.plantagineum* heat-shock element-like sequence (*Cp*-HSE) and at the same time is consistent with the potential transcriptional activation function of the heat-shock transcription factor homologue *Cp*-HSF1.

Fig. 3.17
Gel retardation assay showing the binding activity of E.coli Cp-HSF1 fusion protein to the synthetic oligonucleotide HSE1 and to the Cp-HSE probe.



2,4 µg protein extracts per samples were incubated respectively with the radio-labelled HSE1 or *Cp*-HSE DNA and the samples were separeted on native gel electrophoresis at 4°C.

C :control, free probe

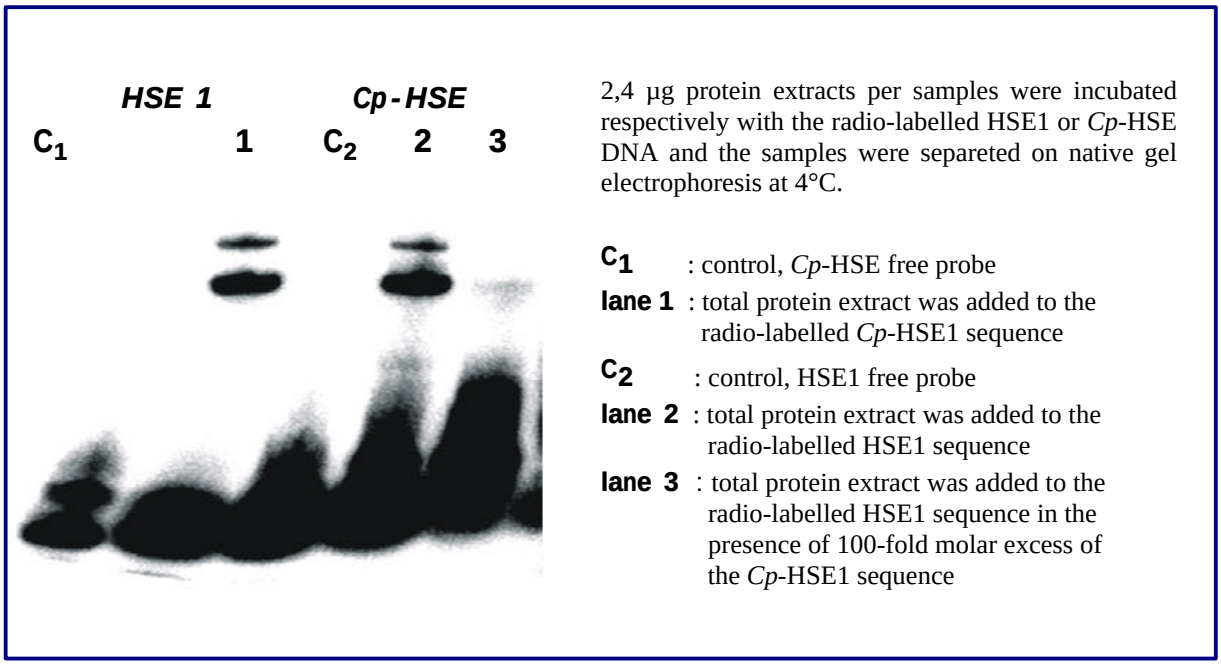
NI :non-induced total cell extract containing the *Cp*-HSF1 fusion protein

I :induced total cell extract containing the *Cp*-HSF1fusion protein

The competition assays were performed using the radio-labelled synthetic oligonucleotide HSE1 in the presence of the recombinant *Cp*-HSF1 protein and of 100-fold molar excess of the *C.plantagineum* heat-shock element (*Cp*-HSE). As reaction conditions control the *Cp*-HSF1:HSE1 forming complex were used (Fig. 3.18, left). After addition of the non-labeled *Cp*-HSE to the reaction containing the radio-labelled synthetic oligonucleotide HSE1, the hybridization band corresponding to the *Cp*-HSF1:HSE1 complex disappeared (Fig. 3.18, right). This result indicated that *Cp*-HSF1 protein specifically binds to the heat-shock element *Cp*-HSE.

Fig. 3.18

Gel competition assays showing the specific binding of the E.coli Cp-HSF1 fusion protein to the Cp-HSE probe.



2,4 µg protein extracts per samples were incubated respectively with the radio-labelled HSE1 or *Cp*-HSE DNA and the samples were separeted on native gel electrophoresis at 4°C.

C_1 : control, *Cp*-HSE free probe

lane 1 : total protein extract was added to the radio-labelled *Cp*-HSE1 sequence

C_2 : control, HSE1 free probe

lane 2 : total protein extract was added to the radio-labelled HSE1 sequence

lane 3 : total protein extract was added to the radio-labelled HSE1 sequence in the presence of 100-fold molar excess of the *Cp*-HSE1 sequence

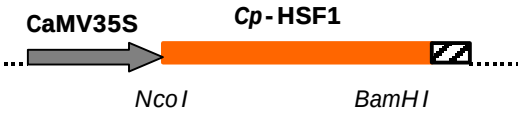
3.3.2 Transient expression assays in tobacco protoplasts

3.3.2.1 Activation of GUS-reporter genes by *Cp*-HSF1

In order to test whether *Cp*-HSF1 was able to bind to the heat-shock element-like sequence *Cp*-HSE and to promote gene transcription *in vivo*, the transient expression of the *CaMV*35S:*Cp*-HSF1 chimeric construct (expression plasmid) was tested in a tobacco mesophyll protoplasts system . The target sequence was the 1096 bp fragment containing the *Cp*-HSE motif and other putative regulatory elements from the *Cp*-Hsp20.6 promoter region (3.2.2.3). This sequence named *Cp*-HSp (for *C.plantagineum* heat-shock promoter) was fused in front of the bacterial β-glucuronidase gene (GUS) in order to obtain the reporter plasmid *Cp*-HSp:GUS. The negative control was the promoterless GUS reporter plasmid pGUS, tested both alone and in pairwise combination with the expression plasmid expressing the full-length *Cp*-HSF1 protein. The reporter and expression plasmids are shown in Fig. 3.19.

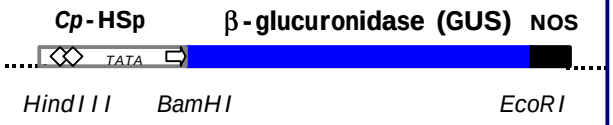
Fig. 3.19

Expression plasmid CaMV35S: Cp-HSF1



The pRT104 vector (Toepfer et al., 1987) was used to create a traslational fusion cassette containing the *CaMV*35S promoter sequence (800 bp) linked to the full-length *Cp*-HSF1(subcloned omitting the start codon, as a 1150 bp *NcoI-BamHI* fragment, *orange* area). The pRT104 vector contains a NcoI site, providing the ATG codon and the poly(A) signal sequence (stripped area).

GUS-reporter plasmid Cp-HSp:GUS

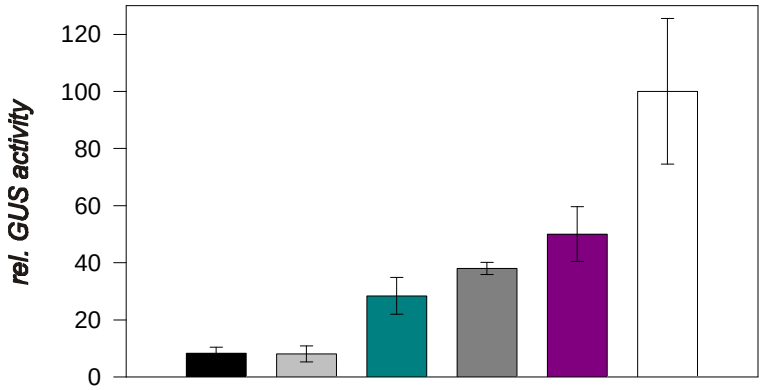


The expression vector pBI101 (Jefferson et al., 1987) was used to create a translational cassette where 1096 bp from the *Cp*-HSp promoter plus 69 bp downstream of the initiation site (the start codon of the *Cp*-Hsp20.6 gene is indicated by the arrow) were fused in frame with the GUS-reporter gene. The NOS fragment (*black* area) respresent the terminal poly(A) sequence The *Cp*-HSp promoter fragment contains multiple and overlapping HSE plus putative cis-regulatory sequences (see Fig.3.12 for the detailed description). Restriction sites *HindIII* and *BamHI* were used for subcloning the cassette in the Bluescript SK vector for protoplasts trasformation.

The evaluation of the pGUS/*CaMV*35S:*Cp*-HSF1 interaction was necessary to estimate how specific the binding of *Cp*-HSF1 to the *Cp*-Hsp20.6 promoter was. As shown in Fig 3.20, the *Cp*-HSF1 protein was able to activate the reporter gene by binding to the *Cp*-Hsp20.6 promoter region (*violet* column): the achieved GUS activity was 1.5-fold higher than that observed in the absence of the *Cp*-Hsp20.6 promoter (*blue* column). The reporter gene basic expression level caused by the endogenous HSF system of the tobacco protoplasts, was indicated by the GUS activity obtained after transformation with the reporter plasmid *Cp*-HSp:GUS alone (*grey* column). The basic GUS activity levels were rather high but in agreement with values occasionally observed for plant heat-shock full-length promoter sequences cloned in front to the β-glucuronidase gene (Treuter et al., 1993; Lyck et al., 1997). Constructs were tested three to five times in duplicate.

Fig. 3.20

Transactivation of the Cp-HSp promoter by the Cp-HSF1 factor.



■	*CaMV*35S:*Cp*-HSF1	: expression plasmid
■	pGUS	: promoterless GUS-reporter pl.
■	pGUS / *CaMV*35S:*Cp*-HSF1	:control interaction
■	*Cp*-HSp:GUS	: GUS-reporter plasmid
■	*Cp*-HSp:GUS / *CaMV*35S:Cp-HSF1	: transactivation

□ Activity of the *CaMV*35S:GUS construct used as reference to normalize the GUS activity (100)

In *vivo* activation of GUS reporters by *Cp*-HSF1 was performed using tobacco mesophyll protoplasts. 10µg expression plasmid (*CaMV*35S:*Cp*-HSF1) plus 10µg GUS-reporter plasmid (*Cp*-HSp) were used to transform $5x10^6$ protoplasts. GUS expression assays were incubated at 37°C for 1 hour. GUS activity is expressed as pmol 4-MUG mg/protein/min.

3.3.2.2 Heat stress inducible expression of the *Cp*-HSF1 protein

Table 3.H summarizes the data on GUS activities measured in tobacco protoplasts after heat-shock treatment. In the test system used for this experiment, the protoplasts were divided in three aliquots: one aliquot was kept at the control temperature (25°C), one was heat stressed at 37°C for 16 hours and the third was shocked for two periods of 2 hours each at 40 °C, separated by a 16 hours recovery phase. Protein extracts were then obtained and the GUS assay was performed according to established protocols (see in Material and Methods).

Table. 3.H

Heat stress inducible GUS expression in tobacco protoplasts

	control	heat shock	
	25°C	37°C	40°C
***CaMV*35S:GUS**	+ + + +	+ + + +	+ + + +
***Cp*-HSp:GUS**	+ +	+ + +	+ +
***Cp*-HSp/*Cp*-HSF1**	+ +	+ +	+ +
PGUS/*Cp*-HSF1	+ +	++ (+)	+

CaMV35S:GUS : GUS activity reference *Cp*-HSp/*Cp*-HSF1 : transactivation

Cp-HSp:GUS : GUS-reporter plasmid pGUS/*Cp*-HSF1 : control interaction (pGUS:promoterless reporter plasmid)

For the transfection, polyethylene glycol-mediated DNA transformation was used (Krens et al., 1982). The *Cp*-HSF1 expression plasmid (10 µg) was co-transformed with 10 µg of the *Cp*-HSp:GUS reporter plasmid. The GUS activity was measured in pmol 4-MUG per mg protein. Transactivation levels are shown as GUS activity relative to the *CaMV*35S:GUS construct: + (5-25%); ++ (25-50%); +++ (50-75%); ++++ (100%). The activity level caused by the endogenous HSF system of the tobacco protoplasts is indicated by the *Cp*-HSp:GUS construct (reporter plasmid where the *Cp*-HSE promoter is fused to the GUS gene).

In respect to the expression levels observed for the assays performed at the control temperature, the heat-shock treatment seemed to increase the β-glucuronidase (GUS) activity in general, but did not modify the relation of the values corresponding to the

different interactions. As shown in Table 3.H, the heat-shock did not increase the GUS activity when the *Cp*-HSp promoter sequence was in the presence of the *Cp*-HSF1 protein (*Cp*-HSp/*Cp*-HSF1 combination) and the values observed by the 37°C treatment were comparable, if even not lower, to the basic GUS activity caused by the endogenous HSF tobacco system (*Cp*-HSp:GUS construct). The activity levels reported for the protoplasts heat-shocked at 40°C were higher for the *Cp*-HSp/*Cp*-HSF1 interactions in respect to the interaction between the promoterless construct and the *Cp*-HSF1 protein (pGUS/*Cp*-HSF1combination); nevertheless the values did not differ very much. Constructs were tested three times in duplicate.

4 Discussion

4.1 The yeast two-hybrid system as method to detect partners of dehydration stress proteins in *Craterostigma plantagineum.*

The investigation of the molecular basis of desiccation tolerance in *C.plantagineum* has mainly focused on characterization of genes which are up regulated during dehydration or rehydration (reviewed by Bartels et al., 1997). Several different approaches have been used to understand the role of the corresponding gene products. On the basis of their putative functions the transcripts so far characterized have been grouped in three major categories: polypeptides with protective function (osmotic adjustment, cellular protecting mechanisms), carbohydrate metabolism-related enzymes and potential regulatory factors (for reviews see Ingram and Bartels, 1996; Bartels et al., 1997). In this study, the *in vivo* yeast two-hybrid system was chosen as experimental strategy to identify proteins interacting with two transcripts previously isolated, in order to define their role in the *C.plantagineum* response to dehydration.

The two-hybrid system has three major applications: testing interactions of known proteins, defining protein domains critical for an interaction, and screening libraries for proteins that bind target proteins. Current systems contain three basic components: a vector expressing the protein of interest, termed “bait”; a vector driving the synthesis of open reading frame encoded proteins, representing the “target” to identify, one or more reporter genes such as *lacZ*, *LEU2* or *HIS3*. Interactions between the bait and the target proteins result in the localization of the transcription

activation domain and in the transcription of the adjacent reporter genes, generating a phenotypic signal. Because selection of positive protein interactions is indirect and relies on activation of reporter genes, two-hybrid screens are potentially vulnerable to artifacts (Bartel et al., 1993b; Fields and Sternglanz, 1994; Serebriiskii et al., 2000). Artifacts or false positives are proteins that cause the activation of reporter genes but not as a result of a specific interaction with the bait (Bartel et al., 1993b) and that frequently occur during the two-hybrid library screening. Despite the diversity of the two proteins used in this study as bait (the Dsp16 dehydrin and the *Cp*-HSF1 transcription factor homologue), considerations to reduce the percentage of artifacts were

common to the two independent screenings.

1. Successful two-hybrid selections require a yeast host strain that contains inducible promoter elements fused to reporter genes. The yeast strain used for this study was the YRG-2 (Callahan et al., 1995), a derivative of the HF7c strain (Feilotter et al., 1994), selected for its ability to generate high-efficiency competent cells. The YRG-2 yeast strain contains two reporter genes (*HIS3* and *lacZ*) for the detection of interacting proteins. This represents a significant advantage by reducing the number of false positives (Mullinax and Sorge, 1995). In addition, the reporter genes *HIS3* and *lacZ* contain different UAS (upstream activating sequence) sequences, GAL1 or GAL4 respectively, which govern their expression. The use of two different UAS promoter sequences decreases the possibility of non-specific interactions because of the expression of both the reporter genes. Due to the advantages offered by the dual-reporter system of the YRG-2 strain, other commercially available yeast two-hybrid hosts were not tested. Even if the strains commonly used do not differ very much in their general features (James et al., 1996), yet some strains could be more sensitive to small changes in reporter activity for detecting weak or transient interactions. This observation could partially explain the missed identification of many positive interactions observed once after the first selection steps and not more occurred. In fact, the failed reproducibility of preliminary positive interactions has been observed by both independent screenings performed in this study.
Finally, host strains such the YRG-2 utilize a *HIS3* reporter that is sensitive but very leaky. This leakness can be reduced by addition of the drug 3-aminotriazole (3-AT) to the media. Incorporation at a concentration of 20-40 mM generally inhibits the basal level of histidine and keeps the strain from growing unless *GAL4-HIS3* gene is activated (Bartel et al. 1993a); but severely inhibits true positives in higher concentrations, reducing the sensitivity of the system

and increasing the time required for the growth and the selection of positive clones (Feilotter et al., 1993; James et al., 1996).

2. A high quality two hybrid cDNA library is required: the domains responsible for interactions may occur anywhere within a protein and many fusion proteins could be non- functional due to problems with folding or stability (James et al., 1996). The target

protein library for this study was constructed in which total cDNA derived from 2 hours dehydrated *C.plantagineum* leaves was fused to the GAL4 activation domain sequence.

Typically, two-hybrid libraries contain $>10^6$ inserts, although only one-sixth of these are likely to be in the correct orientation and reading frame (Chien et al., 1991). The primary titer obtained was $1,5 \times 10^6$ pfu (plaque forming units) and therefore the library was supposed to be sufficient to represent the cDNA population of interest. Because it is critical to test each component before starting the screening, the ability of the plasmid library pAD-GAL4 vector to activate the reporter genes system by itself was tested (3.1.1.2). The inability to produce β-galactosidase activity when alone or in combination with the DNA binding domain (i.e. not fused to a protein coding DNA sequence) appeared to indicate specificity. The possibility to identify artifacts in using this plasmid library vector by the two-hybrid screening was estimated to be extremely low.

3. In using the system, it is critical to test each isolated positive clone against an unrelated protein fused to the DNA binding domain and to eliminate those that do not show specificity for the bait used (Bartel et al., 1993b). Regarding the putative positive targets isolated in this study, plasmids from the library were reintroduced into the original wild type strain YRG-2 with the following combinations: alone or with the plasmid encoding the DNA binding domain alone (pBD-GAL4(Cam)). Some putative targets were transformed also in combination with the plasmid encoding a completely different protein (the murine p53 protein) as DNA binding domain vector. Usually these assays provide a rapid system to eliminate the great majority of false positives (Fields and Sternglanz, 1993), but this has not always been an effective filter against artifacts (see 4.1.1.1 and 4.1.2.1).

4.1.1. The Dsp16 dehydrin used as bait

Studies of their biochemical properties have suggested that some of the *C.plantagineum* genes, which are up-regulated during dehydration, encode late embryogenesis abundant (LEA) proteins (Dure, 1989) or LEA-like proteins (Baker et al., 1988), which have a

protective function during dehydration (Ingram and Bartels, 1996). One abundant class of drought-induced proteins is represented by the dehydrin-related Dsp16 protein, a 16 kDa polypeptide which accumulates abundantly very early during dehydration or in response to ABA treatment (Piatkowski et al., 1990; Schneider et al. 1993). The Dsp16 features match the typical biochemical profile of the dehydrins, known to be very hydrophilic, heat stable proteins, which accumulate in response to any type of environmental stress or developmental change that has a dehydration component, or in response to abscisic acid (Close et al., 1993a). At the present time the biochemical role of the dehydrins is not clear but it has been suggested that they act as stabilizers of nuclear or cytoplasmic macromolecules under low water conditions: it is possible that dehydrins interact with membranes or lipids in a manner similar to apolipoproteins (Campbell and Close, 1997; Wisniewski et al., 1999), or with partially denatured proteins (Close, 1997) in a manner similar to chaperones (Mayhew and Hartl, 1996). Physical properties of recombinant *Cp*-Dsp16 were determinated that confirmed its putative role as cellular osmoprotectans (Lisse et al., 1996).

Dehydrins are characterized by a conserved up to 15 amino acid, lysine-rich sequence that is present near the carboxyl terminus, several times repeated throughout the protein (Dure, 1989). This highly conserved domain is predicted to be involved in hydrophobic interactions (Close, 1996), as it has been suggested for the 17.7 kDa maize dehydrin (Close et al., 1993a). Based on the observation that Dsp16 dehydrin from *C.plantagineum* contains the conserved lysine-rich repeat motif (Piatkowski et al., 1990; Lisse et al., 1996), the ability of the protein to interact with other polypeptides or at least with other dehydrins to form oligomers, should be expected. Thus, the two-hybrid system was chosen as experimental strategy to identify partner proteins interacting with the Dsp16 dehydrin, in order to get some indications about its biological function. The protein expressing the full-length *C.plantagineum* Dsp16 fused to the GAL4 binding domain, named BD-Dsp16, was the bait used for the screening of the cDNA library.

4.1.1.1 Interacting proteins and false positive targets

The problems preventing success of two-hybrid screens are the transactivation by the bait protein itself and the failure to express the bait properly (Golemis et al., 1994). Both these possibilities were tested before the library screening (3.1.2.1) and the control assays indicated that the BD-Dsp16 fusion protein prepared for the system was a reliable bait. Consistent with the temporal expression of the CDeT6-19 gene, which is induced in leaves at the very beginning of the drying process (Bartels et al, 1990), putative interacting partners were supposed to belong to the pool of early dehydration-induced transcripts. Consequently, a cDNA library was prepared, which represented proteins expressed in *C.plantagineum* leaves after two hours dehydration.

The aim of the screening was the identification of true interactions, therefore the selection criteria were mainly the observation of a strong activation of the *HIS3* and *lacZ* reporters and the reproducibility of the assays. In order to maximize the range of interactions detectable, the transformants were plated at first on selective medium which did not contain the histidine inhibitor 3-AT (normally used to eliminate the basic level of the *HIS3* reporter activity and to avoid the isolation of artifacts); this omission probably has reduced the effectiveness of the screening. On the other hand, there are no report that dehydrins have been used as bait for two-hybrid screens and indications about their use by this system are not available in the literature (i.e., if they have any toxic effect on the yeast metabolism that could reduce the transformation efficiency, which could be their putative partners, recurring isolated false positives, etc.).

The primary screening showed an unspecific and massive activation of the *lacZ* reporter and the presence of the histidine inhibitor 3-AT in the medium was necessary to increase the effectiveness of the selection. However, the drug concentration could not exceed 10 mM, a value normally used in the presence of very specific protein interactions (Bartel et al., 1993b; Cayrol et al., 1997; Golemis et al., 1999). In fact when 2-or-3-fold higher 3-AT concentrations were used in the presence of the BD-Dsp16 bait, a drastic reduction of the transformation efficiency was observed.

After two independent library screenings and several rounds of selection by plating and transformation steps, still most of the targets identified by the BD-Dsp16 bait protein were

false positives. Starting initially with two hundred candidates, the number of putative positives was reduced to 22 clones: the sequence analysis showed that two third of them expressed proteins which unlikely could represent true targets. In fact, the putative interacting proteins were encoded by very short nucleotide sequences (the longer one stretched to 150 bp), containing high acidic regions These acidic patches, as well as extended helical sequences and charged regions, are typical of two-hybrid system artifacts and are thought to promote sticky behavior and consequently to activate the reporter genes (Bartel and Fields, 1995; Serebriiskii et al., 2000).

The ability of the Dsp16 dehydrin to interact with other proteins has been finally verified by isolation of three ORF sequences encoding for three different polypeptides. The first identified target was the desiccation stress protein Dsp11-24 isolated from *C.plantagineum* (Velasco et al., 1998). Its amino acid sequence contains a lysine-rich domain and conserved motifs which have been observed in some LEA proteins; because these characteristics, together with other structural features like the high hydrophilicity, has been speculated that the protein could belong to a new LEA protein group (Velasco et al., 1998). The temporal expression of the 11-24 transcript, which accumulated in response to dehydration in leaves, and the subcellular localization of the protein (cytosol), are consistent with those of the Dsp16 dehydrin. This is rapidly accumulated during dehydration and has been localized in the cytosol (Schneider et al., 1993). So the two proteins likely could be present at the same time in the same cellular compartment and interact. The evidence that a lysine-rich region is present in both proteins supports this hypothesis. Studies on plant desiccation proteins containing the 11-mer amino acid conserved motif, originally identified in the D-7 family of cotton LEA proteins but typical of dehydrins, indicate that tandemly repeating 11-mer units result in an amphiphilic helix. It has been speculated that putative interactions between two proteins containing such motifs could take place via binding of the hydrophobic faces of the helix, within or near the lysine-rich basic domain (Dure, 1993a). Because of their temporal expression and amino acid composition the Dsp11-24 protein and the Dsp16 dehydrin should be able to interact with each other. The biological meaning of this interaction is not clear. The function of the Dsp11-24 protein is unknown and a general role in balancing cellular water content has been suggested (Velasco et al., 1998). The

ability to interact with the Dsp16 dehydrin may depend on the high number of dehydration-related proteins expressed in cellular water stress conditions and on the sequence of the biochemical composition of the two proteins.

Another putative candidate to interact with the Dsp16 dehydrin was a predicted 120 amino acid polypeptide, homologous to the *A.thaliana* TRAP-α protein, a membrane protein of the endoplasmic reticulum (Hartmann and Prehn., 1994). This transcript was not induced by dehydration, therefore the characterization of the protein was not continued, but the interaction of the Dsp16 dehydrin with a membrane protein is consistent with the hypothesis that dehydrins would preserve membrane structures in drying cells by acting at the interface
between membrane phospholipids and the cytosol (Close, 1996, 1997; Campbell and Close, 1997). Experimental evidence in support of this hypothesis comes from studies on cold-induced acidic dehydrins from wheat, thought to localize at the plasma membrane (Danyluk et al., 1998) and on onion epidermal cells, where anti-dehydrin antibodies were associated with the endomembrane sheath (Reuzeau et al., 1997). In addition, Ergenton-Warburton et al. (1997) demonstrated the association of dehydrins near the membranes surrounding the protein bodies in maize. Finally, two proteins which are expressed upon low water activity, the desiccation-related protein Dsp34 isolated from *C.plantagineum* (Schneider et al., 1993) and a cold-induced protein from Arabidopsis (Artus et al., 1996; Thomashow et al., 1996), have been identified in the thylakoid membranes of chloroplasts.
Altogether, these observations indicate a general ability of dehydrins to act in reducing the dehydration-induced membrane alterations and perhaps to prevent the interaction between membrane bilayers (Steponkus et al., 1993; Danyluk et al., 1998). Although it has been suggested that dehydrins probably interact with lipids, (Campbell and Close, 1997), the evidence reported to date in the literature is not conclusive and does not exclude other possible interactions.

The third isolated target showed very low sequence homology to other plant proteins: the highest identity (39%) was to a *Sorghum bicolor* 22 kDa kafirin (DeRose et al., 1989). Kafirins are the major storage proteins of sorghum seed endosperm and show extensive homology to maize zeins (Shull et al., 1992). Like for the two other targets previously

isolated, the reporter gene activity was not very high, indicating that the binding to the Dsp16 dehydrin was rather weak. The isolated clone encoded for a 620 bp ORF containing the methionine start codon and did not show highly charged regions or acidic domain which usually are responsible for interactions with non specific baits by the two-hybrid system. In addition, the transcript was induced by dehydration and ABA, indicating a putative role of this protein in the

C.plantagineum response to desiccation. The interaction observed between the Dsp16 dehydrin and this storage-like protein can be explained by the high concentration of dehydration-related proteins that accumulate abundantly in *C.plantagineum* leaves during the early phases of dehydration (Piatkowski et al., 1990; Bockel et al., 1998).

4.1.1.2 No dimerization ability of the Dsp16 dehydrin

It is speculated that dehydrins form dimers. Analysis on the biochemical composition of dehydrins isolated from cotton have shown the presence of a conserved 11-mer amino acid repeating motif thought to be the functional domain for dehydrin dimerization (Dure, 1989). The existence as a dimer of maize dehydrins has been strongly suggested by detailed studies on its biochemical properties and cellular localization (Close et al., 1993a; Ceccardi et al. 1994; Goday et al., 1994). The results obtained from the application of the two-hybrid system for testing the dimerization ability of the Dsp16 dehydrin, were not conclusive. It cannot be excluded that weak interactions sufficient for dimerization are not detectable for those proteins in the two-hybrid system.

4.1.1.3 Conclusions

As mentioned above, the biochemical role of dehydrins has not yet been established and still very little is known about their function in plants (Ismail et al., 1999; Cellier et al., 2000). So far a considerable number of studies have described the biochemical and physiological features, the subcellular localization, the timing of expression and the phenotypic traits associated with dehydrins isolated from several plants and expressed in

E.coli strains (Close, 1997). Yet, there is no experimental evidence on interactions of dehydrins with other proteins. This study has nevertheless at least provided some putative partners.

4.1.2 The use of the transcription factor homologue *Cp*-HSF1 as bait

Previous studies on *C.plantagineum* genes which are transiently activated during dehydration suggested that *Cp*-Hsf1 represents a regulatory gene involved in the early drought response (Bockel C., 2000, Ph.D thesis). The corresponding *Cp*-HSF1 protein shows significant sequence similarity to heat-shock transcription factors (HSFs), which play a central role in the ubiquitous heat stress response (Nover et al., 1996; Scharf et al., 1998). The studies conducted by C. Bockel indicated that *Cp*-HSF1 is involved in HSF regulatory
processes. Through the two-hybrid system, the study of the protein *in vivo* should have confirmed this potential, primarily by identification of interacting proteins presumably involved in gene transcription activity.

By using heat-shock transcription factors as bait, TATA-binding proteins have been identified in yeast (Tillman et al., 1995) and in *A.thaliana* (Reindl and Schoeffl, 1998), as well as several other HSF-binding proteins which are supposed to have trans-regulatory activity (Goff et al., 1992; Staudinger et al.,1993; Shi et al., 1995; Kanei-Ishii et al., 1997; Cormack et al., 1998; Satyal et al., 1998; Eulgem et al., 1999). On the contrary, the two-hybrid system has never been used to test the oligomerization ability of heat-shock factors. In fact, the HSF complexes observed in vertebrates and *Drosophila* have been so far estimated from gel filtration profiles and EGS cross-linking experiments (Clos et al., 1990; Baler et al., 1993; Rabridan et al., 1993; Westwood et al., 1993; Zuo et al., 1995; Zandi et al., 1997; Scharf et al., 1998; Satyal and Morimoto, 1998; Tanabe et al., 1998). The unique application of the two-hybrid system to confirm the trimerization ability of the human HSF1 factor has been reported by Liu (Liu et al., 1997).

4.1.2.1 Assessing the different deletion constructs of *Cp*-HSF1

The analysis of heat-shock transcription factors from a number of organisms has revealed the presence of highly-conserved motifs: a DNA-binding domain (DBD) contained within the amino terminus that is also conserved in its three dimensional structure, an adjacent trimerization domain composed of hydrophobic heptad repeats (HR-A/B) and a stress responsive transcriptional activation domain at the carboxyl terminus (Rabindran et al., 1993; Harrison et al., 1994; Vuister et al., 1994; Green et al., 1995; Shi et al., 1995; Wisniewski et al., 1996). A feature

of the plant and animal HSFs is the presence of an additional hydrophobic repeat (HR-C) located towards the carboxyl terminus. *Cp*-HSF1 shows the modular amino acid arrangement characteristic of eukaryotic heat-shock transcription factors and contains the typical plant HSF domains (Fig. 3.4, page 43) and Bockel C., 2000, Ph.D thesis). The full-length protein (*Cp*-HSF1157) was used as bait for a two-hybrid cDNA library screening. As it has been reported (Bartel et al., 1993, Fields and Sternglanz, 1994), when the protein fused to the DNA-binding domain (the bait) is a transcription factor, it likely activates reporter gene transcription by itself. Therefore, the observed expression of the *HIS3* and *lacZ* reporters by *Cp*-HSF1157 in the absence of any other protein was not surprising. In a test involving two defined proteins, the two proteins could be switched to the opposite vector such that the activating protein is fused to the activator domain instead of the DNA-binding region: in this case a more efficient transcription has been observed (Bartel and Fields, 1995). This change of orientation of the hybrids was obviously not possible for the cDNA library search performed during this study. Thus, in order to define a minimal domain for interaction, three deletions of *Cp*-HSF1 were constructed and assayed in the system.

The HSF domains involved in transactivation activity have been mapped and characterized by use of deletion mutants and chimeric proteins (reviewed by Nover and Scharf, 1997; Scharf et al., 1998). With respect to the importance of the different regions, the three *Cp*-HSF1 deleted proteins (Fig.3.6, page 49) should have represented baits with distinct transactivation ability, because respectively either the activation region (completely or in part) or the DNA-binding domain were missing.

The *Cp*-HSF960 protein was lacking in the HR-C terminal domain. The importance of the

C-terminal region in the regulation of HSF activity has been shown for HSFs of *Drosophila*, plant and chicken. Mutations in the HR-C region lead to trimerization and DNA binding activity; deletion of this domain creates a more active phenotype (Nakai and Morimoto, 1993; Rabindran et al., 1993; Zuo et al., 1994; Lyck et al., 1997). The effect of such deletion has been observed in this study during the yeast selection on medium containing the 3-AT histidine inhibitor. Even at concentrations three times higher than those routinely used for two-hybrid assays, the growth of the transformants expressing the *Cp*-HSF960 protein did not stop. In comparison with the full-length, the *Cp*-HSF960 deleted protein activated the transcription of the *HIS3* reporter gene more efficiently*. In the case of *A.thaliana*, it has been speculated that the C-terminal truncated form of the *At*HSF1 protein interacted more efficiently than the full-length protein, because it is folded

in a conformation more accessible for its TATA-binding protein target (Reindl and Schoeffl, 1998). According to this, it is expected that a shorter protein could interact more easily with putative partners, particularly if they belong to a transcriptional complex formed by several components, as suggested by Struhl (Struhl, 1996).

The strong gene activation ability was the major limit to use the *Cp*-HSF1 protein as bait for the two-hybrid screening, because it could activate the reporter gene transcription by itself. Indeed, even the deletion of most of the activation domain region, as in the *Cp*-HSF775 protein, did not decrease the gene activation ability of the protein, as verified by the *HIS3* and *lacZ* reporter genes expression (Table 3.F, page 44). The observation that for the cDNA library screening, the only suitable bait was represented by the *Cp*-HSF302 protein, which contains just the HR-A/B oligomerization region plus the nuclear localization sequence (NLS), was predictable. The strong transactivation ability shown by *Cp*-HSF1 could be drastically reduced just through the elimination of the DNA-binding domain region, besides that of the activation domain (already tested by the *Cp*-HSF960 and *Cp*-HSF775 deleted proteins). Consistent with this hypothesis, the transcription of both *HIS3* and *lacZ* reporter genes in the presence of the *Cp*-HSF302 alone was not observed. The interactions taking place between *Cp*-HSF302 and putative target proteins were not

*The yeast colonies expressing *Cp*-HSF960 could grow in the presence of 90 mM 3-AT concentration, while the yeast transformed with the full-length protein could not survive up to 75mM.

particularly strong and difficult to reproduce by the standard β-galactosidase assays. The *Cp*-HSF302 protein was able to activate only one (the *HIS3* gene) of the two reporter genes.

The activation of the *lacZ* reporter gene presupposes at least a stable protein interaction, which has been not found by this two-hybrid screening. Because the *HIS3* gene expression is necessary for growing on minimal medium and because was not possible to add the histidine inhibitor 3-AT, even weak interactions between *Cp*-HSF302 and putative targets resulted in the gene activation and surviving yeast phenotypes were obtained. None of the predicted protein sequences from the isolated yeast clones contained hydrophobic regions which could interact specifically with the oligomerization domain of *Cp*-HSF1 used here as bait, as well as amino acid motifs usually present in proteins known to act as transcriptional regulators. All together these observations confirmed that the *Cp*-HSF302 bait did not interact specifically to other proteins.

4.1.2.2 Interactions among different domains of *Cp*-HSF1

The heat-shock transcription factor (HSF) protein exists in the cell as monomer and is reversibly induced upon heat-shock or other physiological stresses to a trimeric state, capable of binding to the DNA (Sorge and Nelson, 1989; Jakobsen and Pelham, 1991). A model used to explain the inactive state of HSFs involves intramolecular interactions between the C-terminal HR-C and the central HR-A/B/NLS region (Rabindran et al., 1993; Zuo et al., 1994, 1995). This part of the protein is required for oligomerization and nuclear transport, which are both steps of the activation process. Thus, intramolecular shielding might efficiently block the HSF function as transcription activator. Deletions and point mutations in these domains were reported to abolish the heat-shock regulation (Chien et al., 1993; Zuo et al., 1994; Shi et al., 1995; Boscheinen et al., 1997; Farkas et al., 1998), confirming the special role of the central part of the protein for the stress response.

The model of an intramolecular interaction between the HR-A/B and HR-C regions in the monomeric HSF and in general speculations about the regulatory role played by the different HSF domains, is to date not supported by experimental evidences, e.g. a two-hybrid test. For this reason, the analysis of the *Cp*-HSF1 conserved domains through

two-hybrid assays of the *Cp*-HSF960, *Cp*-HSF775 and Cp-HSF302 deleted proteins was particularly interesting. The interactions tested included the short hydrophobic oligomerization domain (HR-A/B/NLS) in combination respectively with the full-length and three truncated proteins: all of them contained the same short HR-A/B/NLS region thought to be the core of the HSF trimer formation. Thus, if the central HR-A/B/NLS oligomerization region interacts specifically with the C-terminal HR-C domain, as suggested by several studies and molecular models, this should be observed through the two-hybrid interactions taking place between the *Cp*-HSF302 and the full-length *Cp*-HSF1157, the only protein containing the HR-C region. Consequently, the other protein combinations should be ineffective. The strongest activation of the *lacZ* reporter gene (Fig.3.6, page 49) was observed for the *Cp*-HSF1157 x *Cp*-HSF302 combination. Because in previous assays the full-length protein could function as a positive transactivator by itself, the observed reporter gene transcription could be an artifact and not the proof of true protein interactions. Noteworthy, when *Cp*-HSF302 was in the presence of

the C-terminal truncated *Cp*-HSF960, which showed even stronger self-activation ability than the full-length protein

but was lacking the HR-C region, the β-galactosidase activity observed after the colony lift assay was lower (the colonies turned blue slowly and the final color was not very intense).

A strong interaction was expected also from the *Cp*-HSF302 x *Cp*-HSF302 combination, where the oligomerization domains of the two proteins could easily interact, because, in the absence of the other domains, the normal tridimensional protein folding was missing and the HR-A/B regions were more accessible. The β-galactosidase activity was surprising low and did not indicated this protein combination be more successful that the others in activating the reporter system. However, a positive two-hybrid effect requires dimer formation, whereas the natural interaction of HSFs by their oligomerization domains leads to trimers. Thus, the difficulty to observe stable interactions among the *Cp*-HSF1 functional regions could depend on the experimental conditions that force the proteins to associate in an abnormal way.

4.1.2.3 Conclusions

The knowledge of the processes of transcription in plants is still limited but due to the high level of conservation in the factors involved, the mechanism of transcriptional activation is believed to follow a common scheme in all eukaryotic organisms (Stargell and Struhl, 1996). The use of the *Cp*-HSF1 protein as bait for a two-hybrid cDNA library did not reveal novel interacting partners, but has shown the high transactivation ability of this transcription factor homologue *in vivo*, at least in a yeast system. With regard to the interactions among the conserved functional domains of Cp-HSF1, the yeast clones expressing different protein combinations showed different phenotypes by lift colony assay, demonstrating the ability of the proteins to interact. Yet the quantitative β-galactosidase activity values were very low in comparison with the control reactions. For this reason the presented results must be considered more as experimental indications not yet conclusive.

4.3.1 Limits and pitfalls of the yeast two-hybrid system

The two-hybrid technology has contributed significantly to the discovery of protein interactions in eukaryotes and technical variations of this system have been developed and successfully applied. Yet, limitations in applying the two-hybrid system have been reported by several scientists working with proteins from different organisms. A major difficulty in the two-hybrid screening is the elimination of false positives. These are clones that cause the activation of reporter genes but not as result of a specific interaction with the bait (Bartel et al., 1993b).

Serebriiskii and Golemis have compiled a list of such promiscuous interactors (http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html); unfortunately, the database is not very representative of plant protein interactions, because most of the data come from studies on humans, *Drosophila* or yeast. Even if not complete, this analysis emphasizes the high probability to identify false interactions by using the two-hybrid system, especially for screening of libraries.

Generally false positives are eliminated by verifying that *HIS3* and *lacZ* reporter gene expression is specific for the presence of the target protein. Activation of *lacZ* reporters in yeast is assayed by one of three approaches: growth on plates containing X-gal; growth on standard cultivation conditions following colony lysis and X-gal incubation; growth in liquid, cell lysis and quantitative assay (Bartel and Fields, 1997). Surprisingly, it has been recently demonstrated that the assay can produce dramatic differences in *lacZ* activity levels obtained after the three different assays (Serebriinskii et al., 2000). These results indicated that the *lacZ* readout could not be strictly correlated with the transcriptional activation of the reporter gene and that variables unrelated to transcriptional activation biased the assessment of the gene reporter activity. In addition, a study about the correlation of two-hybrid interactions affinity data with *in vitro* measurements (Estojak et al., 1995) showed that the pool of positive clones selected may be biased by the choice of the reporter gene assay. Proteins that appear to interact strongly on the basis of the *HIS3* phenotype but which activate a *lacZ* reporter only weakly may still be valid (Datta et al., 1995). In other cases, interactions were detected when one partner was the bait (carrying the binding domain) and the other carried an activation domain (AD), but were not observed in the other combination

(i.e. when the bait expressed the AD). All together these observations confirm that successful or failure applications of the two-hybrid system depend on experimental variables whose importance can be unlikely predicted because it is specific to each different test. In fact, comments on troubles found by using the two-hybrid system are increasing, as well as the studies about the limits and new strategies to overcome them have to be developed.

4.2 Characterization of the small heat-shock protein *Cp*Hsp20.6 gene

The discovery of the dehydration-induced heat-shock transcription factor *Cp*-HSF1 raised the question for a putative target. Previously, Alamillo et al. (1995) had discovered the expression of small heat-shock protein (small HSPs) in *C.plantagineum* by using antibodies raised against seed-stored small HSPs from sunflower (Almoguera and Jordano, 1992; Coca et al., 1994). These observations indicated the presence of small heat-shock protein-related

genes in *C.plantagineum*. Small heat-shock proteins (small HSPs) represent the most prominent group of plant heat stress responsive proteins (Key et al., 1981; Vierling, 1991). Besides the expression in unstressed vegetative tissues of *C.plantagineum*, further accumulation of these proteins was induced by heat-shock and water stress (Alamillo et al., 1995). Therefore, small HSPs were potential targets for *Cp*-HSF1.
In the following, the heat stress response in *C.plantagineum* and the role of the small HSP homologue *Cp*-Hsp20.6 gene as putative target for the heat-shock transcription factor *Cp*-HSF1 will be discussed.

4.2.1 Sequence and expression analyses of the *Cp*-Hsp20.6 gene transcript

A considerable number of sequences of diverse plant small HSPs, most of them characterized on the cDNA level, have been reported. A cDNA clone from *C.plantagineum* (kindly provided by Dr. J. Phillips), showed high homology both at the nucleotide and at the amino acid sequence level to plant small HSPs. In addition, the theoretical molecular weight
(20.6 kDa) of the predicted protein (named *Cp*HSP20.6) was in accordance with the size expected for small HSPs, usually smaller than 30 kDa (Vierling, 1991; Waters, 1995). Amino

acid sequence comparisons using public datebases (Pearson and Lipman, 1988; Altschul et al., 1990) indicated that *Cp*HSP20.6 belongs to the mitochondrial small HSPs (MT small HSPs) family. So far, MT small HSPs have been cloned from pea (Lenne et al., 1994), *Chenopodium rubrum* (Debel et al., 1995), soybean (LaFayette et al., 1996), *Arabidopsis* (Willet et al., 1996, Visioli et al., 1997) and maize (Lund et al., 1998). Noteworthy, the highest homology level for *Cp*HSP20.6 was to the pea *Ps*HSP22 protein (Table 3.G, page 54), a small HSP which has been shown to be directly associated with the mitochondria (Lenne and Douce, 1995; Lenne et al., 1995). The detailed analysis of the *Cp*HSP20.6 protein sequence supports the homology to the MT small HSPs family.

First, a putative consensus sequence necessary to enter the mitochondrion is present in the predicted amino acid sequence of *Cp*HSP20.6 (Fig.3.9, page 55). The transit peptides identified in the plant MT small HSP sequences so far analyzed is contained within the first 32-40 amino acids. It has been shown that the N-terminus of the mature protein lies in

the region –FNTNA-, which is highly conserved within the maturation site sequence of all angiosperm mitochondrial small HSPs - (Lenne et al., 1995). The pre-sequence predicted for the *Cp*HSP20.6 protein consists of 35 amino acid and contains the -FN*S*NA- sequence, very similar to the conserved motif (the replacing of the threonine residue with a serine, should not modify the function of this region, because both are neutral, hydrophilic amino acids). So, the presence of the mitochondrial transit peptide sequence suggests the organelle localization of the *Cp*HSP20.6 protein.

Secondly, *Cp*HSP20.6 contains the two C-terminal consensus regions thought to be responsible for the structural and functional properties of the small heat-shock protein family (Caspers et al., 1995). Plant small HSPs share a consensus region (named consensus II) not present in the other eukaryotic small HSPs and a 15 amino acid region (named consensus I) common to all eukaryotic small HSPs (Vierling, 1991). Both these domains are present in its *Cp*HSP20.6 protein sequence. Further analysis of its C-terminus amino acid composition also revealed the presence of the heat-shock motif **GVL**KVT(F)V**P**, which is conserved in all small HSPs from prokaryotes and eukaryotes (Lindquist and Craig, 1988).

The *Cp*-Hsp20.6 expression patterns suggests that the *Cp*HSP20.6 protein is induced both by dehydration and by heat stress in *Craterostigma* roots (Fig.3.13 and 3.14, page 61,62). The expression of a mitochondrial small HSP homologue in *C.plantagineum* is consistent with the

observation that heat stressed plants accumulate much more mitochondrial small heat-shock proteins than other heat-shock related proteins, like HSP60 and HSP70 (Vierling, 1991; Lenne and Douce, 1994; Lund et al., 1998). In fact, very early experiments on heat-shocked organisms demonstrate that the inhibition of the mitochondrial function was one of the first targets in the heat response, which could lead to HSP synthesis (Ritossa, 1962; Lindquist, 1986).

The high tolerance to water stress of *C.plantagineum* has been extensively studied but not very much is known on its response to heat stress. Alamillo et al. (1995) observed the presence of small HSP-related proteins both in vegetative and in water or heat stressed tissues and the importance of these proteins for desiccation tolerance in *C.plantagineum* has been suggested. As defined by Levitt (1980), direct heat injury in plants results from an exposure to extreme temperature (45°C to 60°C). Investigations on the response of

Craterostigma to high temperature were previously performed (Alamillo et al., 1995; Bockel C., 2000, Ph.D thesis); yet, the data coming out from the presented study are the first obtained from plants heat stressed at temperatures higher than 42°C.

The induction of the *Cp*-Hsp20.6 transcript in heat stressed roots suggests that *Craterostigma* responds to overheating by expression of heat-shock related proteins: the more abundant *Cp*Hsp20.6 gene transcription was actually observed in the heat stressed tissues. However, the transcript is induced also by water stress, as indicated by the expression in dehydrated roots and in extremely dehydrated leaves. The rate of HSP synthesis in plants or other eukaryotes has been shown to be directly proportional to the temperature applied during the stress, increasing at the higher temperatures (Nagao et al., 1986; Lindquist and Craig, 1988; Chen et al., 1990). Indeed, the *Cp*-Hsp20.6 expression in the roots increased considerably when the plants were exposed for three hours at 45°C, in respect to the expression observed for plants heat stressed at 42°C for the same length of time (Fig. 3.14, page 43). So, in *C.plantagineum* heat-shock-related gene expression is induced by heating at the same temperature (42°C) which has been shown to promote the heat stress response in most plant species so far studied; yet, for a massive production of heat-shock related proteins this threshold must be overcome.

It can be hypothesized that in *C.plantagineum* an abundant synthesis of heat-shock proteins represents the way for the plant to tolerate high temperature stress. It has been postulated that resurrection plants should be resistant to large fluctuations in temperature (Hartung et al., 1998) and it is known that *C.plantagineum* occurs in regions where arid climate predominates (Dinter, 1918; Gaff, 1977). Therefore, *C.plantagineum* should be expected to be heat tolerant. HSPs are

implicated in the ability to grow at high temperatures and in the development of thermotolerance (McAlister and Finkelstein, 1980; Nagao et al., 1986; Key et al., 1987; Czarnecka-Verner et al., 1994; Nover and Scharf, 1997). Hence, the specific involvement of small HSPs in thermotolerance has been demonstrated in animal systems (Chretien and Landry, 1988; Lavoie et al., 1993), as well as in plants (Vierling, 1991; DeRocher and Vierling, 1994; Lee et al., 1995; Waters et al., 1996; Prasad, 1997; Malik et al., 1999). In addition, the expression of a plant small HSP has been shown to enhance the viability of *E.coli* under heat and cold stress (Soto et al., 1999). According to the evidence that MT small HSPs synthesis is synchronized with the enhancement of the heat tolerance in

different plant species (Chou et al., 1989; Visioli et al., 1997; Lund et al., 1998; Liu and Shono, 1999), it could be hypothesized that the induction of the mitochondrial small heat-shock protein homologue *Cp*-Hsp20.6 gene could be involved in heat tolerance acquisition in *C.plantagineum*.

Besides heat stress, the induction of the *Cp*-Hsp20.6 transcript was observed in roots after prolonged dehydration. Heat and water stress induces the *Cp*-Hsp20.6 transcript in the roots as it was observed for the drought-responsive *Cp*-Hsf1 transcript (Bockel C., 2000, Ph.D thesis), suggesting that in this tissue *Cp*-Hsp20.6 gene could be a potential target for the *Cp*HSF1 protein. *Cp*HSF1 was detected both in heat stressed and dehydrated roots; on the contrary, in dehydrated leaves the *Cp*-Hsf1 expression was transiently activated, but not induced by heat (Bockel C., 2000, Ph.D thesis). According to the observation that *Cp*-Hfs1 gene was differently regulated in leaves and roots, this putative heat-shock transcription factor could activate different targets in the two different tissues: it might be assumed that the *Cp*-Hsp20.6 gene is one of the targets activated in the roots of *Craterostigma*.

Roots are characterized primarily by long-term drought response (Monneveux and Belhasse, 1996): so, it must be expected that in this tissue the expression of important genes can continue for several hours, at least in response to severe stress conditions. The observed *Cp*-Hsp20.6 transcript after prolonged exposition at 45°C is consistent with this hypothesis. The prolonged expression of *Cp*-Hsf1 in the roots at 45°C has not been tested and the protein synthesis was observed only in roots heat stressed at 42°C. Nevertheless, because in roots upon dehydration the *Cp*-Hsf1 expression increased without subsequent decrease, it has been suggested that it can function in this tissue in prolonged synthesis of HSPs. The hypothesis that a dehydration/drought

responsive transacting factor can promote the expression of heat stress-related genes is not surprising.

It is well known that heat-shock proteins are synthesized in the plant cells in response to high temperature (Nover et al., 1984; Schlesinger et al., 1990; Gurley and Key, 1991) and water deficit (Bray, 1993), besides other environmental factors. Data indicate that heat and drought stress in plants are often positively correlated (Wallner at al., 1982) and evidence is accumulating in favor of this hypothesis. Recently, a 45 kDa heat-shock protein from a drought and heat resistant maize line has been identified (Ristic et al., 1999) and increased heat tolerance has been demonstrated in water stressed leaves of geranium (Arora et al.,

1998). In particular, high level expression of homologous small HSPs in response to water stress has been shown in sunflower (Almoguera et al., 1993) and studies on stress responsive proteins from tomato revealed the existence of one small HSP in mitochondria, which was induced by cold and high temperature (Sabehat et al., 1996; 1998) as well as by oxidative stress (Banzet et al., 1998). So, drought can be the signal for the synthesis of stress responsive gene, like *Cp*-Hsp20.6, through the expression of the drought responsive transacting element *Cp*HSF1. The ability of *Cp*HSF1 to bind *in vitro* to the promoter region of Cp-Hsp20.6 (3.3.1) further suggests the putative role of this gene as target for the protein.

In leaves, the expression of the *Cp*Hsp20.6 transcript was observed in response to severe dehydration and to heat stress (Fig. 3.15). The observation that in leaves *Cp*HSF1 is rapidly activated upon dehydration (Bockel C., 2000, Ph.D thesis), whereas *Cp*Hsp20.6 synthesis starts in the tissues after several hours, is consistent with the hypothesis that the trans-factor homologue acts in leaves on different targets than in roots. The absence of the transcript after ABA treatment suggests that the *Cp*Hsp20.6 gene is not exclusively a dehydration responsive gene.

4.2.2 Identification of a heat-shock responsive element-like sequence (*Cp*-HSE) homologous to plant heat-shock cis-regulatory elements.

The thermoinducibility of heat-shock genes has been attributed to the presence of cis-elements designated as heat-shock consensus elements (HSEs), which are located in the 5' flanking sequences (Mirault et al., 1982; Pelham, 1982; Amin, 1988; Lis et al, 1990). The HSE has been shown to serve as binding site for the heat-shock transcription factor (HSF) that mediated the thermal activation of transcription (Parker and Topol, 1984; Goldenberg et al.,

1988; Wu, 1995). A functional heat-shock element includes a minimum of three 5-nucleotide nGAAn (or its complement nTTCn) modules, which do not have to be consecutive (Amin et al., 1988; Sorge, 1991); even arrangements of HSE modules that are interrupted are functional (Amin et al., 1988). However, at least two nGAAn units are needed for high affinity binding of heat-shock factor in vitro, and these can be arranged either head-to-head (nGAAnnTTCn) or tail-to-tail (nTTCnnGAAn; Perisic et al., 1989). The heat-shock element identified in the *Cp*-Hsp20.6 promoter (*Cp*-HSE) is a tail-to-tail motif

very similar to the HSE1 sequence used successfully for binding assays with the soybean heat-shock transcription factor *Gm*HSF34 (Czarnecka-Verner et al., 1995); the similarity to the HSE1 of the *Cp*-Hsp20.6 promoter sequence extends for 25 nucleotides. The gel retardation and competition assays showed the binding of the transcription factor homologue *Cp*HSF1 (3.3.1) to the 25 bp *Cp*-HSE and suggest the putative function of this motif as cis-regulatory element. Hence, the observed *Cp*HSF1 binding activity supports the role of the *Cp*-Hsp20.6 gene as potential target and at the same time is consistent with the potential function as transactivator of the *Cp*HSF1protein.

HSF binds to DNA with high affinity and deviations from the nGAAn consensus sequence may be tolerated in vivo because multiple HSEs foster cooperative interactions between multiple HSF trimers (Bonner et al., 1984; Topol et al., 1985; Xiao et al., 1991). Imperfect HSE motifs, usually found in distal positions in respect to the nGAAn/nTTCn core, have been shown to be equally important for the binding activity of heat-shock factors in *Drosophila* and yeast, (Perisic et al., 1989; Santoro et al., 1998) as well as in plants (Guy and Key, 1991; Barros et al., 1992; Rojas et al., 1999). The occurrence of multiple HSE-like motifs within the TATA upstream region of the *Cp*-Hsp20.6 promoter was observed. Their allocation in a range of 250 nucleotides along the proximal TATA box region of the *Cp*-Hsp20.6 promoter is in accordance with the general observation that heat-shock promoters carry multiple copies of the HSE within a few hundred base pair upstream of the TATA sequence (reviewed in Nover, 1997).

The configuration of HSEs in promoters of genes encoding small HSPs localized either in the cytoplasm or in different cellular compartments are similar, consistent with the observation that all small HSPs are nuclear encoded. So, besides the conserved trinucleotide core nGAAn sequence, the presence of cis-regulatory elements typical of heat-shock promoter genes was expected. Indeed, several conserved transcriptional heat-shock response regions have been identified in the *Cp*-Hsp20.6 promoter.

A structural element featured in some HS gene promoters is the CCAAT box (Czarnecka-Verner et al., 1994), present twice in the *Cp*-Hsp20.6 promoter region. The contribution of this motif to basal expression of HS genes has been demonstrated in the case of HSP70 promoter from human and *Xenopus* (Bienz, 1986; Morimoto and Milarski, 1990; Williams and Morimoto, 1990). It has been also observed the involvement of CCAAT elements in

heat induced expression of small HSP genes from soybean (Czarnecka et al., 1989; Rieping and Schoeffl, 1992).

Further control of *Cp*-Hsp20.6 transcriptional activity upon heat is substantiated by the presence of AT-rich sequences. These repeats are thought to act as transcriptional activators and have been found upstream of a variety of inducible plant genes (Jofuku et al., 1987; Bustos et al., 1989; Jordano et al., 1989), including heat-shock genes. For example, several soybean genes encoding small HSPs (Schoeffl et al., 1984; Czarnecka et al., 1985; 1990; Nagao et al., 1985; Baumann et al., 1987) contain in their promoter contiguous AT-rich blocks located upstream from the TATA-box.

Finally, the *Cp*-Hsp20.6 promoter sequence contains twice the AGCT element, which represent the core of the ABA-responsive element (ABRE) involved in ABA responsive gene expression (Giuliano et al., 1988; Giraudat et al., 1994). However, the presence of the ABRE sequence in the promoter region is not necessary correlated to the ABA regulation of the corresponding transcript. On the one hand, studies employing ABA-insensitive mutants of *Arabidopsis* indicated that there are also ABA-independent signal transduction pathways for dehydration-regulated gene expression (Gilmour and Liss, 1986; Nordin et a., 1991; Yamaguchi-Shinozaki and Shinozaki, 1994). On the other hand, promoters of plant genes, which are not known to be modulated by ABA, contain the AGCT or similar sequences, which in many cases have been demonstrated to be functionally important (Staiger et al., 1989; McKendee et al., 1990). The presence of this motif in the *Cp*-Hsp20.6 promoter but the failed expression of the *Cp*-Hsp20.6 transcript after ABA treatment seems to confirm these observations.

Further investigations on the function as cis-acting sequence of the *Cp*-Hsp20.6 promoter were conducted in a transient expression system using tobacco protoplasts, where the full-length *Cp*-Hsp20.6 promoter sequence was used to drive the GUS-reporter expression in the presence of the *Cp*-HSF1 transcription factor homologue (3.3.2). As described above, the 5'-flanking region of the *Cp*-Hsp20.6 gene, besides the HSE and HSE-like sequences, contains several regions supposed to act as transcription regulators, if even not as enhancers. Because these regions were

present in the GUS-reporter plasmid used for the transient assays, the transcription of the reporter gene could be likely activated by the tobacco endogenous transcriptional system, as suggested by Treutet et al (1993). In that case,

transcription depending on the endogenous HSF system was observed with reporter constructs containing intact HSE but not with truncated promoter sequences. The transcription level of the reporter gene driven by the full-length *Cp*-Hsp20.6 promoter was significant also in the absence of the transactivator *Cp*-HSF1. However, the highest values were observed when the transcription factor-homologue was expressed. Anyway, the role as cis-activating sequence of the *Cp*-Hsp20.6 promoter region was confirmed.

4.2.3 Conclusions

During drought, plants are often affected both by water deficiency and high temperature. The water stress tolerance of *Craterostigma* is well known and because the particular ability of this plant to tolerate severe desiccation, the presence of heat responsive genes that are also induced in dehydrated tissues might be expected. The mechanisms essential for desiccation tolerance in *Craterostigma* are still not completely known; however, cellular protection seems to play a central role (Ingram and Bartels, 1996). LEA genes that are assumed to function as chaperones are strongly expressed upon desiccation (Bartels et al., 1990; Dure, 1993). Small HSPs too are proposed to act as chaperones in desiccation tolerance (Almoguera et al., 1993; Jacob et al., 1993; Alamillo et al., 1994; DeRocher and Vierling, 1994; Coca et al., 1994, 1996).

It has been demonstrated *in vitro* that both the chloroplast small HSP and plant mitochondrial small HSPs protect electron transport during high temperature conditions in these organelles (Downs and Heckathorn, 1998; Heckathorn et al., 1998; Downs et al., 1999). So, the induction of heat and water stress responsive genes (as observed for the *Cp*-Hsp20.6 gene in *C.plantagineum*) with a relevant biochemical function, as for maintaining the activity of mitochondria, certainly belongs to the fundamental mechanisms of cellular protection.

5 Summary

The resurrection plant *Craterostigma plantagineum* (Fam. Scrophulariaceae) is one example of the known desiccation-tolerant plants. Its ability to cope with drought stress has been extensively studied and several dehydration-induced genes have been characterized. The identification and isolation of stress responsive proteins is a central theme in plant stress studies. To investigate the complex network of dehydration-related gene products in *C.plantagineum*, protein-protein interactions were analyzed *in vivo* by the yeast two-hybrid system. A cDNA expression library was generated and screened using two dehydration-induced proteins, a dehydrin-like protein (*Cp*-Dsp16) and a heat-shock transcription factor homologue (*Cp*-HSF1).

Dehydrins and heat-shock proteins represent two very different classes of stress proteins that accumulate upon drought conditions. Little is known about the role of dehydrins in plants and their putative function as chaperones has been suggested; yet, there is no experimental evidence about interactions of dehydrins with other proteins. This study has provided some putative *Cp*-Dsp16 partners, which have been identified from a pool of dehydration-induced proteins from *C.plantagineum* (3.1.2). Heat-shock transcription factors (HSFs) play an important role in the ubiquitous heat stress response. The heat-shock factor homologue *Cp*-HSF1 represent a putative regulatory protein of the early drought response in *Craterostigma.* Through the two-hybrid system, the high transactivation ability of this transcription factor homologue has been shown *in vivo* (3.1.3).

In order to identify potential target genes of the heat-shock transcription factor *Cp*-HSF1, experiments have been extended to the investigation of small heat-shock proteins (small HSPs) in *C.plantagineum*. The small HSPs represent the most prominent group of plant

heat stress responsive proteins. Previously, the presence of small HSPs was observed in vegetative tissues of *C.plantagineum*, where their accumulation was induced both by heat and by water stress. During this study a small HSP homologous cDNA isolated from *Craterostigma* (*Cp*-Hsp20.6) has been characterized (3.2). Phylogenetic and sequence comparison analyses assigned the predicted protein (*Cp*HSP20.6) to the plant mitochondrial small HSP family. The *Cp*Hsp20.6 transcript is induced both by high temperature and by dehydration in *C.plantagineum* roots (3.2.3). Upon heat and water stress the *Cp*-Hsp20.6

expression patterns were similar to those observed in the roots for the drought responsive *Cp*-Hsf1 transcript, suggesting that in this tissue *Cp*Hsp20.6 gene could be a potential target for the *Cp*-HSF1 protein. A heat-shock responsive element (*Cp*-HSE) has been identified in the *Cp*-Hsp20.6 promoter region and the ability of the heat-shock transcription factor homologue *Cp*-HSF1 to bind to this heat-shock element-like sequence and to activate the *Cp*-Hsp20.6 target gene has been tested by *in vitro* binding assays (3.3.1) and by *in vivo* tobacco protoplasts transformation experiments (3.3.2).

6 Zusammenfassung

Die Wiederauferstehungspflanzen *Craterostigma plantagineum* (Fam. Scrophulariaceae) ist ein Beispiel für eine trockentolerante Pflanze. Die Fähigkeit, Austrocknen zu überleben, ist molekularen und physiologischen Ebene gut untersucht worden. Mehere Trocknenstress induzierte Gene sind charakteriziert worden.

Die Identifizierung und Isolierung von Streßproteinen ist ein zentrales Thema in der Streßforschung. Um das complexe Network der Trocknenstreß induzierten Proteine in *C.plantagineum* zu erforschen, sind in der vorliegenden Arbeit Protein-Proteininteraktionen in *vivo* in Hefe-zwei-Hybridsystem untersucht worden. Eine cDNA Bank wurde durchmustert um interagierende Proteine für das Dehydringen (*Cp*-Dsp16) und für den homologen Hitzestreßtranskriptionsfaktor (*Cp*-HSF1) zu identifizieren.

Dehydrine und Hitzeschochproteine repräsentieren zwei sehr verschiedene Klassen von Streßproteinen, die während Trocknenstress in den Geweben von *C.plantagineum* akkumulieren. Über die biochemische Funktion von Dehydrinen in Pflanzen ist wenig

bekannt. Es ist postuliert worden, dass sie als Chaperone fungieren. Bislang gibt es allerdings keinen experimentellen Nachweis, dass Dehydrine mit andere Proteinen wechselwicken. In der vorgeligenden Arbeit sind Proteinen identifiziert werden, die möglichesweise mit *Cp*-Dsp16 interagieren (3.1.2). Hitzeschochtranskriptionsfactoren (HSFs) spielen eine wichtige Rolle in der Hitzestreßreaction, die in allen Organismen zu finden ist. Der homologe Hitzeshoch *Cp*-HSF1 stellt ein putatives regulatorisches Protein dar, das in der frühen Trocknenstreßreaktion beteiligt ist. Mit Hilfe des Hefe-zwei-Hybridsystems kommt das hohen Transaktivierungspotential von *Cp*-HSF1 nachgewiesen werden (3.1.3).

Um mögliche Zielgene des Hitzeschochtranskriptionsfaktores *Cp*-HSF1 zu identifizieren, sind die Gruppe der kleinen Hitzestreßproteine (kleine HSPs) in die experimentellen Untersuchungen miteinbezogen worden. Die kleinen HSPs sind eine sehr prominente Gruppe von Hitzestreßproteinen in Pflanzen. Die Expression der kleinen HSPs in vegetativen Geweben von *C.plantagineum* nach Hitze und Trockenstreß konnte nachgewiesen werden.

In der vorliegenden Arbeit ist ein cDNA Klon (*Cp*-Hsp20.6) charakterisiert worden, der für ein kleines HSP (*Cp*-HSP20.6) kodiert (3.2). DNA Sequenzanalysen und phylogenetisch Vergleiche weisen darauf hin, dass *Cp*-HSP20.6 zur Gruppe der pflanzichen mitochondrialen Hitzeshochproteinen angehört. Das *Cp*-Hsp20.6 transcript wird sowohl durch hohe Temperatur und durch Trockenstreß in Wurzeln induziert (3.2.3). Das Expressionsmuster von *Cp*-Hsp20.6 als Antwort auf Temperaturerhöhung und Wasserstress entspricht dem des Hitzeschochtranskriptionfaktores *Cp*-Hsf1, so daß *Cp*-Hsp20.6 ein mögliches Zielgen von *Cp*-HSF1 sein könnte. Ein typisches Hitzeschoch-Response-Element konnte im Promoter von *Cp*-Hsp20.6 identifiziert werden. Es könnte gezeigt werden, dass *Cp*-HSF1 an dieses Promoterelement bindet. In einem transienten transformationssystem in Tabakprotoplasten wurde untersucht, ob *Cp*-HSF1 die Transkription von *Cp*-Hsp20.6 aktivieren kann.

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8 Appendix

I. Accession numbers of the plant small HSP amino acid sequences used for phylogenetic analyses showing the relationship between the *C.plantagineum* small heat-shock protein *Cp*HSP20.6 and small HSP from diverse plant species.

The protein names are listed in alphabetic order.

Protein Class	*Protein Name*	*Accessions Number*
Cytosolic I	*Ath* HSP 17.4	X17293
	Ath HSP 17.6	X16076
	Cs HSP 17.5	AJ009880
	Dc HSP 18	X53852
	Hv HSP 17	Y078844
	Gm HSP 18.5	X07160
	Ha HSP 17.6	U46545
	Ha HSP 18.2	P30693
	Le HSP 17.8	X56138
	Ms HSP 18.2	X58711
	Nt HSP 18.1	X70688
	Os HSP 16.9	X60820
	Os HSP 17.4	D12635
	Peg HSP 17	X94191
	Ps HSP 18.1	M33899
	Ta HSP 16.9	X64618
	Zm HSP 17.2	X65725
Cytosolic II	*Ath* HSP 17.6	X63443
	Gm HSP 17.9	X07159
	Ha HSP 17.9	Z29554
	In HSP 17.2	M99429
	Pc HSP 17.9	X95716
	Pg HSP 17.1	L47717
	Ps HSP 17.7	M33901
	Ta HSP 17.3	X58279
	Zm HSP 17.5	X54076
	Zm HSP 17.8	X54075

Protein Class	Protein Name	Accessions Number
Chloroplast-localized	*Ath* HSP 21	X54102
	Ps HSP 21	X07187
	Zm HSP26	L28712
Endomembrane-localized	*Ath* HSP 22	U11501
	Gm HSP 22.2	U21723
Mitochondrion-localized	*Ath* HSP 23.5	X98375
	Ath HSP 23.6	U72958
	Gm HSP 23.9	U21722
	Le HSP 23.5	AB017134
	Pg HSP 23.5	L47741
	Ps HSP 22	X86222
	Ta HSP 23.5	104107
	Zm HSP 23.8	AF03560

II. Nucleotide sequence of the *Cp*-Hsp20.6 gene

Translational start and stop codon are highlighted, as well as the HSE-like sequence (position –165). The TATA motif and the putative polyadenilation sequence are underlined. The intro sequence is shown in *italic* (the consensus GT/AG boundaries are underlined). The numbering in based on the traslational start site (**ATG** = 1).

```
- 1245  CTCTGTGGTCTACGTAAATTATAAGGGAGAGAAGAGGCCTATGAGGACTTCAGAGAGTCC
        CAAGTTGTGCGATGGAACCTCCTCATTATACGAAACAAAGCTAAGAAGAATCGCCGAGGA
- 1125  GTTAAGGTTCGAAGAAAATGAAGTCCAAGAAAAGTACCACAACCTAATCAACACCATCTC
        TGCTCGAATATCCTTTTCGAAGAAGGCTGCGATCAATGAATTCATCCTAGGGCTTAGAAG
- 1005  GTCTCCTTTCACCCTGGTGAAACCAAAGAAAAAGAAAACACAAAGGACACCAACAAGGCG
        AAGAGGACACTAAGGGGGAGATTGTTAGGAAATCATTATAGTGTTGGGTTTATTTTTTGT
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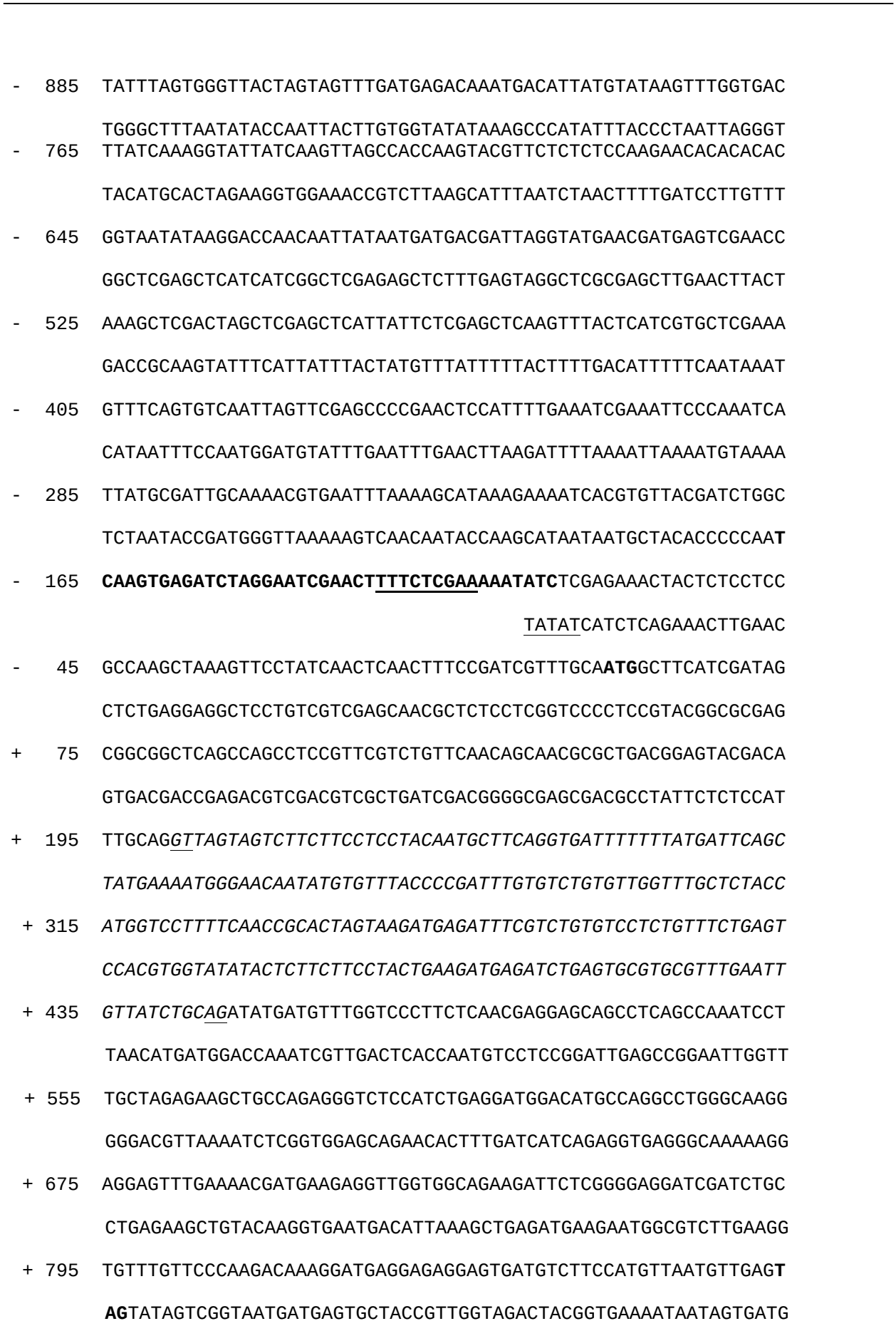
- 885 TATTTAGTGGGTTACTAGTAGTTTGATGAGACAAATGACATTATGTATAAGTTTGGTGAC
TGGGCTTTAATATACCAATTACTTGTGGTATATAAAGCCCATATTTACCCTAATTAGGGT
- 765 TTATCAAAGGTATTATCAAGTTAGCCACCAAGTACGTTCTCTCTCCAAGAACACACACAC
TACATGCACTAGAAGGTGGAAACCGTCTTAAGCATTTAATCTAACTTTTGATCCTTGTTT
- 645 GGTAATATAAGGACCAACAATTATAATGATGACGATTAGGTATGAACGATGAGTCGAACC
GGCTCGAGCTCATCATCGGCTCGAGAGCTCTTTGAGTAGGCTCGCGAGCTTGAACTTACT
- 525 AAAGCTCGACTAGCTCGAGCTCATTATTCTCGAGCTCAAGTTTACTCATCGTGCTCGAAA
GACCGCAAGTATTTCATTATTTACTATGTTTATTTTTACTTTTGACATTTTTCAATAAAT
- 405 GTTTCAGTGTCAATTAGTTCGAGCCCCGAACTCCATTTTGAAATCGAAATTCCCAAATCA
CATAATTTCCAATGGATGTATTTGAATTTGAACTTAAGATTTTAAAATTAAAATGTAAAA
- 285 TTATGCGATTGCAAAACGTGAATTTAAAAGCATAAAGAAAATCACGTGTTACGATCTGGC
TCTAATACCGATGGGTTAAAAAGTCAACAATACCAAGCATAATAATGCTACACCCCCAAT
- 165 CAAGTGAGATCTAGGAATCGAACTTTTCTCGAAAAATATCTCGAGAAACTACTCTCCTCC
TATATCATCTCAGAAACTTGAAC
- 45 GCCAAGCTAAAGTTCCTATCAACTCAACTTTCCGATCGTTTGCAATGGCTTCATCGATAG
CTCTGAGGAGGCTCCTGTCGTCGAGCAACGCTCTCCTCGGTCCCCTCCGTACGGCGCGAG
+ 75 CGGCGGCTCAGCCAGCCTCCGTTCGTCTGTTCAACAGCAACGCGCTGACGGAGTACGACA
GTGACGACCGAGACGTCGACGTCGCTGATCGACGGGGCGAGCGACGCCTATTCTCTCCAT
+ 195 TTGCAGGTTAGTAGTCTTCTTCCTCCTACAATGCTTCAGGTGATTTTTTTATGATTCAGC
TATGAAAATGGGAACAATATGTGTTTACCCCGATTTGTGTCTGTGTTGGTTTGCTCTACC
+ 315 ATGGTCCTTTTCAACCGCACTAGTAAGATGAGATTTCGTCTGTGTCCTCTGTTTCTGAGT
CCACGTGGTATATACTCTTCTTCCTACTGAAGATGAGATCTGAGTGCGTGCGTTTGAATT
+ 435 GTTATCTGCAGATATGATGTTTGGTCCCTTCTCAACGAGGAGCAGCCTCAGCCAAATCCT
TAACATGATGGACCAAATCGTTGACTCACCAATGTCCTCCGGATTGAGCCGGAATTGGTT
+ 555 TGCTAGAGAAGCTGCCAGAGGGTCTCCATCTGAGGATGGACATGCCAGGCCTGGGCAAGG
GGGACGTTAAAATCTCGGTGGAGCAGAACACTTTGATCATCAGAGGTGAGGGCAAAAAGG
+ 675 AGGAGTTTGAAAACGATGAAGAGGTTGGTGGCAGAAGATTCTCGGGGAGGATCGATCTGC
CTGAGAAGCTGTACAAGGTGAATGACATTAAAGCTGAGATGAAGAATGGCGTCTTGAAGG
+ 795 TGTTTGTTCCCAAGACAAAGGATGAGGAGAGGAGTGATGTCTTCCATGTTAATGTTGAGT
AGTATAGTCGGTAATGATGAGTGCTACCGTTGGTAGACTACGGTGAAAATAATAGTGATG

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+ 915  TCCCTACTTTGGTAGATGAGTTGGGAATCTTCCTGCGTCGTAAATTCGTTACTTTCTTGG

       TTAAGCGATATCTACAGATCTGTTAAAAAAAAAAAAAA
```

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10 Eidesstattliche Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig und ohne unzulässige Hilfe angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. F. Salamini betreut worden.

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