

IMMUNOMODULATION OF CYTOSOLIC SMALL HEAT SHOCK PROTEINS
IN TRANSGENIC TOBACCO PLANTS

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Abbreviations

Aa – amino acid

ALP – alkaline phosphatase

BA – 6-Benzyladenine

BCIP – 5-Bromo-4-chloro-3-indolyl phosphate

BSA – bovine serum albumin

bp – base pairs

CaMV – cauliflower mosaic virus

DAPI – 4,6-Diamidino-2-phenylindole

DNA – deoxyribonucleic acid

ELISA – enzyme linked immunosorbent assay

ER – endoplasmic reticulum

g – gram

g – gravity constant

h – hour

HRP – horseradish peroxidase

HSG – heat stress granule, heat shock granule

IMAC – immobilized metal affinity chromatography

IPTG – isopropyl- β -D-1-thiogalactopyranoside

kD – kilo Dalton

KDEL – ER retention signal

l – litre

m - milli

M – molarity

MPBS – Marvel milk powder dissolved in PBS

min – minute

μ - micro

n - nano

NAA – 1-Naphtalene acetic acid

NBT – Nitro Blue Tetrazolium

OD – optical density

PCR – polymerase chain reaction

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

PEG – polyethyleneglycol

pNPP – 4-Nitrophenyl phosphate

RT – room temperature

rpm – rotations per minute

scFv – single chain fragment variable antibody

SDS – sodium dodecyl sulfate

TMB – 3,3',5,5'-Tetramethylbenzidine

v – version

WT – wild type

1. Introduction

1.1. Thermotolerance and heat shock response of plants

The heat stress is one of the common factors in nature taking influence on the plants during their lifetime. Plants, which have a sessile life style, can not avoid stress conditions when the temperature of environment is rising to sublethal or even lethal levels. However, plants have evolved specific features that allow to survive heat stress and to resist temperatures higher than their optimal life temperature to the level called basal thermotolerance. Temperatures, higher than basal thermotolerance are harmful or lethal. However, a short pre-exposition of plants to temperatures close to the lethal ones leads, after recovery period, to an enhanced thermoresistance of plants in comparison to basal thermotolerance. This higher level of induced heat resistance is called acquired thermotolerance. Numerous investigations of the plant response to elevated temperatures revealed that plant cells react to heat shock by reduction of housekeeping gene activity accompanied by reduced synthesis of proteins essential for vital cell functions (rev. Vierling, 1991). In response to heat stress the plant cell starts to synthesize a set of special proteins called heat shock proteins (HSPs) (rev. Vierling, 1991, rev. Waters et al., 1996).

HSPs synthesized in plants and in other eukaryotes have been divided into five classes according to the molecular mass of the proteins: HSP100, HSP90, HSP70, HSP40 and HSP20, termed small heat shock proteins (rev. Waters et al., 1996). Some of these proteins are also constitutively expressed (HSC) in the cell during optimal life conditions, and their synthesis becomes just enhanced by heat shock. Some of them are however exclusively induced during the heat shock. Actually not only heat stress can induce the biosynthesis of all or some HSPs in the plant cell but also such factors as heavy metals, glucose starvation, dehydration, chilling and oxidative stress (rev. Schöffl, 1998). All HSPs create the complex functional network of the heat shock response that enables the plant to survive elevated temperatures. The compilation of HSP sequences suggests that the major HSPs are highly homologous among eukaryotes and similar proteins have also been identified in prokaryotes. High evolutionary conservation of the heat stress response supports the argument that the stress-induced biosynthesis of HSPs is a fundamental component of cell life (rev. Vierling, 1991).

1.2. Gene families of plant sHSPs

Small HSPs (sHSPs) are the class of HSPs with a molecular mass of about 16-30 kDa. With some exceptions, sHSPs are not constitutively synthesized in plant vegetative tissue like HSP70 but they are among the most abundant proteins expressed in plant cells in response to heat stress. In contrast to all other eukaryotes sHSPs of higher plants are referenced by more than 20 different members divided to six different gene families (rev. Waters et al., 1996, Forreiter and Nover, 1998, Scharf et al., 2001). In other eukaryotes, there are only 2-4 representatives of sHSPs. For example, yeast and mammals have only one sHSP gene and four genes are found in *Drosophila* (Lindquist and Craig, 1988). Moreover, plants have not only cytoplasmic sHSPs but also organelle-located sHSPs. With the exception of mitochondria-localized sHSPs in animal cells, no organelle-located sHSPs have been reported outside the plant kingdom. Organelle-located sHSPs are nuclear-encoded and synthesized as cytosolic precursors showing posttranslational processing to their final size during import into the corresponding organelles. Differences between plant and other eukaryotes such as the existence of multiple sHSP gene families can be indicative of the evolutionary development of specific survival mechanisms.

Two gene families encoding cytoplasmic sHSPs of the plant cell are designated as cytosolic sHSP class I, class II (rev. Waters et al., 1996). The third gene family of plant cytoplasmic sHSPs designated as cytosolic sHSP class III was recently described (Scharf et al., 2001). Other families include the genes encoding sHSPs targeted to chloroplasts, mitochondria and to the endoplasmic reticulum (ER), respectively. The proteins encoded by one gene family are similar to each other even in different plant species. Similarity can be up to 93%, and identity up to 85% (Vierling, 1991). However, the proteins of one plant species belonging to different sHSP families show much less similarity – around 50-75%, and identity usually below 50% (Vierling, 1991). Comparisons between the corresponding nucleic acid sequences of sHSPs from different families show even lower percentages of homology.

There is no similar gene family structure for sHSPs of non-plant eukaryotes. Analysis of the relation between DNA sequences coding for animal and plant sHSPs suggests that formation of such a structure occurred only in the lineage of higher plants (Plesofsky-Vig et al., 1992; DeJong et al., 1993). The multiplicity of sHSP genes in higher plants probably evolved by way of gene duplications and subsequent

sequence divergence. Duplicated genes were driven by Darwinian selection to evolve the differentiation of function and intracellular localization (rev. Waters et al., 1996).

1.3. The molecular structure of plant sHSPs

The sHSPs of plants show conserved C-terminal regions homologous to the α -crystalline structural protein of the vertebrate eye lens (rev. Waters et al., 1996). This region is a highly conservative part of all sHSPs and consists of about 100 amino acid residues termed the “ α -crystalline domain” or “heat shock domain” (Plesofsky-Vig et al., 1992). The heat shock domain can be further subdivided into two regions, designated Consensus regions I and II, separated by a hydrophilic region of variable length (Czarneka et al., 1985; Lindquist and Craig, 1988). The N-terminal Consensus region I of plant sHSP (27 amino acids) consists of a conserved **Pro-X₍₁₄₎-Gly-Val-Leu** sequence, that can also be found in all sHSPs of other eukaryotes (Lindquist and Craig, 1988; rev. Vierling, 1991). Motif **Pro-X₍₁₄₎-X/Val/Leu/Ile-Val/Leu/Ile** appears in the 29 amino acid long C-terminal Consensus region II (rev. Vierling, 1991).

The N-terminal regions of plant sHSPs are divergent between different sHSP classes and have only consensus domains typical for sHSPs within the corresponding classes. The class I cytosolic sHSPs have a consensus region in the N-terminal part of the protein sequence (amino acids 107-120), whereas cytosolic class II sHSPs also have a small conserved region (amino acids 143-154) at the end of the N-terminus absent in other sHSP classes (Vierling, 1995).

A common feature of all plant sHSPs is the spontaneous formation of homooligomers, about 200-300 kDa in size, *in vivo* and *in vitro* (Lee et al., 1995; Jinn et al., 1995). Experiments with recombinant truncated sHSP showed that deletion of 15 amino acid residues of the N-terminal domain caused a dramatic reduction of the oligomer size (Leroux et al., 1997). It has therefore been suggested that the N-terminal part of sHSP polypeptide contains the region involved in oligomerization of sHSPs. Moreover, there are at least two sites of interaction between sHSP monomers (Merck et al., 1992; Merck et al., 1993): one is responsible for assembly of monomers to yield the oligomer and is located within the N-terminal domain and the other one is located in the C-terminal domain and allows the formation of only smaller oligomers. A single consistent model concerning sHSP quaternary structure is not yet at hand.

Probably, the large homooligomer complexes assemble cooperatively from the previously formed small oligomers (Leroux et al., 1997). In such a large oligomer complex, the N-terminal domain is buried in the oligomer unit whereas the C-terminal domain is exposed at the surface. All the subunits are in equivalent positions within the spherical aggregate (Leroux et al., 1997). Interestingly, experiments with a C-terminally truncated sHSP showed that deletion of the C-terminal region reduces solubility of the sHSPs oligomers but is without effect on oligomerization (Leroux et al., 1997). The C-terminal extensions of the sHSP molecule, located on the surface of the oligomer, probably increase the solubility and stability of the oligomer complex.

1.4. Putative functions of plant sHSPs

With some exceptions, cytoplasmic sHSPs are not constitutively expressed in vegetative plant tissues under normal conditions but their biosynthesis is preferentially induced by heat stress (rev. Waters et al., 1996). Accumulation of sHSPs starts rapidly in plant tissues in response to heat shock and proportionally to the temperature level and the duration of stress (rev. Waters et al., 1996). Maximal synthesis and accumulation of sHSPs are observed at temperatures just below lethal temperatures. Quantitative analysis reveals that the amount of sHSPs in relation to the total amount of leaf protein can reach 1% of the cytosolic proteins (De Rocher et al., 1991) or 0,02% of chloroplast proteins (Chen et al., 1990). sHSPs are quite stable after stress showing a half-life time of 30-50 h (Chen et al., 1990, De Rocher et al., 1991). This indicates that their function is highly important for processes taking place during the recovery period. Recent data suggest that one of the main functions of sHSPs as well as higher molecular weight HSPs in plant cells is molecular chaperoning. Molecular chaperones are proteins binding partially unfolded protein substrates and promote correct folding or prevent the aggregation or precipitation of denatured substrates. HSPs are thus able to prevent the accumulation and aggregation of heat-denatured proteins and to facilitate protein reactivation during the high temperature stress (Hendrick and Hartl, 1993).

It has been shown *in vitro* that both α -crystallin and sHSPs interact with partially unfolded polypeptides under heat stress conditions, thereby preventing unspecific aggregation of the protein substrates (Jacob et al., 1993). This leads to the

idea that the heat shock domain located at the C-terminal part of the sHSP molecule is responsible for binding of unfolded polypeptides.

The function of sHSPs is ATP-independent contrary to the chaperon activity of high-molecular members of the HSP family. HSP70 and HSP40 function as ATP-dependent chaperones that perform efficient refolding of unfolded proteins (Lee and Vierling, 2000). HSP90 is under discussion as a molecular chaperon involved specifically in processes of folding and maturation of several protein kinases and nuclear steroid hormone receptors (Scheibel et al, 1998). HSP100 probably disassembles already aggregated proteins (Schirmer et al., 1996).

Cytoplasmic sHSPs in cooperation with other HSPs commonly assemble to yield high-molecular-weight aggregates of about 1-2 MDa called heat stress granules (HSGs). HSG formation was observed in all plant cells including embryos and pollen under heat stress conditions (Nover et al., 1989). Probably, during the stress response HSGs function not only as a macromolecular chaperoning machine but also as a depot of HSPs, which dynamically disassemble from HSG during stress response to act as stand-alone factors. Regulatory elements of the plant heat-stress response such as transcription factors HsfA2, which are synthesized in the cell by heat induction in contrast to constitutively expressed HsfA1, also become incorporated into the HSG (Scharf et al., 1998). HSG complexes might also be a storage depot for mRNAs encoding housekeeping proteins that then might become available during the recovery period by HSG disassembly (Nover et al., 1983, Nover et al., 1989). At present it is controversially being discussed whether or not specific subsets of cellular mRNAs are also incorporated into the HSG complex (Nover et al., 1989, Smykal et al., 2000).

Every component of the HSG probably fulfills specific tasks in heat stress response. Plants have two classes of cytoplasmic sHSPs – class I and II. Their functional difference in the process of HSG formation during heat stress is under discussion. Both form stable homo-oligomers *in vivo* (Helm et al., 1997, Lee et al., 1997) and act as molecular chaperones (Löw et al., 2000). Recent data show that cytosolic class I and class II sHSPs take part in HSG formation not only by way of general aggregation to high molecular complexes but also as members of a specific assembly mechanism. This process primarily depends on the autoaggregation to homo-oligomers of cytosolic class II sHSP, which are then able to recruit sHSP class I to HSG formation. The formation of HSG takes place on three levels (Kirschner et al., 2000):

- formation of a homo-dimer complex of cytosolic class I and II sHSPs by interaction of two monomers of sHSP through their α -crystallin domains;
- integration of cytosolic class I and II sHSPs of plants to higher-order oligomers such as homo-dodecamers requiring structural information from their N-terminal and C-terminal extensions of their α -crystallin domains;
- formation of HSG complexes by cytosolic class I and II sHSPs of plants together with other components of the heat-stress response of plant cells.

The C-terminal domain of class II sHSPs and the formation of dodecamers are crucial for the heat stress-induced autoaggregation and thus for the recruitment of class I sHSPs into HSGs. Cytosolic class I and II sHSPs in HSGs are suspected to provide a binding surface for partially unfolded proteins to keep them in competent stage for refolding by interacting with HSP70, perhaps in complex with HSP40 (Forreiter et al, 1997, Lee and Vierling, 2000, Smykal et al., 2000). Nevertheless, the model of interaction between sHSPs and high molecular mass HSPs during heat shock is still under discussion. At present, neither the structure/function relationships between sHSP oligomers and HSGs during the heat stress response and recovery are exactly understood nor is it clear which of the sHSP classes are involved in the processes of basal and acquired thermotolerance.

1.5. Developmentally regulated expression of plant sHSPs

Developmentally regulated expression of sHSP genes during embryogenesis independent of an environmental stress was found in animals and recently also in plants (rev. Waters et al., 1996). Expression of both cytosolic class I and class II sHSPs was demonstrated at the mRNA and protein level in maturing embryos of pea, wheat, sunflower, alfalfa, *Arabidopsis*, tobacco, maize and tomato (rev. Waters et al., 1996). In addition to the expression during embryogenesis, developmentally regulated expression of sHSPs was also detected during pollen development and microspore embryogenesis (Prändl et al., 1995). Furthermore, sHSPs are found in storage organs such as bulbs of *Allium*, *Amaryllus*, *Crocus* and *Hyacinthus*, tendrils of *Aristolochia* and twigs of *Acer* and *Sambucus* during dormant stages (zur Nieden and Neumann, personal communications).

In embryos of pea class I and class II sHSPs appear during the biosynthesis of storage proteins at mid-maturation and increase in abundance as the seed dehydrates

(De Rocher and Vierling, 1994). In sunflower seeds similar accumulation of class II sHSP was found but class I sHSPs accumulated only during late seed maturation (Coca et al., 1994). In *Arabidopsis* seeds class I sHSPs accumulate at mid-maturation and decline during germination (Wehmeyer et al., 1996). In axes of developing pea seeds, class I sHSPs are firstly detected in the period of seed abscission from the pericarp, which coincides with the onset of desiccation. Both sHSP mRNA and protein are present in dry seeds of sunflower and pea. sHSPs are also detected in pea seeds during germination for 2 to 3 days after emergence of the radicle (De Rocher and Vierling, 1994) and for 2 to 3 days in sunflower seeds (Coca et al., 1994). Thereafter, sHSPs are quickly degraded. Such patterns of sHSP expression and degradation may be of specific advantage and seem to be similar in different plant species. Probably class I sHSPs are involved in acquisition of desiccation tolerance of seeds and maintenance of dormancy during seed development or they take part in seed dehydration during germination.

In contrast to other classes of sHSPs and particularly to class I sHSP there is limited information about accumulation and function of class II sHSP. Studies in lily and maize show that class II sHSP mRNAs are absent before meiosis. But HSP mRNAs significantly accumulate in meiotic prophase during pollen development and are present in tetrad microsporocytes (Bouchard, 1990, Kobayashi et al., 1994). Class II sHSP genes are transcribed during pollen development but their mRNAs are absent in mature pollen and only weakly induced during pollen germination (Hopf et al., 1992). In contrast, class I mRNAs have been detected only during maturation of the pollen from the bicellular stage to the mature pollen grain (Zarsky et al., 1995). Such data indicate that cytosolic class I and class II sHSPs take part in plant developmental processes as components with certain regulatory functions (zur Nieden et al., 1995). However, their specific functions in plant development are still unknown and need further investigations.

1.6. Methodical tools to study the functions of plant sHSPs

In vitro systems were used to learn more about the molecular chaperone activity of sHSPs. The principle of such systems is to simulate conditions of physiological stress temperatures for thermosensitive proteins and to investigate the impact of sHSPs on their heat-induced denaturation or aggregation *in vitro*.

In vitro studies of α -crystallins and sHSPs provided evidence that both of them show ATP-independent molecular chaperone activity. Thermal aggregation of several proteins was prevented in the presence of α -crystallin at a ratio of about 20 substrate molecules per crystallin oligomer. Recombinant mammalian sHSPs prevented heat-induced aggregation of proteins *in vitro* and increased the half-life time of heat-inactivated α -glucosidase. They also increased the amount of active citrate synthase and α -glucosidase after dilution from denaturants (Horwitz, 1992, Jakob et al., 1993).

In order to proof the protecting functions of plant sHSPs against the heat-induced aggregation of proteins, an enriched fraction of sHSPs from soybean was used. The sHSP fraction was mixed with total protein extract and heated to 55⁰C. 50% of the proteins were protected from heat-induced aggregation (Jinn et al., 1989, 1995). Results were verified by later experiments using purified recombinant plant cytosolic class I and class II sHSPs produced in bacterial expression systems. The experiments revealed *in vitro* ATP-independent molecular chaperoning activity of PsHSP18.1 (class I) and PsHSP17.1 (class II) from *Pisum sativum* concerning refolding of chemically denatured citrate synthase (Lee et al., 1995).

In vivo systems were also introduced to support the data about chaperon functions of sHSPs. The generation of a transgenic *Arabidopsis* cell line with a high level of firefly luciferase expression was reported as an appropriate *in vivo* system for analysis of sHSP chaperone activity (Forreiter et al., 1997). Convenient genetic manipulation of this cell line by generation of transgenic protoplasts and following PEG-mediated transient transformation with chaperone-encoding plasmids provided a possibility for *in vivo* testing of individual HSPs including sHSPs and combinations of them in a plant cell system. Inactivation of luciferase at 41⁰C with a half-life time of 6-7 min can be compared between control cells and cells with transiently expressed heat stress proteins prior to luciferase denaturation (Forreiter et al, 1997, Forreiter and Nover, 1998). Therefore this system was used to show *in vivo* molecular chaperoning by cytosolic class I Hsp17.6 from *Arabidopsis thaliana* (Forreiter et al., 1997) and later by cytosolic class I Hsp17.7 and class II Hsp17.3 from *Lycopersicon peruvianum* (L w et al., 2000).

Transgenic plants with overexpression of truncated sHSP gene sequences are models to study *in vivo* oligomerization of sHSP and the role of different domains in

this process as well as in molecular chaperoning function (Guo and Cooper, 2000). However, the overexpression of full-length sHSP genes in transgenic plants and inhibition of stress-induced sHSPs by using antisense RNA approaches in transgenic plants had also no effects on the phenotypical alterations of transgenic plants under heat stress conditions (Schöffl et al., 1987). Both methods were ineffective because sHSPs are encoded by a multigene family in plants.

Using genetically engineered *Arabidopsis* plants as a model, regulatory mutations of heat shock transcription factors (HSF) with constitutive synthesis of HSPs were successfully generated (Lee et al., 1995; Prändl et al., 1998). Such regulating mutations can influence the HSP biosynthesis but did not permit to investigate the function of individual HSPs.

Plant mutants that lack stress-response functions could provide a convenient material to investigate the role of sHSPs in stress response and thermotolerance of the plant cell. At present such mutants were found and studied only for high-molecular HSPs (Ludwig-Müller et al., 2000; Burke et al., 2000). But no plant mutants with constitutively repressed or non-stress-inducible sHSP genes were found until now.

Therefore we decided to create a new model to investigate the functions of sHSPs using *in vivo* immunomodulation of sHSP by expression of sHSP-specific single chain fragment variable antibodies in the cells of transgenic plants.

1.7. Immunomodulation of regulatory proteins in plant cells

Immunomodulation is a molecular technique that allows to inhibit or to modulate the functions of corresponding antigens by intracellular ectopic expression of specific antibodies or antibody fragments *in vivo*. The modulation is specifically due to interaction of antibody and antigen by formation of antigen-antibody complexes. Antibody binding can interfere with enzyme-substrate interaction, changes quaternary structure of an antigen, causes allosteric inhibition of active protein, or the antibody binds to substrate or ligand itself (rev. De Jaeger et al., 2000). The problem of a correct assembly of light and heavy chains of antibody by formation of disulphide bridges at ectopic expression in transgenic cells can be resolved by using single chain fragment variable (scFv) antibodies. These genetically engineered antibodies, consisting of variable light and variable heavy chain domains connected by a flexible short linker polypeptide, can be expressed intracellularly without specific requirements for chain assembly as necessary to form a complete immunoglobulin.

ScFvs retain full antigen-binding specificity and activity (rev. Conrad and Fiedler, 1998).

Creation of phage scFv libraries of human or animal variable immunoglobulin gene repertoires using RT-PCR and phage display technology simplified the isolation of specific scFv cDNA sequences (Kramer, 1998). cDNA sequences coding for scFvs can be fused with corresponding target peptides for compartment-specific localization of the scFv in transgenic cells to modulate the corresponding target antigen in different cell compartments as cytosol, ER, chloroplasts and apoplastic space (rev. Conrad and Fiedler, 1998).

In order to study physiological and developmental processes of plants, several strategies are used to analyze the functional activities of regulatory compounds in metabolic or signal pathways. As molecular biological methods, sense and antisense RNA approaches and RNA-mediated interference are useful tools to affect the target gene in the cells of transgenic organism to create mutants. However, these methods are not successful in all cases, require special promoter activities with respect to organ-, tissue- or cell-specificity, and are not suitable for compartment-specific functional analysis or functional investigations of protein encoded by multigene families. In contrary, scFv can be expressed temporally and spatially regulated. Additionally, scFvs specific to gene products encoded by whole gene family are able to immunomodulate functional activities of all encoded proteins, whereas scFv directed specifically against one member of the gene family will inhibit individual isoforms (rev. Conrad and Manteuffel, 2001).

Originally developed for human cells (rev. Marasco, 1995, Cardinale et al., 1998, rev. Cattaneo and Biocca, 1999), immunomodulation was also successfully used to study the functions of the phytohormones abscisic acid and gibberellin in transgenic plants (Artsaenko et al., 1995, Shimada et al., 1999, rev. Conrad and Manteuffel, 2001) and plant regulatory receptor protein phytochrome (Owen et al., 1992). Furthermore, expression of scFvs specific to viral protein in transgenic plants was successfully used for genetic engineering of plant viral resistance (Tavladoraki et al., 1993, Voss et al., 1995).

1.8. The aim of current work

Cytosolic small heat shock proteins are important elements in the complex network of heat shock response in plant cells. They also take part in developmental processes.

The study of cytosolic sHSPs as stand-alone factors and as members of such macromolecular complexes as HSGs allowed to collect a lot of data but mechanisms of their individual functions during stress response and recovery period are still unclear and need further investigation.

In the present study we use single chain variable fragments (scFv) of antibodies that bind specifically to their corresponding target antigen and immunomodulate its function *in vivo*. Therefore, cytoplasmic expression of scFvs with specificity to cytosolic sHSPs in the plant cell are used to analyze the function of sHSP and HSG in processes of basal and acquired thermotolerance.

We perform screening of a scFv phage library with recombinant class I HSP17 from *Lycopersicon esculentum* to select phage clones with scFvs, which are able to recognize the HSP17 target. ScFvs selected with class I HSP17 from tomato will also recognize sHSPs in tobacco cells because of the phylogenetic relation between *L.esculentum* and *N.tabacum* and high similarity of sHSP protein sequences from different plant species of the same family and the same class of sHSP. cDNA sequences encoding the corresponding anti-HSP17 scFvs could be isolated from the selected phages and used to design expression cassettes sufficient for plant transformation with respect to stable overexpression of scFvs in the cytoplasm of plant cells. The cauliflower mosaic virus 35S (35S CaMV) promoter is used to control the ubiquitous expression of scFvs in transgenic plants. The transgenic plants with stable ubiquitous anti-HSP17 scFv expression are used to investigate the effects of immunomodulation of cytosolic sHSPs on the processes of heat stress response of transgenic plants.

We design a recombinant construct for overexpression of anti-HSP17 scFvs under the 35S CaMV promoter control with targeting to the ER. Since cytosolic sHSPs are also found in the protein storage organelles of tobacco seeds, the functions of cytosolic sHSPs during seed development could be investigated.

Ultrastructural analysis by use of immunofluorescence, will be performed to localize the distribution of anti-HSP17 scFv and sHSPs in the cells of transgenic and wild type plants at normal temperature and during heat stress response. The level of basal and acquired thermotolerance of transgenic and control plants will be compared using isolated leaves incubated at different heat stress regimes. Electron microscopic analysis will be performed to compare the intracellular changes in transgenic and control cells during the heat shock response.

2. Materials and Methods

2.1. Materials

2.1.1. Bacterial strains and Phages

Escherichia coli:

BL21 (Studier and Moffat, 1986) *HsdS gal (λI_{tc}857 ind1 Sam7 nin5 lacUV5-T7 gene 1)*

TG1 (Gibson, 1984) *SupE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB⁺ lacI^q lacZΔM15]*

HB2151 (Pharmacia Biotech) *K12, ara, Δ (lac-pro), thi/F, 'pro A⁺B⁺, lacI^q, lacZΔM15*

Agrobacterium tumefaciens:

C58C1 R^f (pGV2260 in C58C1)

Phages:

M13KO7 helper phage Km^r (Amersham Pharmacia Biotech, Uppsala, Sweden)

2.1.2. Phage library

Human Single Framework scFv Libraries, Tomlinson A+B (Tomlinson, T., MRC, Center for Protein Engineering, Cambridge, UK)

2.1.3. Phagemids and Vectors

pIT1	amp ^r
pRTRA7/3 (Artsaenko, 1996)	amp ^r
pTRX (Askari, 1999)	amp ^r
pBIN19 (Bevan, 1984)	km ^r

2.1.4. Plant material

Nicotiana tabacum cv. Samsun NN

2.1.5. Oligonucleotide primers

2.1.5.1. Oligonucleotide primers for PCR amplification

NOT: 5' – TTT TTG TTC TGC GGC CGC CC – 3'

BAM1: 5' – CAG CCG GCC GGA TCC CGA GG – 3'

BAM2: 5' – AAC AGT CTA TGC GGA TCC AT – 3'

2.1.5.2. Oligonucleotide primers for DNA sequencing

LINK: 5' – CGA CCC GCC ACC GCC GCT G – 3'

PHEN: 5' – CTA TGC GGC CCC ATT CA – 3'

KDEL: 5' – GATTTTTGCGGACTCTATCGACGG – 3'

2.1.6. Media

2.1.6.1. Media for plant culture

MS medium (Murashige and Scoog, 1962):

4.49 g l⁻¹ Murashige and Scoog medium basal salt mixture including vitamins and microelements (Duchefa, Haarlem, The Netherlands)

30 g l⁻¹ sucrose

pH 5.8

1% agar (Difco, Detroit, USA) for solid medium

Sterilized by autoclaving.

2.1.6.2. Media for bacterial culture

LB medium:

5 g l⁻¹ Yeast extract (Difco)

10 g l⁻¹ Tryptone (Difco)

10 g l⁻¹ NaCl

10 g l⁻¹ agar (Difco) for solid medium

pH 7.0

Sterilized by autoclaving

SOC medium:

5 g l⁻¹ Yeast extract (Difco)

20 g l⁻¹ Tryptone (Difco)

0.5 g l⁻¹ NaCl

0.186 g l⁻¹ KCl

20 mM glucose

pH 7.0

Sterilized by autoclaving

TYE medium:

10 g l⁻¹ Tryptone (Difco)

5 g l⁻¹ Yeast extract (Difco)

8 g l⁻¹ NaCl

pH 7.0

Sterilized by autoclaving

2×TY medium:

16 g l⁻¹ Tryptone (Difco)

10 g l⁻¹ Yeast extract (Difco)

5 g NaCl

pH 7.0

10 g l⁻¹ agar (Difco) for solid medium

Sterilized by autoclaving

YEB medium:

5 g l⁻¹ Beef extract (Difco)

1 g l⁻¹ Yeast extract (Difco)

5 g l⁻¹ Peptone (Difco)

5 g l⁻¹ Sucrose

2 mM MgSO₄

pH 7.2

Sterilized by autoclaving

2.1.7. Enzymes

NcoI (GIBCO, Karlsruhe, Germany)

NotI (GIBCO)

BamHI (GIBCO)

HindIII (GIBCO)

Shrimp Alkaline Phosphatase (Amersham Pharmacia Biotech)

Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA)

2.1.8. Antibiotics

Ampicillin (ROTH, Karl Roth GmbH & Co, Karlsruhe, Germany)

Kanamycin (Duchefa)

Rifampicin (Duchefa)

Carbenicillin (Duchefa)

Ticarcillin (Claforan, Hoechst AG, Frankfurt, Germany)

2.1.9. Immunochemicals

Anti-polyHistidine monoclonal antibody, clone HIS-1 (Sigma, Deisenhofen, Germany)

Anti-c-myc monoclonal antibody, clone 9E10, (I.Tillack, Research Group Phytoantibodies, IPK, Gatersleben, Germany)

Anti-M13 monoclonal antibody HRP conjugate (Amersham Pharmacia Biotech)

Anti-mouse IgG alkaline phosphatase conjugate (Sigma)

Anti-mouse IgG horse radish peroxidase conjugate (Sigma)

Anti-rabbit IgG biotinylated goat antibody (DAKO Diagnostica, Hamburg, Germany)

Anti-rabbit IgG alkaline phosphatase conjugate (Boehringer Mannheim, Mannheim, Germany)

Anti-tobacco globulin antibody (Dr. R.Manteuffel, Research Group Serology, IPK, Gatersleben, Germany)

Anti-HSP17 antibody (Dr. R.Manteuffel, Research Group Serology, IPK, Gatersleben, Germany)

2.1.10. Molecular markers, kits and other biochemicals

Desoxynucleoside Triphosphate Set (Boehringer Mannheim)

DNA Smart Ladder (Eurogentech, Seraing, Belgium)

0.025 µm Dialysis filter VS (Millipore Corporation, Bedford, MA, USA)

ECL Kit (Amersham Pharmacia Biotech)

Flexiprep Kit (Amersham Pharmacia Biotech)

Gene Images CDP-Star Detection Module (Amersham Pharmacia Biotech)

Gene Images Random Prime Labeling Module (Amersham Pharmacia Biotech)

Hybond N+ nitrocellulose membrane (Amersham Pharmacia Biotech)

Hyperfilm ECL (Amersham Pharmacia Biotech)

MicroSpin S-200 Columns (Amersham Pharmacia Biotech)
Ni-NTA Spin Kit (QIAGEN GmbH, Hilden, Germany)
QIAquick Gel Extraction Kit (QIAGEN)
QIAquick PCR Purification Kit (QIAGEN)
Protein L agarose (AC Tigen, Oslo, Norway)
Rapid DNA Ligation Kit (Roche, F. Hoffmann-La Roche Ltd, Basel, Switzerland)
RediPrime Kit (Amersham Pharmacia Biotech)
See Blue Pre-Stained Standards Protein Marker (Novex, Groningen, The Netherlands)
Triethoxysilylpropylamine (Merck, KgaA, Darmstadt, Germany)
TSA-Direct-Green (Tyramid Signal Amplification Systems, Du Pont, NEN, Boston, Massachusetts, USA)

2.1.11. Buffers

TAE-buffer:

0.04 M Tris-acetate

0.001 M EDTA

pH 8.0

Tris-Saline buffer:

0.01 M Tris-HCl

0.15 M NaCl

pH 7.4

Alkaline phosphatase substrate buffer:

100 mM Tris-HCl

100 mM NaCl

5 mM MgCl₂

pH 9.5

20×SSC buffer:

175.3 g l⁻¹ NaCl

88.2 g l⁻¹ Sodium citrate

pH 7.0 adjusted with 10 M NaOH

Church buffer (Church and Gilbert, 1984):

0.5 M Sodium phosphate buffer (0.5 M NaH₂PO₄ and 0.5 M Na₂HPO₄) pH 7.0

2 mM EDTA

1% BSA

7% SDS

2.1.12. Laboratory equipment and other materials

ELISA reader MR 7000 (Dynatech Burlington, Massachusetts, USA)

Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA)

Agarose gel electrophoresis camera Owl (Owl Separation Systems, Portsmouth, NH, USA)

Power Supply Consort E132 (PEQLAB Biotechnologie GmbH, Erlangen, Germany)

Water Bath GFL 1012 (Schütt Labortechnik, Göttingen, Germany)

Electron microscope EM912OMEGA (Zeiss, Oberkochen, Germany)

PCR amplification UNO-Termoblock (Biometra, Göttingen, Germany)

ALF DNA Sequencer (Amersham Pharmacia Biotech)

2.2. Methods

2.2.1. Preparation of recombinant HSP17 in the bacterial expression system

For phage panning (2.2.2.3.) and monoclonal phage ELISA (2.2.2.5.), recombinant class I HSP17 from *Lycopersicon esculentum* expressed in *E.coli* was used as antigen.

Transformed *E.coli* BL21 cells containing the expression plasmid vector with the cDNA insert encoding the corresponding HSP17, tagged at the C-terminus by 6×His (pQE92), was received from Alexander Bucka (IPB, Halle, Germany).

200 µl overnight culture of *E.coli* BL21, grown in 2×TY medium supplemented with 100 µg ml⁻¹ ampicillin, were added to 100 ml 2×TY Broth containing 100 µg ml⁻¹ ampicillin and incubated for 5 hours at 37°C with shaking. IPTG was added to a final concentration of 1mM for induction of lac-promoter. The culture was incubated for 5 h at 37°C with shaking. Bacterial cells were collected by 10 min centrifugation at 3000 rpm, 4°C.

The expressed recombinant protein containing the C-terminal 6×His tag was purified after cell lysis under denaturing conditions using the Ni-NTA Spin Kit (QIAGEN) according to producer's instructions. Protein eluted with urea under acidic condition was dialysed against PBS in colloidium bags (Sartorius) overnight. The protein concentration of dialysed HSP17 was measured according to Bradford (Bradford, 1976). Gel electrophoretic analysis of the HSP17 preparation was performed on 12.5% SDS polyacrylamide gel according to Laemmli (Laemmli et al., 1970). One part of the gel was stained with Coomassie Blue (Sambrook et al., 1989), the other part was used for Western blot analysis (Sambrook et al., 1989). The recombinant His-tagged protein was detected on Western blot by using anti-His antibodies (Sigma) in a dilution of 1:1000 and anti-mouse IgG conjugated to alkaline phosphatase. The recombinant HSP17 was also identified by use of the anti-sHSP antibody diluted 1:1000 followed by anti-rabbit IgG conjugated to alkaline phosphatase. Color reaction was performed by staining with NBT/BCIP as substrate.

2.2.2. Selection of HSP17-specific display phages from the Phage display libraries

2.2.2.1. Growing of the phage libraries

For phage display library selection of scFvs specific to HSP17 Human Single Framework scFv Libraries A+B (Tomlinson, MRC, University of Cambridge, UK) were used. Screening of libraries A and B was done simultaneously under the same conditions.

Libraries were grown after dilution of an aliquot of library stock in 500 ml 2×TY containing 100 µg ml⁻¹ ampicillin and 1% glucose and shaking at 37⁰ C until OD₆₀₀ was 0.4 (1-2 h). 2.5×10¹¹ helper phage M13KO7 were added to 50 ml of the bacterial culture. After incubation at 37⁰ C for 30 min, the cells were spinned at 3000 *g* for 10 min. Cell pellet was resuspended in 500 ml 2×TY containing 100 µg ml⁻¹ ampicillin and 50 µg ml⁻¹ kanamycin and incubated by shaking overnight at 30⁰ C. Overnight culture was spinned at 10000 *g* for 15 min. Phage particles were precipitated from the supernatant by polyethylene glycol/NaCl (20% polyethylene glycol 6000, 2.5M NaCl). 100 ml PEG/NaCl solution were added to 400 ml of supernatant, mixed and incubated 1h on ice. The mixture was spinned at 10000 *g* for 30 min. The pellet was resuspended in 8 ml TE buffer, followed by supplementation

with 2 ml PEG/NaCl, mixed, incubated on ice for 20 min and spun at 3000 *g* for 30 min. The pellet was resuspended in 5 ml PBS. The solution was cleaned from the bacterial cell debris by centrifugation at 11000 *g* for 10 min.

2.2.2.2. Production of helper phage.

200 μ l of the *E.coli* TG1 culture (OD₆₀₀ of 0.2) were infected with 10 μ l of 100-fold serial dilutions of helper phage M13KO7, incubated at 37⁰ C for 30 min, and plated on TYE medium for overnight plaque growth at 37⁰ C. Small plaques were picked individually into 5 ml of exponentially growing *E.coli* TG1 cultures and incubated with shaking for 2 h at 37⁰C. Phage-infected TG1 culture was diluted with 500 ml of 2 \times TY and grown with shaking at 37⁰ C for 1 h. Thereafter, kanamycin was added to a final concentration of 50 μ g ml⁻¹ and the culture was incubated with shaking overnight at 30⁰C. Cells were spun down at 10 000 *g* for 15 min. Phage particles were precipitated from the supernatant by 100 ml of PEG/NaCl solution and incubated on ice for 1 h. Precipitated phage particles were collected by centrifugation at 10000 *g* for 30 min and then resuspended in 8 ml TE buffer. Phage precipitation was repeated by supplementation with 2 ml of PEG/NaCl solution to the phage suspension in TE buffer on ice for 20 min and the phages were collected by centrifugation at 3000 *g* for 30 min. The phage pellet was resuspended in PBS and respinned at 10000 *g* for 10 min to clean the phage particles from bacterial cell debris. The helper phage particles were resuspended in PBS with 15% glycerol for storage at -70⁰ C.

Titration of the phage stock was done by infection of 1 ml *E.coli* TG1 culture (OD₆₀₀ of 0.4) with 1 μ l of 1:1000 phage stock dilution in PBS. 50 μ l of the infected culture and 50 μ l of its dilutions 1:10², 1:10⁴ and 1:10⁶ with TYE were plated on solid TYE medium containing 50 μ g ml⁻¹ kanamycin and 1% glucose. Phage plaques were counted after overnight incubation at 37⁰ C.

2.2.2.3. Phage panning

Three rounds of panning were done by using solid phase-fixed recombinant HSP17 antigen (2.2.1.) to select HSP17-specific phages.

Immunotubes were coated with 4 ml of 50 μ g ml⁻¹ antigen solution in PBS and incubated at room temperature overnight. Tubes were washed 3 times with PBS and

treated with 2% MPBS at room temperature for 2 h in order to block the free binding sites of the tubes. After blocking, tubes were washed 3 times with PBS, loaded with 10^{12-13} phage particles in 4 ml of 2% MPBS and incubated for 30 min at room temperature and rotation. The tubes were washed 20 times with PBS containing 0.1% Tween 20. Phage particles were eluted by adding 1 ml 100mM triethylamine and rotation for 10 min. The solution containing eluted phages was neutralized by supplementation with 500 μ l 1M Tris pH 7.4. Eluted phage particles were amplified by infection of *E.coli* TG1 for the next round of phage selection. 9 ml of TG1 fresh culture at OD₆₀₀ of 0.4 were infected by 750 μ l of eluted phage particles and incubated 30 min at 37⁰ C without shaking. For phage titration 50 μ l of the stock culture and its dilutions (1:10², 1:10⁴, 1:10⁶) were plated on TYE medium containing 100 μ g ml⁻¹ ampicillin and 1% glucose. Non-infected TG1 culture grown in parallel was used as a negative control. The infected culture was spinned at 3000 **g** for 10 min. Cell pellet was resuspended in 1 ml of 2 \times TY medium and plated on a Bio-Assay dish containing TYE with 100 μ g ml⁻¹ ampicillin and 1% glucose for overnight growth at 37⁰ C. Colonies were scraped with a glass spreader from the agar surface by using 5 ml 2 \times TY supplemented with 15% glycerol. 50 ml of 2 \times TY containing 100 μ g ml⁻¹ ampicillin and 1% glucose were inoculated by 50 μ l of scraped bacterial suspension and grown with shaking at 37⁰ C to OD₆₀₀ of 0.4. 5×10^{10} helper phage particles were added to 10 ml of the bacterial culture and incubated without shaking for 30 min in a 37⁰ C water bath. Cells were spinned at 3000 **g** for 15 min, resuspended in 50 ml 2 \times TY containing 100 μ g ml⁻¹ ampicillin and 50 μ g ml⁻¹ kanamycin and incubated with shaking at 30⁰ C overnight. Cells were spinned at 3000 **g** for 15 min. Phages were precipitated from 40 ml of supernatant by 10 ml PEG/NaCl and incubated for 1 h on ice. Then the mixture was spinned at 3000 **g** for 30 min. The pellet containing phage particles was resuspended in 2 ml PBS and spinned at 11000 **g** for 10 min to remove the bacterial debris. For the next round of selection 1 ml of the received phage stock solution was used. Two additional rounds of selection were done by using the same experimental procedure.

2.2.2.4. Amplification of individual phages

After the third panning round individual colonies from the titration plates were selected and inoculated into 100 μl 2 \times TY medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin and 1% glucose. The 96-well microtiter plates were incubated with shaking (300 rpm) overnight at 37⁰ C. Plates were used to prepare glycerol stocks of individual clones by adding glycerol to a final concentration of 15% for storage at -70⁰ C.

2.2.2.5. Monoclonal phage ELISA

From the 96-well microtiter plate containing in each well individual phage clones grown after the third round of selection about 2 μl of overnight cultures were transferred to a second plate containing 200 μl of 2 \times TY medium supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin and 1% glucose per well. The plate was incubated with shaking (300 rpm) at 37⁰ C for 1 h. After 1 h incubation 20 μl of 2 \times TY medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin, 1% glucose and 10⁹ helper phage M13KO7 particles were added per well. The plate was incubated again for 30 min at 37⁰ C and then shaken (300 rpm) at 37⁰ C for 1h. The plate was centrifuged at 1800 *g* for 10 min and the individual supernatants were collected for phage ELISA. For antigen coating the wells of the 96-well microtiter ELISA plate were incubated with 100 μl of 20 $\mu\text{g ml}^{-1}$ recombinant HSP17 solution in PBS overnight at RT. The plate was washed 3 times with PBS. For blocking, each well of the plate was loaded with 200 μl of 2% MPBS and incubated for 2 h at RT. After washing 3 times with PBS, 10 μl of phage solution from the last phage panning round were added to 100 μl of 2% MPBS per well and incubated for 90 min at RT. Thereafter the plate was washed 5 times with PBS containing 0.05% Tween 20. For detection of the phage particles bound specifically to the coated antigen, 100 μl of 1:5000 diluted anti-M13 antibody, conjugated to HRP in 2% MPBS, were loaded per well, incubated 90 min at RT and washed 5 times with 0.1% Tween 20 in PBS. 100 $\mu\text{g ml}^{-1}$ TMB in 100mM sodium acetate, pH 6.0 was used as substrate for color reaction. 10 μl of 30% hydrogenperoxid were added to 50 ml of substrate solution immediately before use. The plate was incubated at RT until blue color development (about 10 min). Then the reaction was stopped by adding 50 μl of 1M sulfuric acid per well. OD at 650 nm and at 450 nm was measured by using the ELISA reader and the difference between

OD₆₅₀ and OD₄₅₀ was calculated. Results were compared to choose phage clones with high binding to the HSP17 antigen using Microsoft Excel 97.

2.2.3. Preparation of soluble scFvs from display phages

2.2.3.1. Preparation of competent *E.coli* cells

E.coli strain HB2151 (Amersham Pharmacia Biotech) glycerol stock culture was used to inoculate 2 ml of LB medium for overnight growth. 500 µl of overnight culture were diluted with 100 ml of fresh LB medium and incubated at 37⁰C with shaking at 300 rpm till OD₆₀₀ reached 0.8. The cells were spun down at 3000 *g* for 10 min at 4⁰C and resuspended in 10 ml of ice-cold sterile 0.1 M CaCl₂ solution. After centrifugation at 3000 *g* for 10 min at 4⁰C, the cells were resuspended in 2 ml of ice-cold sterile 0.1 M CaCl₂ containing 15% glycerol. The stock solution was subdivided into 50 µl aliquots for storage at -70⁰C.

2.2.3.2. Transformation of competent *E.coli* cells

Stored competent cells were refrozen on ice. DNA samples or aliquots of a ligation mixture (10 µl), chilled on ice, were added to competent cells, mixed and incubated for 30 min on ice. The tubes were transferred to 42⁰C for exactly 90 sec and rapidly transferred to ice for 1-2 min. 800 µl of SOC medium were added to each tube and incubated at 37⁰C for 45 min to allow bacterial cells to recover and to express the antibiotic marker encoded by the plasmid. Aliquots of transformed cells (up to 200µl) were transferred onto LB agar containing the selective antibiotic and grown overnight at 37⁰C.

2.2.3.3. Preparation of soluble scFvs

Phagemid pIT1 was produced as a plasmid in bacterial cells growing on medium with low amount of glucose. Therefore, glycerol cultures of individual clones (see 2.2.2.) were inoculated into 1.5 ml 2×TY containing 100 µg ml⁻¹ ampicillin and 0.1% glucose to grow overnight with shaking at 37⁰ C. Plasmid DNA was purified from overnight bacterial culture by using the FlexiPrep Kit (Amersham Pharmacia Biotech). *E.coli* HB2151 competent cells (2.2.3.2.) were transformed with plasmid DNA from different clones.

After transformation, single colonies from each clone were inoculated into 1.5 ml 2×TY containing 100 µg ml⁻¹ ampicillin and 0.1% glucose and grown overnight with shaking at 37⁰ C. From these overnight cultures about 2 µl were transferred to 200 µl of the same medium in a 96 well microtiter plate and incubated 3 h with shaking at 37⁰ C, until OD₆₀₀ was approximately 0.9. For expression of soluble scFv, 25 µl 2×TY containing 100 µg ml⁻¹ ampicillin and 9 mM IPTG were added per well to a final IPTG concentration of 1 mM. The incubation was continued with shaking (300 rpm) at 30⁰C overnight. Plate with overnight cultures was centrifuged at 1800 *g* for 10 min. 50 µl of the culture supernatant were used as primary antibody to perform scFv ELISA (2.2.3.3.). Simultaneously a glycerol stock was prepared by adding glycerol to a final concentration of 15% and stored at -70⁰ C.

2.2.3.4. ScFv ELISA

Wells of a 96 well microtiter plate were coated with 100µl of 20 µg ml⁻¹ recombinant HSP17 in PBS overnight and washed two times with PBS. For blocking, 100 µl MBPS were loaded per well and incubated for 3 h at RT. Thereafter the plate was washed 3 times with PBS. 50 µl of supernatant containing soluble scFv (2.2.3.) were loaded per well and incubated 90 min at RT. The plate was washed 5 times with PBS containing 0.05% Tween 20 and two times with PBS to remove unbound scFv. 200 µl of monoclonal anti-c-myc antibody 9E10, diluted 1:30, were loaded per well and incubated for 1 h. Plate was washed 3 times with PBS containing 0.05% Tween 20 and two times with PBS. 100 µl of anti-mouse IgG conjugated to ALP diluted 1:2000 were loaded per well and incubated for 1 h. The plate was washed 3 times with PBS containing 0.05% Tween 20 and two times with PBS.

For signal generation, 100 µl of 1 ng ml⁻¹ pNPP solution in alkaline phosphatase substrate buffer were added per well and incubated for 1 h at 37⁰ C. Signals were measured at 405 nm using an ELISA reader. The results were analyzed by Microsoft Excel 97.

2.2.3.5. Sequence analysis of scFv cDNA

The scFv cDNA encoded by plasmid pIT1 and purified as described in 2.2.3.3. was sequenced by use of biotin-labeled primers. For sequencing of cDNA encoding

variable regions of the heavy chains primer LINK, complementary to the linker region of DNA encoding scFv was used. For sequencing of variable regions of light κ -chains, primer PHEN complementary to the DNA sequence of the c-myc tag sequence in pIT1 plasmid was used. Sequence analysis was done by Susanne König (IPK, Gatersleben, Germany) with an automatic ALF DNA sequencer (Amersham Pharmacia Biotech).

2.2.4. Construction of expression cassettes for plant transformation

All the cloning procedures were performed according to standard techniques described by Sambrook et al. (1989). The plasmid pRTRA7/3 (Artsaenko, 1996) containing the 35S CaMV promoter, the legumin B4 signal peptide sequence, the anti-ABA scFv gene sequence, c-myc tag, the ER retention sequence KDEL and poly-A sequence and the plant binary vector pBIN19 were used to construct the recombinant DNA for anti-HSP17 scFv-c-myc-KDEL fusion expression in plants. Expression cassettes were constructed by replacing the anti-ABA scFv gene sequence in pRTRA7/3 by anti-HSP17 scFv gene sequences.

For obtaining the cytoplasmic expression cassette, the anti-HSP17 scFv gene was cut from phagemid pIT1 using restriction enzymes Nco I and Not I. DNA fragments were electrophoretically separated on agarose gel (see 2.2.3.2.). The 800 bp Nco I – Not I fragment containing the scFv coding sequence was extracted from agarose gel using QIAGEN Gel Extraction Kit (QIAGEN) according to manufacturer's instructions and cloned into the Nco I and Not I sites of plasmid pRTRA7/3.

In order to create the ER expression cassette, the anti-HSP17 scFv sequence was PCR-amplified using BAM1 and BAM2 primers containing BamHI sites at their 5'- ends. Reaction was done in 50 μ l of amplification mixture containing 0.2 μ M primers BAM1 and BAM2, 0.2 μ M dNTPs, 1U Pfu Turbo DNA polymerase, 5 μ l 10 \times Pfu Buffer and 50 ng of plasmid pIT1 DNA as a template with following program: 1 min 95 $^{\circ}$ C, 1 min 66 $^{\circ}$ C, 1 min 72 $^{\circ}$ C. The PCR product was digested by use of restriction enzyme BamHI and cloned into BamHI sites of plasmid pRTRA7/3. Prior to ligation, dephosphorylation of 3'-ends of pRTRA7/3 DNA was performed by adding 1 U of shrimp alkaline phosphatase during digestion of plasmid DNA with restriction enzyme BamHI.

All constructed plasmids containing the scFv expression cassettes were digested with restriction enzyme Hind III. Subsequent to restriction, DNA fragments were separated on the agarose gel. 1 kb fragment containing the expression cassette and the anti-HSP17 scFv gene sequence was eluted from the agarose gel as described above and cloned into the Hind III site of the plant binary vector pBIN19. All DNA ligation reactions were performed using Rapid DNA Ligation Kit (Roche) according to the instructions of the manufacturer.

5 µl of ligation mixture were used to transform competent cells of *E.coli* strain HB101 (see 2.2.3.2.). Single colonies of transformed bacterial cells were propagated in LB medium supplemented with 100 µg ml⁻¹ of ampicillin. Plasmid DNA was purified from 2 ml of a bacterial overnight culture using FlexiPrep Kit (Amersham Pharmacia Biotech). Purified DNA of the plant binary vector was used to transform *A.tumefaciens* strain C58C1. After each step of the procedure the recombinant DNA was analyzed by sequencing using biotin-labeled primer 35S to prove the presence of scFv sequence and to check the reading frame.

2.2.5. Transformation of *Agrobacterium tumefaciens* cells

2.2.5.1. Preparation of transgenic *Agrobacterium tumefaciens* cells

The overnight culture of *Agrobacterium tumefaciens* in YEB medium was diluted with fresh medium 1:100 (final volume 1 l) and grown at 37⁰C till OD₆₀₀ reached approximately 0.5-0.8. Cells were collected by centrifugation for 15 min at 4000 rpm and resuspended in 1 l of sterile millipore water. The cells were spinned down for 15 min at 4000 rpm and resuspended in 500 ml of sterile millipore water. After centrifugation for 15 min at 4000 rpm, cells were resuspended in 20 ml of sterile 10% glycerol, spinned down for 10 min at 4000 rpm and resuspended in 2 ml of sterile 10% glycerol. 40 µl aliquotes of the cell suspension were frozen in liquid nitrogen for further storage at -70⁰C.

1 µg plasmid DNA (2.2.4.) dissolved in 10 µl of water was drop dialysed for 1 h. The DNA solution was pipetted on a millipore filter floated on the surface of distilled water in a Petri dish. Subsequently, the DNA solution was mixed with an aliquote of competent *A.tumefaciens* cells in the ice-cold electroporation cuvette. Electroporation was done at 2.5V, 2 kΩ, 250 µF using a Gene Pulser (Bio-Rad). 800 µl of SOC medium were added to the cells. The cells were mixed and transferred to a

microcentrifuge tube for 1 h incubation at 37⁰C with shaking. Then 200 µl of the cell suspension were spread onto selective YEB plates and incubated at 28⁰C for two days to receive visible colonies for further propagation.

2.2.5.2. Southern blot analysis of DNA from transgenic *Agrobacterium tumefaciens* culture

2 ml of YEB medium containing 50 µg ml⁻¹ kanamycin, 100 µg ml⁻¹ rifampicin and 100 µg ml⁻¹ carbenicillin were inoculated with transformed *Agrobacterium tumefaciens* cells and incubated for 2 days at 28⁰C. The bacterial culture was centrifuged for 5 min at 5000 *g* at RT and the cell pellet resuspended in 300 µl TE buffer. 100 µl of 5 mg ml⁻¹ Proteinase K and 100 µl of 5% Sarkosyl in TE were added to the bacterial culture and incubated for 1 h at 37⁰C. The solution was treated 3 times with 500 µl phenol, 3 times with 500 µl phenol-chloroform and one time with chloroform. After centrifugation for 15 min at 10000 *g* at RT, the DNA was precipitated from the supernatant by 2 volumes of ethanol and 0.05 volumes of 5M NaCl. DNA was spun down by 10 min centrifugation at 10000 *g*, 4⁰C, washed by 70% ethanol, dried and dissolved in water. 5 µg of DNA were taken for restriction digestion in a total volume of 50 µl for overnight incubation at 37⁰C. The restriction mixture complemented with a 6× gel loading buffer was separated on 1% agarose gel in TAE buffer, containing 0.5 µg ml⁻¹ ethidium bromide. Gel electrophoresis was performed at 5 V cm⁻¹ during 5 h. DNA was transferred from the agarose gel to Hybond N+ membrane (Amersham Pharmacia Biotech) using alkali capillary blotting overnight.

The DNA fragment used as hybridization probe was diluted to 25 µg ml⁻¹ in distilled water. 20 µl of DNA solution were denatured by boiling for 5 min in a water bath and then chilled on ice. Labelling of the DNA probe, prehybridization of the membrane and hybridization with the labeled probe was performed by using the ECL Random Prime Labelling Kit (Amersham Pharmacia Biotech) according to standard procedure described in the instruction of the producer. After hybridization the membrane was washed for 15 min at 60⁰C with 2×SSC containing 0.1% SDS followed by 15 min washing with preheated 1×SSC containing 0.1% SDS. Signal generation by the labeled DNA probe hybridized with DNA fragments on the

membrane was performed using Gene Image CDP-Star Detection Module. The developed signal was detected by exposing the membrane to Hyperfilm ECL (Amersham Pharmacia Biotech).

2.2.6. Transformation of *Nicotiana tabacum*

2.2.6.1. Leaf disk method

For preparation of leaf disks *in vitro*, cultivated plants of *Nicotiana tabacum* cv. Samsun NN were used. The leaf disks (0.5 cm²) were cut under sterile conditions and placed on MS medium supplemented with 0.1 µg ml⁻¹ NAA and 1 µg ml⁻¹ BA. After 2 days of incubation at 25⁰C in darkness, the leaf disks were used for infection by the transgenic *Agrobacterium tumefaciens* (2.2.5.). 5 ml of YEB medium, supplemented with 50 µg ml⁻¹ kanamycin, 100 µg ml⁻¹ rifampicin and 100 µg ml⁻¹ carbenicillin, were inoculated by transformed *Agrobacterium tumefaciens* clones (2.2.5.2.) and incubated for 2 days at 28⁰C with shaking. The 2-day culture of *Agrobacterium tumefaciens* was diluted 1:20 by liquid MS. Each leaf disk was treated with the diluted bacterial culture and placed on MS containing 0.1 µg ml⁻¹ NAA and 1 µg ml⁻¹ BA for 2 days at 25⁰C. After 2 days, leaf disks were transferred onto fresh MS, containing 50 µg ml⁻¹ kanamycin and 250 µg ml⁻¹ ticarcillin, 0.1 µg ml⁻¹ NAA and 1 µg ml⁻¹ BA, and incubated at 22⁰C in darkness for plant regeneration. Regenerated plantlets were placed onto solid MS, supplemented with 50 µg ml⁻¹ kanamycin, and 250 µg ml⁻¹ ticarcillin and incubated at 22⁰C (16 h light/8 h darkness cycle) for root formation. After root formation plantlets were cultivated in the greenhouse.

2.2.6.2. Regeneration of transgenic plants

From plants grown in the greenhouse seeds were collected and sterilized by treatment with 10% NaOCl solution. After washing 3 times 10 min with sterile water the seeds were planted on 0.5 MS medium containing 50 µg ml⁻¹ kanamycin. Drug resistance was recognized by ability of plants to survive antibiotic selection. Resistant plantlets were propagated to establish transgenic plant lines.

2.2.7. Characterization of transgenic plants

2.2.7.1. Characterization of transgenic plants by Western blot analysis

For Western blot analysis, leaves or seeds of plants were extracted with 50 mM Tris-HCl buffer pH 7.6 supplemented with 150 mM NaCl, 5mM EDTA, 0.1% SDS, 0.1% β -mercaptoethanol and 1mM PMSF. The fresh weight / buffer ratio was 1:3 and the extraction procedure was repeated twice at 4⁰C. Protein extracts were centrifuged at 10000 *g* for 10 min and both supernatant fractions were combined. The protein concentration of the supernatant was measured according to Bradford (1976). In order to check the efficiency of the extraction procedure the pellet fraction was extracted once more by using gel loading buffer of the used SDS-PAGE system.

10 μ g of proteins per slot were separated on 12.5% SDS polyacrylamide gels according to Laemmli (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membranes (0,4 μ m, BA85, Schleicher and Schuell) as described by Borisjuk et al. (Borisjuk et al., 1998). After electrotransfer the membranes were washed in TBS and blocked by incubation for 1 h in Roti-Block solution (ROTH). The wash step was repeated and the membranes were incubated with the primary antibody in TBS supplemented with 0.5% BSA and 0.05% Tween 20 for 1 h at RT. The membranes were washed 3 times, each for 5 min, in washing solution supplemented with 0.5% Triton X-100 and one time without detergent for 10 min. Membranes were incubated with the secondary antibody conjugated to ALP (color reaction) or to horseradish peroxidase (ECL technique). After additional washing as described above, labeling was detected by staining with NBT/BCIP (color reaction) or by chemiluminescence using the ECL Kit (Amersham Pharmacia Biotech) according to the producer's instructions.

To detect the expressed anti-sHSP scFv in plant material as primary antibody, the monoclonal anti-c-myc antibody 9E10 was used. The endogenous sHSP expression was checked by using a polyclonal anti-sHSP antibody produced in rabbits.

2.2.7.2. Quantitative detection of scFv expression by Western blot analysis

Preparative amounts of the soluble scFv were prepared by way of IPTG-induced expression in *E.coli* as described in 2.2.3.3. The supernatant of bacterial culture containing soluble scFvs was loaded on a Protein L agarose (AC Tigen) column.

After 1 h incubation the column was washed with PBS to remove unbound proteins. ScFvs bound to Protein L agarose were eluted with glycine buffer at pH 2.2. The eluate containing scFvs was immediately neutralized with Na₃PO₄ and concentrated by using collodium bags and PEG 20000. The protein concentration of the scFv preparation was measured according to Bradford (Bradford, 1976). Protein extraction from the leaf samples of transgenic plants was performed as described in 2.2.7.1. The extracted fractions were combined and used for determination of total protein concentration according to Bradford (Bradford, 1976). The pellet fractions of the samples were extracted with Laemmli buffer to check the efficiency of the extraction procedure as described in 2.2.7.3. Extracts from the leaves of transgenic plants with known protein content and scFv preparations in a concentration range of 5 µg, 10 µg and 20 µg were simultaneously separated on 12.5% polyacrylamide gel followed by Western blot analysis as described in 2.2.7.3. using anti-c-myc antibody as primary antibody and the ECL Kit for signal generation. The Hyperfilm image of the Western blot membrane was scanned to create a digital picture for further computer analysis. The signal intensity, generated by known amounts of scFv standard on the membrane, was determined by Tina v2.08 software (Raytest, Sprockhow, Germany) and used to calculate the calibration curve. The signal intensity generated by protein extracts from transgenic plants was also measured by Tina v2.08 software and used to calculate the content of scFv in the leaf protein extracts.

2.2.7.3. Southern blot analysis of genomic DNA from transgenic plants

The total DNA was extracted from young fresh leaves by using the QIAquick Plant DNA Kit (QIAGEN). 10 µg of plant DNA were digested to completion with restriction enzyme BamHI. Cleaved DNA was separated on 1% agarose gel in TAE buffer and transferred to Hybond N+ membrane (Amersham Pharmacia Biotech) with 20×SSC. Restriction digestion, electrophoretic separation and capillary transfer of DNA onto Hybond N+ membrane with 20×SSC were performed according to Sambrook (Sambrook et al.,1989).

DNA containing the scFv DNA sequence was cut out from plasmid pIT1 isolated from HSP17-specific clones (2.2.3.) using restriction enzymes NcoI and NotI. 25 ng of DNA fragments containing the scFv DNA sequence were labeled using RediPrime Kit (Amersham Pharmacia Biotech) and ³²P-dCTP according to

manufacturer's manual. ^{32}P -labelled DNA was separated from unbound ^{32}P -dCTP by using MicroSpinTM S-200 Columns. After capillary transfer of genomic plant DNA, the nylon membrane was washed in $6\times\text{SSC}$, prehybridized for 2 h at 65°C in Church buffer supplemented with $100\ \mu\text{g ml}^{-1}$ of salmon sperm DNA and hybridized with the ^{32}P -labelled denatured scFv DNA probe (95°C for 5 min). Incubation was continued at 65°C overnight. After hybridization, stringency washes of the membrane were done with $1\times\text{SSC}$ and 0.1% SDS for 20 min at 65°C . The membrane was exposed to Fuji Imaging Plate Type BAS III to obtain radiographic image and analyzed with the Fuji BioImaging Analyzer, BAS 2000 software (Fuji Foto Film Co., LTD, Japan) and Tina v2.08 software (Raytest, Sprockhow, Germany).

2.2.8. Heat stress treatment of plant leaves

Leaves of the same age were isolated from the plants and treated for different time periods in preheated plastic boxes in order to analyze basal and acquired thermotolerance of transgenic and control plants. During heat treatment the leaves were placed on water-saturated paper towels to avoid desiccation of the leaf tissue. To check the basal thermotolerance of transgenic and control plants the leaves were incubated for 2 h at 42°C , 48°C and 50°C , respectively. In order to induce acquired thermotolerance the leaves of transgenic and control plants were preincubated for 15 min at 42°C , recovered for 2 h at 25°C and, after the recovery period, heat-treated for 2 h at 42°C , 48°C and 50°C , respectively. After temperature treatment visual morphological alterations of the leaves were photographically documented.

2.2.9. Determination of the total chlorophyll content

Measurement of the total chlorophyll amount in the leaf tissues of stressed and non-stressed plants was performed by use of 100 mg of plant material. After stress treatment the leaf material was immediately frozen in liquid nitrogen and grounded in a ceramic mortar. 500 μl of pure acetone were added to the grounded leaf samples. The cell debris was removed from the mixture by centrifugation at 5000 g for 10 min. The acetone phase containing dissolved chlorophyll pigments was collected. The pellet was resuspended two times in 500 μl of pure acetone to extract quantitatively the chlorophylls. The acetone fractions from all three procedures were combined. The combined acetone fraction was diluted with pure acetone. 1 ml extract diluted 1:20,

1:50 and 1:100 was used for OD measurement at 645 nm and 662 nm. The concentration of total chlorophylls in the diluted leaf extracts was calculated according to Lichtenthaler (Lichtenthaler, 1987):

$$C_{a+b} = 7.05 \times OD_{662} + 18.09 \times OD_{645},$$

C_{a+b} is the concentration of the total chlorophylls in the diluted leaf extract [$\mu\text{g ml}^{-1}$]. The concentration of total chlorophylls in the leaf extract was calculated by use of the following equation:

$$C = C_{a+b} \times D,$$

C is the concentration of the total chlorophyll pigments in the leaf extract in $\mu\text{g ml}^{-1}$, C_{a+b} is the concentration of total chlorophylls in dilution of the leaf extract in $\mu\text{g ml}^{-1}$ and D is dilution of extract. Concentrations of the total chlorophylls in three individual extract dilutions (1:20, 1:50 and 1:100) were used to calculate the final approximate concentrations of total chlorophylls in the leaf extract. Total content of chlorophyll pigments in leaf tissue was calculated by the following equation:

$$M_{\text{total}} = (C_{\text{appr.}} \times V_a) / m_{\text{fresh}},$$

M_{total} is the total amount of chlorophylls in fresh leaf tissue in $\mu\text{g g}^{-1}$ fresh weight, $C_{\text{appr.}}$ is the approximate concentration of chlorophylls in the leaf extract in $\mu\text{g ml}^{-1}$, V_a is the volume of acetone in ml used for the extraction procedure, m_{fresh} is the mass of fresh leaf tissue in g used as leaf material.

2.2.10. Immunofluorescence

Plant tissue was fixed in PBS containing 3% paraformaldehyde and 0.01% Triton X-100 for 2 h, dehydrated in ethanol and embedded in PEG (van Lammeren et al., 1985). 3 μm sections were affixed to silanized (10% Triethoxysilylpropylamine, Merck) slides and blocked with 1% BSA in PBS for 30 min. After incubation with the primary anti-HSP17 antibody for 12 h at 4°C, labelling was performed by using a second, biotinylated goat anti-rabbit IgG antibody (DAKO Diagnostica, Hamburg, Germany) for 30 min at 20°C and streptavidin-HRP conjugate. To visualize the

sHSPs the fluorescein tyramide reagent was used according to the manufacturer's instructions.

To detect the intracellular distribution of the scFv in transgenic plant material the monoclonal anti-c-myc antibody 9E10 was used as a primary antibody followed by anti-mouse IgG antibody from rabbit, biotinylated goat anti-rabbit IgG antibody and streptavidin-HRP conjugate. Visualization of scFv was performed as described above for HSP. After fluorescence staining the sections were counter-stained with DAPI (in final concentration of 1 mg l⁻¹). (Immunofluorescence was performed by Dr. U. zur Nieden, IPB, Halle).

2.2.11. Electron microscopy

For ultrastructural investigations by transmission electron microscopy the plant leaf material was prefixed with 3% glutaraldehyde in phosphate buffer, pH 7.2 for 2 h, fixed with OsO₄ in the same buffer for 1 h, dehydrated in a series of acetone and embedded in ERL epoxy resin. Staining was performed with 1% uranylacetate in 30% acetone during dehydration and with lead on the grids.

For immunolocalization of scFv by use of the immunocolloid technique the plant material was fixed with 3% formaldehyde in phosphate buffer for 2 h, dehydrated in ethanol and embedded in Lowicryl K4M. Ultrathin sections were incubated with the monoclonal anti-c-myc antibody 9E10. Bound anti-c-myc antibody was labeled by using a bridge anti-mouse IgG antibody from rabbit followed by gold-labeled protein A (Electron microscopy was performed by Dr.habil. D.Neumann and Dr. U. zur Nieden, IPB, Halle, Germany).

3. Results

3.1. Expression of recombinant sHSPs in bacteria

Preparative amounts of pure class I sHSP were required as antigen for phage panning, ELISA and Western blotting. In order to avoid the time consuming preparation of pure class I sHSP from plant material by using biochemical methods, class I HSP17 from *Lycopersicon esculentum* was expressed as a fusion protein containing 6×His residues as a C-terminal tag in the *E.coli* QIAexpress™ system. The pQE32 vectors of this bacterial expression system are expression plasmids for *E.coli* that permit high IPTG inducible expression of a cloned foreign gene sequence in bacterial cells; the product accumulates in the cytoplasm in soluble form or aggregates into inclusion bodies as insoluble form. The C-terminal His-tag of the expressed fusion protein is able to bind to an activated nickel matrix so that the recombinant protein can be dissociated from the binding sites of the matrix by protonation under reduced pH conditions. As competitive substances of the chelate complex formation imidazol or nickel ion chelators, EDTA or EGTA are used under native elution conditions. When the fusion protein is expressed as insoluble product it must be extracted from the bacterial cells under denaturing conditions. Denaturing agent, such as urea, combined with a pH shift can be used to elute the bound recombinant protein from the Ni-matrix. Denatured recombinant protein can be refolded using dialysis under non-denaturing conditions.

Vector pQE92 containing the class I HSP17 gene sequence was constructed using plasmid pQE32 (QIAGEN, Fig.1) and transformed into *E.coli* strain BL21. The DNA sequence encoding class I HSP17 was cloned to the Hind III and Sma I site of the polylinker from pQE32 (kindly provided by Dr. A. Bucka, IPB, Halle, Germany).

Exponentially growing cultures of transgenic *E.coli* BL21 were used for IPTG induction of foreign gene expression. The bacterial cultures were grown under inducing conditions for only 5 h to avoid degradation of recombinant HSP17 by bacterial proteases or toxic effects caused by expressed foreign proteins. Thereafter, bacteria were collected by centrifugation and extracted by using the Ni-NTA Spin Kit buffer systems. Bacterial cell lysis performed under native conditions using imidazol was not sufficient to extract recombinant HSP17 from the bacterial cells. Therefore

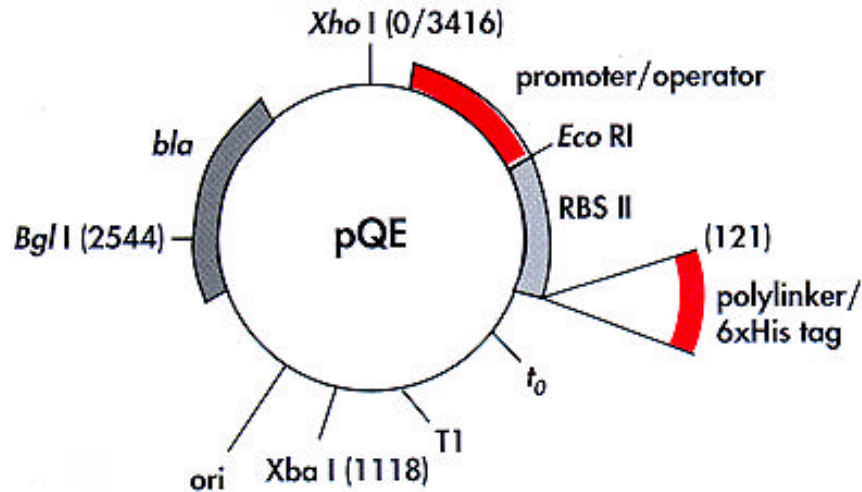


Fig.1 Restriction map of plasmid pQE32.

The DNA fragment encoding class I HSP17 from *L. esculentum* was cloned to Sma I and Hind III sites of the polylinker (145-187 bp region). **Promoter/operator** – element consisting of the phage T5 promoter and two lac operator sequences increasing *lac* repressor binding for repression of T5 promoter; **RBS II** – synthetic ribosomal binding site for high translation rates; **Polylinker/6 His tag** – 6 His tag coding sequence and multiple cloning site; **t₀** – transcription terminator of phage lambda; **T1** – transcription terminator of the *rrnB* operon of *E. coli*; **ori** – ColE origin of replication; **bla** – β-lactamase gene conferring resistance to ampicillin.

lysis of bacteria was performed under denaturing conditions with 8M urea. The recombinant HSP17 was presumably located in inclusion bodies and not secreted into bacterial periplasm as soluble protein. After cell homogenization using urea-containing cell lysis buffer the protein extract from IPTG-induced transgenic *E. coli* cells was loaded on Ni-NTA spin columns to bind the recombinant HSP17 by the 6×His tag as chelate complex on the matrix. The column was washed with urea-containing buffer at pH 5.8 to remove unbound proteins. After washing the bound recombinant HSP17 was eluted with urea-containing buffer at pH 4.0 from the Ni-matrix. Eluted recombinant HSP17 was dialyzed against PBS. 5 μg of protein per slot were loaded on SDS polyacrylamide gel to analyze the molecular mass and purity of the IMAC-purified HSP17 preparation (Fig.2). Only one polypeptide could be detected after Coomassie staining of the polyacrylamide gel verifying the purity

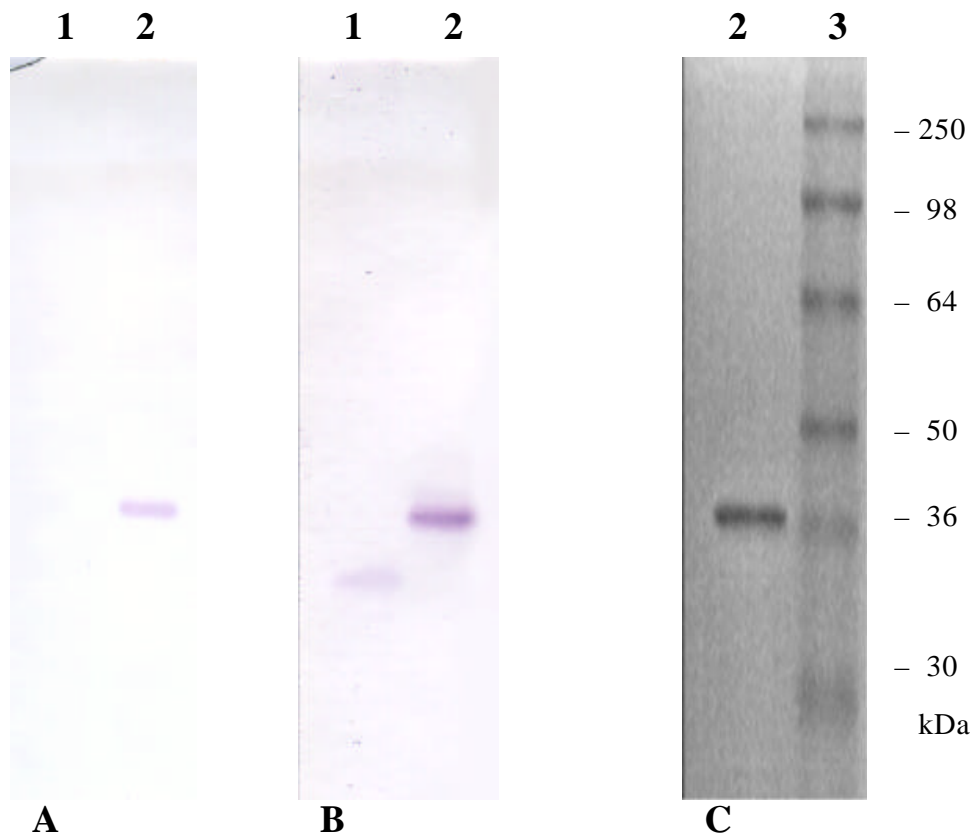


Fig. 2 Detection of recombinant HSP17 expressed in *E.coli* using Western blot analysis and Coomassie staining.

1 – endogenous HSP17 isolated from a heat stress treated suspension cell culture of *L.esculentum*, 2 – recombinant HSP17 expressed in *E.coli* and purified by IMAC, 3 – See Blue Standard protein molecular mass marker. **A** – detection of recombinant HSP17 by using monoclonal anti-His antibody followed by anti-mouse IgG antibody conjugated to ALP; **B** – detection of endogenous and recombinant HSP 17 by using polyclonal anti-HSP17 antibody followed by anti-mouse IgG antibody conjugated to ALP. Signals shown on images **A** and **B** have been generated by color reaction of ALP with BCIP/NBT substrate. About 1 μ g of recombinant HSP17 and native HSP17 were loaded per slot **C** - Coomassie stained protein pattern of the purified recombinant HSP17 preparation after IMAC. 5 μ g of purified recombinant HSP17 were electrophoretically separated on 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. The molecular masses of the standard protein marker in kDa are shown on the right side.

of the HSP17 preparation by using IMAC (Fig.2C).

The recombinant HSP17 preparation was additionally checked immunochemically by using Western blot analysis. HSP17 was immunochemically

detected by using anti-His antibodies that specifically recognize the His-tag of the recombinant protein as shown in Fig. 2A. Additionally, polyclonal antibodies produced against enriched preparation of endogenous HSP17 from heat stressed tomato suspension cell culture recognized also purified recombinant HSP17, as it is shown on Fig.2B. The molecular mass of recombinant protein was about 36 kDa. This is two times higher than the molecular mass of HSP17 monomer from *L.esculentum* (17 kDa). It suggests that recombinant HSP17 produced in *E.coli* using the QIAexpress™ system probably associates to dimers. The mechanism of dimerisation of recombinant HSP17 is unclear. However, according to the results of Western blot analysis described above, dimerisation of recombinant HSP17 does not prevent specific recognition of HSP17 by anti-HSP17 antibodies produced against the native endogenous HSP17 from *L.esculentum*.

Therefore, purified recombinant protein was used as coating antigen for phage library screening by phage panning to select specific anti-HSP17 scFvs and for ELISA to check the specific recognition of HSP17 by selected phages and scFvs.

3.2. Phage library screening

In order to isolate gene sequences coding for anti-HSP17 scFv libraries Tomlinson A and B were screened by using support-fixed recombinant class I HSP17 (see 3.1.) as coating antigen for phage panning. ScFv phage library was used because of its relative simplicity as to the screening procedure and the possibility to receive phage clones producing monoclonal scFvs with very high affinity and specificity to the antigen of interest in comparison with immunization of animals and hybridoma technology. The phage libraries Tomlinson A and B contain cDNA sequences coding for the variable regions of heavy and light human immunoglobulin chains, prepared using PCR amplification. Artificial genes encoding scFvs were genetically engineered by random combination of cDNA fragments encoding variable regions of heavy and light variable chains connected by a short linker sequence and cloned into phagemid pIT1 for scFv display on the surface of the phage particles.

The antigen-specific phage selection procedure was repeated three times. By the elution of phage particles in the first panning round a pool of phages was received displaying on their surface anti-HSP17 scFvs with different affinity and specificity to the HSP17 antigen. Second panning was performed to enrich the phage pool for

phages displaying anti-HSP17 scFvs with high antigenic specificity and affinity. In the third panning round, the phage pool was separated to single phage clones. The specificity of the expressed scFvs on the phage surface was checked by using monoclonal phage ELISA (2.2.2.5.). Several phages selected from the third panning round show relatively high binding capacity to the HSP17 antigen, in comparison with all other populations of tested phages, and were used to prepare soluble scFvs.

The ability of soluble scFv to recognize recombinant HSP17 was checked by scFv ELISA. The principle of scFv ELISA is schematically demonstrated in Fig.3. Soluble scFvs from individual phage clones were used as primary antibody in order to bind to the immobilized recombinant HSP17 used as coating antigen. The antigen bound scFv was specifically recognized by the monoclonal anti-c-myc antibody 9E10 followed by anti-mouse IgG conjugated to ALP. The OD of ALP reaction with substrate pNPP was measured at 405 nm. The diagram shown on Fig.4 presents the results of OD measurement. As may be seen from Fig.4, scFvs from phage clones A14 (library Tomlinson A) and B8 (library Tomlinson B) showed the highest binding capacity to the coating antigen when compared with scFvs from other clones. Phage clones A14 and B8 were selected for further experiments.

In order to characterize cDNA sequences of the individual scFv clones A14 and B8 their size and the sequence were checked. For this approach the plasmid pIT1 DNAs with the insertion of the scFv DNA sequence were isolated from bacterial cultures, infected with corresponding individual phages and grown on the medium with low content of glucose (see 2.2.3.2.). Under these conditions the pIT1 phagemid is present as a plasmid in bacterial cells. The DNA fragments of the clones A14 and B8 coding for the corresponding scFvs were cut from plasmid pIT1 with restriction enzymes Nco I and Not I and electrophoretically separated on 1% agarose gel to determine their sizes. In the Library Tomlinson A+B clones with incomplete scFv-coding fragments were also present. The correct size of 800 bp coding for complete variable regions of the heavy and light chains was therefore used as criterion of scFv cDNA selection. Both clones showed correct the 800 bp size of the scFv cDNA sequence (Fig. 5). Additionally, scFv coding DNA fragments of phage clones A14 and B8 were sequenced by use of primers PHEN and LINK followed by analysis with BLAST Search (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/BLAST/>). High homology of A14 and B8 cDNA

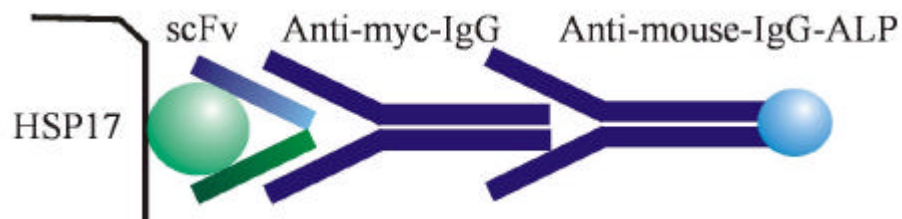


Fig. 3 Scheme of scFv ELISA.

HSP17 – solid fixed recombinant class I HSP17 from *L.esculentum* as coating antigen; **scFv** – soluble anti-HSP17 scFv; **anti-c-myc-IgG** – anti-c-myc antibody 9E10 to recognize c-myc tag of the scFv; **anti-mouse-IgG-ALP** – anti-mouse IgG antibody conjugated to ALP to detect bound monoclonal anti-c-myc antibody.

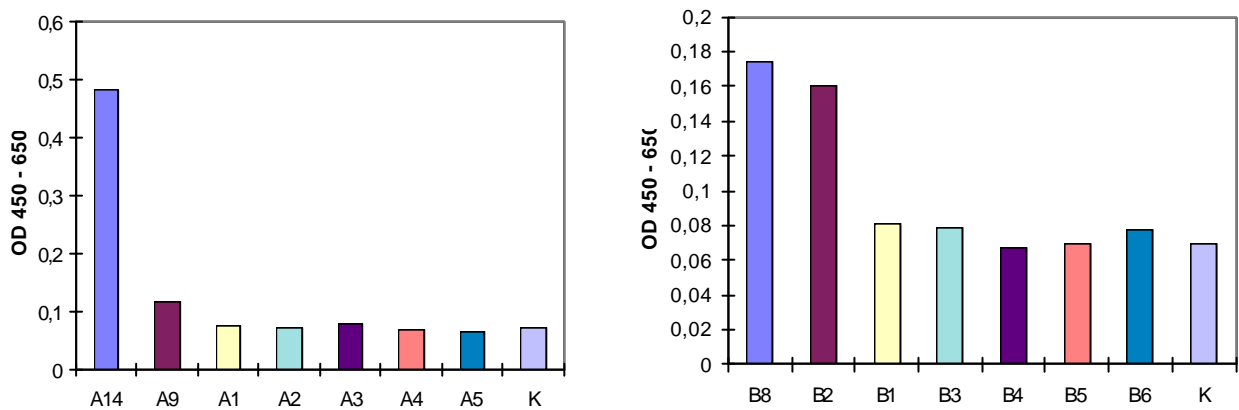


Fig. 4 Specific recognition of the HSP17 coating antigen by selected anti-HSP17 scFvs detected by scFv ELISA.

100 μ l of 20 μ g ml⁻¹ recombinant HSP17 in PBS, 50 μ l of bacterial culture supernatant containing soluble scFv, 200 μ l of monoclonal anti-c-myc antibody 9E10 diluted 1:30 and 100 μ l of anti-mouse IgG conjugated to ALP (diluted 1:2000) were loaded per well. For signal generation 100 μ l of 1 ng ml⁻¹ pNPP solution in alkaline phosphatase substrate buffer were added per well. Optical density measurement at 650 and 450 nm; OD values at 650 nm were subtracted from OD values at 450 nm. **A** – individual scFv clones isolated from phage library Tomlinson A; **B** – individual scFv clones isolated from phage library Tomlinson B. **K**- BSA used for coating as background control. The results were analyzed by Microsoft Excel 97 software.

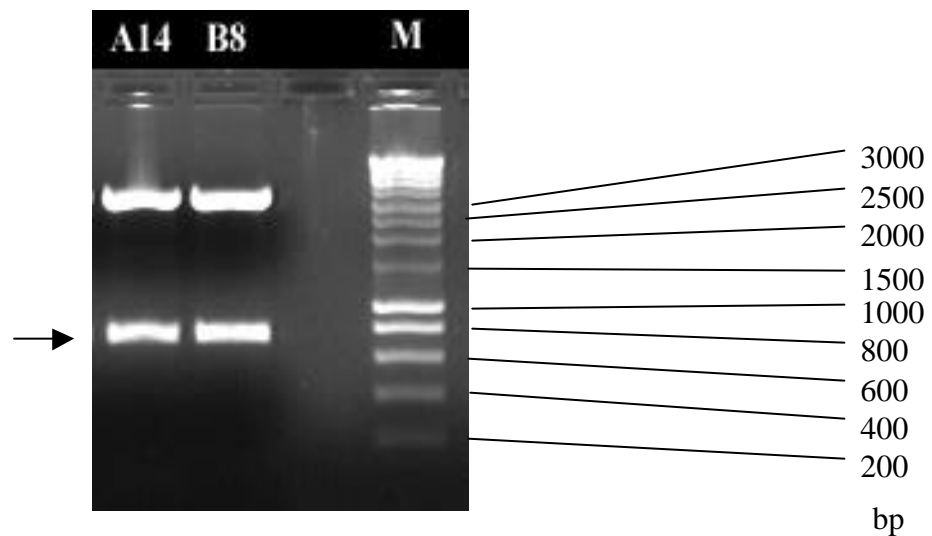


Fig. 5 Restriction patterns of plasmid DNA isolated from clones A14 and B8.

Plasmid DNA was digested with restriction enzymes *Nco*I and *Not*I and electrophoretically separated on 1% agarose gel. Arrow on the left shows the size of scFv fragments from clones A14 and B8. Lane M – DNA Smart Ladder.



Fig. 6 Recognition of endogenous HSP17 from heat-treated tomato cell suspension culture by soluble scFvs on Western blot.

Protein extracts (10 μ g per slot) from tomato suspension cultures stressed (1) and non-stressed (2) by heat shock were separated on 12.5% SDS polyacrylamide gel and electrophoretically blotted on a nitrocellulose membrane. ScFvs clones A14 (A) and B8 (B) were used as a primary antibody. Bound scFvs were detected with anti-c-myc antibody 9E10 followed by anti-mouse IgG conjugated to HRP. Signal generation was done by chemiluminescence.

sequences with *Homo sapiens* mRNA coding for the immunoglobulin heavy chain variable (accession numbers Z98716, AB021526) and kappa light chain variable (accession numbers L01279, Z37336) regions was found (Appendix A and B).

To be sure that selected scFvs are able to recognize not only recombinant HSP17 but also endogenous HSP17 of plants, suffered heat stress, soluble scFvs from clones A14 and B8 were prepared and used as a primary antibody in Western blot analysis. Western blot analysis was performed after electrophoretic separation of protein extracts prepared from heat-treated and non-treated cell suspension cultures of *L. esculentum* as antigens. The HSP17-bound scFvs were detected by anti-c-myc antibodies 9E10 followed by anti-mouse IgG conjugated to HRP (Fig. 6). Both scFvs from clones A14 and B8 recognized sHSPs from heat-stressed tomato suspension culture, whereas no signals were detected using protein extracts from suspension cultures without heat stress as antigen. The scFv cDNA isolated from phage clones A14 and B8 were used to construct the expression cassettes for gene transfer to express anti-HSP17 scFv in transgenic plants.

3.3. ScFv-expression cassettes for plant transformation

In order to immunomodulate cytosolic sHSPs *in vivo* several recombinant DNA constructs for ubiquitous expression of anti-HSP17 scFvs in the vegetative tissues of transgenic plants were designed (see 1.4.). Two recombinant DNA constructs were created for cytosolic expression of anti-HSP17 scFv in plant cells. Recombinant constructs for expression of anti-HSP17 scFvs targeted into ER were designed because sHSPs are not only stress-induced and synthesized in the cytoplasm of vegetative cells but also developmentally-dependent accumulated in specific storage organelles of parenchyma cells of mature seeds (D. Neumann, personal communications).

Phagemid vector pIT1 (Fig. 7) and plasmid pRTRA7/3 (Fig. 8) were used as a basis for construction of expression cassettes responsible for cytoplasmic biosynthesis of the scFv-KDEL fusion protein in vegetative cells after plant transformation. The vector pRTRA7/3 contains the cassette for expression of an anti-ABA scFv under the transcriptional control of the CaMV 35S promoter. Additionally, the expression cassette contains the legumin B4 signal peptide sequence at the 5'-end as well as the tetrapeptide KDEL and polyadenine sequences at the 3'-

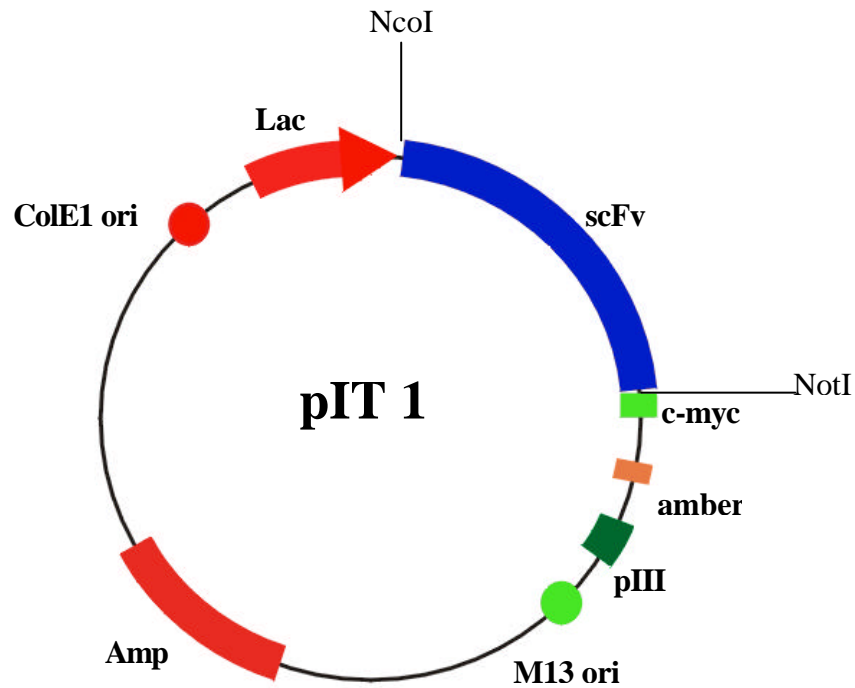


Fig. 7 Schematic presentation of phagemid vector pIT1 from scFv phage library.

ScFv – scFv cDNA sequence, **Lac** – Lac Z promoter of E.coli, **pIII** – sequence of M13 phage protein pIII, **c-myc** – c-myc-tag, **amber** – amber stop-codon, **ColE ori** – ColE1 origin of replication, **M13 ori** – phage M13 origin of replication, **Amp** – ampicillin resistance gene. Restriction sites NcoI and NotI used for recloning of anti-HSP17 scFv cDNA sequence into the expression cassette pRTRA7/3 (Fig.8).

end of scFv DNA sequence. The legumin B4 signal peptide sequence and the KDEL sequence are responsible for biosynthesis on membrane-bound polysomes and retention of synthesized scFv in the ER. The c-myc tag sequence is also part of all created recombinant constructs as a sequence coding for a reporter tag specifically recognizing expressed scFvs at the protein level by using immunochemical or immunohistological techniques.

Fig. 10 shows the strategies of construction and the schemes of expression cassettes containing the anti-HSP17 scFv-KDEL DNA sequence. Cassettes containing the KDEL sequence to stabilize cytoplasmically-expressed anti-HSP17 scFv in plant cells were created by recloning of A14 and B8 scFv cDNA sequences to pRTRA7/3

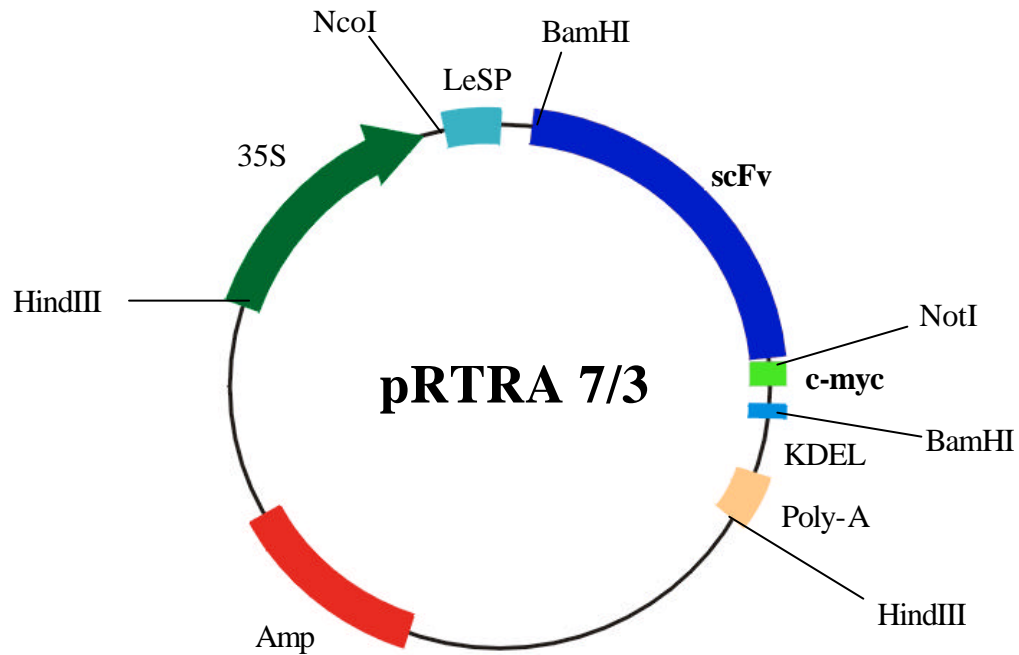


Fig. 8 Schematic presentation of plasmid vector pRTRA 7/3 (Artsaenko, 1996).

ScFv – scFv sequence, **35S** – 35S CaMV promoter, **c-myc** – c-myc tag, **LegSP** – legumin B4 signal peptide sequence, **KDEL** – ER retention sequence, **poly-A** – polyadenine sequence. Restriction sites NcoI, NotI and BamHI used for cloning of anti-HSP17 scFv cDNAs into the expression cassettes pHSP17-CA14/CB8 and pHSP17-RA14/RB8 (Fig. 10).

using restriction sites NcoI and NotI (compare restriction maps of pIT1 and pRTRA 7/3 shown on Fig. 7 and 8). At first, scFv sequences were cleaved out from the phagemid vector pIT1 with restriction enzymes NcoI and NotI and cloned at the same restriction sites into plasmid pRTRA7/3. The legumin signal peptide sequence was thus removed from the original plasmid pRTRA7/3 and the anti-ABA scFv sequence was substituted by the anti-HSP17 scFv sequence, whereas c-myc, KDEL and poly-A sequences were preserved in the new constructs. Newly prepared plasmids were designated as pHSP17-CA14 and pHSP17-CB8, respectively. The designed expression cassettes pHSP17-CA14 and pHSP17-CB8 contain the CaMV 35S promoter sequence to control the ubiquitous transcription of the scFv sequence, the KDEL sequence to stabilize the secondary structure of cytoplasmically synthesized

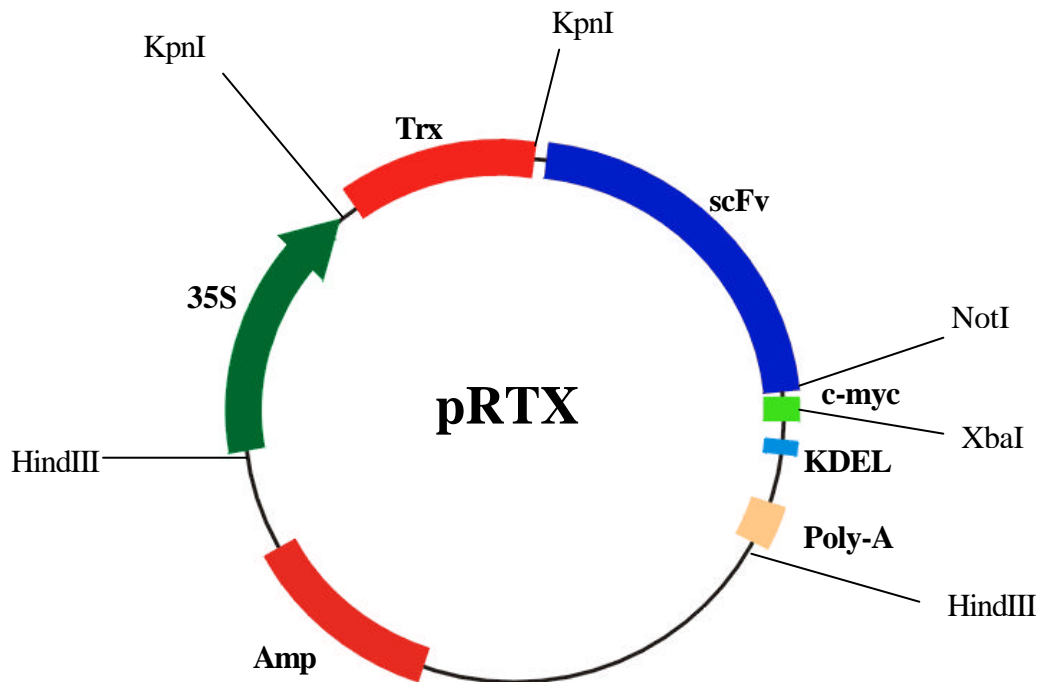


Fig. 9 Schematic presentation of plasmid vector pTRX.

ScFv – scFv sequence, **35S** – 35S CaMV promoter, **c-myc** – c-myc-tag, **KDEL** – ER retention sequence, **TrxA** – E.coli thioredoxin A sequence, **poly-A** – polyadenine sequence, **Amp** – ampicillin resistance gene. Restriction site KpnI used for cloning of anti-HSP17 scFv cDNAs into the expression cassettes pHSP17-TA14/TB8 (Fig.10).

scFv, and the c-myc tag sequence for immunochemical recognition of the scFv by use of tag-specific antibody.

Plasmid pTRX shown in Fig. 9 was used to construct a second cassette for cytoplasmic expression of anti-HSP17 scFvs as Thioredoxin A fusion protein. Plasmid pTRX contains a part of the Thioredoxin A sequence combined in frame with the cDNA sequence coding for anti-ABA scFv under the control of 35S CaMV promoter. The Thioredoxin A part of the fusion protein is also responsible for stabilization of the secondary structure and increases the solubility of the expressed scFv. pTRX was cut with restriction enzyme NotI and then partially digested by

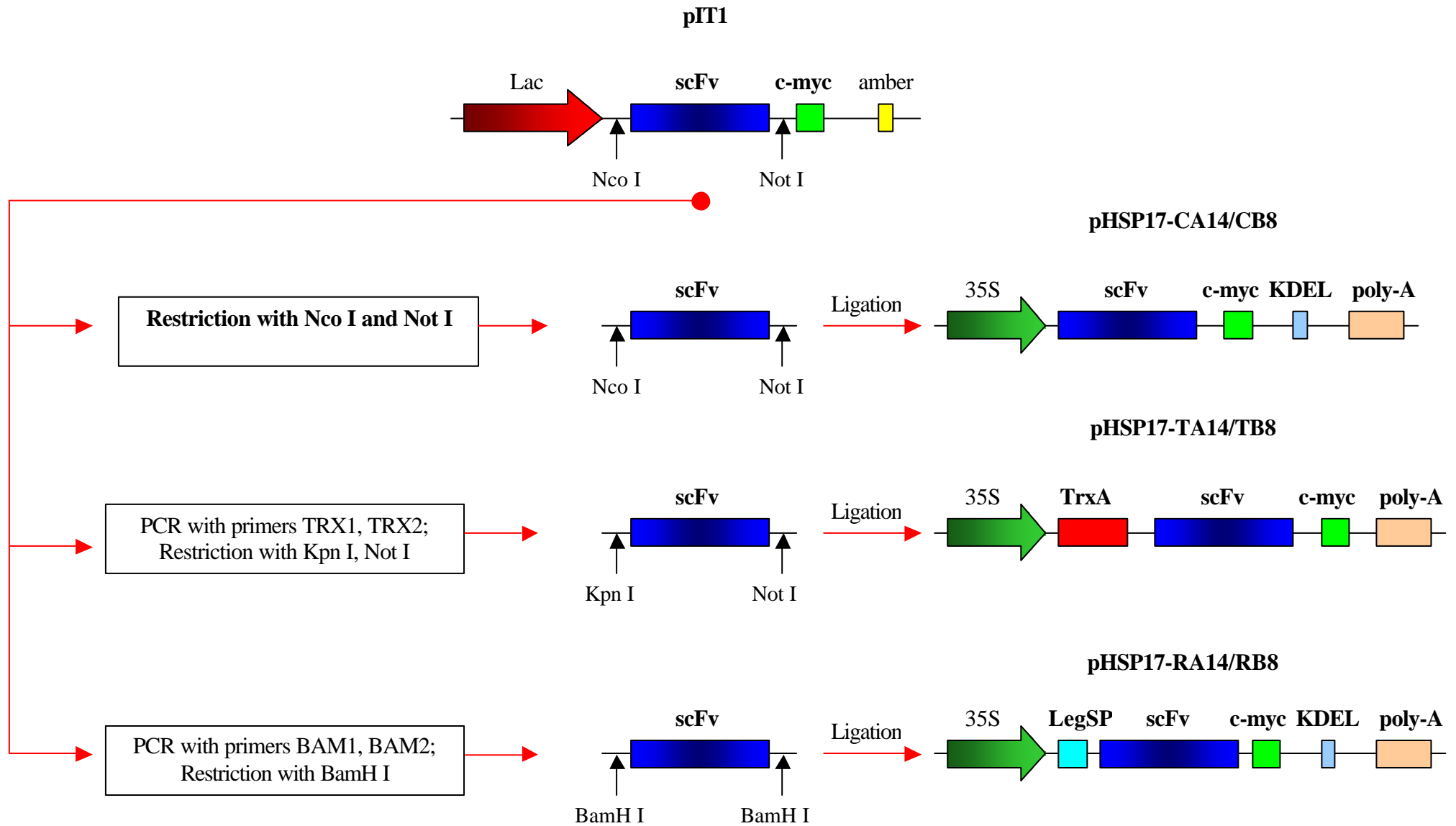


Fig.10 Construction of expression cassettes containing anti-HSP17 scFv DNA sequences.

ScFv – anti-HSP17 scFv sequence, **Lac** – Lac Z promoter of E.coli, **35S** – 35S CaMV promoter, **c-myc** – myc-tag, **amber** – amber stop-codon, **LegSP** – legumin B4 signal peptide sequence, **KDEL** –ER retention sequence, **TrxA** – E.coli thioredoxin A sequence, **poly-A** – polyadenine sequence.

restriction enzyme KpnI to obtain the DNA fragment consisting of pTRX with the Thioredoxin A sequence. Thereafter, DNA fragments were electrophoretically separated on agarose gel and the pTRX-Thioredoxin A DNA fragment was eluted from the gel. The scFv cDNA fragment from phagemid pIT1 was PCR-amplified using the specific primers TrxK and TrxB containing KpnI and NotI sites. The PCR product was cleaved with restriction enzymes KpnI and NotI and ligated with the pTRX-Thioredoxin A DNA fragment. The created plasmids were designated pHSP17-TA14 and pHSP17-TB6, respectively. The schematical presentation of the preparation of these expression cassettes is shown in Fig. 10, explaining the strategies used to design different expression cassettes for scFv expression in transgenic plants.

In order to design the recombinant constructs for ER targeting of the expressed scFvs it was necessary to change the restriction sites flanking the DNA sequences of scFv, to BamHI. For this purpose PCR amplification of scFv genes was done using the specific primers BAM1 and BAM2 that contain BamHI sites at the 5'-ends of their sequences. The amplification products were cleaved with BamHI and cloned into the BamHI site of vector pRTRA7/3 (compare restriction map of pRTRA 7/3 shown on Fig. 8). Vectors with correct orientation of scFv sequences were designated pHSP17-RA14 and pHSP17-RB8, respectively. These two cassettes contain legumin B4 signal peptide and the KDEL sequences of the original vector pRTRA7/3 and the c-myc-tag sequence of phagemid pIT1. They were created for retention of the expressed scFvs in the ER lumen of the transgenic plant cells. Since this kind of scFv expression is also under control of the 35S promoter, it was expected that scFv not only accumulate in the ER of all vegetative cells but also in the ER of storage parenchyma cells in matured seeds.

For *Agrobacterium*-mediated gene transfer the expression cassettes were recloned from *E.coli* vectors to the binary vector which can be amplified in *A.tumefaciens* cells and in *E.coli* cells. Cassettes for cytoplasmic scFv expression and for accumulation of scFv in the ER were recloned into HindIII site of plasmid pBIN19. The correct reading frame of the scFv-KDEL-c-myc fusion sequence under CaMV 35S promoter control in pBIN19 was verified by sequence analysis using 35S and KDEL primers before *Agrobacterium*-mediated transformation of *N.tabacum* leaf disks was performed.

3.4. Production of transgenic *N.tabacum* plants

Transgenic plants were produced in three steps: transformation of *A.tumefaciens* by binary vectors containing recombinant constructs for the expression of anti-HSP17 scFvs in transgenic plant cells, infection of *N.tabacum* leaf disks with transgenic *A.tumefaciens* cultures and selection of antibiotic-resistant plants.

A.tumefaciens strain C58C1 was transformed by use of electroporation. Several single colonies of transgenic *A.tumefaciens* grown on selective medium were transferred to fresh medium to obtain a bacterial culture for total DNA isolation. To proof the presence of scFv DNA sequences in transformed bacterial cells total bacterial DNA was isolated, cleaved with restriction enzyme and used for Southern blot analysis. The total DNA from *A.tumefaciens* cells, digested with restriction

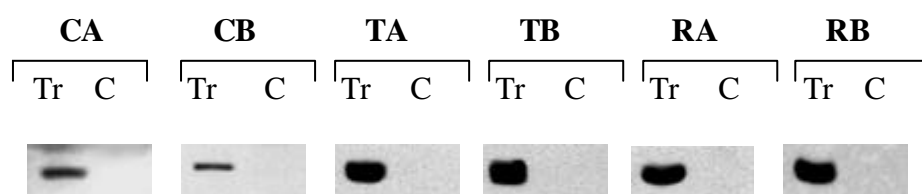


Fig. 11 Southern blot analysis of genomic DNA from transgenic *A.tumefaciens*.

Total bacterial DNA was cleaved with restriction enzyme HindIII, separated electrophoretically and hybridized with the scFv specific CDP-labeled scFv probes. Signals were generated by chemiluminescence and detected using exposition of the membrane to ECL Hyperfilm. **Tr** – DNA of transgenic *A.tumefaciens*; **C** – DNA of non-transformed *A.tumefaciens*. **CA, CB** - bacterial cultures transformed by pBIN19 containing expression cassettes for cytoplasmic expression of anti-HSP17 scFv as c-myc-KDEL fusion protein (**CA** – scFv A14, **CB** – scFv B8). **TA, TB** - bacterial culture transformed by pBIN19 containing expression cassettes for cytoplasmic expression of scFvs as Thioredoxin fusion protein (**TA** – scFv A14, **TB**– scFv B8). **RA, RB** - bacterial culture transformed by pBIN19 containing expression cassettes for ER retention of the expressed anti-HSP17 scFv as c-myc-KDEL fusion protein

enzyme HindIII was electrophoretically separated on 1% agarose gel and the DNA fragments were blotted onto a nitrocellulose membrane. The 800 bp DNA fragments

isolated from phagemid pIT1 after digestion by restriction enzymes NcoI and NotI were used as specific hybridization probes. Fragments consisting of scFv DNA sequences from phage clones A14 and B8 were labeled with biotin for specific detection of scFv sequences inserted into the bacterial DNA pattern of transgenic *A.tumefaciens*. The hybridization signal was generated by chemiluminescence after enzyme-substrate reaction by exposition of the membrane to ECL Hyperfilm. Fig.11 shows Southern blot analysis of DNA from transformed and non-transformed *A.tumefaciens*. All the DNA samples from transgenic *A.tumefaciens* generated strong signals, whereas the DNA from non-transformed *A.tumefaciens* cultures, used as negative control, generated no signals after hybridization. Transformed *Agrobacterium* bacterial clones containing the anti-HSP17 scFv sequences in their total DNA were used to infect leaf disks of *in vitro* cultivated *N.tabacum* plants.

0.5 cm² leaf disks were infected with a freshly grown bacterial culture of transgenic *A.tumefaciens* and after 2 days transferred to the selective medium containing antibiotic and plant hormones NAA and BA. During incubation of the leaf disks on the medium containing plant hormones callus formation was observed. The regeneration of plants from the callus culture started after 3 weeks of *in vitro* cultivation. Regenerated plants were cut from the callus and grown on the selective medium without plant hormones. Rooted plants were able to survive on the selective medium and can potentially be counted as transgenic plants. However, not all regenerated plants were also able to survive on the selective medium. Probably regenerates were formed from the upper part of the leaf disk. That may work as a filter preventing entrance of the selective antibiotic from the medium. Thus, plants regenerated from non-infected cells of the leaf disk, are not resistant to the selective antibiotic.

25 kanamycin resistant primary plant transformants from each transformation experiment using 6 different expression cassettes were planted in the greenhouse and propagated for flowering and seed development.

With respect to the intracellular distribution of expressed scFvs the groups of transgenic plants were designated as follows: **CA** – cytoplasmic expression of anti-HSP17 scFv **A14** – c-myc – KDEL fusion protein; **CB** – cytoplasmic expression of anti-HSP17 scFv **B8** – c-myc – KDEL fusion protein; **TA** – cytoplasmic expression of Thioredoxin A – anti-HSP17 scFv **A14** – c-myc protein fusion; **TB** – cytoplasmic expression of Thioredoxin A – anti-HSP17 scFv **B8** – c-myc fusion protein; **RA** –

Legumin Signal Peptide and KDEL mediated ER accumulation of the anti-HSP17 scFv **A14** – c-myc – KDEL fusion protein; **RB** – Legumin Signal Peptide and KDEL mediated ER accumulation of the anti-HSP17 scFv **B8** – c-myc – KDEL protein fusion.

Seeds from the primary plant transformants were collected and used for further propagation of the next plant generations after drug selection of germinating seeds on selective MS medium containing kanamycin followed by propagation of drug-resistant plants in the greenhouse.

3.5. ScFv expression in transgenic plants

3.5.1. Western blot analysis of scFv expression in primary transgenic plants

In order to select the primary transgenic plants with high levels of anti-HSP17 scFv expression under the transcription control of the CaMV 35S promoter, leaf material from 25 of each CA, CB, RA, RB, TA and TB plants growing in the greenhouse were semiquantitatively analyzed by Western blotting using the anti-c-myc monoclonal antibody 9E10 as primary antibody followed by anti-mouse IgG-HRP conjugate. The signals were generated by chemiluminescence using ECL Kit and detected by exposition of the blotting membrane to ECL Hyperfilm. The results of Western blot analysis of protein extracts isolated from plants with a high level of anti-HSP17 scFv expression are shown in Fig.12.

The level of cytoplasmic scFv expression greatly varied in the leaves of individual primary transformants, but the highest scFv amounts were found in leaf extracts from the plants CA8, CB6 and CB11. Therefore, primary transgenic plants CA8, CB6 and CB11, expressing the anti-HSP17 scFv – c-myc – KDEL fusion protein, were preferred for further experiments. In comparison to CA and CB plants the expression of the scFv as Thioredoxin-scFv fusion protein was low in primary transgenic TA and TB plants. Additionally, the relatively high molecular mass of the Thioredoxin-scFv fusion protein, in comparison to the scFv-c-myc-KDEL fusion protein, could cause unexpected complications in the process of antigen-antibody interaction between endogenous sHSPs and cytoplasmically expressed scFvs in transgenic cells. Both facts favored the use of transgenic plants with the expression of the scFv-KDEL fusion protein for further investigations to immunomodulate the heat shock response of plants.

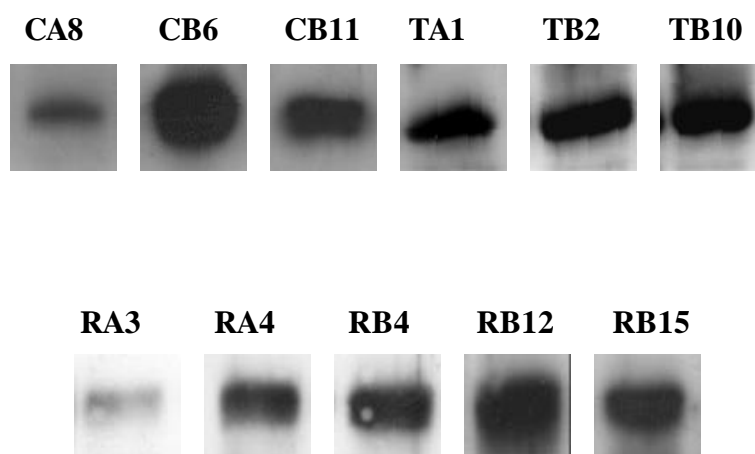


Fig. 12 Western blot analysis of scFv expression in leaves of primary transformants.

Protein extracts from the leaves of primary transgenic plants CA, CB (10 μ g per well) and TA, TB, RA and RB (20 μ g per well) were electrophoretically separated on 12.5% SDS polyacrylamide gel. After blotting scFv was semiquantitatively detected by monoclonal anti-c-myc antibody 9E10 followed by anti-mouse IgG conjugated to HRP. Signals were generated by chemiluminescence. **CA8** – transgenic plant with cytoplasmic expression of anti-HSP17 scFv from clone A14 as KDEL fusion protein; **CB6**, **CB11** - transgenic plants with cytoplasmic expression of anti-HSP17 scFv from clone B8 as KDEL fusion protein; **TA1** - transgenic plant with cytoplasmic expression of anti-HSP17 scFv from clone A14 as Thioredoxin A fusion protein; **TB2**, **TB10** - transgenic plants with cytoplasmic expression of anti-HSP17 scFv from clone B8 as Thioredoxin A fusion protein ; **RA3**, **RA4** – transgenic plants with ER retention of expressed anti-HSP17 scFv from clone A14 as KDEL fusion protein; **RB4**, **RB12**, **RB15** - transgenic plants with ER retention of expressed anti-HSP17 scFv from clone B8 as KDEL fusion protein.

For transgenic plants with anti-HSP17 scFvs targeted into ER the highest scFv levels were found in protein extracts from the leaves of plants RA3, RA4, RB4, RB12 and RB15.

Generally, the level of cytoplasmic scFv expression in primary transgenic CB plants was higher than in CA plants. This corresponds to a lower level of scFv accumulation in RA primary transgenic plants as compared to the scFv level in RB plants. The highest level of scFv among all the primary transgenic plants was found in the primary transgenic plant CB6.

The primary transgenic plants CA8, CB6, CB11, RA3, RA4, RB12 and RB15 were grown in the greenhouse for seed maturation. The seeds were used to obtain T₁ and T₂ generations of the respective transgenic plants.

3.5.2. Selection of transgenic plants with stable scFv expression

Seeds from T₀ transgenic plants CA8, CB6, CB11, RA3, RA4, RB4, RB12 and RB15 expressing a relatively high level of scFv were germinated on a selective MS medium containing 50 µg ml⁻¹ kanamycin. The results of segregation analysis are presented on Tab.1. 50 seeds from each individual T₀ plant were planted on two independent plates. The numbers of antibiotic resistant plantlets were counted after about 30 days of growing on the medium and compared to the total seed number used for each transgenic plant. The seeds from the wild type plants were simultaneously incubated on selective medium to check the efficiency of drug selection. Seeds collected from primary transgenic plants exhibited segregation of kanamycin-resistant and kanamycin-sensitive plants with ratios 3:1 and 15:1 indicating that active copies of the scFv gene were integrated at a single (segregation 1:3) or double (segregation 1:15) locus in the plant genome and were inherited as a simple Mendelian trait. Selected germinated kanamycin-resistant T₁ plantlets were transferred to soil and grown in the greenhouse to obtain the next generations of transgenic plants with stable scFv expression. T₁ generation plantlets developed from the seeds of plants RB4 and RB15 were unable to survive on kanamycin containing MS medium, whereas the seeds from primary transgenic RA3, RA4 and RB12 plants germinated and developed plantlets under conditions of drug selection.

Plants CA8, CB6 and CB11 from the T₁ generation were also analyzed by Southern blotting to confirm scFv gene integration and to check the copy number of scFv gene in the transgenic plant genome. Isolated genomic DNA samples from plant leaves were digested with restriction enzyme BamHI, electrophoretically separated, transferred to nitrocellulose membrane and hybridized with a ³²P-labelled DNA probe containing anti-HSP17 scFv sequence. As shown in Fig.13, genomic DNA fragments from plants CA8, CB6 and CB11 hybridized specifically with the scFv DNA probe and generated strong positive signals. indicating integration of the recombinant constructs into genome of plants CA8, CB6 and CB11. According to the number of labeled bands on the hybridization

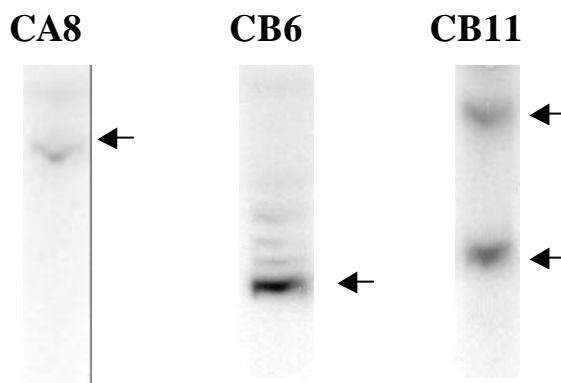


Fig. 13 Southern blot analysis of genomic DNA isolated from transgenic plants.

Genomic DNA samples isolated from leaves of transgenic plants CA8, CB6 and CB11 were cleaved with BamHI, electrophoretically separated and hybridized with the radioactive-labeled scFv DNA probe. Signals were detected after exposition of the membrane to Fuji Imaging plate Type BAS III which was analyzed with Fuji BioImaging Analyzer BAS 2000 software (Fuji Foto Film Co., LTD, Japan) and Tina v2.08 software (Raytest, Sprockhow, Germany). Arrows show genomic DNA fragments that specifically hybridized with the scFv DNA.

image, shown in Fig.13, transgenic plant lines CA8 and CB6 have only one copy of the foreign gene whereas transgenic plant line CB11 has two scFv gene copies integrated into the genome.

The T₁ progenies of plants CA8, CB6 and CB11 were also analyzed at the protein level by use of Western blotting. Protein extracts isolated from leaves were electrophoretically separated on polyacrylamide gel and transferred to a nitrocellulose membrane. Anti-HSP17 scFvs were detected by using the anti-c-myc monoclonal antibody 9E10 as a primary antibody followed by anti-mouse IgG conjugated to HRP. Enriched preparations of the anti-ABA scFv-c-myc fusion protein, extracted from leaves of transgenic *N.plumbaginifolia* plants with expression of the corresponding scFv (Senger , 2000), were used as a positive control. The molecular mass of the anti-ABA scFv fusion protein is a little bit higher than the molecular mass of anti-HSP scFv. However, anti-ABA scFv is also expressed as c-myc-tagged scFv-KDEL fusion protein in transgenic *N.plumbaginifolia* and can be detected independently of its antigenic specificity by using the same immunochemical assay as the c-myc-tagged anti-HSP17 scFv. The results of Western blot analysis

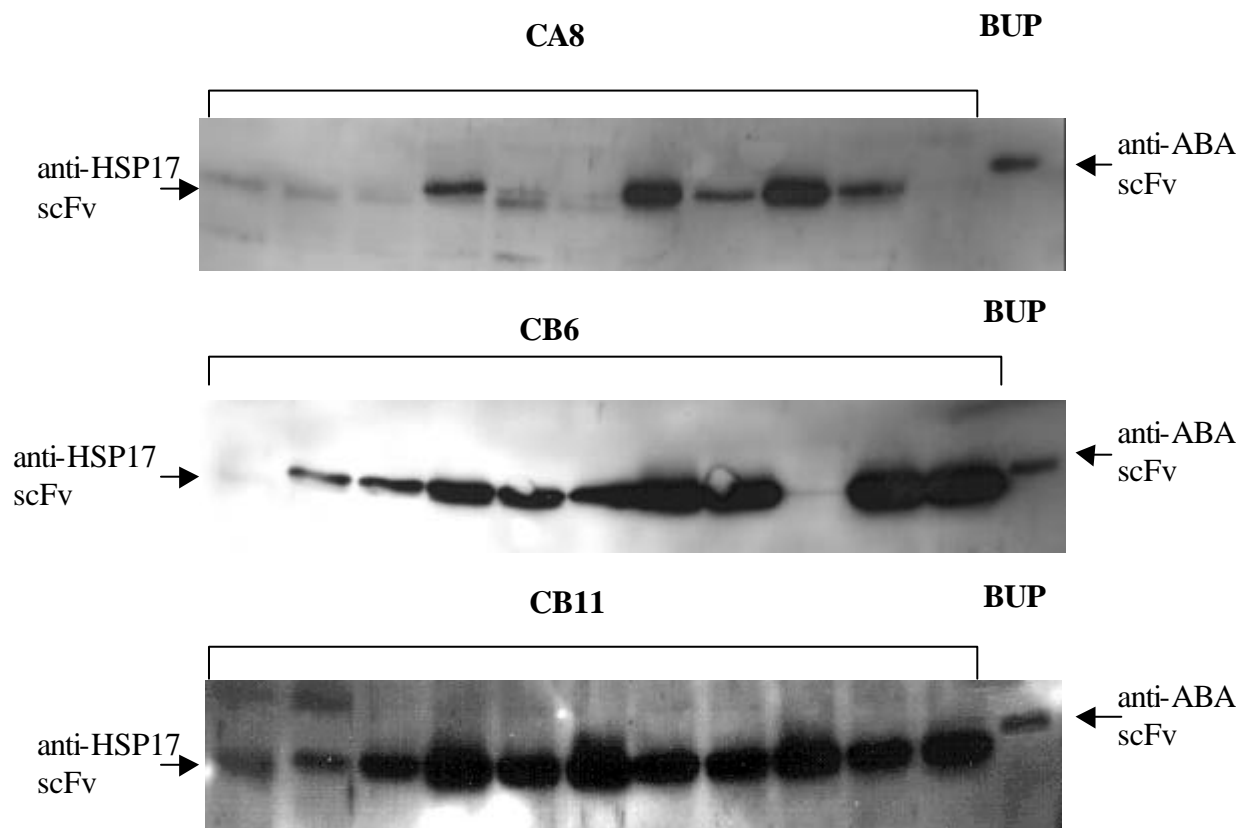


Fig. 14 Semiquantitative detection of the scFv content in protein extracts from leaves of transgenic plants of T_1 generation.

10 μ g protein per well were separated on 12.5% polyacrylamide gel. Western blot analysis was performed using monoclonal anti-c-myc antibody 9E10 as primary antibody followed by anti-mouse IgG conjugated to HRP. Signals were generated by chemiluminescence using ECL Kit. **BUP** – anti-ABA scFv as reference antigen. **CA8**, **CB6**, **CB11**– protein extracts from leaves of individual plants of transgenic lines CA8, CB6 and CB11.

concerning the levels of scFvs in the leaves of individual transgenic plants from the T_1 generation of the lines CA8, CB6 and CB11 are shown in Fig 14. The levels of anti-HSP17 scFv expression are very different in individual plants that originated from the same transgenic line of the T_1 generation. Seeds from individual plants CA8, CB6 and CB11 of the T_1 generation with the highest level of scFv expression were collected, germinated and cultivated on drug selective medium followed by propagation in the greenhouse to receive stable transgenic plants of the T_2 generation.

50 seeds of each of 4 individual T_1 transgenic plants CA8 with the highest level of scFv expression were germinated and cultivated on kanamycin-containing

MS medium. All the T₂ plantlets originating from plant CA8-4 were able to survive on selective medium (see Tab.2) and 10 of them were grown under greenhouse conditions. But all T₂ plants of line CA8 had to be discarded because of complete absence of scFv expression in the plants of this line in generation T₂.

50 seeds of each of 4 individual T₁ transgenic plants CB6 with the highest level of scFv expression were germinated and cultivated on kanamycin containing MS medium. All the T₂ plantlets, also those grown from the seeds of plant CB6-1, were able to survive under drug selection (see Tab.2) and 10 of them were propagated in greenhouse. Leaf extracts obtained from 5 individual plants of transgenic line CB6 of the T₂ generation were semiquantitatively analyzed using Western blot. The result of Western blot analysis as to the scFv level in leaves of transgenic plants is shown in Fig.15. All the 5 plants showed a high expression of scFv. Further generations of plants grown from the seeds of transgenic plant CB6-1 were designated as plant line CB6.

50 seeds of each of 4 individual T₁ transgenic plants CB11 with the highest levels of scFv expression were germinated and cultivated on MS medium under drug selection. The plantlets showed segregation into kanamycin-sensitive and kanamycin-resistant ones with a ratio of 1:15 or 1:3 (see Tab.2). Due to the time-consuming procedure for selection of plant line CB11, containing two active copies of scFv genes integrated into genome it was decided to use transgenic plant line CB6 for further investigations (Fig.15).

Tab. 1 Segregation analysis of seeds from T_0 transformants of *Nicotiana tabacum*. Segregation of kanamycin-resistant and kanamycin-sensitive plantlets was performed on selective MS medium containing $50 \mu\text{g ml}^{-1}$ of kanamycin. The seeds were germinated and cultivated for 3 weeks in two independent experiments. Numbers of plantlets used for the second experiment are shown in brackets.

Plant	Number of resistant plantlets	Number of non-resistant plantlets	Ratio of resistant/non-resistant plantlets
CA8	46(49)	4 (1)	1 : 15
CB6	38(34)	12 (16)	1 : 3
CB11	47(48)	3 (2)	1 : 15
RA3	52(48)	8 (12)	1 : 3
RA4	45(41)	5 (9)	1 : 3
RB4	0(0)	0 (0)	-
RB12	49(47)	1 (3)	1 : 15
RB15	0(0)	50 (50)	-

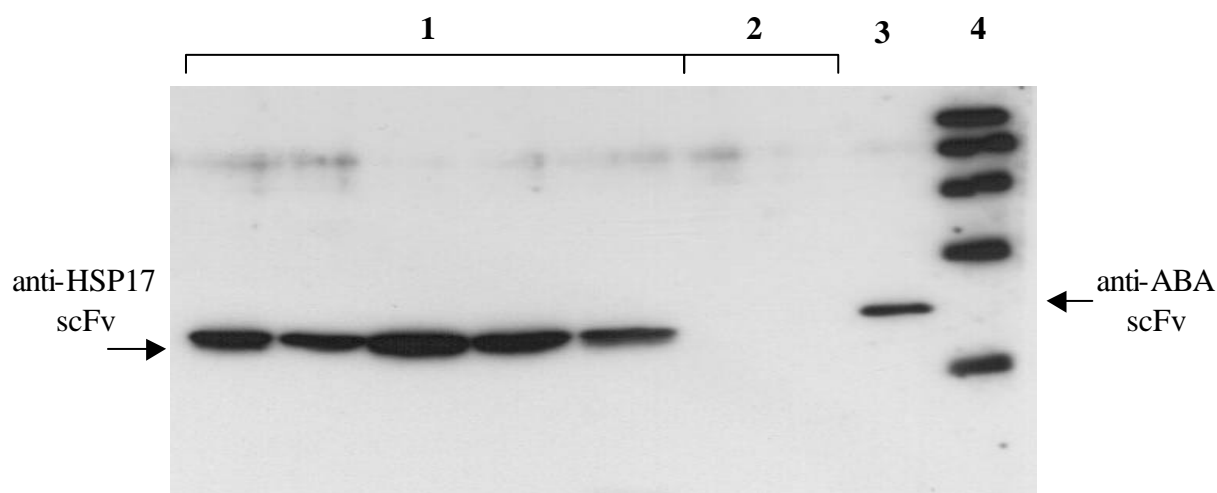


Fig. 15 Semiquantitative detection of the scFvs in protein extracts from the leaves of transgenic plant line CB6 of generation T_2 using Western blot analysis.

1 – protein extract of leaves from individual plants of transgenic line CB6, **2** – protein extracts from leaves of wild type plants, **3** – anti-ABA scFv as reference antigen, **4** – See Blue Standard protein marker. 10 μg of protein were loaded per slot. Primary antibody – monoclonal anti-c-myc antibody clone 9E10 was used prior to anti-mouse IgG conjugated to HRP. Signal generation was performed by using the ECL Kit.

Tab. 2 Segregation analysis of seeds collected from individual CA8, CB6 and CB11 plants from T₁ generation.

Segregation of kanamycin-resistant and kanamycin-sensitive plantlets was performed on selective MS medium containing 50 µg ml⁻¹ of kanamycin. About 50 seeds from individual plants were germinated and cultivated for 3 weeks. Two independent experiments were performed. Numbers of plantlets from the second experiment are shown in brackets.

Plant	Number of resistant plantlets	Number of non-resistant plantlets
CA8-1	46 (48)	6 (2)
CA8-2	36 (37)	14 (13)
CA8-3	43 (39)	7 (11)
CA8-4	50 (50)	0 (0)
CB6-1	50 (50)	0 (0)
CB6-2	31 (46)	9 (4)
CB6-3	34 (38)	16 (12)
CB6-4	39 (38)	11(12)
CB11-1	38 (38)	12 (12)
CB11-2	47 (48)	3 (2)
CB11-3	50 (49)	0 (1)
CB11-4	37 (38)	11 (12)

3.5.3. Quantitative estimation of scFv content in transgenic plants

Quantitative estimation of scFv content in the leaf tissue of CB6 plants and transgenic control plants (GM and JC) was performed to compare their levels of scFv expression.

The buffer-soluble proteins were quantitatively extracted from leaves of seven individual transgenic CB6 plants (see 2.2.6.3.). 10 and 5 µg of protein per slot were electrophoretically separated on polyacrylamide gel and blotted on nitrocellulose membrane. Anti-HSP17 scFvs were detected quantitatively by using an excess of anti-myc monoclonal antibody 9E10 as a primary antibody followed by an excess of anti-mouse IgG conjugated to HRP. The signals were generated by chemiluminescence using the ECL Kit and documented by exposition with ECL Hyperfilm. The images of generated signals on the ECL Hyperfilm were scanned and

digitally measured by using Tina v2.08 software (Raytest, Sprockhow, Germany). The concentration of anti-HSP17 scFv in the protein extracts was estimated using the calibration curve calculated from the signal intensity produced by known amounts of the pure scFv preparation. For this purpose the anti-HSP17 scFv–c-myc fusion protein was expressed as recombinant protein in *E.coli* and purified by affinity chromatography on Protein L agarose column. The protein concentration of the pure scFv preparation was determined according to Bradford (Bradford, 1976). A concentration range from 0.5 to 5 ng per slot was loaded simultaneously with the leaf protein

Tab. 3 Quantitative estimation of the anti-HSP17 scFv content in leaves of transgenic plant line CB6.

Protein extracts (5 and 10 μ g) from transgenic leaves and concentration range (0.5-5 ng) of the scFv standard were used for Western blotting. The specific signals of scFv on the ECL Hyperfilm image were scanned and digitally measured by using Tina v.2.08 software. Standard detection for average volume of the scFv content was calculated using Microsoft Excel 97 software.

Number of transgenic CB6 plants	Anti-HSP17 scFv, ng mg⁻¹ per μg soluble protein
1	1.09
2	0.77
3	0.74
4	1.15
5	1.03
6	1.11
7	0.89
Average content	0.97\pm 0.168

extracts on the polyacrylamide gel, electrophoretically separated and immunochemically detected. Table 3 shows the calculated amounts of scFvs in the protein extracts from leaves of seven individual CB6 plants. The scFv content in the leaves isolated from individual CB6 plants did not vary to a high degree and reaches

about 0.097% of soluble leaf protein. Plants with cytosolic expression of scFv specific to transcription factor Fus3 (GM plants), which is absent in leaf tissue (Dr. G.Mönke, IPK, Gatersleben), were used as one of the transgenic controls. Soluble proteins were extracted from leaves of three individual transgenic GM plants (see 2.2.6.3.). The estimation of scFv content was performed in the same way as described above for CB6 plants. The calculated average amount of anti-Fus scFv in the leaves of transgenic GM plants was $0.508 \pm 0.338 \text{ ng } \mu\text{g}^{-1}$ or 0.0508% of soluble leaf protein. Thus, scFv level in transgenic CB6 and GM plants is about on the same level. GM plants can therefore be used as transgenic controls. Furthermore, an additional transgenic control was introduced in our investigations. This transgenic control plant expressed cytoplasmically an anti-jasmonate scFv under transcriptional control of the CaMV 35S promoter (JC plants) at the level of $0.337 \pm 0.058 \text{ ng } \mu\text{g}^{-1}$ or 0.0337% of soluble leaf protein (P.Bartoskova, IPK, Gatersleben). Since jasmonate has no functional activity during the heat shock response of plants, we used the leaves of JC plants as well as GM plants to verify the results on immunomodulation of sHSP by the cytoplasmically expressed anti-HSP17 scFv during the heat shock response of plants.

3.5.4. Intracellular distribution of scFvs in transgenic cells

Electron microscopic analysis was performed in order to look for the intracellular distribution of expressed anti-HSP17 scFv in transgenic plant cells. Mesophyll cells from vegetative tissue of transgenic CB6 plants were analyzed using immunolabeling of the sections with anti-c-myc antibody. Bound scFvs were labeled by using a bridge anti-mouse IgG antibody followed by gold-labeled protein A. Fig.16 shows the intracellular localization of expressed scFv in mesophyll cells of mature transgenic leaves. The intracellular distribution of scFv produced in mesophyll cells of the leaves is restricted to the cytosol and the nucleus of transgenic plant cells. The most pronounced labeling of scFvs was seen in the cytosol, but labeling was also observed in the nuclei. No scFv was detected in the vacuoles, in the cell wall and in other organelles. Sections A and C of Fig.16 were used to compare the signal density in the cytosol and in the nucleus of transgenic cells. The signal spots were calculated on three squares of $1 \mu\text{m}^2$ taken randomly from the areas of the cytosol (sections A) and

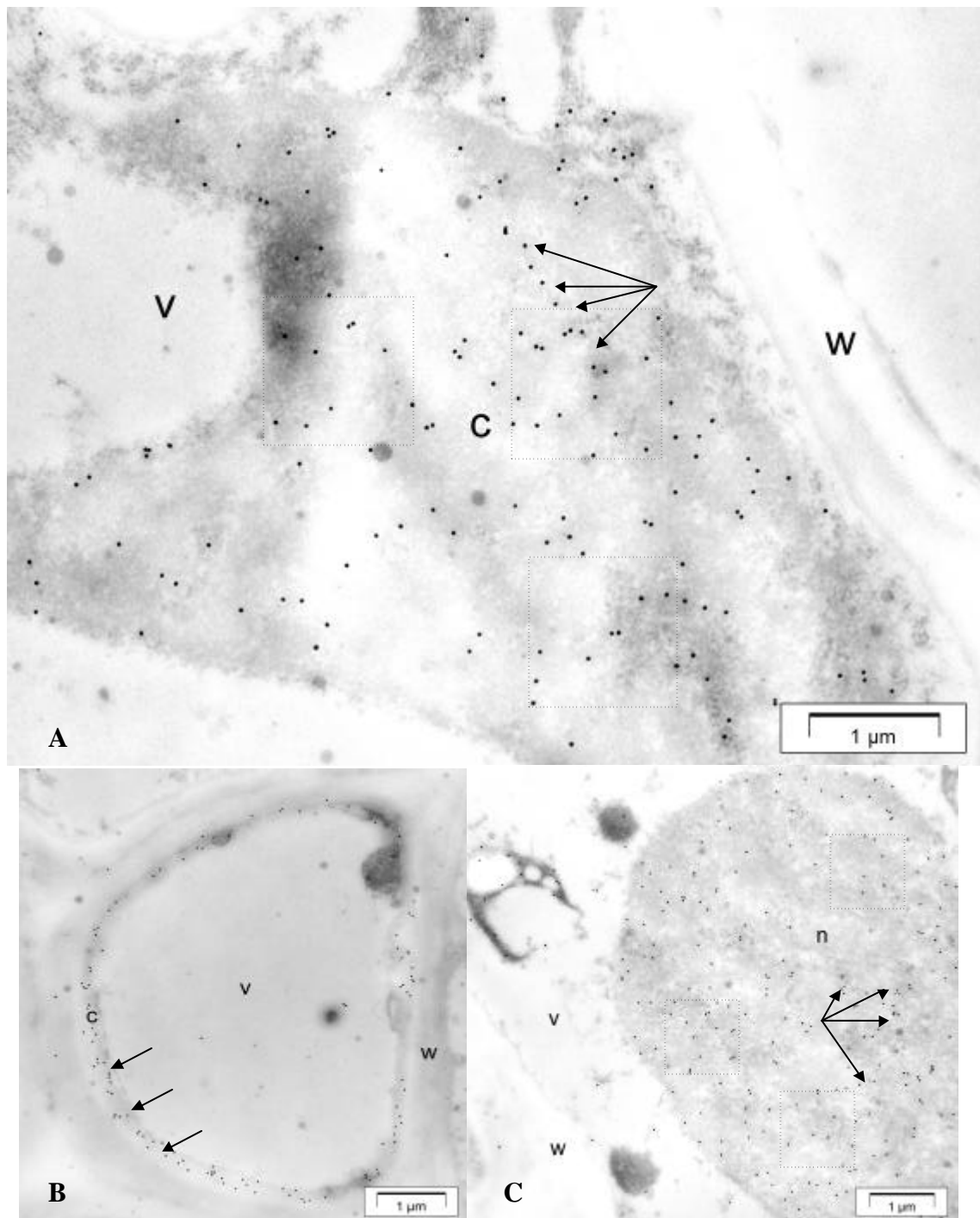


Fig. 16 Intracellular distribution of anti-HSP17 scFvs in mesophyll cells of the transgenic CB6 plant.

Anti-HSP17 scFv was immunolabeled by using monoclonal anti-c-myc 9E10 antibody followed by the bridge anti-mouse IgG antibody and gold-labeled protein A. **A, B** – distribution of anti-HSP17 scFv in the cytosol of transgenic plant cell. **C** – localization of anti-HSP17 scFv in the nucleus of the transgenic plant cell. Arrows show the specific scFv labeling. Areas marked with squares were used to calculate the average labeling density detected in the cytosol and nucleus. **v** - vacuole, **w** – cell wall, **n** - nucleus, **c** – cytosol.

the nucleus (sections B). Average numbers of signal spots were calculated as shown in Tab. 4. The density of signal spots specifically detecting scFvs in the cytosol of CB6 mesophyll cells is 1.2 times higher than the labeling density in the nucleus. This indicates a similar or a little bit higher distribution of scFvs in the cytosol in comparison with nucleus.

Table 4 Comparison of the labeling density in cytoplasm and nucleus of transgenic cells.

The number of signal spots was counted on three squares of $1 \mu\text{m}^2$ taken randomly from areas of the cytosol and the nucleus on the electron microscopic images.

	Number of signals from randomly taken $1 \mu\text{m}^2$ squares			Average number of signals per $1 \mu\text{m}^2$
Cytosol	9	13	8	10 ± 2.6
Nucleus	11	7	7	8.3 ± 2.3

3.6. Phenotypic and ultrastructural alterations of transgenic plants in response to heat stress

3.6.1. Phenotypic alterations of plant leaves during heat treatment

Transgenic CB6 and transgenic control plants showed no differences concerning phenotype when compared to wild type plants at normal temperature. However, if the cytoplasmically expressed anti-HSP17 scFvs are able to immunomodulate the function of stress-elicited sHSPs *in vivo* during stress response, then thermosensitivity of transgenic CB6, transgenic controls and wild type plants may be different.

In order to check the basal thermotolerance of transgenic plants with cytoplasmic anti-HSP17 scFv expression and of control plants, a stress regime for 2 h starting from sublethal and finishing with lethal temperatures was chosen. Wild type plants and transgenic plants expressing cytoplasmically scFvs without sHSP specificity (GM plants with anti-Fus scFv expression and JC plants with anti-jasmonate scFv expression) or plants accumulating anti-HSP17 scFv in the ER of mesophyll cells (RB plants) were used as control plants.

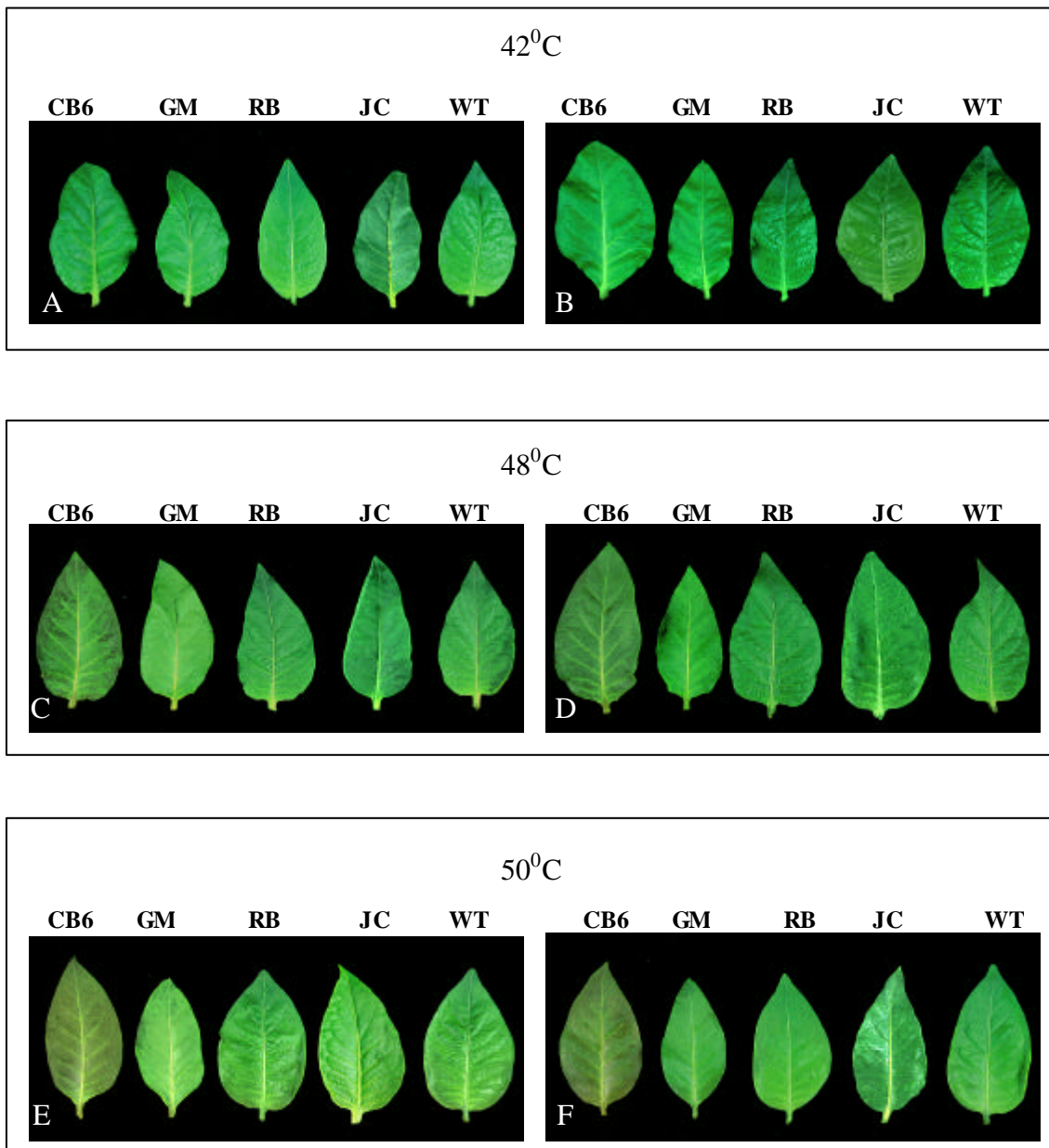


Fig. 17 Phenotypic alterations of **CB6**, transgenic (**GM**, **RB**, **JC**) and wild type (**WT**) control leaves due to heat stress.

For comparison of basal thermotolerance, leaves were stressed for 2h at 42°C (**A**), 48°C (**C**) and 50°C (**E**). For comparison of the ability to acquire thermotolerance the leaves were pre-treated for 15 min at 42°C with a subsequent recovery period of 2 h at 25°C and then challenged for 2 h at 42°C (**B**), 48°C (**D**) and 50°C (**F**).

All investigated plants were grown at the same light and temperature regimes in the greenhouse. Leaves of about the same age were collected from the plants and stress-treated for 2 h at 42°C, 48°C and 50°C, respectively. After stress treatment the phenotype of the leaves was photographically documented.

Fig. 17 shows representative example of phenotypical alterations of the leaves caused by the temperature treatments of the transgenic immunomodulated plants (CB6) as compared to transgenic control (RB, GM and JC plants) and wild type plants. The leaves of transgenic immunomodulated and control plants as well as the wild type did not show any visible alterations of their phenotypes after 2h of stress treatment at 42°C (Fig. 17, A). Transgenic control leaves and wild type leaves also showed no visible changes after 2h of heat stress at 48°C, while local development of multiple brownish necrotic spots was observed on the leaves of immunomodulated CB6 plants with anti-HSP17 scFv expression (Fig. 17, C). After 2h heat stress at 50°C the leaves of immunomodulated transgenic CB6 plants changed their color to brownish (Fig. 17, E). No such stress damage symptoms as for CB6 leaves were observed for transgenic control and wild type plants. The stronger phenotypic alterations of the leaves from transgenic plants with anti-HSP17 scFv expression in comparison to the transgenic control and wild type plants caused by the stress regime are indicative of a lower basal thermotolerance of immunomodulated CB6 plants than that of the corresponding controls.

In order to check this immunomodulation phenomenon more in detail, the content of chlorophyll pigments in the leaves of transgenic CB6 plants and wild type plants was measured after heat shock. Heat stress treatment of the leaves was performed at 42°C, 48°C and 50°C, respectively. After each stress treatment the fresh weight of the leaf samples was determined and the chlorophyll pigments were extracted from the samples with acetone. Optical density of the chlorophyll extracts was measured at 645 nm and 662 nm to calculate the contents of chlorophyll in transgenic CB6 and wild type leaves. The experiment was repeated three times. The average amount of chlorophyll pigments in CB6 and wild type leaves and the standard deviation were calculated using the Microsoft Excel 97 software and are presented as a diagram in Fig.18. No change of the chlorophyll content was found in CB6 and wild type leaves after 2h of 42°C stress treatment, whereas the chlorophyll content in CB6 leaves was diminished after 2h at 48°C stress treatment in comparison to the wild type leaves. Decrease of the chlorophyll content in CB6 leaves in comparison to wild type

leaves confirmed visually observed stress effects: only CB6 leaves showed brownish necrotic spots; no stress damage was observed on wild type and transgenic control leaves after the same stress treatment (Fig. 17). The content of chlorophyll pigments in leaves of transgenic plants that expressed anti-HSP17 scFv decreased continuously with increasing stress temperatures although the pigment content of transgenic control and wild type leaves were stress-protected at all the used temperatures from 42°C to 50°C. We concluded that the basal thermotolerance of immunomodulated CB6 plant is diminished ($\leq 48^{\circ}\text{C}$) in comparison to transgenic control and wild type plants ($\geq 50^{\circ}\text{C}$).

The ability to acquire thermotolerance of transgenic CB6 leaves with anti-HSP17 scFv expression, transgenic control and wild type plants was compared after a short pretreatment of the leaves for 15 min at 42°C, 2h recovery at 25°C and 2h of stress treatment at 42°C, 48°C or 50°C. The transgenic CB6 leaves and the control plant leaves pretreated 15 min at 42°C did not show any visible effects caused by renewed stress treatment at 42°C after the recovery period (Fig. 17, B). Pretreated transgenic control and wild type leaves also showed no visible changes after 2h heat shock at 48°C (Fig. 17, D), whereas a weak development of brownish colored spots was found on pretreated transgenic CB6 leaves after the same stress treatment regime at 48°C (Fig. 17, D). After 2h stress at 50°C, pretreated CB6 leaves changed their color to brownish and showed wilt symptoms in contrast to pretreated control plants on which no such stress-elicited phenomena were observed (Fig. 17, C).

These results were confirmed by analysis of the chlorophyll pigment content in the leaves from CB6 plants expressing anti-HSP17 scFv as compared to control plants. The chlorophyll content remained unchanged in both pretreated CB6 leaves and control wild type plants by stress treatment at 42°C. However, CB6 leaves pretreated 15 min at 42°C followed by a recovery period and renewed stress treatments for 2h at 48°C or 50°C showed a lower decrease of chlorophyll pigments than non-pretreated CB6 leaves (compare Fig. 17 and 18). As revealed by the decrease of pigment content in CB6 leaves when compared to pretreated leaves of control plants, treated with the same stress regime mentioned above, CB6 leaves showed a restricted ability to acquire thermotolerance. In contrast to this, the wild

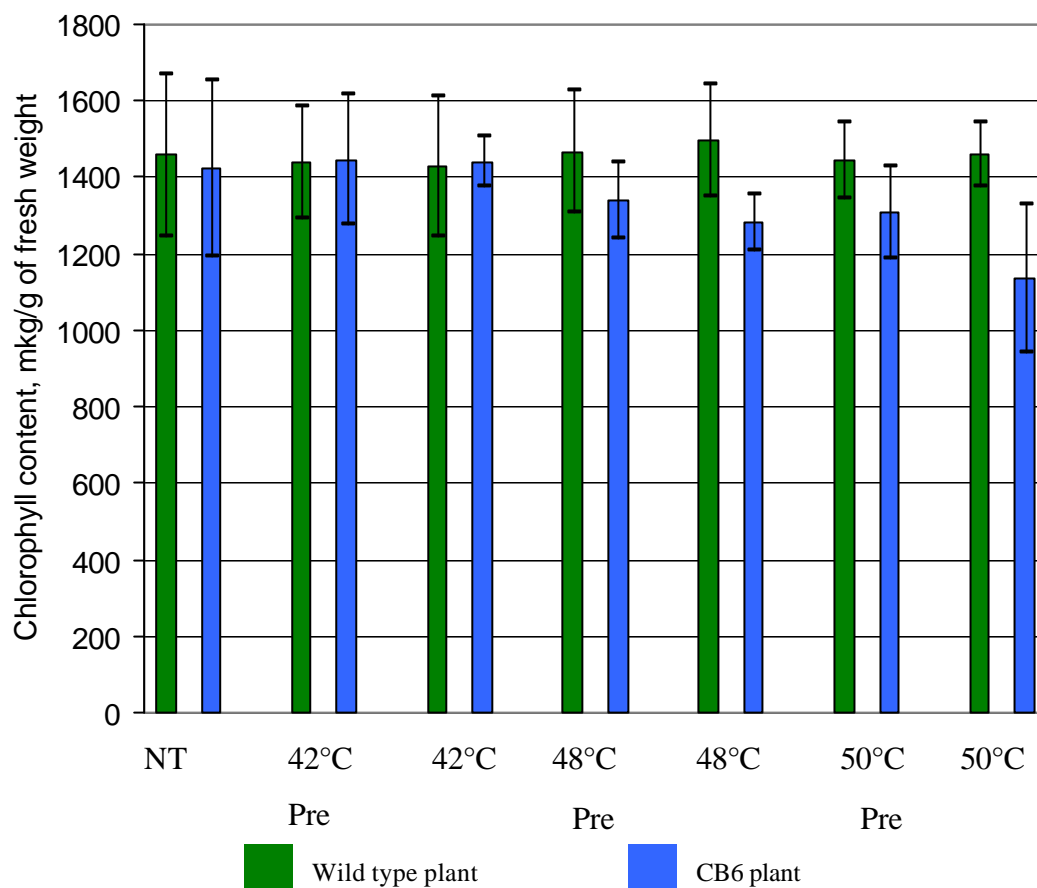


Fig. 18 Chlorophyll content in the leaves of transgenic CB6 and wild type plants in dependence on heat treatment.

Non-pretreated leaves were immediately stressed for 2 h at 42°C, 48°C and 50°C, respectively. **Pre** – pre-treated leaves were pre-stressed for 15 min at 42°C followed by 2 h of recovery at 25°C and heat stress treatment for 2 h at 42°C, 45°C and 50°C, respectively. **NT** – chlorophyll content of non-treated leaves at 25°C. Vertical lines show the standard deviation of average chlorophyll content.

type leaves were able to resist at 48°C and 50°C after a short stress pretreatment at 42°C and showed stress-protection at 48°C and 50°C independent of the treatment regime (compare Fig. 17 and 18).

No significant changes of chlorophyll content were observed in pretreated wild type leaves after renewed heat stress at 50°C in comparison to heat-treated wild type leaves without pretreatment. Observed phenotypical alterations caused by heat stress of the immunomodulated CB6 plants were in agreement with changes of chlorophyll content in their stress damaged leaves. Immunomodulated CB6 leaves

revealed a lower basal thermotolerance ($\leq 48^{\circ}\text{C}$) in comparison to the basal thermotolerance of the leaves from control plants ($\geq 50^{\circ}\text{C}$). The ability of CB6 leaves to acquire thermotolerance was limited, and acquired thermotolerance of CB6 leaves did not attain the level of basal thermotolerance of controls.

3.6.2. Stress-dependent distribution of anti-HSP17 scFv and HSP17 in leaf tissue of transgenic and wild type plants

Stressed and non-stressed mesophyll cells from the leaves of transgenic plants with cytoplasmic anti-HSP17 scFv expression were analyzed and compared to transgenic control and wild type plants by using immunofluorescence microscopy. Tag-specific monoclonal anti-c-myc antibodies were used as primary antibodies in order to detect intracellular distribution of scFvs. Transgenic GM plants with cytoplasmic anti-FUS scFv expression were used as transgenic control, wild type plants as non-transgenic control. After immunofluorescence staining, cell sections were counter-stained with DAPI for visualization of nuclei. Differential interference contrast images of the stained section of the leaves from CB6 plants were made and also used to demonstrate the histological structure of leaf tissue. Fig.19, a1 shows the distribution of anti-HSP17 scFv in non-stressed cells of a transgenic CB6 plant. ScFv is detected in the cytosol and also in the nucleus but not in the vacuoles of epidermal, palisade and spongy parenchyma cells. These results partly confirm previously described results of immunolabeling analysis of scFv expression in mesophyll cells of non-stressed CB6 plants (see 3.6.1.). A similar intracellular distribution of anti-FUS scFv in the cytosol and the nucleus of non-stressed transgenic GM control was observed as shown in Fig.19, c1. Mesophyll cells of the wild type showing no immunolabelling confirm the specificity of the immunochemical scFv detection procedure (Fig.19, b1). Only the c-myc tag of the expressed scFv fusion protein is specifically recognized by used monoclonal anti-c-myc antibody.

The intracellular distribution of cytosolic sHSPs in mesophyll cells of transgenic and wild type plants after heat stress was visualized with anti-sHSP17 antibodies followed by polyclonal biotinylated goat anti-rabbit IgG

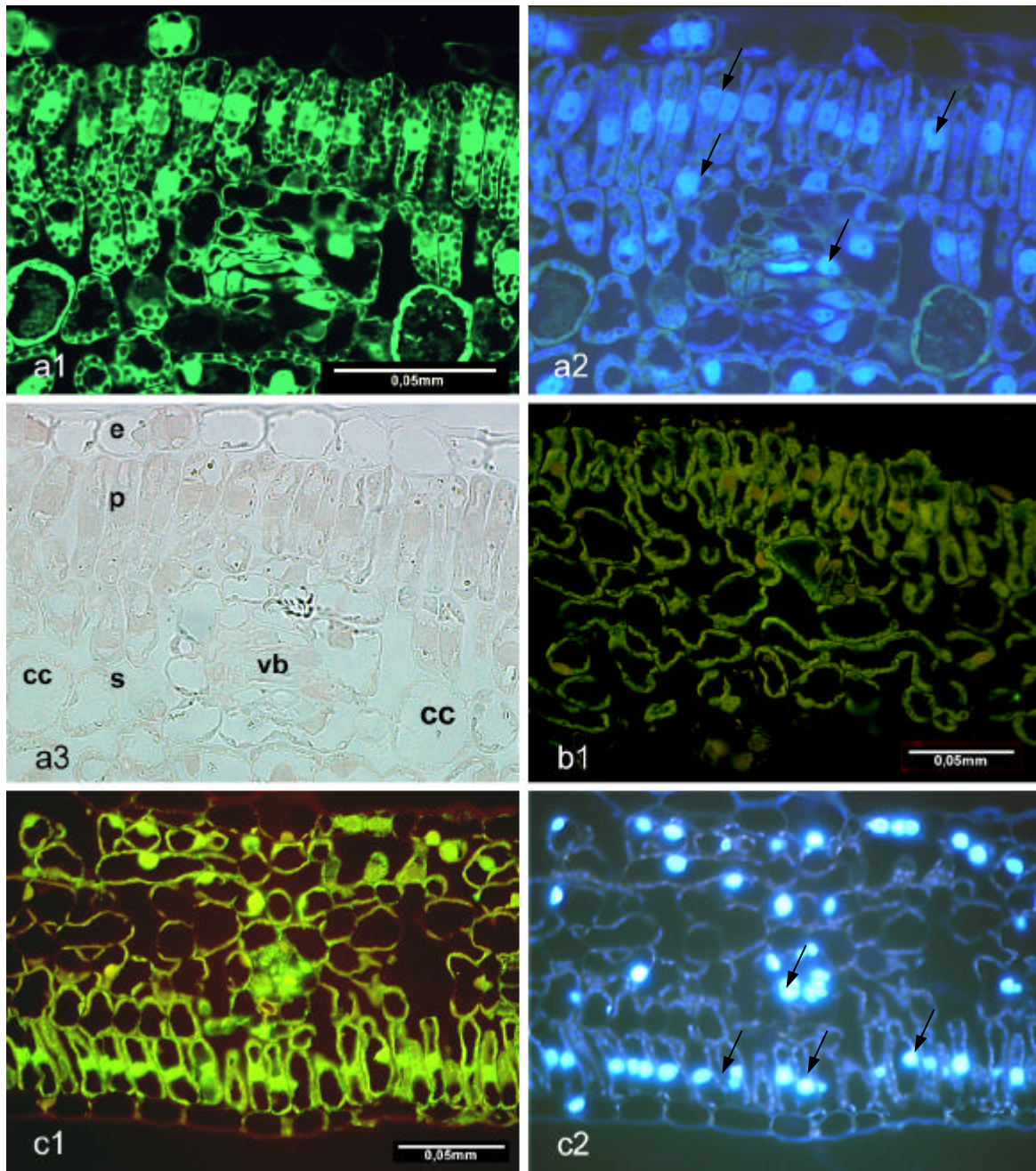


Fig. 19 Intracellular distribution of scFvs in mesophyll cells of anti-HSP17 scFv immunomodulated CB6 and transgenic GM control plants visualized by immunofluorescence.

a1 – localization of anti-HSP17 scFv in the leaf tissue of CB6 plant. **a2** – DAPI staining of the section shown in a1. **a3** – differential interference contrast microscopy of the section shown in a1. **b1** – section of wild type leaf tissue immunostained for fluorescence microscopy. **c1** – localization of anti-FUS scFv in leaf tissue of a transgenic GM control plant. **c2** – DAPI staining of the section shown in c1. The arrows show DAPI-stained nuclei. **cc** – crystal containing cells, **e** – epidermis, **p** – palisade parenchyma, **s** – spongy parenchyma, **vb** – vascular bundle.

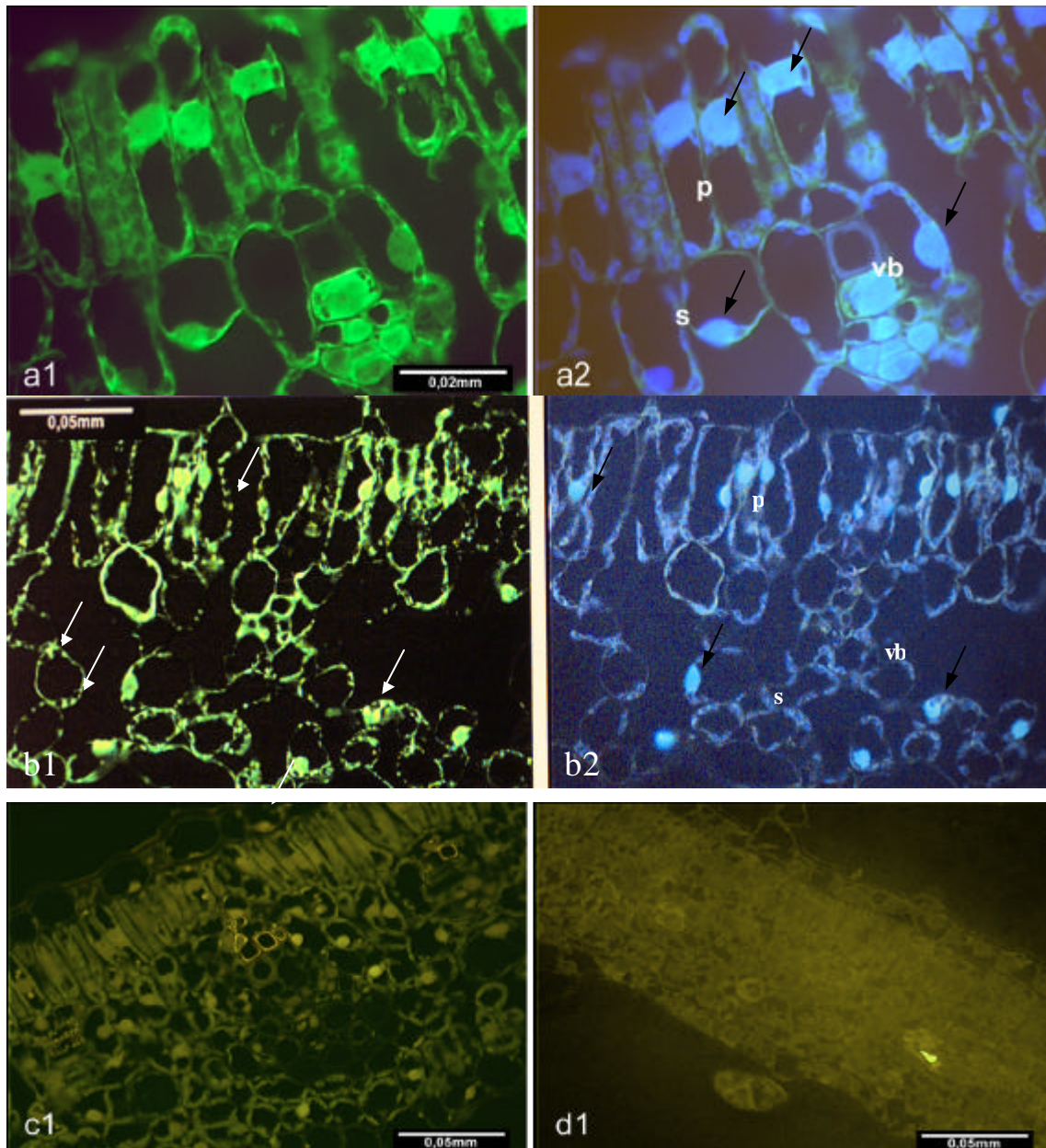


Fig. 20 Intracellular distribution of HSP17 in mesophyll cells of immunomodulated CB6 and wild type plants after heat stress.

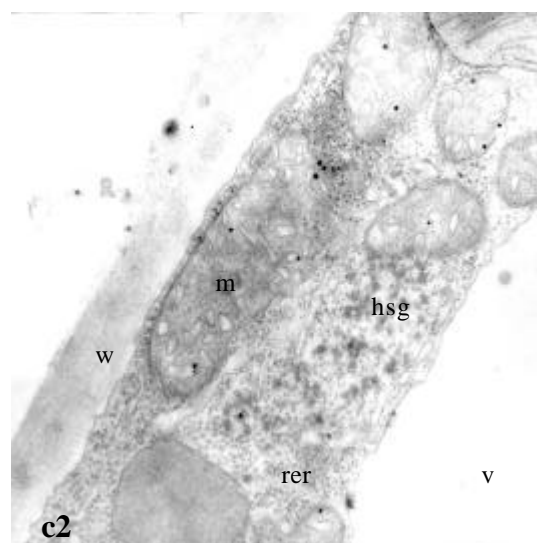
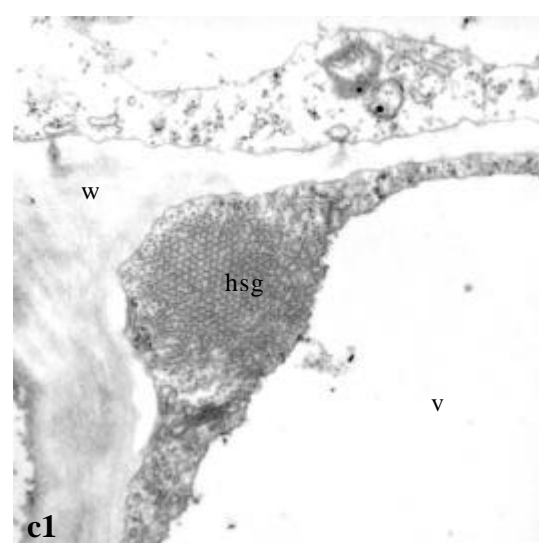
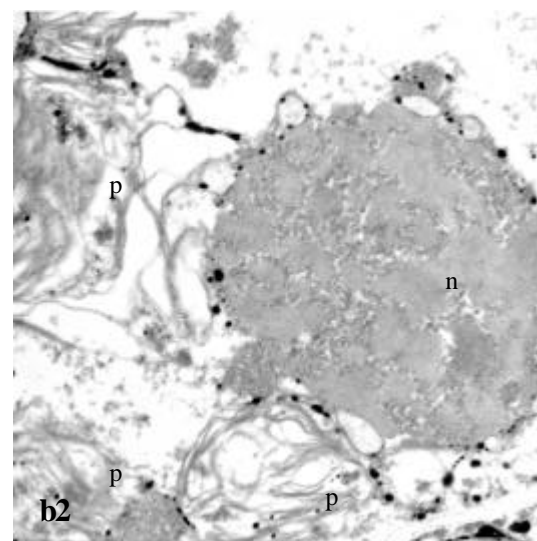
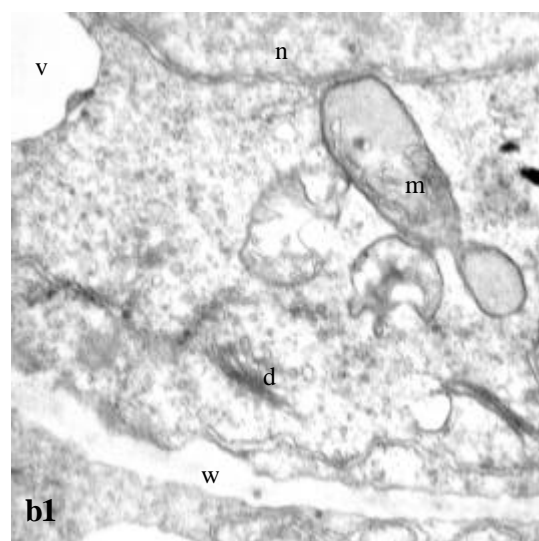
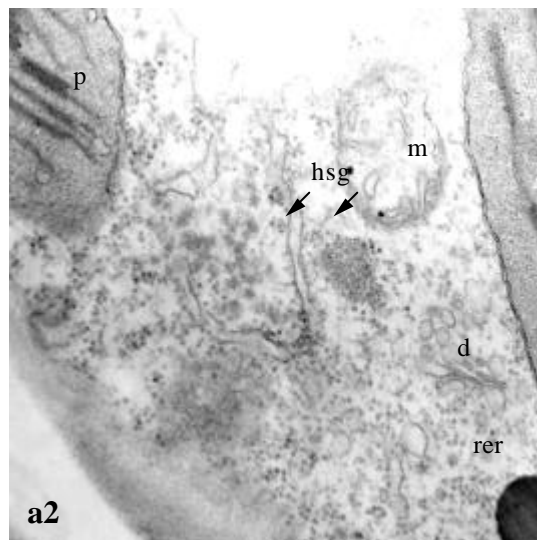
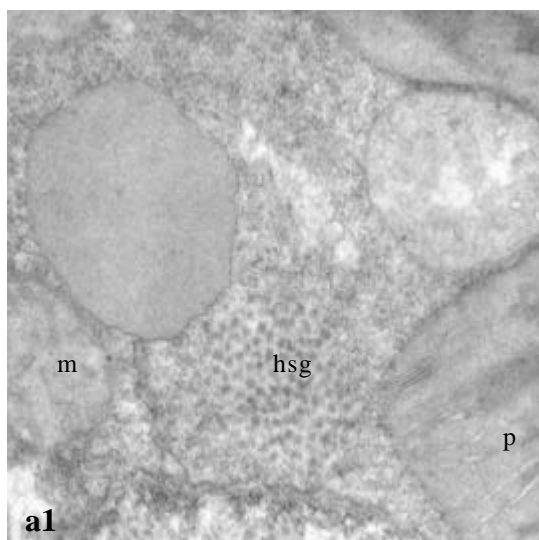
a1 – localization of HSP17 in leaf tissue of the transgenic CB6 plant after 2 h of heat stress. **a2** – DAPI staining of the section shown in a1. **b1** – localization of HSP17 in the leaf tissue of wild type plants after 2h of heat stress. **b2** – DAPI staining of the section shown in b1. **c1** – section of non-stressed immunofluorescence-labeled leaf tissue of the CB6 plant (negative control). **d1** – section of immunofluorescence-labeled non-stressed leaf tissue of the wild type plant (negative control). The black arrows show DAPI-stained nuclei, the white arrows show HSGs. **p** – palisade parenchyma, **s** – spongy parenchyma, **vb** – vascular bundle.

antibodies and a streptavidin-horse radish peroxidase conjugate by using the TSA-Direct-Green Kit. Leaves of transgenic CB6, transgenic controls and wild type plants were treated for 2h at 42°C to trigger the sHSP biosynthesis. Thereafter the leaves were immediately fixed and labeled for immunofluorescence microscopy. Unstressed leaves of transgenic CB6 and wild type plants were introduced as a control to detect the specificity of the immunolabeling procedure (Fig.20, c1 and d1). The intracellular distribution of HSP17 in the mesophyll cells of heat-stressed transgenic and control leaves is shown in Fig.20, a1 and b1. Cytosolic HSP17 is detected in the cytosol and in the nuclei of palisade and parenchyma cells of the transgenic CB6 plant (Fig.20, a1) and control plants (Fig.20, b1) under heat stress conditions. However, no formation of HSGs could be detected in the cytosol of CB6 cells after heat stress treatment; clearly visible HSGs, however, have been observed in the cytosol of heat-stressed wild type mesophyll cells (shown on Fig. 20, b1).

3.6.3. Influence of heat stress on the ultrastructure of immunomodulated mesophyll cells

Electron microscopic analysis was performed to investigate the impact of the expression of anti-HSP17 scFv on the ultrastructure of mesophyll cells after 2h and 4h of heat shock. Stress-treated leaves of transgenic RB plants accumulating anti-HSP17 scFv in the ER, transgenic GM plants, accumulating anti-Fus scFv in the cytosol, and wild type plants were used as controls.

Fig.21, a1 and a2 represent ultrastructural changes in mesophyll cells of the wild type plant after 2h and 4h of heat stress treatment at 42°C. HSGs are formed in the cytosol of wild type cells after 2h of heat stress, as shown in Fig.21, a1. HSGs partially disappear after prolonged heat stress (4h) and dictyosomes and rough ER accumulate in the vicinity of decomposing HSGs (Fig.21, a2). Mesophyll cells of RB plants, accumulating anti-HSP17 scFv in the ER, and transgenic control GM plants show an ultrastructure very similar to that of mesophyll cells of the wild type: HSGs are formed after 2h of heat stress treatment (Fig 21, c1 and d1). Degradation of HSGs occurs after 4h of heat stress (Fig. 21, c2 and d2). This is identical to the stress-elicited alterations in wild type cells.



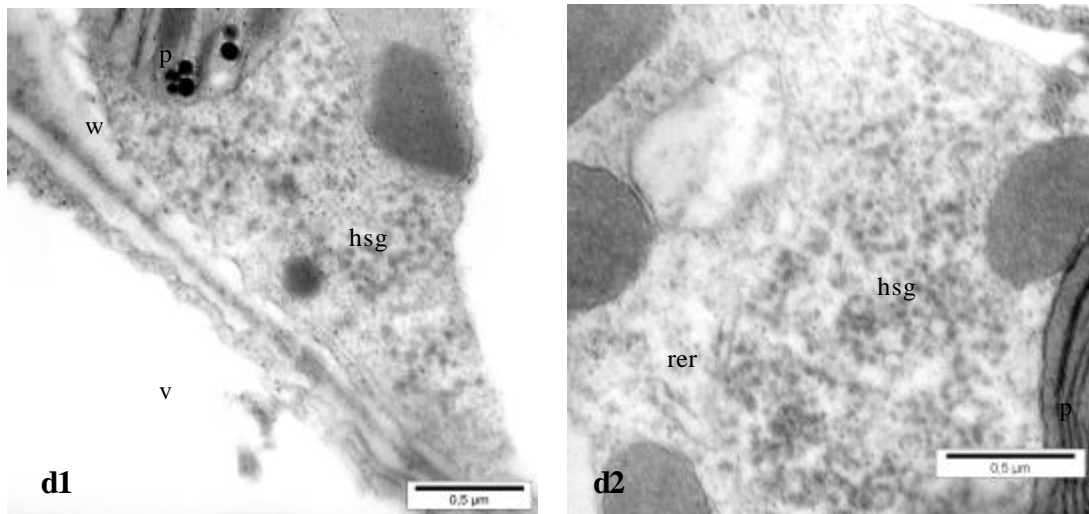


Fig. 21 Ultrastructural alterations in mesophyll cells of transgenic CB6, RB, GM and wild type plants after heat shock.

The heat shock treatment was performed for 2 h and 4 h at 42°C. **a1** and **a2** – wild type plant cells, **b1** and **b2** –transgenic CB6 cells, **c1** and **c2** – transgenic RB cells, **d1** and **d2** – transgenic GM cells after 2 h and 4 h of heat shock, respectively. **d** – dictyosomes, **hsg** – heat shock granule, **m** – mitochondrion, **n** – nucleus, **p** – plastid, **rer** – rough ER, **v** – vacuole, **w** – cell wall.

Fig.21, b1 and b2 represent results of the electron microscopic analysis of ultrastructural changes in mesophyll cells of transgenic CB6 plant under stress conditions. No HSG formation was observed in the cytosol of anti-HSP17 scFv expressing mesophyll cells after 2 h of stress treatment (Fig.21, b1). After 4 h heat stress transgenic CB6 mesophyll cells were found to be dead, as shown on Fig.21, b2. The integrity of all the membranes of cell compartments is destroyed. Plasmamembrane, nuclear membrane and envelopes of chloroplasts and mitochondria show osmiophilic lipid droplets formed by oxidation of the membrane lipids. The ultrastructural alterations of CB6 mesophyll cells expressing anti-HSP17 scFv under both heat stress conditions are substantial and not comparable with the ultrastructural changes in stress-protected transgenic control and wild type cells. Apparently, the formation of HSGs in non-immunomodulated (RB, GM and wild type) cells under

moderate stress conditions (2h of heat treatment) is a prerequisite for stress protection of the cellular ultrastructure and for survival under prolonged stress conditions.

The ultrastructure of immunomodulated mesophyll cells after prolonged heat stress is comparable to the ultrastructure of apoptotic cells. The accumulation of anti-HSP17 scFv in the ER is without influence on the thermotolerance and stress-induced ultrastructural alterations of transgenic RB plants. Mesophyll cells of RB and GM plants show the same ultrastructural alterations as the cells of wild type plants under the used stress conditions, and confirm the phenotypic appearance of transgenic control RB and GM plants after stress treatment (see 3.6.1.) However, immunomodulated mesophyll cells of the transgenic plant CB6, which are not able to form HSGs, showed stress-elicited alterations of the ultrastructure and proved to be unable to survive prolonged heat stress.

3.7. Influence of anti-HSP17 accumulation in the ER lumen on the developmental processes of transgenic plants

Expression of sHSPs is also developmentally regulated during plant life, for instance during seed development (Coca et al., 1994, De Rocher and Vierling, 1994, Wehmeyer et al., 1996). They are synthesized in the seeds at the same time as the storage proteins of seeds and accumulate also in the endomembrane compartment of the storage parenchyma cells (Neumann and zur Nieden, personal communications). It is postulated that they are functionally involved in seed desiccation (De Rocher and Vierling, 1994, Wehmeyer et al., 1996), but their functional activity during seed development is not clear up to now. Therefore, it was examined whether or not anti-HSP17 scFv antibodies constitutively expressed and targeted to the ER lumen are able to affect developmental processes of the transgenic plant.

Contrary to the immunomodulated plants with cytoplasmic anti-HSP17 scFv expression, transgenic RB plants with compartment-specific accumulation of anti-HSP17 scFv in the ER lumen showed not only unaltered thermosensitivity (see 3.6.1.) as compared to the controls but also no phenotypical alterations during organ development. RB plants developed simultaneously flowers and seeds as wild type plants. Their flowers were fertile, the seeds showed no morphological alterations and were able to germinate. Vivipary of the seeds from RB plants was never observed.

In order to characterize ultrastructural alterations in the embryo tissue of mature RB seeds, electron microscopy analysis was performed using mature seeds of

the wild type as a control. Fig. 22 shows the electron microscopic comparison of the embryo ultrastructure of mature seeds from transgenic RB (Fig. 22, a) and wild type (Fig. 22, b) plants. No differences between the tissue ultrastructure of RB and wild type plants were observed. Protein storage organelles (protein bodies) and lipid vacuoles are formed comparable in number, shape and size in transgenic cells as well as in wild type cells.

The sHSP and storage protein patterns of RB and wild type plant seeds were comparatively analyzed by Western blotting. sHSPs and the total amount of salt soluble storage proteins was extracted from the seeds and separated electrophoretically on 12.5% PAAG. The sHSP (Fig. 23) and the storage globulin (Fig. 24) patterns from mature seeds of transgenic RB plants showed no qualitative and quantitative differences in comparison with the protein patterns of wild type control.

It is concluded that the cytoplasmic sHSPs in the ER compartment of the storage parenchyma cells are neither involved in the processes of globulin and lipid storage during seed development nor functionally take part in the processes of thermotolerance.

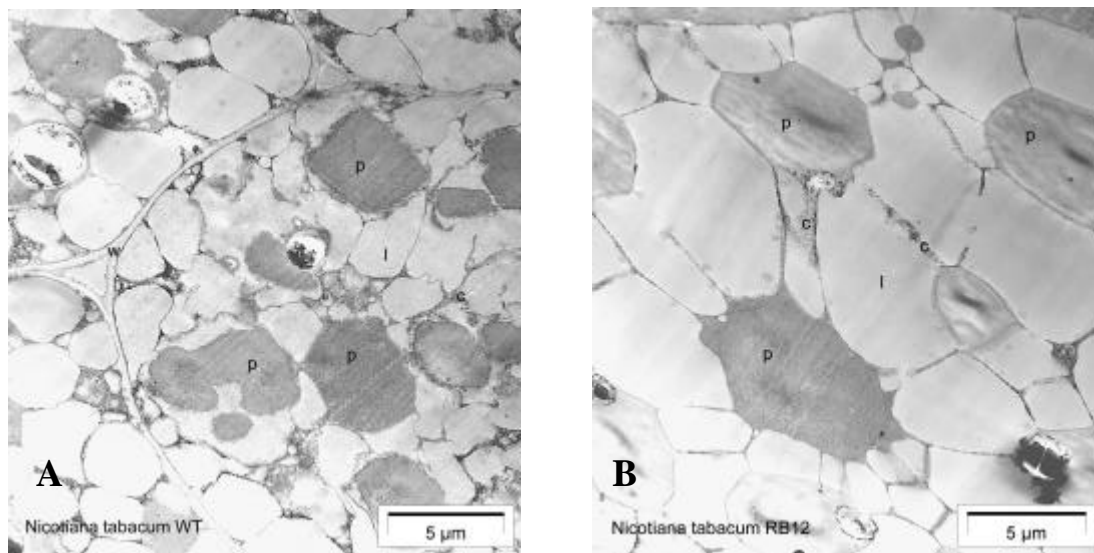


Fig. 22 Ultrastructure of embryo tissue from mature seeds of wild type (A) and transgenic RB (B) plants.

p – protein body, l – lipid vacuole, c – cytoplasm, w – cell wall.

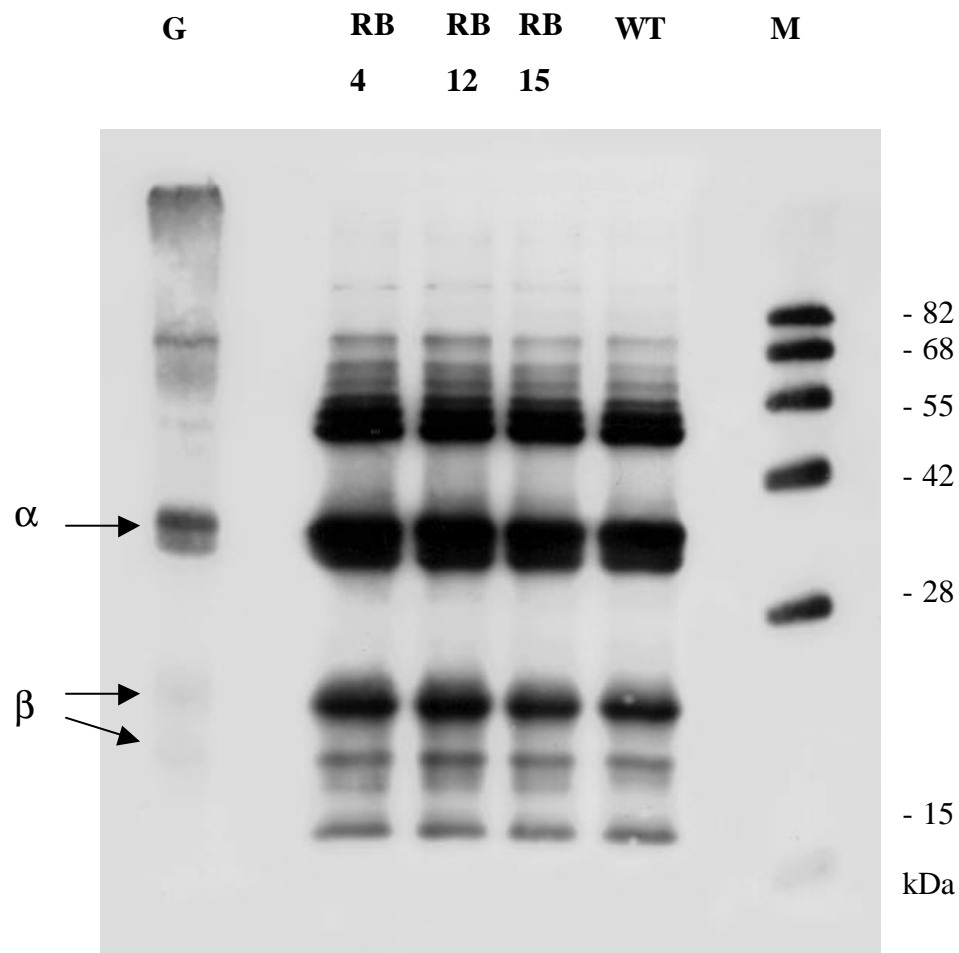


Fig. 23 Western blot analysis of storage globulins from mature seeds of transgenic RB and wild type (**WT**) plants.

Total protein was extracted from 5 mg of mature seeds from individual transgenic RB (**RB4**, **RB12** and **RB15**) and wild type (**WT**) plants. The protein extracts (15 μ l per slot) were separated under denaturation and reducing conditions on 12.5% polyacrylamide gel. After blotting the storage globulins were detected by anti-tobacco-globulin antibody followed by anti-mouse IgG conjugated to HRP. Signals were generated by chemiluminescence. **M** – molecular mass marker in kDa on the right side, **G** – preparation of 12S tobacco storage globulins as reference. The α - and β -polypeptides of the 12S globulin are indicated with arrows on the left.

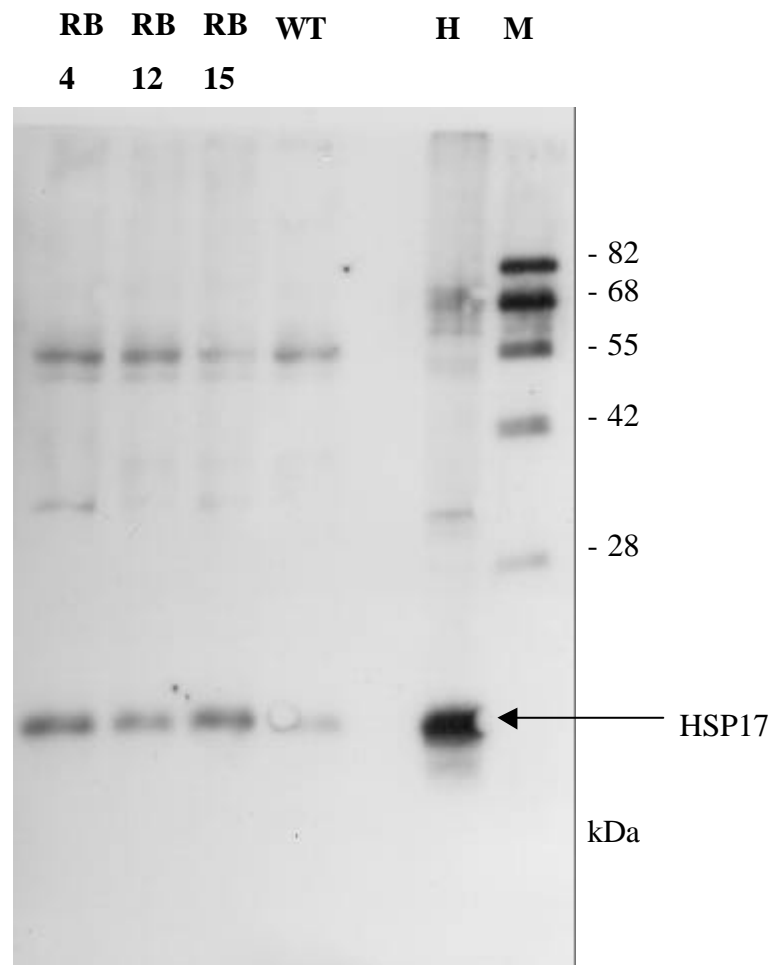


Fig. 24 Western blot analysis of HSP17 from mature seeds of transgenic RB and wild type (WT) plants.

Protein was extracted from 5 mg of mature seeds from individual transgenic RB (**RB4**, **RB12** and **RB15**) and wild type (WT) plants. The protein extracts (15 μ l per slot) were separated under non-reducing conditions on 12.5% SDS polyacrylamide gel. After blotting, HSP17 was detected by anti-HSP17 antibodies followed by anti-mouse IgG conjugated to HRP. Signals were generated by chemiluminescence. **M** – molecular mass marker in kDa on the right side, **H** – HSP17 preparation from *L. esculentum* as reference. The position of HSP17 is indicated by arrow.

Resume :

- two phage clones producing anti-HSP17 scFvs were selected during scFv phage library screening, using solid-fixed recombinant HSP17 from *L.esculentum* as antigen;
- the specificity of soluble scFvs to native sHSPs isolated from heat-stressed tomato cell cultures was proved, the cDNA sequences encoding selected scFvs were characterized by restriction analysis and DNA sequencing;
- recombinant DNA constructs for expression of anti-HSP17 scFvs in the cytosol or targeting to the ER lumen of plant cell were designed;
- transgenic plants with expression of anti-sHSP scFvs were generated by *Agrobacterium*-mediated transformation of *Nicotiana tabacum*;
- stable transgenic plant lines CB6 and RB with cytosolic expression or ER-targeting of anti-HSP17 scFvs were propagated;
- the content of anti-HSP17 scFvs in the plants of CB6 line was determined as 0.09% of total soluble leaf protein;
- transgenic plants with about equivalent expression levels of scFvs without specificity to sHSPs (GM and JC plants) were chosen as positive controls to check artificial effects caused by foreign gene expression;
- distribution of anti-HSP17 scFvs in the cytosol and in the nucleus of CB6 plant cells was demonstrated using fluorescence microscopy and immunocolloid technique combined with transmission electron microscopy;
- the leaves of CB6 plants showed immunomodulation of thermoresistance detectable by lowering the levels of basal and acquired thermotolerance in comparison with transgenic control and wild type plants;
- prevention of HSG formation in the cytosol of immunomodulated CB6 plants cells during heat stress demonstrated the participation of sHSPs in the thermotolerance and importance of HSG to survive stress conditions;
- scFvs with sHSP specificity localized in the ER lumen do not alter basal and acquired thermotolerance, stress-elicited HSG formation and seed development of RB plants.

4. Discussion

From the results received during the experimental work, following questions deserve discussion:

- the functional activities of sHSPs during heat stress response of plants;
- the stability and specificity of anti-sHSP scFvs in the cytosol as a prerequisite requirement for successful immunomodulation of cytosolic sHSPs *in vitro*;
- the participation of sHSPs in the processes of basal and acquired thermotolerance;
- the role of cytosolic sHSPs in the formation of HSGs and possible functional activities of HSG during heat stress and recovery;
- perspectives of immunomodulation as a methodical approach for further investigations of the heat stress response in plants.

4.1. Probable functional activity of cytosolic sHSPs in plant thermotolerance

Thermotolerance of plants is correlated with stress-elicited biosynthesis of numerous heat shock proteins belonging to different HSP families. HSPs protect the structural and functional cell proteins from heat denaturation and aggregation by acting as molecular chaperones (Parcell and Lindquist, 1993, rev. Vierling, 1991, rev. Waters, 1996).

Presumably, a complex functional HSP network exists that includes interactions between high-molecular HSPs, low-molecular HSPs and their endogenous substrates. This network protects essential cell structures against damaging effects of elevated temperatures and leads to acquisition of thermotolerance.

Individual sHSPs act as molecular chaperones *in vitro* (rev. Waters, 1996) and *in vivo* (L6w et al., 2000). However, the endogenous substrates of cytosolic sHSPs are still unknown. At present it is also not known whether they act as chaperones like higher molecular weight HSPs during heat stress or only bind stress-defolded, misfolded or aggregated proteins for subsequent renaturation by high molecular weight HSPs. Stress-elicited cytosolic sHSPs autoaggregate *in vitro* and *in vivo* to homo-oligomers (Lee et al., 1995; Jinn et al., 1995). The homo-oligomers consist of

12 subunits and possess chaperone activity. Presumably, after biosynthesis in the cell cytosolic sHSPs aggregate immediately and do not exist in the form of monomers in the cytosol. Furthermore, during heat stress highly ordered cytosolic complexes are assembled with the formation of class II sHSP oligomers as a prerequisite for stress-induced autoaggregation and recruitment of class I sHSPs and other components in the HSGs (Nover et al., 1989, Kirschner et al., 2000).

HSGs are able to provide chaperoning activity *in vitro* (Smykal et al., 2000), but it is unknown, whether or not HSGs act as more effective chaperones as the oligomers or have another function. HSGs may also be storage depots for mRNAs, HSFs and essential cell proteins, which will be released by disaggregating of HSGs and permit to restore very quickly the metabolic activity of the cells during recovery.

sHSPs represent about 50% of the HSG complex (Smykal et al., 2000). Their contribution to the functions of the HSG machinery is still under question. It is unknown, either sHSPs have also a real chaperone activity inside the HSG or they are only stored in the HSG and are able to shuttle between HSG and cytoplasm. Moreover, direct involvement of sHSPs in the processes of basal and acquired thermotolerance of plants is as yet poorly understood in detail. Mutants with reduced or blocked biosynthesis of sHSPs have not been found until now. Furthermore, functional analysis of sHSP activity by molecular biological approaches such as the use of antisense or overexpression techniques were unsuccessful, presumably due to the multigene nature of sHSPs in the plant genome. Overexpression of HSFs was used to generate transgenic plants with a higher level of thermotolerance (Prändl et al., 1998). However, the HSFs are known to regulate the activity of different heat-shock genes in plants (Schöffl et al., 1998). Use of this strategy does not allow to investigate the activity of individual HSPs.

Therefore, immunomodulation of sHSPs by intracellularly expressed anti-HSP17 scFv was used as a new methodical tool to create mutants with partial or complete antibody-mediated inhibition of sHSP functions presumably excluding them from the oligomerization process or from formation of HSG complexes during the heat shock response. Using immunomodulated sHSP mutants, we investigated the role of sHSPs in basal and acquired thermotolerance of plants and characterized the ultrastructural alterations of immunomodulated cells in dependence on their stress response.

4.2. Intracellular expression of anti-HSP17 scFv as a tool to immunomodulate the heat shock response

The success of *in vivo* immunomodulation depends on such features of scFvs as a high antigen specificity, stability and a relatively high expression level in transgenic cells.

The population of scFv phage clones after three rounds of scFv phage library screening contained a large number of clones with incomplete scFv cDNA sequences. All the incomplete scFv cDNA sequences were about 400 bp in size, whereas the correct size of the complete scFv cDNA sequence is about 800 bp according to producer's description of Tomlinson A and B phage library. It has previously been reported that the scFv phage libraries can, for an unknown reason, contain a large number of clones with incomplete or even absent scFv cDNA inserts (De Bruin et al., 1999, Eeckhout et al., 2000). De Bruin et al. showed that incomplete scFvs have low affinity to the antigen (De Bruin et al., 1999). We therefore decided to search for cDNAs encoding only the complete scFv fragments. Restriction analysis of phagemid DNA from individual phage clones was used to select the clones containing complete cDNA of correct size (800 bp) coding for full variable regions of the heavy and light antibody chains. 20 individual phage clones with full-length anti-HSP17 scFv cDNA inserts were selected.

To immunomodulate sHSPs *in vivo*, anti-HSP17 scFvs must retain antigen specificity not only being displayed on the surface of the phage particle but also in a soluble form. Therefore, not only phages but also soluble scFvs from 10 selected individual phage clones were tested for their ability to recognize specifically recombinant HSP17. Two phage clones A14 and B8 produced soluble scFv with the highest binding capacity to the antigen and could be accepted as candidates for immunomodulation approach in transgenic plants (Fig. 4).

The preparation of recombinant class I HSP17 from *L. esculentum* allowed us to avoid the time-consuming isolation of pure cytosolic sHSP from the plant. Since the recombinant HSP17 was synthesized as a fusion protein with a short 6xHis-tag in *E. coli*, it could be easily purified using IMAC. Nevertheless, the anti-HSP17 scFvs favored for the immunomodulation experiment must recognize not only recombinant HSP17 but also the endogenous sHSP elicited by heat stress in plant cells. The soluble scFvs prepared from the phage clones, fulfill this requirement of the

immunomodulation approach as shown by Western blot analysis (Fig. 6). Both soluble scFvs recognized specifically endogenous sHSP in protein extracts from heat-treated cell suspension culture of *L. esculentum*. The double bands detected on the Western blot membranes by soluble scFvs indicated that the scFvs recognized both classes of sHSPs, which are very similar in size. The cross-reaction of anti-HSP17 scFvs with class II sHSPs may be caused by recognition of identical or similar epitopes in the highly conservative C-terminal heat shock domains, shared by both classes of sHSPs (Plesofsky-Vig et al., 1992, rev. Waters et al., 1996). Since no epitope mapping was performed, it has not been decided where the epitopes recognized by the scFvs are located on the sHSP molecule. The use of cytosolic class I HSP17 as antigen for scFv library screening did not guarantee the selection of class-specific anti-sHSP scFvs. Only a differential screening of the library by use of cytosolic class I and II sHSPs or truncated recombinant sHSPs as antigens might be successful to select class-specific anti-sHSP scFvs. However, no recombinant or purified endogenous cytosolic class II sHSP was available at the time of library screening.

Since the chosen sHSP target for immunomodulation is a cytosolic protein, the scFv must be also functional in the cytoplasm of transgenic plant cells. Additionally, immunomodulation of sHSP requires a stable level of anti-HSP17 scFv expression, high enough to bind the stress-elicited endogenous sHSP in transgenic plant cells. For that reason the pool of ubiquitously expressed anti-HSP17 scFv must immediately disable the stress-elicited sHSP in the cytoplasm. Stability of the scFv is also important for *in vivo* interaction between scFvs and appropriate antigens (rev. Wörn and Plückthun, 2001). We designed recombinant expression cassettes for cytosolic expression of anti-HSP17 scFvs in all vegetative tissues of transgenic plants, where sHSPs are elicited in response to elevated temperatures (see 1.4.). For this purpose, the 35S CaMV promoter was chosen because its activity is relatively high in all vegetative tissues of plants (Odell et al., 1985).

The stable expression of functional scFvs at a relatively high level in the cytoplasm of plant cells is problematic. Correct folding of scFv is hindered by the reducing conditions and the lack of chaperones in the cytosol (rev. De Jaeger et al., 2000). Nevertheless, high accumulation of scFvs in the cytosol of plant cells has been reported (Owen et al., 1992, Tavladoraki et al., 1993, De Jaeger, 1999). In order to secure a sufficient level of scFv expression, two different expression cassettes were

designed and probed for cytoplasmic expression of each anti-HSP17 scFv. Firstly, the C-terminal KDEL tetrapeptide sequence was included into the expression cassette in order to increase the scFv expression level and to stabilize the scFv in the plant cytosol (Schouten et al., 1996). Secondly, the alternative constructs were prepared for cytoplasmic expression of scFvs as Thioredoxin A fusion protein. *E.coli* Thioredoxin A as a fusion partner was used to improve the solubility and activity of expressed scFvs in the cytoplasm (La Vallie et al., 1993). It was necessary to select transgenic plants with scFv expression level high enough to disable the pool of cytosolic sHSPs or at least its major part. The amount of scFv-KDEL fusion expression in selected primary transgenic plants was found to be much higher when compared to the amounts of scFv-Thioredoxin fusion expression. For this reason the primary plants with cytosolic expression of scFv as KDEL-fusion protein were preferred for further propagation of stable transgenic plant line.

The expression cassettes with cDNA inserts encoding two different scFv-KDEL fusion proteins were both being integrated into the genome of transgenic plants CA8 and CB6 as single copy genes and both were expressed under control of the CaMV 35S promoter in the cytosol. But despite the similar expression conditions the amounts of individual scFvs in transgenic plants CA8 and CB6 were not equal. This is in agreement with data showing that scFv accumulation and degradation in the cytosol of plant cells is highly dependent on the intrinsic properties of individual scFvs (De Jaeger, 1999, rev. De Jaeger et al., 2000). Possibly, the position of the integrated scFv gene in the plant genome may also influence the activity of its expression. It is not possible to decide, whether individual scFvs are expressed with different activity or the individual scFvs are accumulated and degraded in different ratios in transgenic CA8 and CB6 plants. The amount of anti-HSP scFvs in transgenic CB6 plants was relatively high also under conditions of heat stress when total plant cell protein biosynthesis decreased (data not shown). Either expression of anti-HSP17 scFv in the transgenic plant takes place also under heat stress conditions or the scFv has a high stability in the cytoplasm of the transgenic cells.

The concentration of anti-HSP17 scFv in the leaves of transgenic CB6 plants is about 0.097% of total soluble protein. The amount of stress-elicited cytosolic sHSPs reaches about 1% of total leaf protein (rev. Waters et al., 1996). However, the amount of scFvs accumulated in the cytosol was sufficient to immunomodulate the thermoresistance of transgenic plants and to inhibit the ability of cytosolic sHSP to

aggregate in HSG complexes. The optimal scFv/sHSP ratio for the complete immunomodulation of sHSPs is unknown. Cytosolic sHSPs auto-aggregate immediately to dodecamers after synthesis (see 1.4.). Either scFvs bind to the monomers without prevention of their oligomerization or scFvs bind to already formed sHSP oligomers. It is also unknown, how many scFv molecules must bind to one oligomer to prevent HSG formation. Furthermore, it is not known, whether or not scFv binding to the oligomers influences its chaperone activity. Perhaps, different amounts of scFv are necessary to inhibit chaperone activity of the oligomers or to prevent HSG formation. It is also possible that the pool of cytosolic sHSPs is not completely immunomodulated by insufficient amount of scFv, but even partial inhibition of cytosolic sHSP activity interrupts HSG formation and caused visible alterations of thermotolerance.

The expressed anti-HSP17 scFvs are located in transgenic cells at the same positions as stress-elicited cytosolic sHSPs. The same distribution of scFv and stress-elicited sHSPs in the cytosol of transgenic cells enables physical antigen-antibody interaction between scFv and sHSPs. Anti-HSP17 scFvs, targeted into the cytosol, were detected also in the nuclei of non-stressed and stressed transgenic plant cells. Since cytoplasmatically expressed anti-Fus scFvs were also detected in the cytosol and nuclei of mesophyll cells from GM control plants, the transport of scFvs from the cytosol to the nucleus seems to be independent of scFv specificity. The cassettes constructed for cytosolic expression of anti-HSP17 and anti-Fus scFvs do not contain any target sequences for protein transport into the nucleus. Up to now we have no explanation as to the nuclear localization of scFvs.

Use of transgenic control plants with expression of scFvs without sHSP-specificity demonstrated that visible alterations of thermotolerance and ultrastructural changes in the cells of transgenic CB6 plants after heat treatment are really caused by the specific immunomodulation of sHSPs and are not effects of the expression of scFv as foreign protein. Fus3 is a developmentally regulated seed-specific transcription factor, not present in plant leaves. The plant hormone jasmonate is not involved in the process of heat stress response. Cytosolic expression of anti-Fus3 or anti-jasmonate scFvs did not alter plant thermotolerance. The transgenic control plants (GM and JC) which accumulated scFvs in amounts 0.05% or 0.03% of their total soluble protein in the cytosol showed the same thermotolerance as wild type plants, whereas the sHSP immunomodulated transgenic CB6 plants revealed a significantly higher

thermosensitivity. Therefore, we postulated that the expressed amounts of scFvs in the cytosol had no artificial influence on the thermotolerance and ultrastructural alterations of transgenic plants under stress conditions. Obviously, the specific antigen-antibody interaction between sHSPs and anti-HSP17 scFvs caused decreased thermotolerance and stress-elicited ultrastructural alterations in the cells of CB6 plants.

Cytoplasmic sHSPs are also involved in seed development (zur Nieden et al., 1995, rev. Waters et al., 1996, Wehmeyer et al., 1996, De Rocher and Vierling, 1994) and accumulate in the endomembrane compartment of storage parenchyma cells of seeds (Neumann, personal communication). In order to investigate the function of cytosolic sHSPs in seed development, cytosolic sHSPs localized in the ER of seed storage cells were immunomodulated by anti-HSP17 scFv accumulated in the ER lumen. Anti-HSP17 scFvs were detected in leaves and seeds of transgenic RB plants using Western blot analysis. However, accumulation of scFv in the ER of transgenic plants has not been immunohistologically proved. No visible alterations of plant and seed development were observed. Interaction of anti-HSP17 scFvs with ER-localized cytosolic sHSPs seems to be without effects on thermoresistance and seed development.

4.3. Immunomodulation of sHSPs during basal and acquired thermotolerance

Due to the lack of natural or genetically engineered plant mutants with reduced or completely blocked sHSP biosynthesis it was never proved that sHSPs are directly involved in the cellular processes of basal thermotolerance, recovery and acquired thermotolerance. The results of our experiments with immunomodulated CB6 plants provide clear direct evidence for the involvement of stress-elicited cytosolic sHSPs in basal and acquired thermotolerance of plant.

In contrast to wild type and transgenic controls, the phenotypical alterations of the immunomodulated CB6 leaves such as necrosis, destruction of vegetative tissue and changes of the leaf color were observed after 2 h heat treatment at elevated temperatures (48°C and 50°C). The phenotypical alterations of stressed transgenic CB6 plants with ubiquitous cytosolic expression of anti-HSP17 scFvs indicate a reduced level of basal thermotolerance in comparison to WT and transgenic GM and JC controls. Isolated leaves of CB6 plants were not able to survive 2h heat treatments at sublethal temperatures (48°C–50°C), although the control plants showed no visible

phenotypical alterations at this temperature regime. This suggests that the observed effects on the thermosensitivity of immunomodulated CB6 plants were apparently generated by the specific interaction of anti-HSP17 scFvs and sHSPs and not by unspecific impact of intracellular scFv expression (Fig.17). The absence of phenotypical alterations of CB6 leaves after 2h of heat treatment at 42°C may suggest that our immunomodulation approach only incompletely blocks sHSP functions, or that basal thermotolerance of CB6 plants is also supported by other factors. Although the HSG formation is blocked in immunomodulated CB6 plants, oligomers of sHSPs bound to the anti-HSP17 scFv might mediate some thermotolerance. On the other hand, constitutive (HSCs) and on stress-induced high molecular weight HSPs such as HSC/HSP70 and HSP40 might presumably provide thermotolerance at lower than sublethal temperatures (42°C). However, phenotypical alterations of CB6 plants such as wilt and boiled-like structure of leaf tissue observed after prolonged heat treatment of CB6 leaves at 42°C suggest that functional sHSPs are really essential for the improvement of basal thermotolerance during prolonged heat shock.

The immunomodulation approach was also sufficient to demonstrate that the ability of transgenic CB6 leaves to acquire thermotolerance was restricted when compared with wild type and transgenic GM and JC control plants (Fig.17). Acquired thermotolerance was generated by short pre-treatment of plant material at 42°C for 15 min to induce biosynthesis of cytoplasmic sHSPs in the mesophyll cells of leaves. Pretreatment was followed by a 2h recovery period at 25°C to permit active sHSP biosynthesis and normalization of cellular metabolic activities. Afterwards a subsequent 2h stress regime was applied at 42°C, 48°C or 50°C. Only after severe heat stress for 2h at 48°C and 50°C the transgenic CB6 leaves were damaged comparable to the experiment concerning basal thermotolerance. Contrary to this, transgenic and wild type control leaves resisted the stress regimes at 48°C and 50°C without visible phenotypical alterations. However, the comparison of visual phenotypical alterations of CB6 leaves under the temperature regime of basal and acquired thermotolerance revealed that pre-treated CB6 leaves were somewhat more temperature-resistant than non-pretreated CB6 leaves. The higher thermoresistance of preheated CB6 leaves indicates that immunomodulated CB6 leaves have a limited competence to acquire thermotolerance, presumably also mediated by incomplete antibody-mediated blocking of sHSPs by the scFv sHSP oligomers with chaperone activity or by high molecular weight HSPs acting as chaperones.

In order to verify the visual phenotypical alterations caused by heat stress, a biochemical approach was used. To estimate damage of green leaves by elevated temperature, the measurement of chlorophyll content was introduced into the thermotolerance studies (Queitsch et al., 2000). The decrease of chlorophyll content in the stress-treated leaves was used to demonstrate the increased thermosensitivity of immunomodulated CB6 leaves and to compare the heat damages of CB6 leaves at different temperature regimes of basal and acquired thermotolerance. The extent of chlorophyll destruction in the leaves during heat stress (Fig. 18) leads to the conclusions described above. Increasing of the temperature from 48°C to 50°C caused more pronounced chlorophyll destruction in CB6 leaves than in stress-resistant wild type leaves during the temperature regimes used to measure the basal thermotolerance. CB6 leaves were still able to develop acquired thermotolerance to a certain extent, as seen from the lower chlorophyll destruction in pre-treated CB6 leaves than in non-pretreated ones after 2h of heat stress at the same temperature. However, even the level of acquired thermotolerance of CB6 leaves was not sufficient to resist severe heat stress conditions as observed for leaves of wild type plants. Comparative analysis of the phenotypical alterations of CB6 leaves after short (2h) and prolonged heat stress (6-10 h at 42°C) was not possible using measurement of chlorophyll content. Since observed wilting and softening of CB6 leaf tissue was not accompanied by a significant chlorophyll degradation after prolonged heat stress.

The measurement of chlorophyll content is an approximate method allowing the comparison of visual phenotypical alterations of stress-damaged leaves with biochemical data of chlorophyll degradation. Therefore, chlorophyll degradation is not only a stress-specific metabolic reaction in heat-stressed leaf tissue but can also be used as an effective method to measure chloroplast destruction as a consequence of stress damage of cells by severe heat stress conditions.

The leaves of transgenic RB plants with accumulation of anti-sHSP scFvs in the ER lumen were also checked as to their basal and acquired thermotolerance. The leaves of RB plants never showed any visible phenotypical alterations during heat stress. This indicates that anti-HSP17 scFvs accumulation in the ER did not influence the basal and acquired thermotolerance of transgenic plants. Since anti-HSP17 scFvs are localized in the ER lumen, interaction of scFvs with stress-elicited cytosolic sHSPs is not possible. Furthermore, ER-localized anti-HSP17 scFvs are probably unable to interact with the structurally different ER-localized sHSPs, or this

interaction has no visible influence on plant thermotolerance. Therefore, the transgenic plants with ER targeting of expressed anti-HSP17 scFvs were taken as additional positive controls in the thermotolerance investigations.

The specific alterations of basal and acquired thermotolerance of immunomodulated CB6 plants demonstrate that cytosolic sHSPs are directly involved in both basal and acquired thermotolerance. It was already reported from experiments with thermal denaturation of firefly luciferase expressed in transgenic *Arabidopsis* protoplasts that co-expressed heterologous sHSPs alone can stabilize luciferase activity to almost the same extent as endogenous chaperones at light (20 min at 42°C) stress conditions (Löw et al., 2000). However, our experiments with immunomodulated CB6 plants demonstrate for the first time that sHSPs are really prerequisite elements for basal and also acquired thermotolerance at sublethal stress conditions. Resistance of CB6 leaves to light heat stress conditions (2h at 42°C) and their partial ability to develop acquired thermotolerance can probably be realized by constitutive and heat-elicited high molecular weight HSPs. However, it is also possible that immunomodulation of sHSPs was incomplete.

4.4. Functions of HSGs in plant heat shock response

sHSPs usually form oligomer complexes, each probably consisting of 12 subunits. During heat stress they assemble together with high molecular weight HSPs and other cell proteins into large aggregates called HSGs (see 1.4.). HSG formation represents a specific assembly process that depends on formation of class II sHSP oligomers as a prerequisite for the auto-aggregation under stress conditions. HSGs are 1-2 MDa large cytosolic complexes (Kirschner et al., 2000, Smykal et al. 2000). They are found in all plant tissues during heat stress (Nover et al., 1989). The functional role of sHSPs in this process is probably to form the core of the complex and to provide binding of defolded, misfolded or aggregated cell proteins for further renaturation by high molecular weight HSPs during recovery (Smykal et al., 2000).

Immunofluorescence and electron microscopic analysis were performed in order to investigate the intracellular distribution of anti-HSP17 scFv and sHSPs as well as HSG formation in immunomodulated CB6 and transgenic controls under heat stress. Although sHSPs are present in the cytosol of CB6 mesophyll cells after 2h of heat stress. They are not able to form any HSG-like complexes in the cytosol of CB6 mesophyll cells. In contrast to transgenic control and wild type cells, cytosolic sHSPs

in mesophyll cells of CB6 plants did not participate in the process of HSG formation obviously due to their specific interaction with anti-HSP17 scFv.

Disintegration of HSGs and formation of polysomes, dictyosomes and rER in the cells of wild type, GM and RB plants was observed during prolonged heat stress (4-5h), indicating reconstitution of the metabolic activity in the stressed mesophyll cells. Contrary to the wild type and transgenic controls, prolonged (4-6 h) heat stress leads to the death of immunomodulated CB6 plant cells as evident lipid peroxidation and destruction of integrity the cell compartments (Fig. 21).

The ultrastructural data indicate that functional oligomers of cytosolic sHSPs are essential elements for HSG formation. Probably, scFv-bound sHSP oligomers are still able to act partially as molecular chaperones, providing thermotolerance at light stress conditions. Presumably, a restricted extent of acquired thermotolerance can be established by soluble scFv-sHSP oligomer complexes in interaction with high molecular weight HSPs (see previous chapter). However, the binding of the anti-HSP17 scFvs to the sHSP oligomers completely inhibits the auto-assembly of the sHSPs to HSG (see Fig. 25). The immunomodulation of sHSPs by anti-HSP scFv at the level of HSG formation caused decreased basal and acquired thermotolerance, stress damage and death of CB6 mesophyll cells. Therefore, the HSGs seem to be a prerequisite for survival under lasting stress conditions.

The functions of sHSP oligomers and HSGs in the mechanisms of stress response remains quite unclear. Probably, plants developed such a highly ordered mechanism of stress response involving the HSGs in order to survive those environmental conditions that cannot be avoided due to their sessile life style.

The isolated HSGs act as molecular chaperones *in vitro* (Smykal et al., 2000). Presumably, aggregation of several different stand-alone molecular chaperones (HSPs) to macromolecular HSG complex allows efficient reactivation of heat denaturated cell proteins. Such macromolecular complexes of HSPs, whose chaperoning activity is probably somehow more complexed or synchronized, could function during prolonged or high temperature stress as an extraordinarily effective mechanism of protein renaturation.

The molecular mass of HSGs is not constant and can range from 1 to 2 kDa (see 1.4.). Probably, the HSGs do not represent a stable structure but a complex consisting of many elements whose composition changes dynamically during heat stress. Therefore, it is supposed that the specific assembly mechanism described for

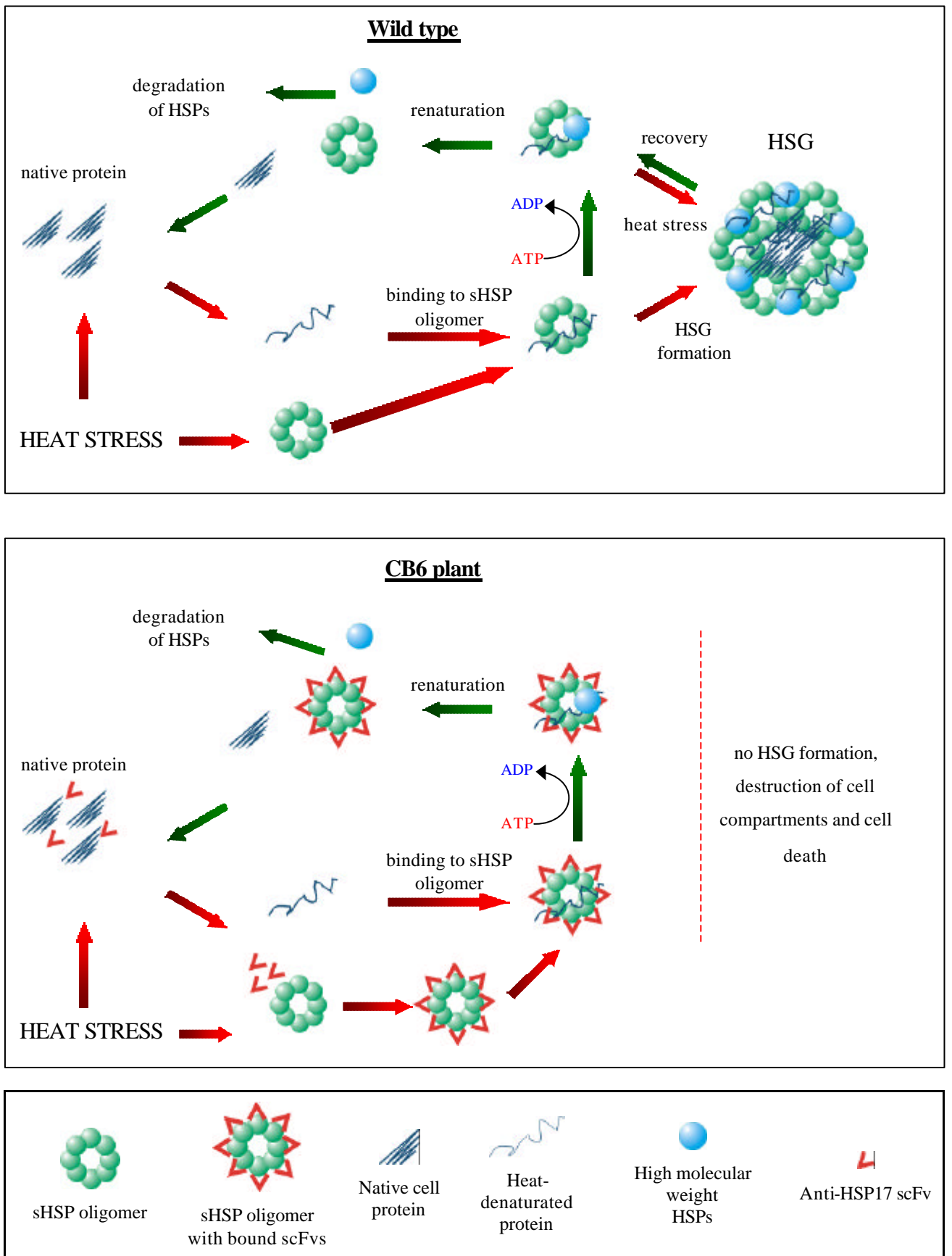


Fig. 25 Schematic representation of processes involved in the heat stress response of wild type and immunomodulated CB6 cells.

sHSPs in the plant cytosol (Forreiter and Nover, 1998, Smykal et al., 2000, Kirschner et al., 2000) leading from monomers to HSGs could be a mechanism of dynamic balance between the free form of functional chaperone (sHSP mono- and/or oligomers) and their stored form in HSGs. Probably, plant cells produce rapidly high amounts of HSPs in the beginning of the heat stress and then store them in HSGs. Afterwards the balance between free and stored cytosolic sHSPs and other HSPs can be mediated by dynamic shuttling of sHSPs between cytosol and HSGs. This process could possibly be regulated, by the concentration of ATP, necessary for the high molecular weight HSP chaperoning activity, or by the ATP/ADP ratio. This might explain such phenomena as rapid stress-elicited accumulation of cytosolic sHSPs in high (up to 1% of total protein) amounts (De Rocher et al., 1991), HSG dissociation *in vitro* in presence of ATP/Mg and further reassembly after removal of ATP/Mg (Smykal et al., 2000).

mRNAs of housekeeping genes encoding essential cell proteins and stress-elicited HSFs were also found to be incorporated into HSGs (Nover et al., 1989, Scharf et al., 1998). This observation allows also to speculate that HSGs may function as storage depots for the compounds immediately available to reconstitute normal cell metabolism during the recovery period. RNA and essential cell proteins may be stored in HSG to protect them from damage during heat stress. mRNAs of housekeeping genes and regulatory factors, which are necessary for recovery processes, cannot be produced immediately in stress-depressed plant cells *de novo*. Therefore, their release from dissociated HSG complexes would be a benefit for rapid reconstitution of the metabolic activity of the stress-depressed cells. However, it is not proved that RNAs are really genuine compounds of HSGs (Smykal et al., 2000).

It is unclear until now, which one of the described hypothetical models of HSG activity during heat stress is more obvious. It cannot be also excluded that HSGs act as multifunctional complexes, simultaneously combining enhanced chaperoning activity and storage/release of HSPs and other essential cellular compounds during heat stress and recovery.

4.5. Immunomodulation as a new methodical tool to analyze the functions of sHSPs in future work

For the first time it has been shown that immunomodulation can be used as methodical tool to analyze the functions of plant regulatory proteins. However,

immunomodulation can be successfully used for more detailed functional analysis of different HSPs during the heat stress response and plant development.

A prerequisite of functional analysis of sHSPs is selection and expression of scFvs with well characterized epitope specificity. ScFvs specific to the individual regions of sHSP molecules, such as C-terminal heat shock domain and N-terminal Consensus regions I and II (see 1.3.), could be expressed in plant cells to determine the sHSP domains responsible for oligomerization and chaperoning activity *in vivo*. Domain-specific scFvs can be selected using recombinant truncated sHSPs as antigens for phage library screening for this purpose.

Class-specific anti-sHSP scFvs will be obtained by differential phage library screening using cytosolic class I and II sHSPs as antigens. Expression of class-specific anti-sHSP scFvs in transgenic plants may reveal the functions of cytosolic class I and II sHSPs in the process of thermotolerance.

The functions of sHSPs in chloroplasts, mitochondria and ER during heat stress can be investigated by compartment-specific expression of scFvs with specificity to different organelle-localized sHSPs.

The role of sHSPs in developmental processes is still an open question and can be studied by using tissue-specific and developmental-regulated expression of anti-sHSP scFvs in plants.

ScFvs specific to different constitutive and stress-elicited HSPs may be selected from the phage library and used for the *in vivo* immunomodulation of HSP40, HSP70 and HSP90 in order to study their activities and specific interactions during heat stress response in plant cells.

Thus, and in addition to the results described here, the technique of immunomodulation is a method that allows to investigate a number of other problems of the heat stress response that are presently unsolved.

5. Abstract

The aim of the work was to investigate the function of cytosolic sHSPs in the heat stress response of plant. As methodical tool cytoplasmic expression of single chain variable fragment (scFv) antibodies directed against cytoplasmic sHSPs were used to immunomodulate the functional activities of sHSPs *in vivo*.

Phage scFv libraries were screened with recombinant cytosolic class I HSP17 from *L. esculentum* as a target to rescue cDNA sequences coding for scFv which recognize the target antigen. cDNA sequences from two phage clones, displaying anti-HSP17 scFv, have been selected and characterized by DNA sequencing.

The scFv cDNA sequences were used to construct the expression cassettes for cytosolic expression of the anti-HSP17 scFvs under CaMV 35S promoter control in transgenic plant cells.

After *Agrobacterium*-mediated gene transfer to *N. tabacum*, the regenerated drug-resistant plantlets were used for subsequent selection of stable transgenic plant lines with a high expression level of anti-HSP17 scFv in the cytosol of mesophyll cells.

Southern blot analysis was performed to confirm the integration of the scFv cDNA sequences into the plant genome and a transgenic line (CB6) with one scFv copy per genome was selected for further investigations.

The scFv content in the leaf tissue of the transgenic line CB6 was quantitatively estimated as 0.09% of the soluble leaf protein by Western blot analysis.

The anti-HSP17 scFv were intracellularly distributed in the cytoplasm and nuclei of mesophyll cells as detected by use of immunocolloid technique and transmission electron microscopy.

In addition to the transgenic CB6 plants, transgenic controls with appropriate cytosolic expression of the scFv without sHSP specificity or with ER-localized anti-

HSP17 scFv accumulation were comparatively analyzed to probe for artificial alterations of thermotolerance caused by foreign gene expression in transgenic cells.

At elevated temperatures isolated leaves of the immunomodulated CB6 plants exhibited remarkably less basal thermotolerance than the leaves of transgenic control and wild type plants as detected by visible phenotypical alterations and decreased chlorophyll content in mesophyll cells under stress conditions.

A short protective pretreatment with subsequent challenge at elevated temperatures of immunomodulated plants resulted in a profoundly diminished capacity to acquire thermotolerance whereas transgenic and wild type controls tolerated shifts to extreme temperatures without phenotypical alterations and decreased chlorophyll content in their mesophyll cells.

Immunohistological analysis of leaf tissue by use of fluorescence microscopy revealed regular cytoplasmic distribution of sHSPs and anti-HSP17 scFv in mesophyll cells of heat-stressed immunomodulated CB6 plants, whereas intensive HSG formation was found in mesophyll cells of transgenic and wild type controls.

The incapacity of mesophyll cells from immunomodulated CB6 plants to form HSG in response to heat shock was verified by ultrastructural analysis using transmission electron microscopy. During prolonged heat stress mesophyll cells of CB6 plants suffered destruction of plasmamembrane and endomembranes, showed osmophilic lipid droplets, and finally apoptosis-like cell death was occurring. In contrast, stress-elicited formation of HSGs was observed in mesophyll cells of transgenic and wild type controls.

ScFv expression cassettes were also constructed to target the anti-HSP17 scFvs to the ER and stable transgenic line RB with scFv retention in the ER was established.

No alterations of temperature sensitivity of RB plants could be observed in comparison to the wild type. Mesophyll cells of RB plants were able to form HSGs under heat stress and showed an ultrastructure identical to the control plants. The

accumulation of anti-HSP17 scFv in the endomembrane compartments of parenchyma cells did not affect seed development and storage globulin accumulation.

It is concluded that the intracellular expression of scFvs in plant cells is a useful tool to immunomodulation functions of regulatory proteins *in vivo*.

Obviously, cytosolic sHSPs are involved in the process of basal and acquired thermotolerance in which the formation of HSGs plays a pivotal role for survival of severe or prolonged stress conditions. Blocking of HSG formation by immunomodulation of cytosolic sHSPs causes stress damage and cell death at sublethal temperatures.

6. Zusammenfassung

Die Arbeit zielte auf Untersuchungen der Funktionen cytoplasmatischer sHSP in der Hitzestressantwort der Pflanze. Als Untersuchungsmethode wurde die Immunmodulation der funktionellen Aktivitäten von sHSP durch cytoplasmatische Expression von Einzelketten-Antikörpern (scFv) mit sHSP-Spezifität gewählt.

Zur Selektion von cDNA-Sequenzen, die für anti-sHSP-scFv kodieren, wurde eine Phagen-scFv-Bibliothek mit rekombinantem HSP17 der Klasse I der Tomate als Antigen gescreent. Aus 2 selektierten Phagenklonen konnten anti-sHSP-scFv spezifische cDNA-Sequenzen isoliert und durch Sequenzierung charakterisiert werden.

Die scFv kodierenden cDNA-Sequenzen wurden zur Konstruktion von Expressionskassetten verwandt, die cytoplasmatische Expression der anti-HSP17-scFv unter Kontrolle des CaMV 35S Promotors in transgenen Zellen vermitteln.

Nach *Agrobacterium* vermitteltem Gentransfer in *Nicotiana tabacum* wurden Antibiotika resistente Pflanzenregenerate selektiert und zur Etablierung stabil transgener Pflanzenlinien mit hohem scFv-Expressionsniveau genutzt.

Die stabile Integration der scFv-spezifischen cDNA-Sequenzen in das Genom der transgenen Pflanzen wurde durch Southern-Blotting nachgewiesen. Die stabil transgene Linie CB6 enthielt 1 Transgenkopie pro Genom und wurde für die nachfolgenden Thermotoleranzuntersuchungen eingesetzt.

Durch Western-Blotting wurde der scFv-Gehalt im Blattgewebe der transgenen Linie CB6 quantitativ mit 0,09% des löslichen Blattproteins bestimmt. Durch Immunkolloid-Technik und Transmissions-Elektronenmikroskopie wurde die cytoplasmatische und Zellkernlokalisierung des exprimierten scFv in den Mesophyllzellen nachgewiesen.

Um artifizielle Beeinflussung der Thermotoleranz durch Transgeneffekte auszuschalten, wurden zusätzlich zur transgenen Linie CB6 transgene

Kontrollpflanzen mit adäquater cytoplasmatischer Expression von scFv ohne sHSP-Spezifität bzw. mit Misstargeting des scFv in das ER-Lumen in die Untersuchungen einbezogen.

Bei erhöhten Temperaturen wiesen isolierte Blätter der transgenen Linie CB6 im Vergleich zu transgenen Kontrollen und Pflanzen des Wildtyps eine Erniedrigung der basalen Thermotoleranz auf, die durch phänotypische Veränderungen sowie Reduktion des Chlorophyllgehaltes in den Mesophyllzellen gemessen werden konnte.

Ein kurzzeitiger Hitzestress mit nachfolgender Erholungsphase und erneuter Stressbehandlung führte an isolierten Blättern der transgenen Linie CB6 zu starker Erniedrigung der erworbenen Thermotoleranz, während transgene Kontrollen und Wildtyp Pflanzen bei identischem Stressregime den extremen Temperaturschift ohne phänotypische Veränderung oder Reduktion des Chlorophyllgehaltes überstanden.

Immunhistologische Untersuchungen des Blattgewebes mittels Fluoreszenzmikroskopie ergaben eine reguläre cytoplasmatische Verteilung von sHSP und scFv in Mesophyllzellen Hitze-gestresster, immunmodulierter CB6 Pflanzen, während intensive Bildung von HSG in Mesophyllzellen der transgenen Kontrollen und Pflanzen des Wildtyps nachgewiesen wurde.

Die Unfähigkeit von Mesophyllzellen der immunmodulierten CB6 Pflanzen unter Hitzestress HSG zu bilden konnte durch detaillierte ultrastrukturelle Untersuchungen unter Verwendung der Transmission-Elektronmikroskopie verifiziert werden. Bei verlängertem Hitzestress wiesen Plasmamembran und Endomembranen unter Bildung osmophiler Lipidtropfen Verlust ihrer Integrität auf und besaßen Apoptose-ähnliche Zellstrukturen. Im Gegensatz dazu zeigten Mesophyllzellen transgener Kontrollen und Wildtyp Pflanzen nach kurzzeitiger stress-elicierter HSG-Bildung durch Auflösung der HSG-Komplexe und intensiver Ausbildung von rauhem ER und Golgi-Vesikeln Rekonstitution der normalen Zellstrukturen.

Außerdem wurden Expressionkassetten kreiert, um ein Misstargeting des anti-HSP17-scFv durch Akkumulation im ER-Lumen zu erzielen. Nach Gentransfer in *N.tabacum*

konnte eine stabile transgene Linie RB mit scFv-Retention im ER lumen etabliert werden.

Im Vergleich zum Wildtyp konnte keine Beeinflussung der Temperaturempfindlichkeit der transgenen RB Pflanzen nachgewiesen werden. Mesophyllzellen der RB Pflanzen zeigten unter Hitzestress intensive HSG-Bildung in Cytoplasma und besaßen eine den Kontrollpflanzen identische Ultrastruktur nach Stressbehandlung.

Die Akkumulation von anti-HSP17-scFv im Endomembrancompartment der Parenchymzellen der RB Pflanzen hatte keinen Einfluss auf Samenentwicklung und Reserveproteinakkumulation.

Die Untersuchungsbefunde zur Immunmodulation von sHSP durch cytoplasmatische scFv-Expression dokumentieren, dass die intrazelluläre scFv-Expression auch in pflanzlichen Zellen eine geeignete Methode zur in vivo Funktionsanalyse regulatorischer Proteine ist. Durch Einsatz dieser Methodik konnte erstmalig gezeigt werden, dass sHSP an Prozessen der basalen und erworbenen Thermotoleranz beteiligt sind. Dabei ist für das Überleben von extremen bzw. andauernden Stressbedingungen die Bildung von HSG eine Grundvoraussetzung, da die Blockade der HSG-Bildung durch Immunmodulation der Funktionalität cytoplasmatischer sHSP zu Stressschäden und Zelltod unter sublethalen Temperaturen führt.

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A

L01279	MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDR	40
Z37336DIVMTQSPSSLSASVGDR	18
B8	0
A14STDIQMTQSPSSLSASVGDR	20
L01279	VTLTCRASQSISSYLNWYQQKPGKAPKLLIYAASLQSGV	80
Z37336	VTLTCRASQSISSYLNWYQQKPGKAPKLLIYAASLQSGV	58
B8	.TLTCRASQSISRRRLNWYQQKPGKAPKLLIYAASLQSGV	39
A14	VTLTCRASQSISSYLNWYQQKPGKAPKLLIYAASLQSGV	60
L01279	PSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPPWT	120
Z37336	PSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPR.T	97
B8	PSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPRTS	79
A14	PSRFSGSGSGTDFTLTISSLQPEDFATYYCQQPGMLPPTF	100
L01279	FGQGTKVEIKRTVAAPSVFIFPP	143
Z37336	FGQGTKVEIKRTVAAPSVF.....	116
B8	AKGPRWKSNGRPNQKNSS.....	97
A14	GQGTKVEIKRAA.....	112

B

Ab021526	...EVQLLES G EGLVQ P GGSLKLS C .AASGFTFSSYAMSW	36
Z98716	...EVQLLES G EGLVQ P GGSLRLS C .AASGFTFSSYAMNW	36
B8	.MAEVQLLES G EGLVQ P GGSLRLS C .AASGFTFSRYKMNW	38
A14	HGPRCSCWSL G EAWYEPGGSPGD S PXAAASGFTFSSYAMSW	40
Ab021526	VRQAPGKGLEWVSAISGSGESTIYADSVKGRFTISRDNK	76
Z98716	VRQAPGKGLEWVSGISASGESTIYADSVKGRFTISRDNK	76
B8	VRQAPGKGLEWVSAISGSGESTIYADSVKGRFTISRDNK	78
A14	VRQAPGKGLEWVSTISPRGDRTIYADSVKGRFTISRDNK	80
Ab021526	NTLYLQMNSLRAEDTAVYYCAK G ELAFDY W GQGTLVTVSS	116
Z98716	NTLYLQMNSLRAEDTAVYYCAK G YGLDC W GQGTLVTVSS	115
B8	NTLYLQMNSLRAEDTAVYYCAK S SRAFDY W GQGTLVTV..	116
A14	NTLYLQMNSLRAEDTAVYYCAK K NRRFDY W GQGTLSPXRA	120
Ab021526	116
Z98716	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS	155
B8	116
A14	VE.....	122
Ab021526	116
Z98716	WNSGALTSGVHTFPAVLQS	174
B8	116
A14	122

Multiple alignment of kappa light (**A**) and heavy (**B**) chain variable regions of human immunoglobulines and anti-HSP17 scFv. L01279, Z37336 – light kappa chain variable regions of human immunoglobulines (Acc.Nr. L01279, Z37336), Ab021526, Z98716 – heavy chain variable regions of human immunoglobulines (Acc.Nr. Ab021526, Z98716), A14, B8 – anti-HSP17 scFvs produced by phage clones A14 and B8, respectively.

Erklärung

Hiermit erkläre ich, dass ich mich mit der vorliegenden wissenschaftlichen Arbeit erstmals um die Erlangung des Doktorgrades bewerbe, die Arbeit selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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Lebenslauf

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