Non-stress induced small heat shock proteins in higher plants

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2D..... two dimensional
°C.....degree Celsius
βME.....β-mercaptoethanol
µl..... microlitre(s)
µmol.....micromolar
µg ..... microgram(s)
A..... adenine
ABA..... *cis*-abscisic acid
ANS.....8-anilino-1-napthalene sulfonate
ATP.....adenosine-5'-triphosphate
bis-ANS..... 1,1'-bi(4-anilino) naphthalene-5,5'-disulfonic acid
BSA..... bovine serum albumine
C..... cytosine
cDNA.....complimentary DNA
Ci.....Curie
DAP.....day after pollination
DAI.....days after imbibition
dATP.....deoxy adenosine triphosphate
DEPC.....diethylpyrocarbonate
DNA.....deoxyribonucleic acid
dNTP.....deoxyribonucleoside triphosphate
EDTA.....ethylendiamin-N,N,N',N'-tetraaceticacid
EGTA..... 1,2-Bis-(2-aminoethoxyethan)-N,N,N',N'-tetraaceticacid
ELF..... enzyme labeled fluorescence
ELISA..... enzyme-linked immunosorbent assay
ER..... endoplasmic reticulum
ERL.....epoxy resin low viscosity
Fig..... figure
G..... guanine
g..... gram or gravitation force
Gly.....glycine
GUS.....beta-glucuronidase
h..... hour
HS.....heat shock
HSE.....heat shock element
HSF..... heat shock factor
HSG..... heat shock granula
HSP..... heat shock protein
Ile..... isoleucine
kDa..... kilodalton
l.....liter
Leu..... leucine
M..... mol
mM..... micromolar
mA.....milliamper
mg.....milligramm

ml milliliter
MOPS N-morpholinoprppane sulfonic acid
MW molecular weight
MRNA messenger RNA
NBT 4-Nitro blue tetrazolium chloride
Nm nanometer(s)
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PEG polyethyleneglycol
Pro proline
RNA ribonucleic acid
Rpm revolutions per minute
RT room temperature
scFv single-chain Fv antibodies
SDS sodiumdodecylsulfat
Sec second(s)
SOD superoxide dismutase
T thymine
TEMED *N*,*N*,*N'*,*N'*- Tetramethylethyleneamine
Tris Tris-(hydrxymethyl)-amino methane
TSA tyramide signal amplification
TTC triphenyltetrazolium chloride
UV ultraviolet light
V volt
Val valine
Vol volume

1. Introduction

A wide variety of biotic and abiotic stress factors influence plants during their growth. These factors trigger different stress responses. The main consequence of these stress responses is the increase of stress tolerance by preventing and/or repairing the injuries produced by the stressor. Since exposure to high temperature represents a serious threat to cellular viability, all organisms have developed a wide range of anatomical (thick cuticle, bark, cortical tissues), morphological (small and narrow leaves, spines, reflective trichomes on the upper leaf surface) and metabolic (thermal tolerance of the enzymes, increased membrane fatty acid unsaturation, repair mechanisms) adaptations to adverse thermal conditions. The synthesis of heat shock proteins (HSP's) is one component of the heat-induced response of cells and organisms to elevated temperatures.

1.1 The discovery of heat shock response

The heat shock response and the HSP's were first discovered in *Drosophila*. In 1962 it was shown that brief exposure of fruit fly (*D. buschkii*) larvae to high but non lethal temperatures caused the appearance of new puffs on the salivary gland polythene chromosomes, which result from the activation of heat shock-inducible genes (Ritossa, 1962). The proteins which are synthesised in response to heat stress (heat shock proteins-HSP's) were discovered ten years later (Tissieres et al., 1974). At that time it was shown that an increase in environmental temperature by 5° to 10° C above normal growth temperature led to dramatic changes in gene expression in a wide range of organisms, from bacteria to the higher vertebrates (review Nover, 1991). This response was referred to as heat-shock. It is widely conserved in living cells and in various model systems that have been used to study the molecular mechanisms responsible for the stress-dependent regulation of gene expression. Heat shock response results in a decrease in the transcription of most previously active genes, repression of the synthesis of most normal proteins, and the expression of a new set of proteins - HSP's.

Although HSP's were first identified by the dramatic increase in their synthesis during heat treatment, the high temperature is not the only factor that leads to elevated expression of HSP's. Other inducers of heat shock protein synthesis include several

potentially cytotoxic chemicals, such as ethanol, heavy metals ions, amino acids analogues, sodium arsenite, etc., as well as physiological states which may cause generation of highly reactive free radicals: osmotic and water stress, UV illumination, gamma irradiation, nutrient starvation, anoxia and a number of other treatments (Nover, 1991). It is not clear whether the synthesis of HSP's is due directly to the effect of these factors, e.g. the primary stress, or because of a secondary stress which is produced subsequent to the primary one. Heat shock and other inducers probably share the ability to cause intracellular accumulation of aberrant or partially denatured proteins, which it is thought to be able to trigger the induction of the heat shock response. HSP's have been found to be expressed also in the absence of external stress factors, either constitutively, or under cell cycle or developmental control in some cells (Lindquist and Craig, 1988). This shows that they participate in basic cellular processes in the absence of stress.

According to their approximate molecular weights, heat shock proteins synthesised by eukaryotes have been designated in five classes: HSP100 (MW 104-110 kDa), HSP90 (MW 80-95 kDa), HSP70 (MW 63-78 kDa), HSP60 (MW 53-62 kDa), the small or low molecular weight proteins (MW 17-30 kDa) and the ubiqutin family (MW 8.5 kDa) (Neumann et al., 1989; Nover, 1991).

1.2 Plant small heat shock proteins (sHSP's)

In higher plants the heat shock phenomenon was first discovered at the level of protein synthesis in soybean (Barnett et al., 1980; Key et al., 1981). Different tissues of a plant species usually synthesise identical sets of HSP's. One of the peculiarities of the plant heat shock response is the extremely abundant synthesis of the low molecular weight (ca 20 kDa) proteins which are usually not detectable in plants grown at optimal temperatures. Some plant species may have as many as 40 different sHSP's (Vierling, 1991). In contrast, most other organisms have one or only a few small heat-shock proteins (Arrigo and Landry, 1994). The diversification of plant sHSP's may reflect heat stress response unique to plants. HSP's accumulate rapidly during temperature stress and the accumulation is proportional to the temperature and duration of the stress. Maximum synthesis and accumulation of small heat shock proteins is observed at temperatures just below lethal levels (Howarth, 1991). Some members of sHSP's are also quite stable following stress, with half-lives of 30 - 50 h (Chen et al., 1990; DeRocher et al., 1991).

In addition to their expression during stress, plant sHSP's are also expressed independently of it during meiotic prophase (Bouchard, 1990; Dietrich et al., 1991), microsporogenesis (Atkinson et al., 1993; Zarsky et al., 1995), seed development, and in somatic embryos (Zimmerman et al., 1989; Györgyey 1991; Hernandez and Vierling, 1993; Coca et al., 1994; DeRocher and Vierling, 1994, zur Nieden et al., 1995; Dong and Dunstan, 1996). There are few examples of constitutive accumulation of sHSP's in vegetative organs: in roots and lower parts of the shoots of the desiccation-tolerant plant *Craterostigma plantagineum* (Alamillo et al., 1995) and in cortical parenchyma cells of mulberry in winter (Ukaji, 1999).

Plant small heat shock proteins are encoded by different gene families and are targeted to different cellular compartments, including cytosol, chloroplasts, mitochondria, and endoplasmic reticulum (for a review, see Waters et al., 1996). This diversification of the sHSP's is completely unique to plants, and plants are the only eukaryotes in which organelle-localized sHSP's have been described. Based upon subcellular localisation of small heat shock proteins, amino acid sequence homology and immunocrossreactivity, plant small heat shock proteins have been divided into five classes: class I cytosolic, class II cytosolic, chloroplast-localised, endoplasmic reticulum (ER)-localised and mitochondria-localised. Proteins of these classes have been identified in several species (Vierling, 1991; Helm et al., 1993, 1995; Lenne and Douce, 1994; Lenne et al., 1995;). Recently a cDNA clone encoding small heat shock protein, which may be a potential member of a sixth class, was isolated from *Glycine max* (LaFayette et al., 1996). The analysis of the predicted amino acid sequences showed that this protein has a signal peptide at the amino terminus typical for endomembrane-directed proteins. Moreover, the mRNA from this sHSP is translated on membrane-bound polysomes, however this protein has no ER retention signal and it final intracellular location is not known.

1.2.1 Plant cytosolic sHSP's

At present cytosolic class I and class II sHSP's have been shown to be the only known sHSP's induced in plants both under stress treatment and during development (DeRosher and Vierling, 1994). Developmental expression of these proteins was observed during pollen and seed maturation. The presence of proteins or mRNA of cytosolic sHSP's have been reported for a variety of seeds (Hernandez and Vierling, 1993; Coca et al., 1994;

DeRocher and Vierling, 1994; zur Nieden et al., 1995; Wehmeyer et al., 1996; Collada et al., 1997) and for pollen of different species (Bouchard, 1990; Dietrich et al., 1991; Hopf et al., 1992; Atkinson et al., 1993; Kobayashi et al., 1994). Under heat shock these proteins are synthesised in all cells and accumulate to more than 1.0 % of total proteins (DeRocher et al., 1991). The isoform pattern of developmentally and stress induced cytosolic sHSP's are different and are represented by several polypeptides (DeRocher and Vierling, 1994; Coca et al., 1994; zur Nieden et al., 1995; Wehmeyer et al., 1996). Heat shock induced cytosolic sHSP's were found to be localised in the cytosol and nuclei. Their distribution in the cytoplasm depends on the length of the stress. After short heat treatments they are distributed uniformly in the cytoplasm. If the time of heat stress is increased, they form „heat shock granules" (HSG's) (Nover et al., 1983; 1989; Neumann et al., 1984, 1987). It was shown that HSG's contain both class I and class II sHSP's and that class II sHSP's are necessary for proteins of class I to incorporate in HSG's (Nover, personal communication). In stressed soybean seedlings (Lin et al., 1984), in cell cultures of *Lycopersicon peruvianum* (Wollgiehn et al., 1994) and in developing seeds (zur Nieden et al., 1995) the localisation of cytosolic small heat shock proteins in the nuclei was shown. How cytosolic sHSP's are translocated into the nuclei is still not clear. It could be that they are transported into the nucleus passively through the nuclear pores or/and in a complex with other nuclear proteins, or that they may possess a nuclear localisation sequence. Recently it was shown that a tomato cDNA clone coding for the cytosolic class II sHSP has two sequence motifs which could be responsible for the translocation of sHSP from the cytosol to the nucleus during stress, or during definite stages of plant development (Kadyrzhanova et al., 1998). One of these sequences corresponds to the Xenopus type nuclear localisation signal and the second one contains a putative SV40 large T-antigen nuclear targeting signal. In seeds of *Lycopersicon esculentum, Nicotiana rustica, Vicia faba,* and *Pisum sativum* the accumulation of cytosolic sHSP's was also observed in protein bodies (zur Nieden et al., 1995).

The specificity of the regulation of cytosolic sHSP's in response to stress and during development suggests that they may have distinctive functions (Waters et al., 1996). Both *in vitro* and *in vivo* it has been shown that some members of cytosolic sHSP's can act under stress treatment as molecular chaperones (Lee et al., 1995, 1997; Forreiter et al., 1997), however the role of developmentally induced cytosolic sHSP's *in planta* is still not understood.

1.3 sHSP genes and their regulation

1.3.1 sHSP genes

The plant small heat shock protein genes have evolved from a single gene found in most animals and fungi into a large super gene family (Waters et al., 1996). The phylogenetic relationships of sHSP's reveals that gene duplication, sequence divergence and gene conversation have all played a role in their evolution (Waters et al., 1996). In comparison to the large HSP genes, small heat-shock protein genes have evolved much more quickly. Plants have six sHSP's gene families which are nuclear encoded. Evolutionary analysis shows that these classes arose prior to the divergence of the major groups of angiosperms sHSP's are more related to proteins of the same class from divergent species than to other small heat shock proteins of the same species (Waters et al., 1996). The analysis of rate of evolution showed that sHSP gene families have evolved at unequal rates (Waters, 1995).

In the early publications concerning the structure of HSP genes it was shown that some genes are free of introns (Yost and Lindquist, 1986). It was also demonstrated that abrupt heat stress interrupts intron processing of several gene transcrips in *Drosophila* (Yost and Lindquist, 1986) and *S.cerevisiae* (Yost and Lindquist, 1991). These facts led to the assumption that absence of introns in HSP genes is a mechanism employed to avoid heat-induced block in inhibition of splicing of HSP transcripts (Yost and Lindquist, 1986). However, several other heat-inducible HSP genes contain introns which are spliced efficiently under heat stress conditions (Russnak and Candido, 1985; Czarnecka et al., 1985; Bond, 1988; Minchiotti et. al., 1991; Takahashi et al., 1992). The first intron-containing small heat shock gene of plants with molecular weight 26 kDa was identified from soybean (Czarnecka et al., 1988). Later it was shown that chloroplast-localised sHSP's from *Arabidopsis thaliana, Nicotiana tabacum, N. sylvestris* and *N. tomentosiformis* also possess a single intron (Osteryoung et. al., 1993; Lee et al., 1998a).

1.3.2 Gene regulation

The heat shock promoters have several different *cis*-acting regulatory promoter elements. One of these sequences is a heat shock element (HSE) located in the TATA box-proximal 5'-flanking regions. This element is involved in heat shock response and also required for developmental regulation of sHSP genes in embryos (Coca et al., 1996; Prändl et al., 1995).

Most eukaryotic heat-shock genes have multiple HSE's present within a region of

a few hundred base pairs, which are alternating units of 5'-nGAAn-3', and several of them overlap over four nucleotides (Schöffl et al., 1998; Raschke et al., 1988). In plants the optimal HSE consensus was shown to be 5'-aGAAg-3' (Barros et al., 1992).

There are a few upstream regulatory elements which have been shown to participate in regulation of HS gene expression. In plants there is evidence for involvement of CCAAT-box elements and AT-rich sequences. The AT-rich repeats are located upstream from the HSE-containing region and are represented by different simple repeats-(A)n, (T)n, and (AT)n. The AT-rich repeats were also found downstream from the HSP genes, however their function is not known.

Heat shock transcription factors (HSF's) are *trans*-regulators of all heat shock genes. The synthesis of most HSF's is not regulated by high temperature. In several organisms including *Arabidopsis* HSF's are present in an inactive form in cytosol under normal conditions (Hübel and Schöffl, 1994; Wu, 1995). However, it was reported that in tomato in addition to a constitutively expressed HSF there are two heat shock inducible HSF's (Scharf et al., 1990). Under heat stress HSF can recognise the heat shock signal and becomes activated. Activation of HSF occurs through the conversion of a monomeric to a trimeric form with high binding affinity for HSE (Clos et al., 1993; Morimoto, 1993; Westwoord and Wu, 1993), but the mechanism by which the trimerization is regulated is not known in detail. The finding that *Arabidopsis* HSF1 is constitutively active in *Drosophila* and in human cells lead to the suggestion that the regulation of HSF depends on a specific factor (Hübel et al., 1995). There is also a possible involvement of HSP70 in the negative regulation of HSF in *Arabidopsis* (Lee and Schöffl, 1996). The formation of trimers of HSF is due to the oligomerization domain located next to the DNA-binding domain in the N-terminal region of HSF. Both domains are conserved in primary structure throughout the HSF protein family. In contrast to the single HSF in yeast and *Drosophila melanogaster*, all investigated plant species contain multiple HSF's which have molecular weights of 32.2 to 57.5 kDa (Scharf et al., 1990; Hübel and Shöffl, 1994; Gagliardi et al., 1995; Nover et al., 1996; Prändl et al., 1998). Based on sequence homology and domain structure, plant HSF's can be subdivided into the two classes, A and B (Nover et al., 1996).

The expression of HSP's is primarily regulated at the transcriptional level. The heat induction of HSP gene transcription is initiated by the binding of activated heat shock factor to heat shock elements. TATA-proximal HSE are usually more important in heat-

induced activation of plant sHSP promoters than more distal elements (Gurley and Key, 1991).

In addition to HSE's, a number of *cis*-elements have quantitative effects on the expression of certain heat-shock genes. In plants CCAAT-box elements, AT-rich sequences and scaffold-attachment regions, affecting the chromatin structure, are involved in regulation of HSP gene transcription (Czarnecka et al., 1989; Rieping and Shöffl, 1992; Schöffl et al., 1993). It was suggested that the chromatin structure may be important for efficient binding of transcription factors and/or transcription activator proteins. A model for the activation of heat-shock gene expression was proposed. According to this model the binding of a chromatin-modifying factor, e.g. a GAGA-sequence binding factor (Giardina et al., 1992; Tsukijama et al., 1994), or scaffold attachment affects chromatin structure so that the transcription factor TBP, the first promoter binding component of transcription complex (Pugh, 1996), has access to the TATA-box. This is the initial step for the subsequent assembly of the basal transcription complex.

Under HS the synthesis of HSP's is also regulated at the translation level. As temperatures are increased, HSP mRNA translation increases and the synthesis of most normal cellular proteins ceases. However, this type of regulation has not yet been investigated in detail.

Heat shock genes, including those encoding small heat shock proteins, are expressed not only in response to heat stress but also during developmental processes in the absence of significant temperature changes. It was shown that not all sHSP genes activated by heat stress are developmentally regulated (Wehmeyer et al., 1996; Coca et al., 1996) and that some sHSP genes expressed during zygotic embryogenesis are noninducible by heat stress (Carranco et al., 1997). Such diversity of stress and developmentally induced HSP's suggests specificity in the regulation of HSP genes. However, the information concerning developmental expression of plant HSP's is limited at present. In other organisms it was shown that the developmental regulation of HS genes depend on the same *cis*-acting elements that are involved in heat stress response (Fernandes et al., 1994), although a functional specialisation of different *trans*-acting factors have been demonstrated for stress and developmental regulation of HS genes (Morimoto et al., 1994; Wu, 1995). In plants, during seed maturation the activation of the sHSP promoter involves at least two distinct regulatory mechanisms: one is dependent on HSE and presumably mediated by

the HSF, and the other, observed at the early stages of seed maturation, is not dependent on HSE (Almoguera et al., 1998). It was suggested that in this case HSP genes are regulated by developmentally specific *trans*-activator proteins. Recently it was shown that ABI3, a seed-specific transcription factor from *Arabidopsis*, regulating various seed-specific genes (Giraudat et al., 1992; Parcy et al., 1995), activates the small heat shock protein promoter (Rojas et al., 1999). It was suggested that ABI3 functions through heat shock factors. Interaction between HSF and other transcription factors has also been demonstrated in animal systems (Kanei-Ishii et al., 1997; Stephanou et al., 1999).

1.4 Structure and biochemistry of sHSP's

The plant sHSP's are related to small heat shock proteins of other organisms and to vertebrate alpha-crystalline proteins (Plesofsky-Vig et al., 1992; Jong et al., 1993). All members of the sHSP family share a characteristic C-terminal sequence of about 100 amino acid residues that has also been conserved in the α-crystallin proteins of the vertebrate eye lens (Plesofsky-Vig et al., 1992; Jong et al., 1993; Waters et al., 1996; Gaestel et al., 1997). This sequence is called the α-crystalline domain, or small heat-shock-protein domain, and comprises two consensus regions (I and II) separated by a variable length region (Vierling et al., 1991). Consensus I is 27 amino acids long with nine identical amino acids and seven conservative replacements. The conserved motif Pro-X(14)-Gly-Val-Leu within consensus I is also present in all sHSP's of other eukaryotes (Lindquist and Craig, 1988). Consensus region II is 29 amino acids long and has a similar motif found in consensus I, Pro-X(14)-X-Val/Leu/Ile-Val/Leu/Ile (Waters et al., 1996). The poorly conserved sequence between consensus I and II is part of a highly hydrophilic domain present in all small heat shock proteins (Czarnecka et al., 1985; Nagao et al., 1985; Rashke et al., 1988). The amino-terminal domains of the plant sHSP's are quite divergent between the different classes. The chloroplast-, mitochondrial- and endoplasmic reticulum-localised proteins all have transit sequences that are specific for each organelle (Chen and Vierling, 1991; Waters, 1995). Additionally the chloroplast-localised proteins have a methionine-rich region in the N-terminal domain (Vierling, 1991; Waters, 1995). The cytosolic sHSP's also have a conserved region which is characteristic of each class and is not present in the other sHSP's. These sequences motifs are present in the N-terminal domain of proteins.

In vivo, sHSP's from many different organisms have an oligomeric quaternary structure. In the native state they form high molecular weight complexes ranging in size from 200 to 800 kDa (Vierling, 1991; Lenne and Douce, 1994; Jinn et al., 1995; Suzuki et al, 1998). The complexes are homo-oligomers of sHSP's. The formation of such structures is also common for the plant organelle-localised small heat shock proteins (Osteryoung and Vierling, 1994). The complexes are homo-oligomers of sHSP's. It was suggested that this complex formation is due to the α-crystalline domain, but the N-terminal regions also appear to be necessary for oligomerization because the minimal α-crystallin domain alone fails to form oligomers (Merck et al., 1993; Leroux et al., 1997). The quaternary structure of the sHSP complexes have been shown only for recombinant proteins: human αB-crystalline, HSP16.5 from *Methanococcus jannaschii* and murine HSP25 (Haley et al., 1998; Kim et al., 1998a; Wieske et al., 1999). Cryoelectron microscopy of recombinant human αB-crystalline aggregates have demonstrated an asymmetric, variable quaternary structure and revealed a large central cavity within the complexes and regions of low density within the protein shell (Haley et al., 1998). Using the same method it was demonstrated that recombinant murine HSP25 particles form a hollow sphere with several openings on the surface and additional material in the centre (Wieske et al., 1999). The crystal structure of HSP16.5 from *Methanococcus jannaschii* is also a hollow sphere with eight trigonal and six square „windows" (Kim et al., 1998a). It was proposed that the formation of such complexes are necessary for chaperone or other stress-related activities of sHSP's. It has also been suggested that the oligomeric form is a storage form from which sHSP's can be disassembled quickly in response to the external stress and protect proteins (Kim et al., 1998a). At more severe temperatures sHSP complexes together with other proteins (HSP70, heat shock factor) and RNA form cytoplasmic particles, which have been referred as „heat shock granules" (Nover, 1983, 1989; Neumann et al., 1984; Scharf et al., 1998).

Using the hydrophobic dyes 8-anilino-1-napthalene sulfonate (ANS) and 1,1'-bi(4-anilino) naphthalene-5,5'-disulfonic acid (bis-ANS), which demonstrate the presence of hydrophobic sites on the surfaces of proteins, it was shown that α-crystallin and sHSP undergo a temperature-dependent structural change that increases surface hydrophobicity (Raman et al., 1995; Das and Surewicz, 1995; Lee et al., 1995, 1997). In contrast to mammalian sHSP's, which are phosphorylated in response to stress and developmental factors (Gaestel et al., 1991; Freshney et al., 1994; Rouse et al., 1994) plant sHSP's are

not phosphorylated and possess no recognisable phosphorylation motifs (Nover and Scharf, 1984; Waters et al., 1996).

1.5 Possible sHSP function

The function of the small heat shock proteins, during both defined stages of plant development and in response to different kind of stress, is not understood at present. The correlation of HSP expression with cellular resistance to high temperature has led to the hypothesis that HSP's protect cells from the effects of high temperature. However, the mechanism by which HSP's may effect such protection has not been clearly defined. Several different hypotheses have been proposed to explain the function of sHSP's under heat stress. In mammalian systems it has been shown that the expression of sHSP's increases cellular thermoresistance concomitant with the stabilisation of cytoskeletal elements such as actin (Lavoie et. al., 1993, 1995). It was proposed that sHSP's interact with the actin cytoskeleton to protect and restore cellular structure (Arrigo and Landry, 1994). The finding of RNA in plant heat shock granules has led to the hypothesis that sHSP's can protect and store mRNA during stress condition (Nover et al., 1989). Subsequent *in vitro* experiments have demonstrated that some members of sHSP's can function as molecular chaperones in an ATP independent manner (Jinn et al., 1989, 1995; Horwitz, 1992; Jakob et al., 1993; Lee et al., 1995, 1997; Collada et al., 1997; Kim et al., 1998b). Molecular chaperones are proteins binding to partially folded or denatured proteins and thereby preventing their irreversible aggregation or promoting their correct folding (Hartl et al., 1992; Hendrick and Hartl, 1993; Landry and Gierasch, 1994). The *in vivo* chaperone function of plant sHSP's was recently demonstrated by the protection and reactivation of luciferase in *Arabidopsis* cells (Forreiter et al., 1997). The renaturation processes need HSP70 and ATP (Forreiter et al., 1997; Lee et al., 1997; Lee and Vierling, 2000). The high stability of sHSP's following stress may indicate that their function is important for the recovery period.

The mechanism of chaperone activity of sHSP's is still poorly understood. It was assumed that the highly conserved α-crystalline domain may be important for chaperone activity. This assumption is not supported, however, by the observation that *Escherichia coli*, expressing a deleted rice sHSP, where the C-terminus two-thirds of the α-crystallin domain is missing, is protected from heat shock (Yeh et al., 1997). Moreover, it was shown that the α-crystallin domain alone has no chaperone activity *in vitro* (Merck et al., 1993; Leroux et al., 1997). A mutation within the phenylalanine-rich region of α-B-

crystallin, located N-terminal to the α-crystalline domain, abolished chaperone activity *in vitro* without altering the size of the oligomeric complex (Plater et al., 1996). This observation seems to suggest that the N-terminal residues, located upstream of the α-crystalline domain, are necessary for chaperone activity.

Recently it was shown that *bis*-ANS is incorporated into the consensus region II in the C-terminus of pea recombinant HSP18 (Lee et al., 1997). The binding of *bis*-ANS is blocked by prior incubation with the substrate protein (malate dehydrogenase) suggesting that the substrate binds to the hydrophobic sites of sHSP's. Since sHSP's undergo a temperature-dependent structural change that increases surface hydrophobicity (Raman et al., 1995; Das and Surewicz,1995; Lee et al., 1995, 1997) without self-aggregation, the hydrophobic sites may be localised within clefts that prevent self-association (Lee et al., 1997). Such a mechanism may prevents non-productive interactions with native proteins at normal temperatures.

Based on the crystal structure of sHSP from *Methanococcus jannaschii*, possible mechanisms by which sHSP's might protect proteins from denaturation were proposed (Kim et al., 1998a). According to this model certain proteins or RNA important for cell survival under stress may be trapped within or on the outer surface of the hollow spheres during their *in vivo* assembly. The openings are large enough to allow small molecules such as enzyme substrates and even extended peptide chains to diffuse in and out of the sphere.

The finding that in orthodox seeds (seeds which are able to withstand complete loss of cellular water) sHSP's are developmentally induced led to the hypothesis that their function is a protection of the cellular components during desiccation and/or rehydration (Almoguera and Jordano, 1992; Coca et al., 1994; DeRosher and Vierling, 1994). However, subsequently it has been shown that the *Arabidopsis* mutant, abi3-1, which is desiccation tolerant, has 10 - fold lower levels of sHSP's than the wild type (Wehmeyer et al., 1996). It was assumed that sHSP's are not required for desiccation tolerance, or they can function at significantly reduced levels. In contrast to this is the finding of a high level of sHSP expression in recalcitrant (sensitive to desiccation) chestnut seeds (Collada et al, 1997). In this case it was suggested that the presence of sHSP's is required for protection against environmental damage, since high-moisture seeds are more sensitive to certain types of environmental factors.

More recently it has been reported that the synthesis of sHSP mRNAs and/or their translations products occurs in response to low-temperature stress (van Berkel et al., 1994; Sabehat et al., 1996; 1998; Soto et al., 1999). Previously, it was also shown that preheating increased the tolerance of the tissues to subsequent chilling (Lurie and Klein, 1991; Saltveit, 1991; McCollum et al., 1995, Sabehat et al., 1996). These facts led to the assumption that sHSP's might contribute to chilling resistance. The finding that recombinant chestnut sHSP, expressed in *E. coli*, enhanced cell viability at chilling temperature (Soto et al., 1999) is in agreement with a possible role of sHSP's in protection against chilling injury.

1.6 The aim of current work

It is important to understand the mechanisms by which plants tolerate environmental stresses, since this information can be efficiently used in plant acclimation and in agriculture to develop varieties of stress resistant plants. The synthesis of sHSP's seems to be one of the basic components of the plant heat stress response. Since these proteins were found to be also induced during some developmental stages independently of stress, the investigation of non-stress induced sHSP's could contribute to the understanding of their function in plants. The aim of the present work is to investigate developmentaly induced sHSP's using tobacco seeds as a model system.

2. Materials and methods

2.1 Plant growth and development

Tobacco plants (*Nicotiana tabacum* L.) were grown in a mixture soil:sand (2:1) in the greenhouse at 24° C and 60 % relative humidity. Light intensity was approximately 600 - 800 μmol m^{-2} sec^{-1}. Mature seeds and those of different development stages were collected and used directly for analysis or stored at - 20° C. Freshly collected seeds were weighed dried overnight at 40° C followed by re-weighing to determine the water content.

For germination, tobacco seeds that have been grown as described above were placed in Petri dishes on filter paper moistened with tap water and kept in the growth chamber on a 16 h day / 8 h night cycle at 24° C.

Mature tubers of *Solanum tuberosum* L. c.v. Desiree, grown in greenhouse were used immediately, after harvesting, or after storage at 12° C. Tubers of field grown plants with well-developed overground parts were harvested in June. Garden grown bulbs of *Narcissus pseudonarcissus* L. were collected in November and March. One year old twigs of *Acer platanoides* L. and *Sambucus nigra* L., were harvested in original location in January and April each and one year old tendrils of *Aristolochia macrophylla* LAMK - in January and in May.

Seeds of transgenic tobacco plants with anti-ABA single-chain Fv (scFv) gene under control of the seed-specific USP promoter from *Vicia faba* was a gift from Dr. Conrad (IPK, Gatersleben, Germany).

2.2 Heat-stress treatment

Heat stressed samples were prepared from plant or excised leaves placed between water-saturated paper towels. The three step heat stress regime was performed under light as follows: pulse heat shock for 15 min at 39° C, followed by 2 h incubation at 24° C and a second heat treatment at 41° C for 2 h. High humidity was maintained during the heat treatment to prevent transpiration cooling. Unstressed control samples were taken prior to the heat stress.

2.3 Antisera

Monospecific, polyclonal antibodies raised against tomato cytosolic HSP17 were prepared and characterised as described (Neumann et al., 1987). The antiserum cross-reacts only with cytoplasmic members of class I and class II sHSP's.

Vicia globuline antiserum was a gift from Dr. Manteuffel (IPK, Gatersleben, Germany). The antiserum cross-reacts with tobacco globulins.

Antibody against cytosolic CuZnSOD was a gift from Dr. Grimm (IPK, Gatersleben, Germany). This antibody cross-reacts only with cytosolic CuZnSOD, but not with CuZnSOD from chloroplasts.

2.4 ABA assay

2.4.1 ABA extraction

Nicotiana seeds were ground in liquid nitrogen and extracted two times with 10 - 20 ml of 70 % methanol for 3 h at 4° C. After centrifugation (10 min, 12 000 rpm) the methanolic extract was passed through a SepPak C18 cartridge to remove lipids and most pigments. The solution was then acidified to pH 2.5 by 0.1 M HCl and extracted with an equal volume of ethyl acetate (3 times). After removal of the organic solvent, the sample was dissolved in 100 mM Tris-HCl, 150 mM NaCl (pH 7.5) with 5 % methanol and used for ABA measuring.

2.4.2 ABA immunoassay

The ABA content in tobacco seeds was measured by an ELISA using monoclonal antibodies as described by Weiler (1986). For ABA analysis ELISA microtitration plates were coated with rabbit anti-mouse immunoglobulin (Sigma) diluted in carbonate solution (CS) and incubated overnight at 4° C. After washing with distilled water, mouse anti-ABA monoclonal antibody, diluted with buffer 2 (B2) were added and the plates were incubated 24 h at 4° C. The plates were then washed with tap water and samples and standard ((+)-cis, trans-ABA (Sigma) solution (1-10 pmol)) both mixed with ABA-alkaline phosphatase conjugate, prepared according to Weiler (1986) in B2, were applied. After incubation for 4h at 4° C in the dark, the enzymatic reaction was performed with p-nitrophenylphosphate (1 mg/ml CB) at 37° C for 1-2 h. The optical density was measured at 405 nm.

Solution used:

CS: 50 mM $NaHCO_3$ (pH 9.6)

B2: 1 mM $MgCl_2$, 150 mM NaCl, 50 mM Tris-HCl (pH 7.8)

2.5 Superoxide dismutase (SOD) assays

For superoxide dismutase activity plant material was homogenized in ice-cold extraction buffer (EB) and centrifuged at 13 000 rpm for 10 min. Supernatant was used for SOD activity assays using the *in situ* staining technique of Beauchamp and Fridovich (1971). Proteins (200 µg/lane) were separated by native PAGE (117 V at 4° C). Gels were then soaked in 100 ml of solution A for 25 min. Then solution A was replaced by solution B, and the gels were shaken for 30 min. After incubation in solution B gels were illuminated for 20 min or until white bands appeared. The gels were then fixed in solution C for 1 h and dried between gel-drying membranes (Bio-Rad, USA). The three types of SOD were distinguished by their different sensitivity to inhibitors (Asada et al., 1975; Britton et al., 1978). Inhibitors (2 mM KCN or 5 mM H_2O_2) were applied 30 min before gel incubation in solution A. CuZnSOD which is sensitive to both inhibitors, and FeSOD which is resistant to KCN and sensitive to H_2O_2 were detected. MnSOD is resistant to both inhibitors.

Solution used:

EB: 50 mM potassium phosphate buffer (pH 7.8)
BSA.....0.1 %
ascorbate.....0.1 %
βME.....0.05 %
Triton X-100.....0.2 %

Solution A: 25 mg NBT and 10 mg riboflavine in 100 ml distilled water (kept absolutely dark in the cold)

Solution B: TEMED .....1 % in distilled water

Solution C: methanol.....30 %
glycerol.....2 %

2.6 Protein techniques

2.6.1 Protein isolation

Plant proteins were extracted in SDS sample buffer (SB) or as described in Lehmann et al. (1995). For protein extraction according to Lehmann, frozen plant

material was homogenized under liquid nitrogen and extracted with phenol extraction buffer (PB). After centrifugation (20 000 x g for 10 min) the phenolic phase was used for protein precipitation by 0.1 M ammonium acetate in methanol (1:3) overnight at -20° C. To remove remaining amounts of phenol, the protein pellet was washed with the 0.1 M ammonium acetate/methanol solution five times. The final pellet was air dried and dissolved in sample buffer.

Solution used:

SB: 50 mM Tris-HCL (pH 6.8), 20 % glycerol, 1.5 % SDS, 5 % βME

PB: Phenol saturated with 0.1 M Tris-HCL (pH 8.0), 5 % βME

2.6.2 Determination of protein concentration

Protein concentration was determined according to Esen (Esen, 1978) or Bradford (Bradford, 1976). For the Bradford method 10 or 20 µl of the protein solution to be determined was mixed with 1 ml of Bradford reagent (Sigma) and the absorbance at 595 nm was measured. Protein concentration was determined by interpolation with a standard curve prepared for BSA.

For the Esen method 5-10 µl of protein solution were dotted onto FN7 filters (Filtrak, Niederschlag, Germany). The proteins were fixed in fixing solution for 5 min and shaken for 15 min in the staining solution. Excess stain was removed by rinsing in distilled water (2 times, RT), twice by boiling in water and again two rinses with water (RT). The filters were then air dried. Following drying, the dye-protein complex was eluted by filter incubation in 3 ml 0.5 % (w/v) SDS for 20 min at 55° C or overnight at RT. The absorbance at 578 nm was measured and the protein amount was determined by comparison with a standard (2µg bovine serum albumin) and a blank (protein sample buffer) that have been similarly treated as the protein samples to be measured.

Solution used:

Fixing solution: deionized water:isopropanol:acetic acid (10:65:25)

Staining solution: 0.1 % (w/v) Coomassie brilliant blue R-250 in fixing solution

2.6.3 SDS-PAGE and Western blot

SDS-PAGE was performed using 12 % or gradient (10 - 20 %) gels as described by Laemmli (1970). After electrophoresis, gels were used for Western blots. The proteins were transferred onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) by semi-dry blotting for 40 min at 240 mA using blotting buffer as describe by Kyhse-Anderson (1984). To assess bloting efficiency the membranes were stained with Ponceau red. After protein blotting membranes were dried and stored at 4° C or used directly for immunodetection of sHSP's according to the following scheme:

- blocking with 1 % I-Block (Tropix Inc., Bedford, Mass., USA) or 1 % BSA in PBS.....**1 h**
- incubation with crude antiserum against tomato cytosolic HSP17 at 1:20000 dilution in blocking buffer (BB).....**1 h**
- washing (3 times) with PBS to remove excess of primary antibody.....**5 min**
- incubation with biotinilated secondary antibody (a goat anti-rabbit antibody, Tropix Inc., Bedford, Mass., USA) at 1:2000 dilution.....**45 min**
- washing (3 times) with PBS.....**5 min**
- incubation with streptavidin-HRP (1:20000) in blocking buffer.....**15 min**
- washing (3 times) with PBS.....**5 min**
- visualization of the immunoreactive component of the protein extract by incubation with Super Signal™ ULTRA chemiluminescent substrate (Pierce).....**5 min**

Solution used:

BB: 1 % I-Block or 1 % BSA in PBS, 0,1 % Tween 20

PBS: 580 mM Na_2HPO_4, 170 mM $NaH_2PO_4 \cdot H_2O$, 680 mM NaCl (pH 7.4)

Ponceau red: 0.2 % Ponceau S, 3 % TCA in water

2.6.4 To-dimensional SDS-PAGE

Two-dimensional gels were run according to O`Farrell (1974). Isoelectric focusing gels contained pH 5 to 8 and pH 3 to 10 ampholines (Pharmacia Biotech, Freiburg, Germany) at a four-to-one ratio. 150-300 μg protein was used per tube. Ampholines pH 3-10, urea and Nonidet NP40 were added to protein samples just before electrophoresis. Isoelectric focusing gels were run overnight according to the following schedule: 300 V for 1 h; 500 V for 14 h and 800 V for 1 h. After isoelectrofocusing gels were equilibrated in SDS sample buffer by shaking for 30 min at RT and frozen (-20° C) or used immediately for the second dimension electrophoresis. The second dimension protein separation were performed using 12 % or a 10 to 20 % linear gradient acrylamide gels as described by Laemmli (1970).

2.7 Nucleic acid techniques

2.7.1 RNA isolation

All stock solutions for RNA preparation were treated with DEPC (diethylpyrocarbonate) and autoclaved. The equipment was autoclaved or treated with RNase Erase (ICN Biomedicals,Inc., USA) to render them ribonuclease-free.

RNA isolation was performed as described by Goldberg et al., (1981) with modifications. Plant material was homogenized in liquid nitrogen and suspended in lysis buffer (LB). After centrifugation (5000 rpm 10 min, 4° C) the supernatant was used for RNA isolation by extraction with an equal volume of phenol-chloroform and isoamylalcohol (25:24:1). The RNA was precipitated by incubation with 0.6 volumes of isopropanol overnight at -20° C or for 1 h at -80° C, followed by centrifugation. The nucleic acids were resuspended in water and RNA was precipitated overnight with an equal volume of 4 M LiCl at 0° C. After centrifugation the final pellet was washed with 100 % ethanol and 70 % ethanol, dried briefly and dissolved in TE (pH 7.5). The concentration of RNA was determined by its optical density at 260 nm.

Solution used:

LB: 100 mM Tris-HCl (pH 8.6), 2 % sarkosyl, 25 mM EDTA, 25 mM EGTA
100 mM βME

TE: 10 mM Tris-HCl, 1mM EDTA (pH 7.5)

2.7.2 Northern blot

Sixty µg per lane of each total RNA sample in a 25 µl volume containing 50 % formamide, 6.3 % formaldehyde and 1xMOPS buffer was denatured at 55° C 15 min and separated on agarose gels (containing 6.3 % formaldehyde) in a 1xMOPS buffer. Equal sample loading was verified by ethidium bromide staining of rRNA in the gel. After electrophoresis the gel was rinsed briefly with water, following washing with 10xSSC buffer for 20 min and capillary blotting to a positively charged nylon membrane (Hybond-N+, Amersham) using 10xSSC buffer (Sambrook at al., 1989). RNA was fixed onto the filters by UV crosslinking with a Stratalinker apparatus (Stratagene).

Solution used:

MOPS buffer:	20 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA
20xSSC:	3 M NaCl, 300 mM sodium citrate (pH 7.0)

2.7.3 Preparation of the probe and hybridisation conditions

The hybridisation probe, tomato cytosolic class I HSP17 cDNA, was a gift from Dr. A. Bucka (Institute of Plant Biochemistry, Halle, Germany). cDNA was isolated from a pUC18 plasmid by digestion with the appropriate restriction endonucleases and labelled with α-[^{32}P]dATP (ICN Radiochemicals, Irvine, CA, 3000 Ci/mmol) by random priming kit (Amersham, Life Science). Approximately 25 ng of DNA were denatured at 95° C for 5 min, cooled rapidly and the reaction mixture (12 µl dNTP mix, 5 µl α-[^{32}P]dATP and 1 µm Klenow enzyme) was added. After 1 h incubation at 37° C un-incorporated nucleotides were removed by either ethanol precipitation or gel filtration chromatography on ProbeQuanat™ G-50 columns (Pharmacia Biotech, USA).

All filters were prehybridized for 4-12 h in hybridization buffer (HB), the labeled probes were added to fresh hybridization buffer, and blots were incubated overnight at 62° C. The filters were rinsed in 2xSSC/0.5 % SDS twice for 20 min at room temperature, followed by two washes with 1xSSC/0.1 % SDS for 1h at 60° C and one wash with 0.1xSSC containing 0.1 % SDS for 10 min.

Solution used:

HB: Roti®-Hybri-Quick (ROTH)
20xSSC: 3 M NaCl, 3 M sodium citrate (pH 7.0)

2.8 Microscopical techniques

2.8.1 Embedding in ERL

For electron microscopy plant materials were prefixed with 3 % glutaraldehyde in 0.1 M sodium-phosphate buffer (PB, pH 7.4) for 2 h at RT, following washing with 0.1 M PB (1 time - 15 min; 1 time - 30 min; 2 times - 1 h). After washing plant material was fixed in 1 % OsO_4 in buffer A for 60 min at RT. After fixation specimens were washed in tap water (4 times - 20 min each) and dehydrated in a graded series of acetone (20 % - 30 min; 50 % - 10 min; 75 % - 10 min; 90 % - 10 min; absolute acetone-two times for 40 min). Dehydrated plant tissues were infiltrated with aceton-ERL mixture (1:1) fo 20 min, then with aceton:ERL (1:2) for 20 min and immersed in ERL first for 1h and then overnight. Infiltration was performed on a rotator to increase penetration of the resin. Specimens were then transferred into embedding moulds. The polymerization was carried out in an oven for 1-3 days at 50-70° C.

Solution used:

PB: 580 mM Na_2HPO_4, 170 mM $NaH_2PO_4 \cdot H_2O$ (pH 7.4)

Buffer A (1 l): Na-veronal-29.4 g, Na-acetat·3 H_2O-19.4 g, 0.1 N HCl

2.8.2 Embedding in polyethylene glycol (PEG)

The embedding in PEG (Merck, Germany) was carried out according to the original procedure of van Lammeren et al. (1985) with some modifications. Plant material was fixed in 3 % paraformaldehyde, 0.25 % glutaraldehyde in PBS buffer for two hours. After fixation specimens were rinsed in PBS for 15 min (2 times) and processed through a graded series of ethanol (10 %, 30 %, 50 %, 70 %, 95 % - 10 min in each solution; absolute ethanol - 2 times for 30 min). All steps were carried out at 20° C. After incubation in absolute ethanol dehydrated plant tissues were immersed in a mixture of PEG:ethanol (1:3) for 1 h and temperature was raised to 55° C. The following steps were carried out at this temperature. Then specimens

were infiltrated in mixture PEG:ethanol (1:1). After 1 h incubation the mixture was replaced by PEG:ethanol (3:1) for 1 h and then samples were transferred in pure PEG. After 2 h of incubation the specimens were transferred to the mould with pure PEG and allowed to solidify at RT.

Solution used:

PBS: 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.2

PEG: PEG 1500:PEG 4000 (2:1) at 55° C

2.8.3 Specimens staining

2.8.3.1 Uranium and lead staining

For ultrastructural investigation, two-step staining of plant tissues was performed using uranyl acetate and lead salts (Reynolds, 1963). For uranium staining specimens were incubated in 5 % uranyl acetate in 30 % acetone for 30 min during the dehydration of the tissue. Treatment with lead citrate solution was carried out for 30-50 min on ultrathin section fixed on grids. Grids were then washed thoroughly by distilled water for 1-2 min and were allowed to dry on filter paper. Sections were viewed with a transmission electron microscope (EM912 Omega; Zeiss, Oberkochen, Germany).

2.8.3.2 Azure II-methylene blue staining

For azure II-methylene blue staining specimens were dried 2 h at 60° C, cooled to RT and stained with solution C for 3-4 min following washing in water.

Solution used:

Solution A: 1 % azure II in water

Solution B: 1 % methylene blue in 1 % $Na_2B_4O_7x10\ H_2O_2$

Solution C: Solution A+solution B (1:1)

2.8.4 Immunolabelling

For immunocytochemistry semi-thin sections (3-10 μm) were carefully tipped onto silanized (10 % triethoxysilylpropylamin, Merck, Germany) slides for adhesion. Slides were then rinsed twice in TBS for 5 min to remove the PEG prior to application of antiserum.

For immunolabelling the TSA™-Direct green kit (Du Pont, NEN, Boston, Massachusetts) or the ELF kit (Molecular Probes Europe BV, Leiden, the Netherlands) were used. For labelling by means of the TSA kit remaining aldehyde groups were blocked with buffer A (BA) for 15 min and after TBS washing 3 times-5min), blocking buffer B (BB) was used for 30 min followed by incubation with the the primary antiserum in BB (4° C) over night at 4° C. The slides were then rinsed with TBS (3 times - 5 min) and subsequently exposed to biotinylated goat anti-rabbit antibody (DAKO Diagnostica, Hamburg, Germany), diluted (1:500) in TBS for 30 min at RT. After washing with TBS (3 times - 5 min) sections were blocked with TNB buffer for 30 min and then incubated with streptavidin-horseradish peroxidase diluted 1:500 in TNB (30 min, RT). The signal was visualized by fluorescein tyramide reagent (Tyramid Signal Amplification Systems, TSA-Direct-Green, Du Pont, NEN, Boston, Massachusetts) according to the manufacturers instructions.

For the immunolabelling with the ELF system, slides were incubated with BA and after washing with PBS and incubating with blocking buffer C (BC) primary antibody diluted in BC was applied for 12 h at 4° C. After washing (3 times - 5 min) in PBS slides were incubated with a secondary, biotinylated goat anti-rabbit antibody (DAKO Diagnostica, Hamburg, Germany) diluted (1:300) in BB for 30 min at RT and washed briefly in ELF wash buffer. The slides were then incubated for 15 min with streptavidine alkaline phosphatase conjugate in ELF blocking reagent and after washing with ELF wash buffer (3 times - 5 min) the ELF substrate working solution was applied to each sample (Molecular Probes Europe BV, Leiden, the Netherlands) according to the manufacturers instructions. For fluorescence microscopy an Axioskop 2 (Fa. Zeiss, Jena, Germany) was used.

Solution used:

TBS: 100 mM Tris-HCl, 150 mM NaCl (pH 7.5)
BA: 50 mM Glycin in TBS
BB: 1 % BSA in TBS
TNB: 100 mM Tris-HCl, 150 mM NaCl (pH 7,5) 0,5 % Du Pont Blocking reagent
BC: 1 % BSA in PBS, 0,05 % Tween 20
PBS: 580 mM Na_2HPO_4, 170 mM $NaH_2PO_4 \cdot H_2O$, 680 mM NaCl (pH 7.4)

3. RESULTS

3.1 ABA and water content in developing tobacco seeds

To measure the ABA amount in tobacco seeds during the course of maturation monoclonal antibody ELISA assay with probes prepared at different time points of seed development was performed.

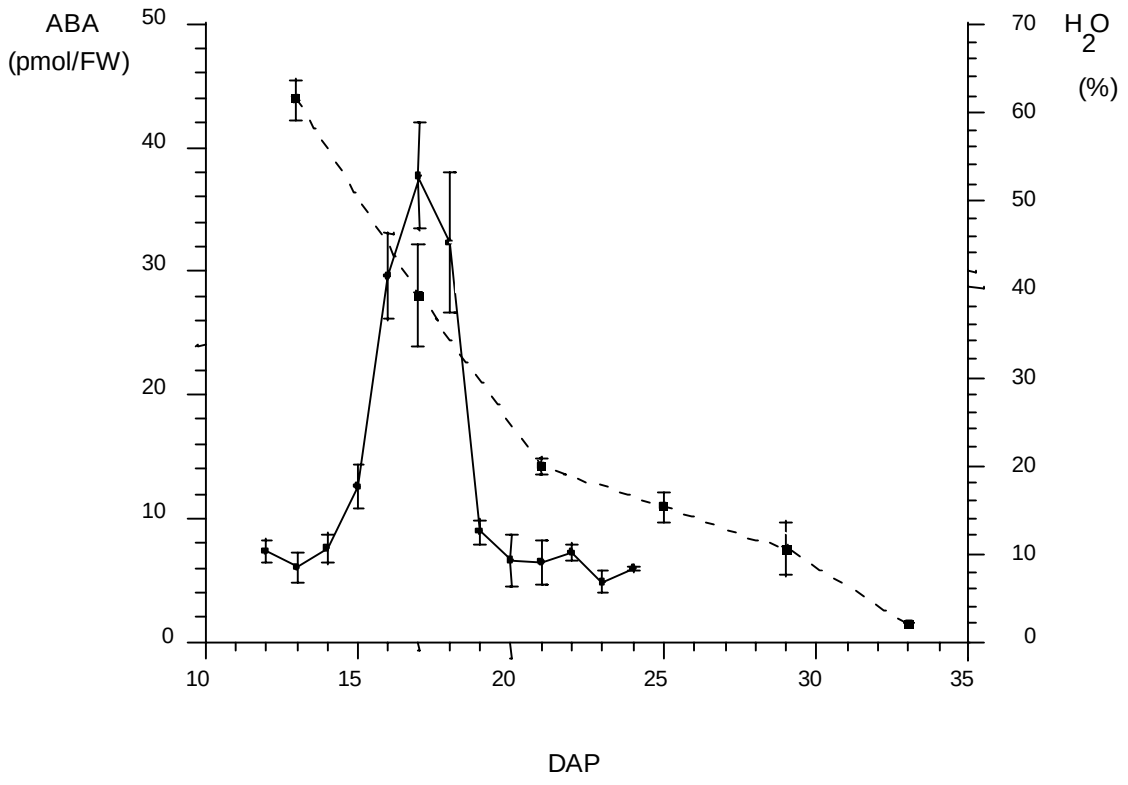

Figure 1. ABA (–●–) and water (- - ? - -) contents in developing tobacco seeds.

Figure 1 represents the pattern of ABA accumulation in tobacco seeds during embryogenesis. It resembled a typical pattern that is frequently found in seeds - with the highest content at about one-third to one-half of the time from seed initiation, and relatively low, or even absent, early in development and at maturity (Napier et al., 1989; Garsia-Maya et al., 1990; Xu et al., 1990; Groot et al., 1990). In the young *Nicotiana tobacco* seeds (11-15 DAP) the ABA level was about 12 pM/gFW (gram fresh weight). As development proceeded, the content of ABA

started to rise rapidly and reached a maximum value at mid-maturation stage (16-18 DAP). At this time of development tobacco seeds had about 4-fold higher ABA levels than seeds of the early stages. Starting from 17 DAP the ABA level rapidly declined and at 20 DAP reached a level which is similar to that observed at 11-15 DAP. During the following stages of seeds development the ABA level did not change significantly.

To characterise the metabolic status of tobacco seeds, which depends on the hydration level, the water content was measured during *Nicotiana* embryogenesis starting at 13 DAP (Fig. 1). At this stage of development the water amount in seeds was about 60 % and it rapidly decreased to about 20 % during the mid-maturation stage of tobacco seeds (13-21 DAP). Starting from 21 DAP the water content was reduced slowly and full dehydration (about 10-3 %) was observed by 30-32 DAP.

Seed dehydration affects the metabolic status of tissues. It was shown that in plants water stress is accompanied by the production of activated oxygen species and result in increased activity of antioxidant enzymes (Smirnoff and Colombe, 1988; Tanaka et al., 1990; Mittler and Zilinskas, 1992, 1994). To prove whether the water loss during embryogenesis represents a stress situation for seed tissues, the expression of SODs was investigated, since these proteins are induced by different stresses and play

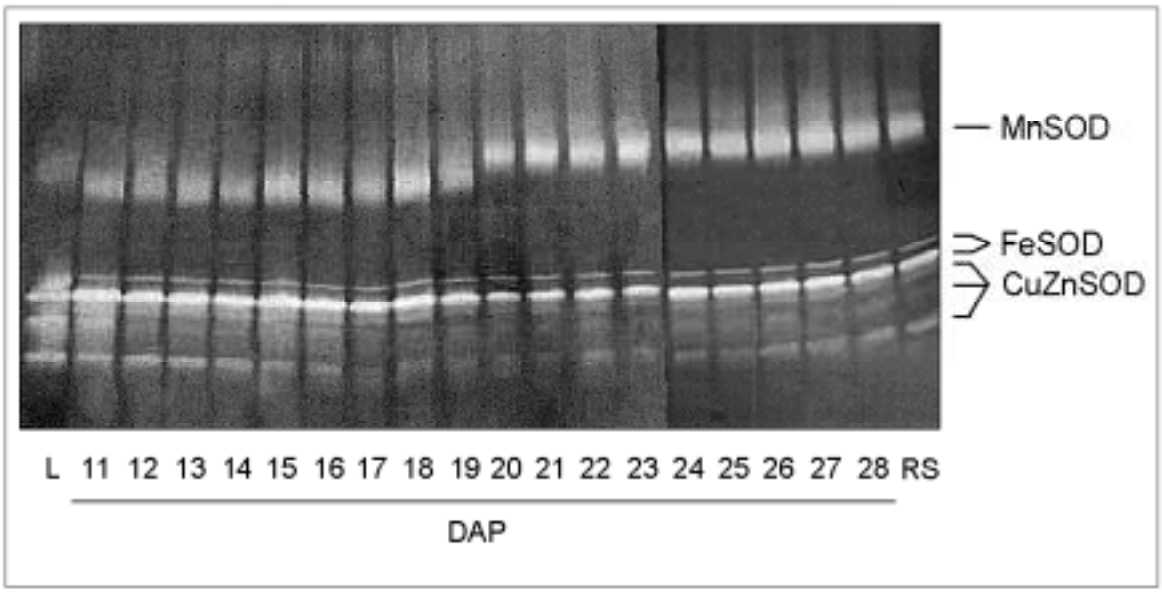

Figure 2. Pettern of SOD enzymes in *Nicotiana tabacum* seeds.
200 µg/lane of total protein were separated on native gels and stained for SOD activity. L - leaves; RS - ripe seeds; numbers - days after pollination (DAP).

an important role in the removal of reactive oxygen species formed during stress. The seed tobacco SOD pattern was defined by *in situ* staining technique on native protein gels according Beauchamp and Fridovich (1971). Electrophoresis of the seed samples indicated that six major SOD bands can be identified by their activity (Fig. 2). The same bands were observed in tobacco leaves (Fig. 2, L).

Different kinds of SODs (according to their metal cofactor) were reported to be expressed in plants - CuZnSOD (cytosolic and plastidal), MnSOD and FeSOD. They could be distinguished by differential sensitivity to KCN and H_2O_2. CuZnSODs are sensitive to both inhibitors. FeSOD is sensitive to H_2O_2 and resistant to KCN. MnSOD is resistant to both KCN and H_2O_2. The determination of tobacco seed SODs revealed the presence of all SOD types (Fig. 3, 4). Three lower bands observed in Figure 2 were identified as CuZnSODs being sensitive to KCN and H_2O_2. FeSODs were represented by two bands of different intensity. The upper band of SOD activitiy was determined as MnSOD.

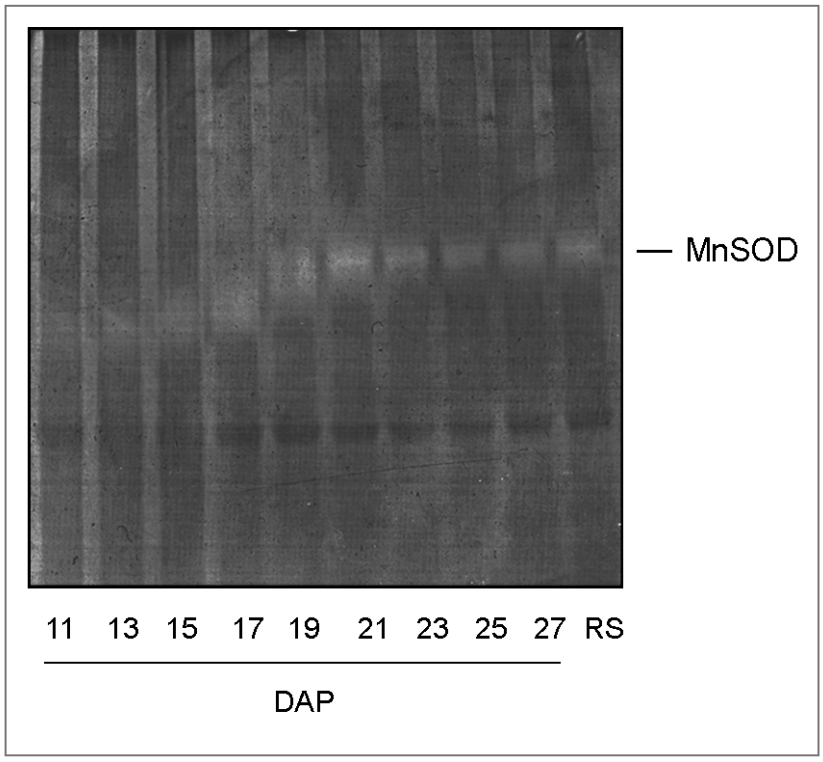

Figure 3. *Nicotiana tabacum* seed SODs after H_2O_2 inhibition.

200 μg/lane of total protein were separated on native gels and after incubation in H_2O_2 (5 mM) stained for SOD activity. RS - ripe seeds; numbers - days after pollination (DAP).

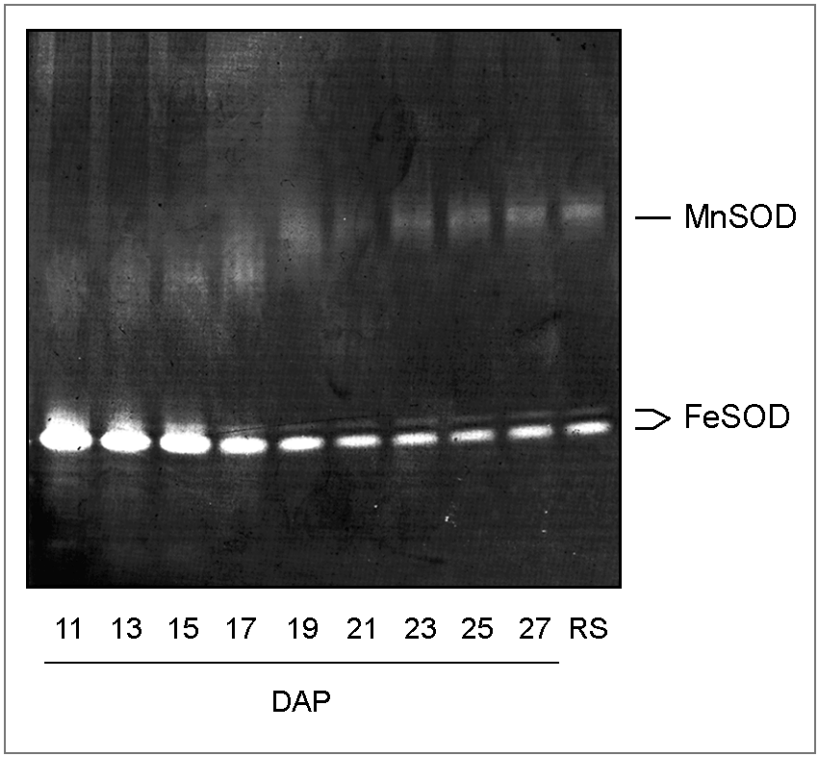

Figure 4. *Nicotiana tabacum* seed SODs after KCN inhibition.
200 µg/lane of total protein were separated on native gels and after incubation in KCN (2 mM) stained for SOD activity. RS - ripe seeds; numbers - days after pollination (DAP).

The analysis of the cytosolic CuZnSOD was choosed to estimate the physiological state of the tobacco seeds during embryogenesis since it was shown that both its mRNA and its activity significantly increase during drought stress (Perl-Treves and Galun, 1991; Mittler and Zilinskas, 1994).

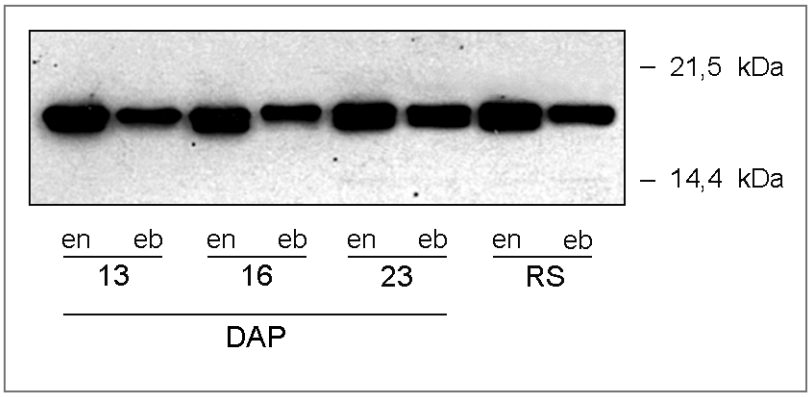

Figure 5. Developmental expression of cytosolic CuZnSOD in *Nicotiana tabacum* seeds.
Western blot analysis of proteins isolated from equal number of the seeds; 13, 16, 23 - days after pollination; RS - ripe seeds. eb - embryo; en - endosperm.

To detrmine tobacco seed cytosolic CuZnSOD activity on native gel is difficult since on the one hand high protein amount (200 μg) is required, and on the other hand, an equal seed number should be used. The measuring of cytosolic CuZnSOD activity by absorbency determination *in vitro* is not possible due to the presence of plastidal CuZnSOD in protein samples. To overcome this Western blot analysis of cytosolic CuZnSOD was used instead of their activity determination. This method requires less protein content. Figure 5 shows that there is no significant difference in expression of cytosolic CuZnSOD in tobacco seeds both in embryos and the endosperm during the course of seed maturation.

3.2 sHSP's in tobacco seeds

3.2.1 sHSP mRNA expression during embryogenesis

To investigate the accumulation pattern of cytosolic sHSP mRNAs during tobacco zygotic embryogenesis, total RNA was isolated from seeds at different developmental stages (11, 17, 22, 27, DAP and ripe seeds) and analysed by Northern blots probed with class I tomato HSP17 cDNA (Fig. 6).

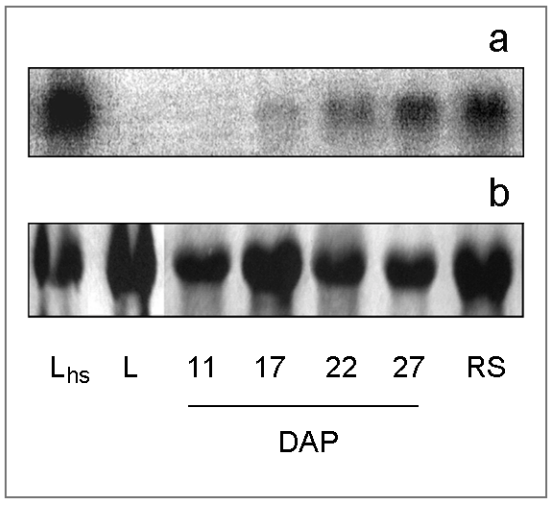

Figure 6. Accumulation of HSP17 mRNAs during zygotic embryogenesis.
a - Northern blot analysis of total RNA (60 μg/lane) during zygotic embryogenesis. Tomato cDNA of class I HSP17 was used as hybridisation probe.
b - ethidium bromide stained 25S rRNA. 11, 17, 22, 27 - days after pollination; RS-ripe seeds; L-leaves; L_{hs}-heat stressed leaves.

sHSP transcripts were initially detected in tobacco seeds at 17 DAP. However, at this stage of seed development the level of sHSP mRNAs was very low. During seed maturation the amount of accumulated sHSP mRNA increased and reached its highest level in ripe seeds, however it was substantially less than in heat-stressed leaves.

3.2.2 sHSP and storage globulin expression during seed development

The presence of cytosolic class I and II sHSP's in *Nicotiana tabacum* seeds during the course of maturation was analysed by Western blotting with antiserum against tomato HSP17 (Fig. 7, a).

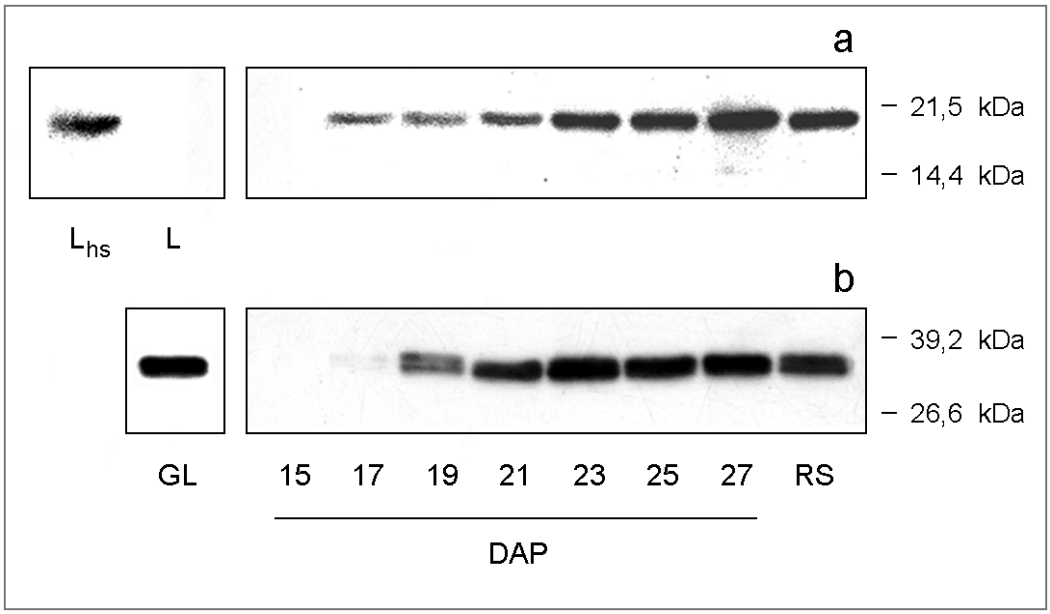

Figure 7. Developmental expression of cytosolic sHSP's and globulins in *Nicotiana tabacum* seeds.

a - Western blot analysis of proteins (10 µg/lane) from tobacco seeds collected at 15, 17, 19, 21, 23, 25, 27 days after pollination, ripe seeds (RS), non-stressed (L) and heat stressed leaves (L_{hs}) using antibodies against tomato HSP17.

b - Western blot analysis of protein (10 µg/lane) samples prepared from seeds collected at 15, 17, 19, 21, 23, 25, 27 DAP and ripe seeds (RS) using antibodies against *Vicia* globulins. Tobacco globulins (GL) (2,5 µg/lane) were used as control.

sHSP's in tobacco seeds were expressed concurrently with their mRNAs and were first detected at 17 DAP. During seed ripening the level of sHSP's expression increased. The accumulation of sHSP's in mature *Nicotiana* seeds were observed in both the embryo and the endosperm (Fig. 8, a).

Since in tobacco seeds the expression of sHSP's was observed about the time of the maximal ABA level, and in seeds of many species the deposition of storage proteins coincides with the presence of high levels of ABA (Kigel and Galili, 1995), the accumulation of storage globulins in tobacco seeds was investigated to determine whether a correlation exists between the synthesis of both proteins. The western blot analysis of tobacco seed proteins separated by gel electrophoresis

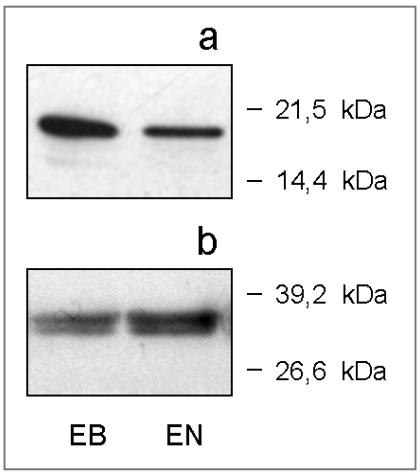

Figure 8. Cytosolic sHSP's and globulins in embryos and the endosperm of mature tobacco seeds.
Protein samples were prepared from embryo and endosperm of ripe tobacco seeds. Proteins isolated from an equal number of embryos and the endosperm were separated by SDS-PAGE, blotted and probed with antibodies against tomato HSP17 (**a**) and *Vicia* globulin (**b**). EB - proteins isolated from embryos; EN - proteins isolated from the endosperm.

and probed with antibodies against globulins demonstrated that expression of globulins in tobacco seeds started about 17 DAP (Fig. 7, b). Tobacco globulins were present in both the embryo and the endosperm (Fig. 8, b). To prove the correlation between the expression of small heat shock proteins and storage proteins, seeds of transgenic tobacco plants expressing a single-chain Fv (scFv) antibody against abscisic acid were analysed. It was shown that in transgenic plants the ABA activity is blocked due to the binding of the scFv to ABA and tageting to the ER. This causes the dramatic reduction of the 12S globulin in embryos and to a lesser degree in the endosperm (Phillips et al., 1997).

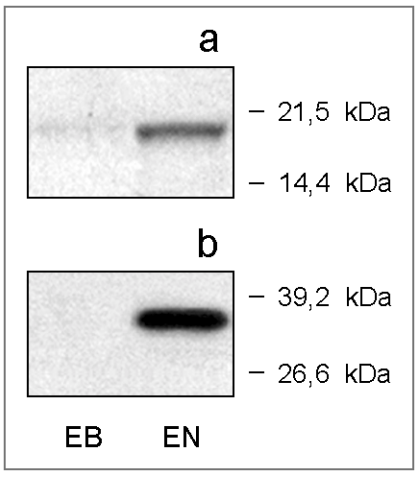

Figure 9. Cytosolic sHSP's and globulins in embryos and the endosperm of mature anti-ABA-scFV tobacco seeds.
Protein samples were prepared from ripe tobacco seeds divided into the endosperm and embryos. Proteins isolated from an equal number of the endosperm and embryos were separated by SDS-PAGE, blotted and probed with antibodies against tomato HSP17 (**a**) and *Vicia* globulin (**b**). EB - proteins isolated from embryos; EN - proteins isolated from the endosperm.

The Western blot analysis of anti-ABA-scFv expressing plants demonstrated that neither sHSP's, nor globulins are synthesised in embryos of transgenic seeds, however in the endosperm both proteins are expressed (Fig. 9). So, there would appear to be a correlation between storage protein synthesis and sHSP expression.

3.2.3 2-D pattern of tobacco seed sHSP's

Cytosolic sHSP's are members of multigene families (DeRocher et al., 1991; Vierling, 1991). It was shown that under stress treatment, and during seed development, plants expressed several isoforms (Cocca et al., 1994; DeRocher and Vierling, 1994; Wollgiehn and Neumann, 1995; zur Nieden et al., 1995). To characterise the isoform pattern of developmentally induced sHSP's in tobacco and to compare to that induced under heat stress treatment, two-dimensional (2D) Western blot analysis of proteins isolated from *Nicotiana* seeds and heat stressed leaves were performed (Fig. 10).

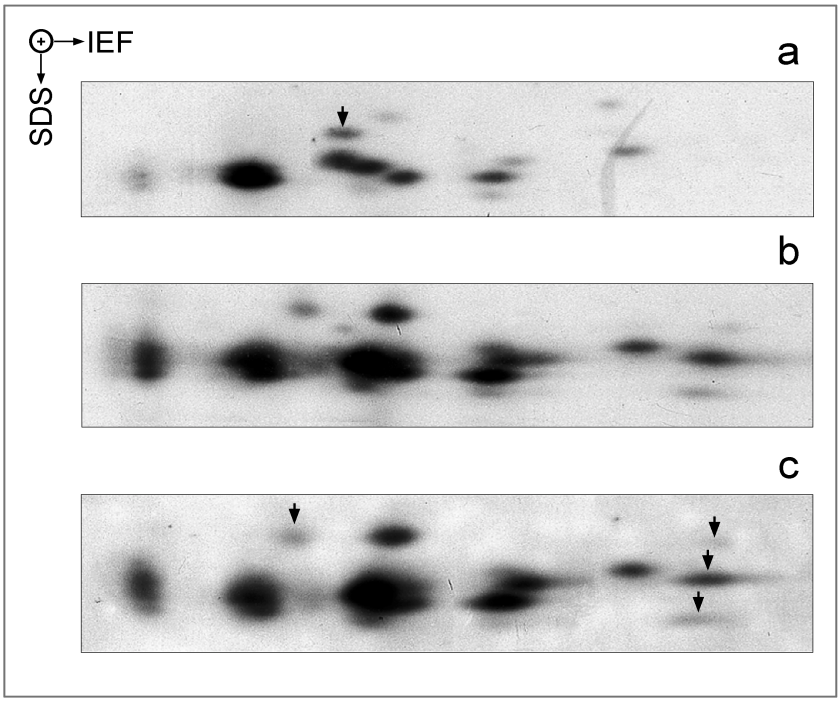

Figure 10. Two-dimensional analysis of cytosolic sHSP expression in mature tobacco seeds and heat stressed leaves.
a - ripe seed proteins; **b -** mature seed proteins mixed with heat stressed leaf proteins; **c -** heat stressed leaf proteins. In all cases 150 µg protein was separated by isoelectric focusing followed by SDS-PAGE and Western blot analysis using tomato HSP17 antiserum. Arrows indicate seed (**a**) and heat stressed leaf (**c**) specific isoforms.

Total protein from seeds and heat stressed leaves were also mixed and separated by two-dimensional electrophoresis to determine the differences in sHSP expression. In both samples the isoform pattern was complex due to the formation of several polypeptides of different molecular weight and different isoelectric points. In

leaves under heat stress treatment about sixteen isoforms were expressed (Fig. 10, c). Thirteen developmentally induced polypeptides were detected in seeds (Fig. 10, a). Nine of these seem to be the same as after heat stress. Some of these polypeptides were more abundant in heat stressed leaves. One seed sHSP isoform prominently reacting with antibodies (Fig. 10, a, arrow) seems to be seed specific since it was not expressed under heat stress treatment. Four polypeptides were

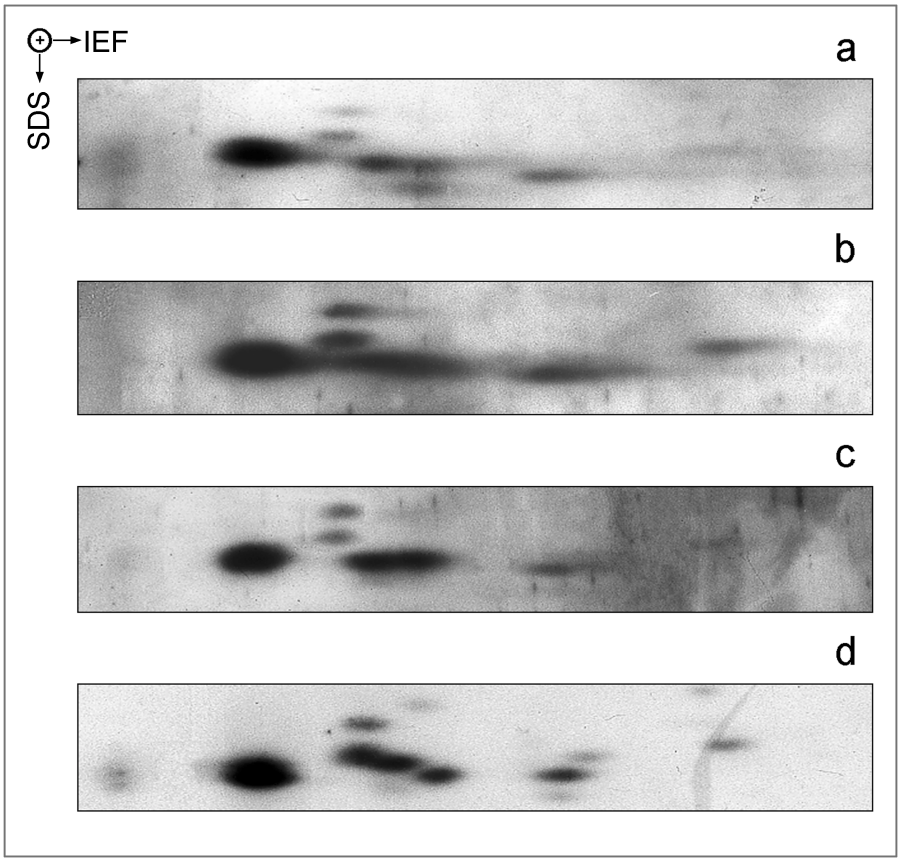

Figure 11. Two-dimensional analysis of cytosolic sHSP expression in tobacco during zygotic embryogenesis.
a, b, c - protein samples prepared from seeds at 18, 22, 27 DAP, respectively; **d -** mature seeds. Proteins of each sample (150 µg) were separated by isoelectric focusing followed by SDS-PAGE and Western blot analysis using tomato HSP17 antiserum.

found to be expressed only in heat stressed leaves (Fig. 10, c, arrows). To determine the isoform pattern of developmentally induced cytosolic sHSP's during the course of seed maturation, protein samples from several stages of development (18, 22, 27 DAP, ripe seeds) were analysed by two-dimensional Western blotting

(Fig. 11). The sHSP isoform pattern during zygotic embryogenesis was not different from that of mature seeds.

3.2.4 sHSP pattern of embryos and the endosperm

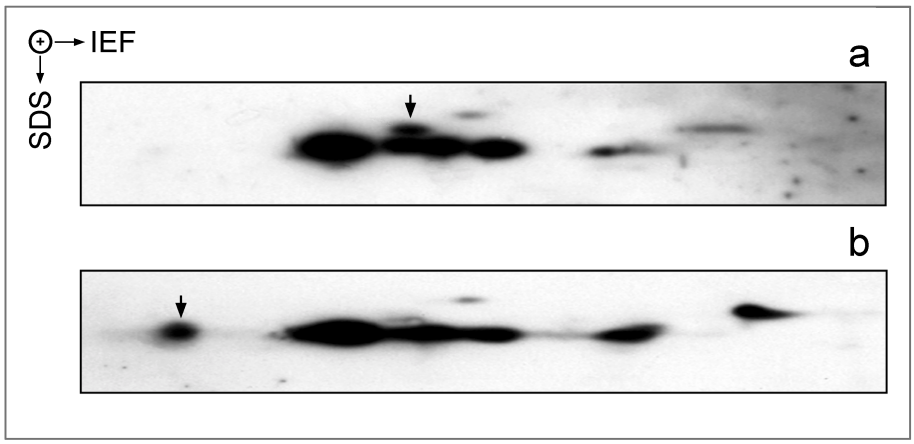

Figure 12. Two-dimensional analysis of cytosolic sHSP's accumulated in embryos and the endosperm of mature tobacco seeds.
a - sHSP's of embryos; **b -** sHSP's of the endosperm. Proteins isolated from an equal number of embryos and the endosperm were separated by isoelectric focusing followed by SDS-PAGE and Western blot analysis. Arrows indicate embryo (**a**) and endosperm (**b**) specific isoforms.

The Western blot immunodetection following 2D SDS-PAGE was performed for total protein isolated from embryos and the endosperm of mature seeds, in order to compare the pattern of sHSP's. Figure 12 shows that embryos and the endosperm had a similar isoform pattern with the exception of two polypeptides. However, differences in the relative amount of embryo and endosperm sHSP's were observed

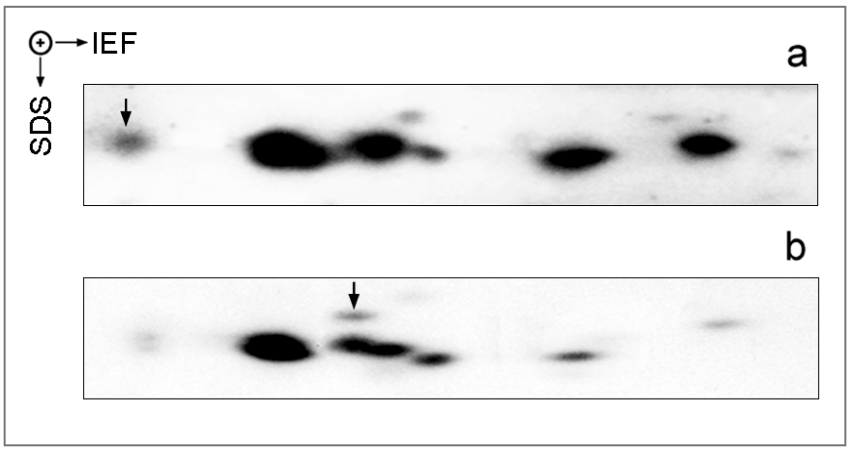

Figure 13. Two-dimensional analysis of cytosolic sHSP's in anti-ABA-scFv transgenic (a) and wild type (b) mature seeds. 150 µg proteins of each sample were separated by isoelectric focusing followed by SDS-PAGE and Western blot analysis. Arrows indicate anti-ABA-scF **(a)** and wild type seeds **(b)** specific isoforms.

for some polypeptides. One specific sHSP has been found to be expressed in embryos (Fig. 12, a, arrow), the second - in the endosperm (Fig. 12, b, arrow). The molecular weight of the embryo specific isoform is higher than that of other isoforms. This polypeptide has been defined as seed specific and was not induced under heat-stress treatment. To prove the specificity of embryo and endosperm isoforms, seeds of transgenic tobacco plants expressing single-chain antibody against abscisic acid were analysed. The seeds of these plant did not accumulate sHSP's in embryo (Fig. 9). The comparison of sHSP isoforms that accumulated in seeds of transgenic and wild type plants, shows that the embryo specific isoform was not expressed in the seeds of transgenic plants (Fig. 13, a). However, the isoform determined as endosperm specific was present. Thus, two developmentally induced sHSP isoforms in seeds seem to be organ specific.

3.3 sHSP localisation in tobacco seeds and heat stressed leaves

The information concerning the localisation of developmentally induced sHSP's in *Nicotiana* seeds may help to understand the function of small heat shock proteins in these organs. In this regard it is also of interest to compare the tissue and cell localisation of developmentally and heat stressed induced sHSP's. In order to address these questions immunohistolabelling of tobacco heat stressed leaves and *Nicotiana*

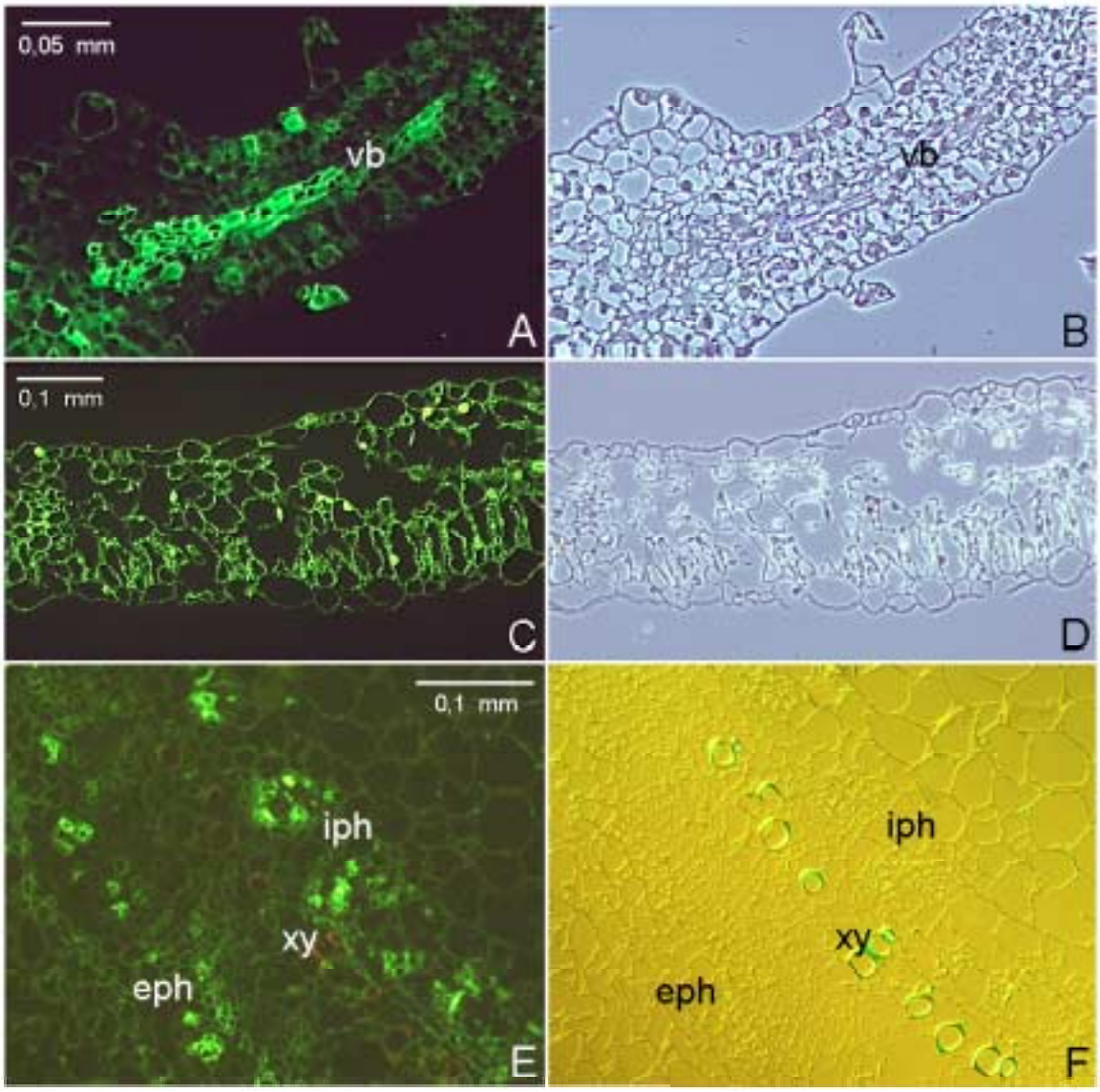

Figure 14. Localisation of sHSP's in heat stressed tobacco leaves (I).
A, C - immunohistochemical staining of sHSP's (green colour) in transverse section of heat stressed young and mature leaves, respectively; **E** - immunohistochemical staining of sHSP's in transverse section including midvein of heat stressed young leaf; **B, D, F** - phase-contrast photographs of a section shown in **A, C, E,** respectively; **eph** - external phloem; **iph** - internal phloem; **vb** - vascular bundle; **xy** - xylem.

seeds were performed using antiserum against tomato HSP17. Figure 14 shows the localisation of sHSP's in sections of young and mature tobacco leaves under heat stress treatment. In both samples sHSP's accumulated in all tissues. In young heat stress leaves the strongest immunolabelling signals were observed in vascular bundles (Fig. 14, A, E). This is in agreement with GUS staining of heat stressed leaves of transgenic tobacco, containing a β-glucuronidase reporter gene driven by a soybean

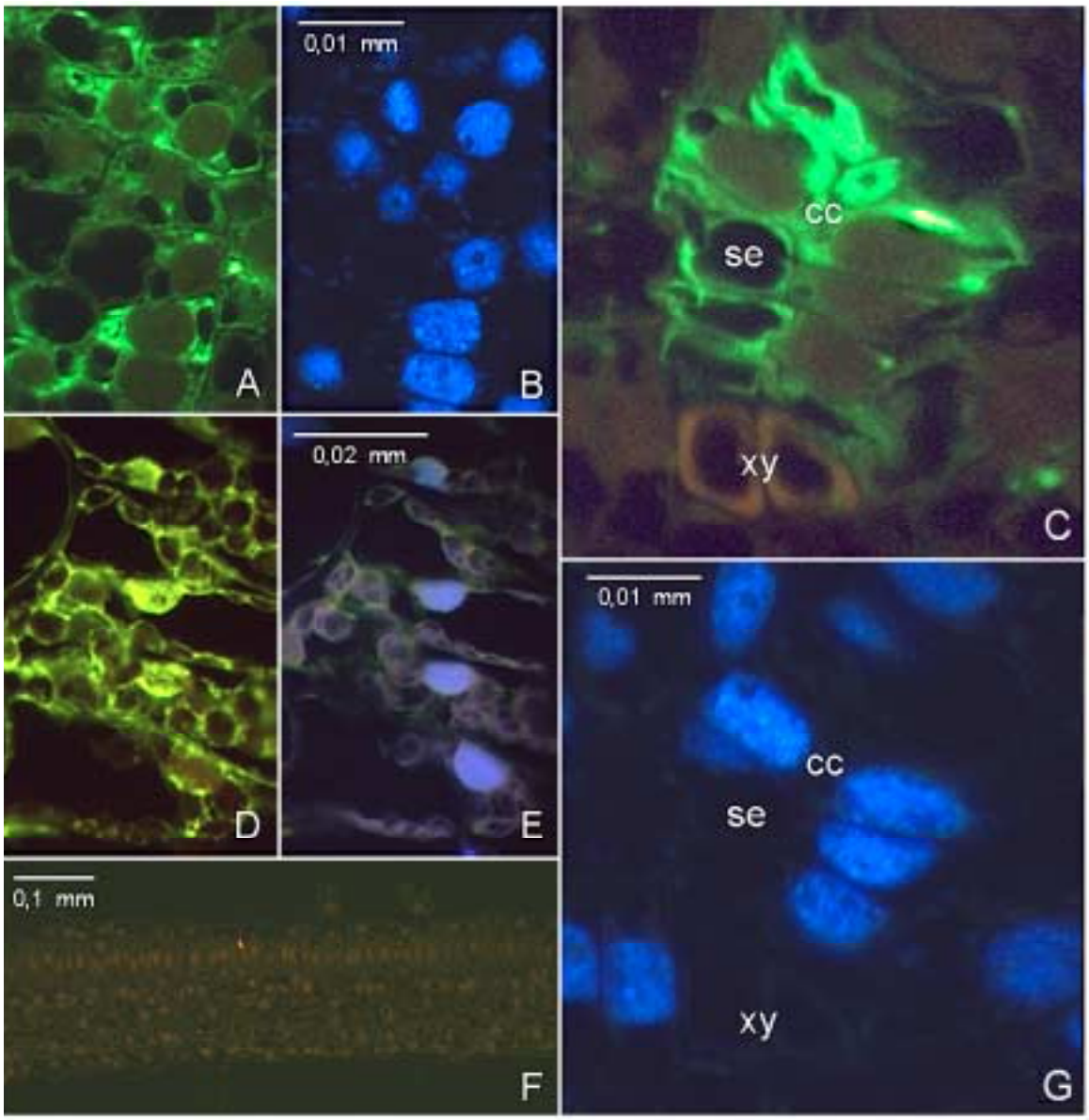

Figure 15. Localisation of sHSP's in heat stressed tobacco leaves (II).
A, D - cellular localisation of sHSP's in heat stressed young and mature leaves, respectively (note the labelling of nuclei in **D**); **C** - immunohistochemical staining of sHSP's in transverse section of vascular bundle of a young heat stressed leaf; **B, E, G** - DAPI staining of section shown in **A, D, C**; **F** - control with non heat stressed leaf. **cc** - companion cells; **se** - sieve element; **xy** - xylem.

heat shock promoter (Prändel et al., 1995). In vascular bundles sHSP's were detected in the phloem, where companion cells and sieve elements were labelled (Fig. 14, E and Fig. 15, C). A difference in the cellular localisation of sHSP's in heat stressed young and mature leaves was observed. In both leaf samples sHSP's were detected to be

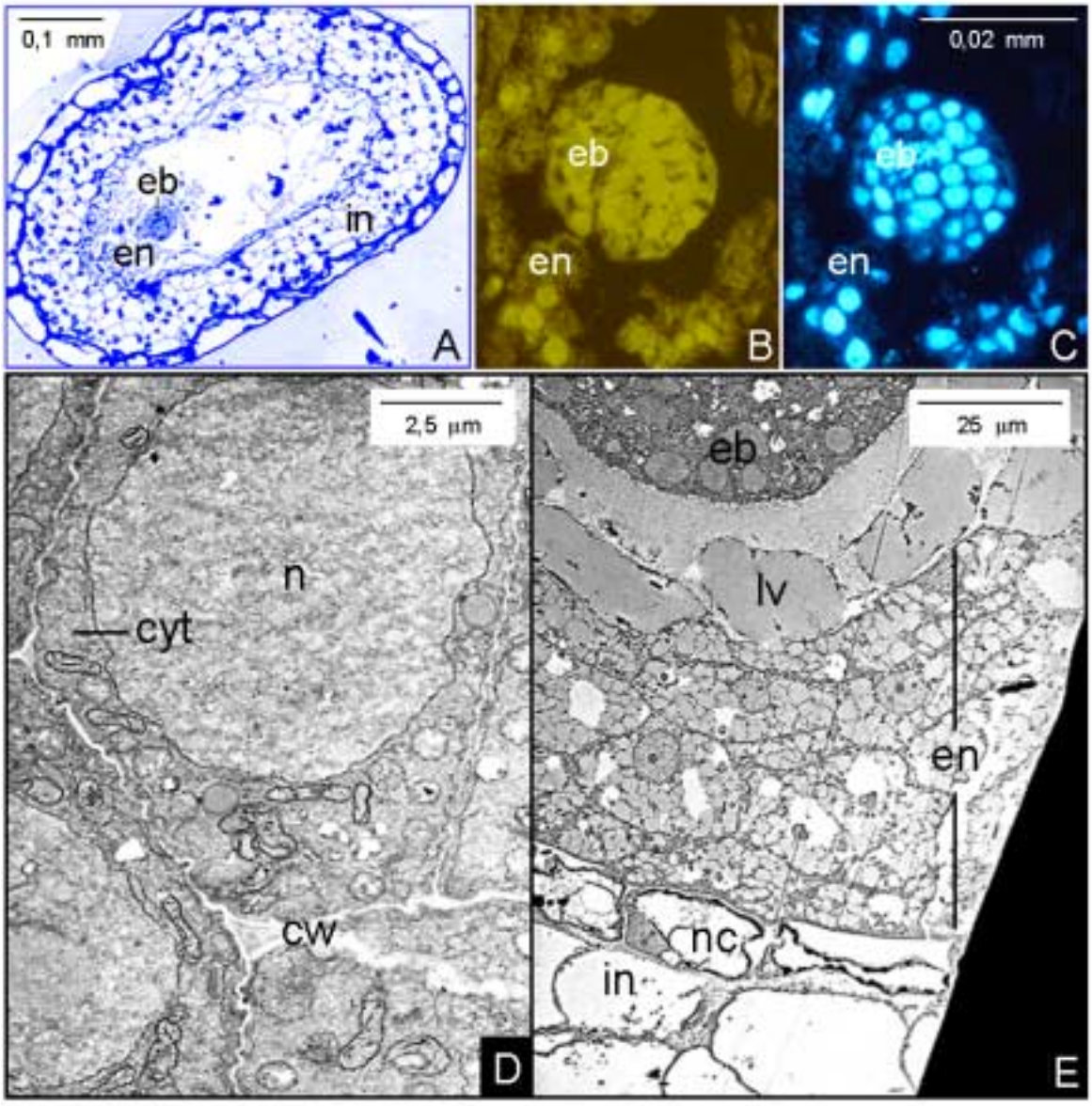

Figure 16. sHSP immunohistochemical staining and structure of tobacco seeds (globular stage).

A - azure/methylene blue staining of longitudinal seed section; **B** - immunohistochemical staining of sHSP's in seeds with a globular stage embryo; **C** - DAPI staining of section shown in **B**; **D, E** - electron micrographs of embryo and endosperm cells, respectively. **cw** - cell wall; **cyt** - cytoplasm; **eb** - embryo; **en** - endosperm; **in** - integument; **lv** - lipid vacuole; **n** - nucleus; **nc** - nucellus.

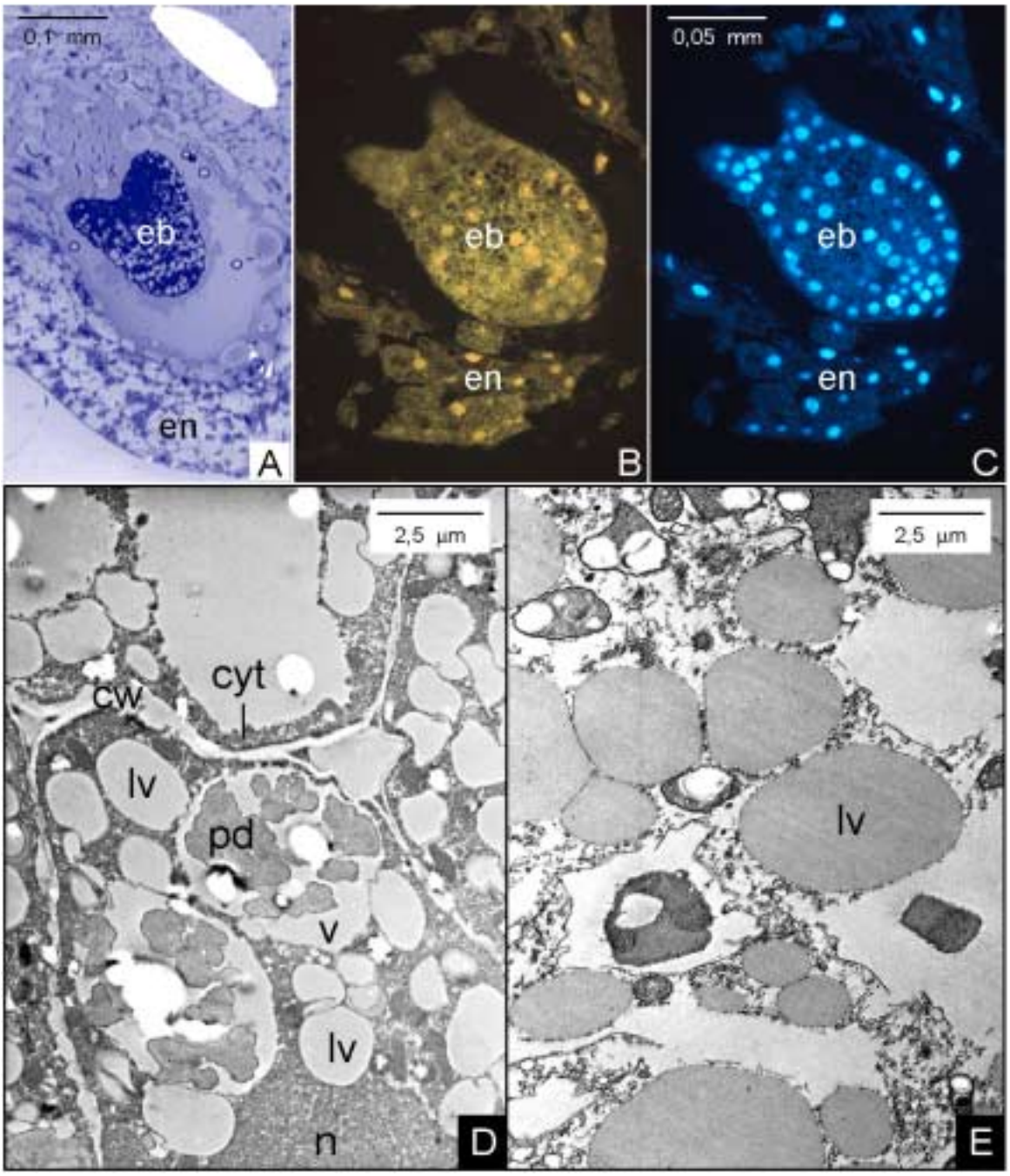

Figure 17. sHSP immunohistochemical staining and structure of tobacco seeds (heart stage).

A - azure/methylene blue staining of longitudinal seed section; **B** - immunohistochemical staining of sHSP's in seeds; **C** - DAPI staining of section shown in **B**; **D, E** - electron micrographs of embryo and endosperm cells, respectively. **cw** - cell wall; **cyt** - cytosol; **eb** - embryo; **en** - endosperm; **lv** - lipid vacuole; **n** - nucleus; **pd** - protein deposits; **v** - vacuole.

localised in the cytosol (Fig. 15, A, D). However, in mature leaves sHSP's beside the cytosolic localisation were found also

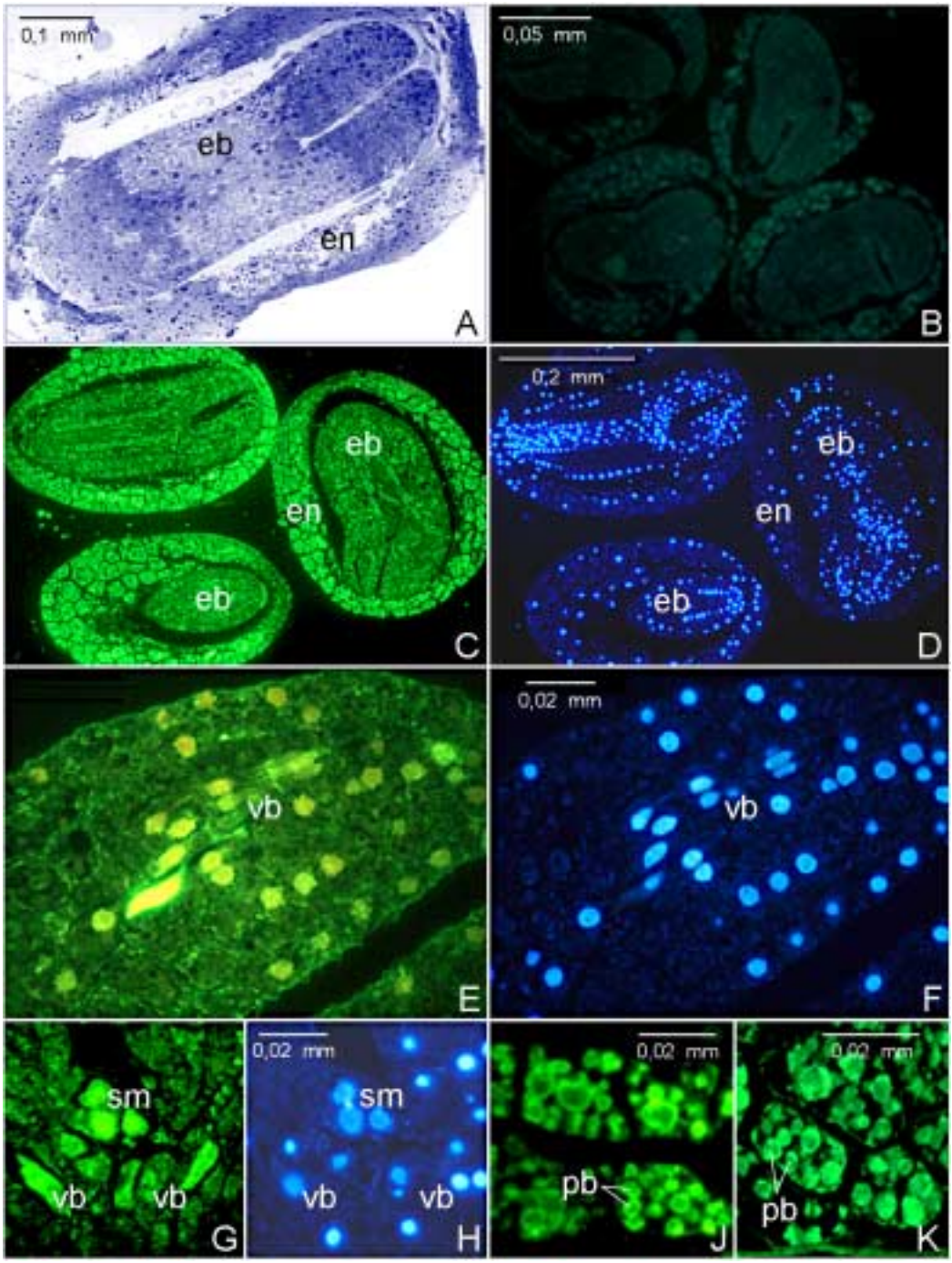

Figure 18. Localisation of sHSP's in tobacco seeds (cotyledon stage).

A - azure/methylene blue staining of longitudinal seed section; **B** - preimmune serum control for unspecific binding; **C** - immunohistochemical staining of sHSP's (green colour) in seeds with a cotyledon stage embryo; **E, G** - immunohistochemical staining of sHSP's in cotyledon (yellow colour in case of high sHSP amount) and shoot meristem, respectively; **J** - immunohistochemical staining of sHSP's in embryo parenchyma cells; **K** - sHSP's in endosperm cells; **D, F, H** - DAPI staining of section shown in **C, E, G**. **eb** - embryo; **en** - endosperm; **pb** - protein bodies; **sm** - shoot meristem; **vb** - vascular bundle.

in the nuclei (Fig. 15, D). Thus, the cellular localisation of sHSP's seems to depend on the physiological state of stressed tissues

The *in situ* immunodetection of sHSP's in seeds containing globular (Fig. 16), heart (Fig. 17) and cotyledon (Fig. 18) stage embryos shows that sHSP's accumulated at high levels only within seeds with cotyledon stage embryos (Fig. 18, C). At this stage sHSP's were observed in both the endosperm and embryos. In the endosperm the distribution was uniform throughout (Fig. 18, C). At higher magnification the granular immunolabelled regions, representing protein bodies, were visualised in endosperm cells (Fig. 18, K). By contrast, in embryos the distribution of sHSP's was differential. The cells of provascular bundles and shoot meristeme were labelled more intensive then the cells of storage parenchyma (Fig. 18, E, G). In embryos the immunological signal within parenchyma cells was distributed in the cytosol and within the nuclei. A similar distribution was observed in provascular bundle and shoot meristem cells, however in these cells the immunological signal was more intense. Moreover, in some bundle cells the labeling was extremely

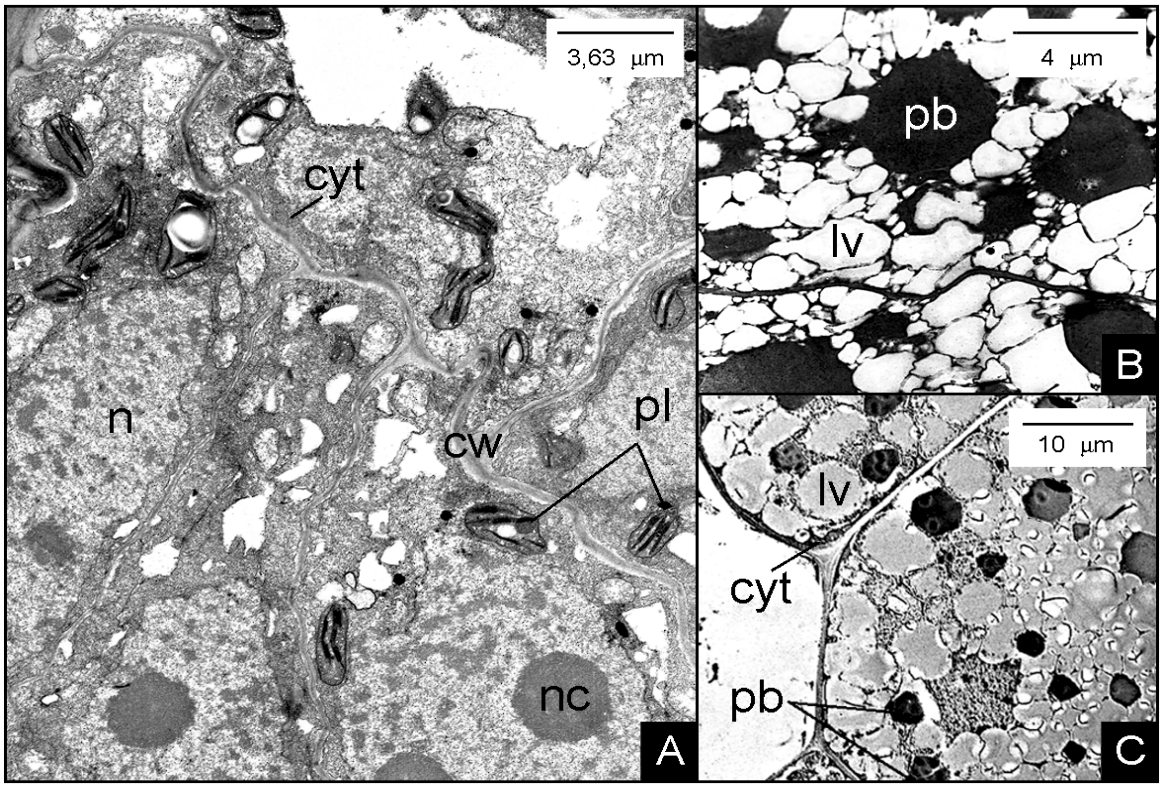

Figure 19. Ultrastructure of tobacco embryo and endosperm cells (cotyledon stage).

A-shoot meristem cell; **B**- embryo parenchyma cell; **C**-endosperm cell. **cw** - cell wall; **cyt** - cytoplasm; **lv** - lipid vacuoles; **n** - nucleus; **nc** - nucleolus; **pb** - protein bodies; **pl** - plastids.

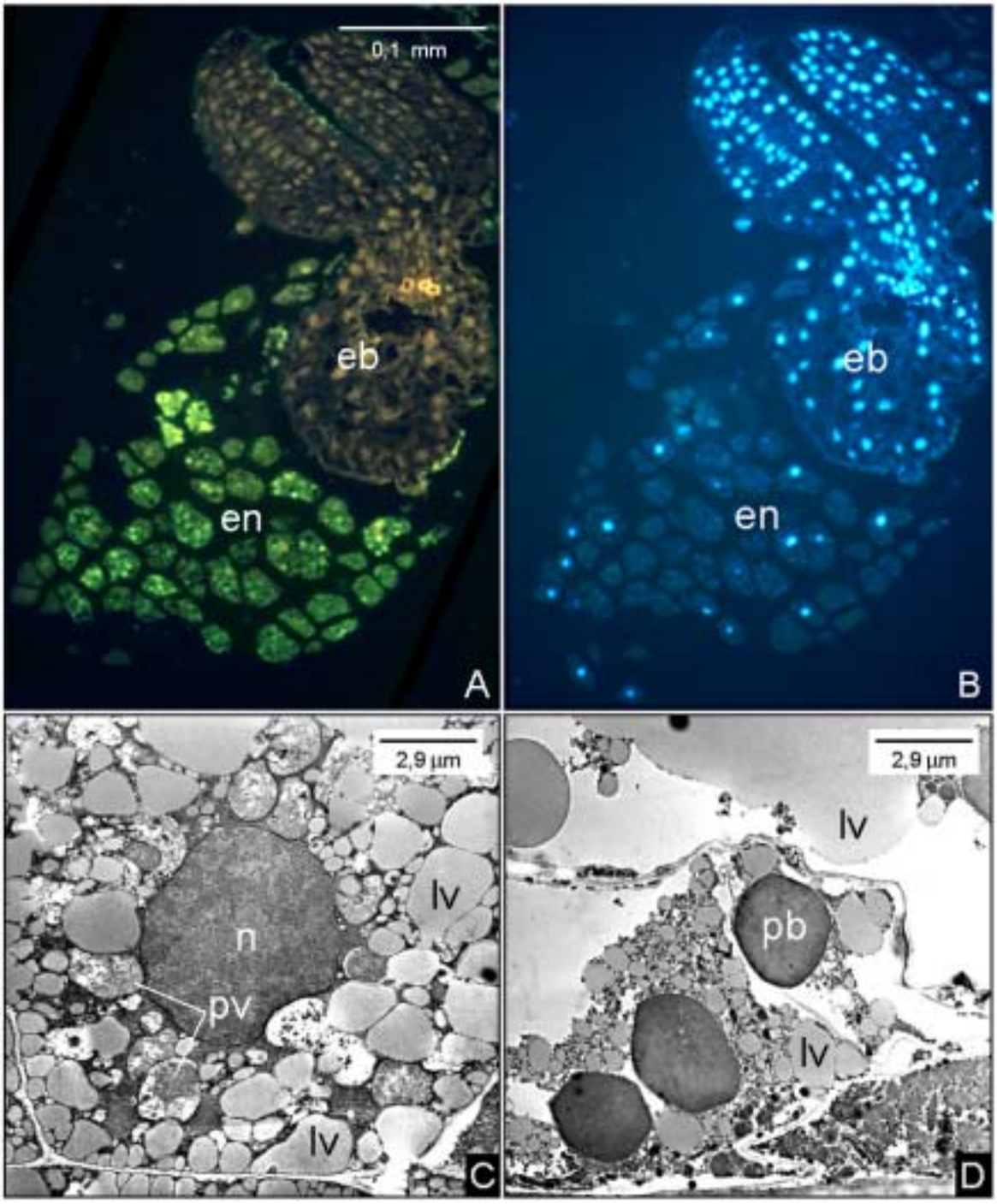

Figure 20. sHSP localisation and ultrastructure of anti-ABA-scFv tobacco seeds.
A - immunohistochemical staining of sHSP's in mature seeds; **B** - DAPI staining of section shown in **A**; **C, D** - electron micrographs of embryo parenchyma and endosperm cells, respectively. **eb** - embryo; **en** - endosperm; **lv** - lipid vacuoles; **n** - nucleus; **pb** - protein bodies; **pv** - protenaceous vacuoles.

intense and distributed uniformly within the cell (Fig. 18, E). In storage parenchyma cells of embryos sHSP's were also detected in protein bodies (Fig. 18, J). The ultrastructure of cotyledon stage seeds showed that embryo and endosperm

cells were filled with typical protein bodies and lipid vacuoles (Fig. 19, B, C). The cytosol in these cells occupied only the small part. The meristematic parts of the shoot (Fig. 19, A) and root apex and the provascular tissues (not shown) showed the typical structure of embryonal cells with large nuclei and a dense cytoplasm without protein bodies and lipid vacuoles. In embryos and the endosperm of globular and heart stage seeds, protein bodies were never detected (Fig. 16, 17). A globular embryo consisted of meristematic cells with a large nuclei and a dense cytoplasm (Fig. 16, D). In heart stage embryo cells lipid vacuoles and vacuolar protein deposits were observed (Fig. 17, D). Lipid vacuoles were found in the endosperm cells of globular and heart stage seeds (Fig. 16, E and Fig. 17, E). In anti-ABA-scFv transgenic seeds sHSP’s were detected only in the endosperm (Fig. 20, A). Electron micrographs of scFv embryos and endosperm cells shows that protein bodies are formed in the endosperm and are absent in embryo cells (Fig. 20, C, D).

3.4 sHSP's in germinating tobacco seeds

3.4.1 sHSP mRNAs during seed germination

To investigate the time course for disappearance of sHSP mRNA during germination, Northern blot analysis was performed for samples prepared at 2, 4, 6 days after imbibition (DAI) (Fig. 21). A small amount of sHSP mRNAs was detectable in germinating seeds for no longer than the second DAI. It can be assumed that these mRNAs remain from the ripe seeds.

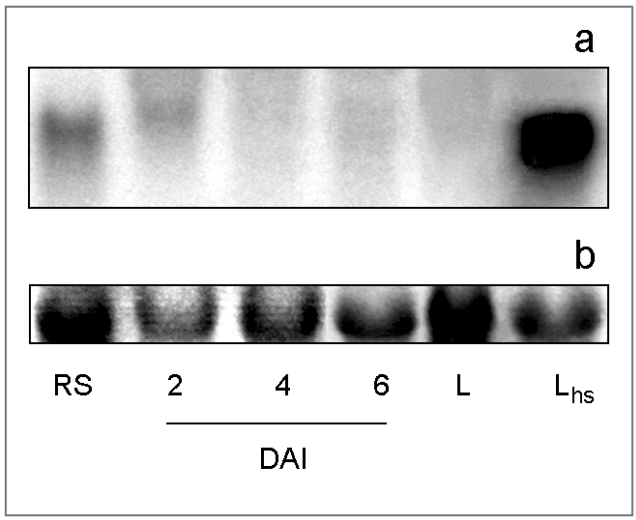

Figure 21. sHSP mRNAs during tobacco germination.
a - Northern blot analysis of total RNA (60 µg/lane) probed with tomato cDNA of class I HSP17.
b - ethidium bromide stained 25S rRNA.
2, 4, 6 - days after imbibition (DAI); RS-ripe seeds; L-leaves; L_{hs}-heat stressed leaves.

3.4.2 Seed sHSP's during tobacco germination

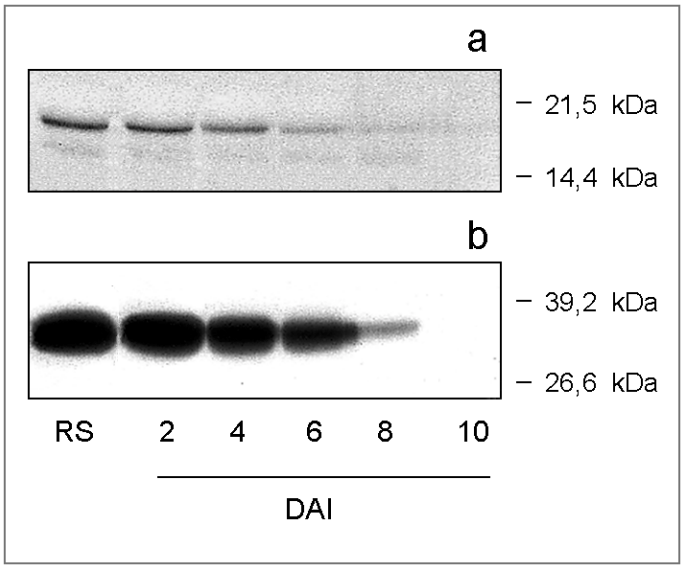

Figure 22. Cytosolic sHSP's and globulins during tobacco seed germination.
a - Western blot analysis of proteins (10 µg/lane) from tobacco seeds collected at 2, 4, 6, 8, 10 days after imbibition and ripe seeds (RS) using antibodies against tomato HSP17.
b - Western blot analysis of proteins (10 µg/lane) from seeds collected at 2, 4, 6, 8, 10 days after imbibition and ripe seeds (RS) using antibodies against *Vicia* globulin.

To examine sHSP's in germinating seeds and to investigate a connection between sHSP's and storage proteins levels, Western blot analysis of tobacco proteins isolated from seedlings of 1 to 10 DAI were performed. Figure 22 (a) shows that sHSP's persisted in the germinating seeds and seedlings for several days. sHSP's were no longe observed after approximately 10 DAI. Alike during seed development, the presence of sHSP's in germinating seeds coincided with that of tobacco globulins (Fig. 22). Seedlings collected at a definite day after imbibition represent a group of plants at diverse stages of development. Therefore their morphology was used to determine more exactly the stage of plant development

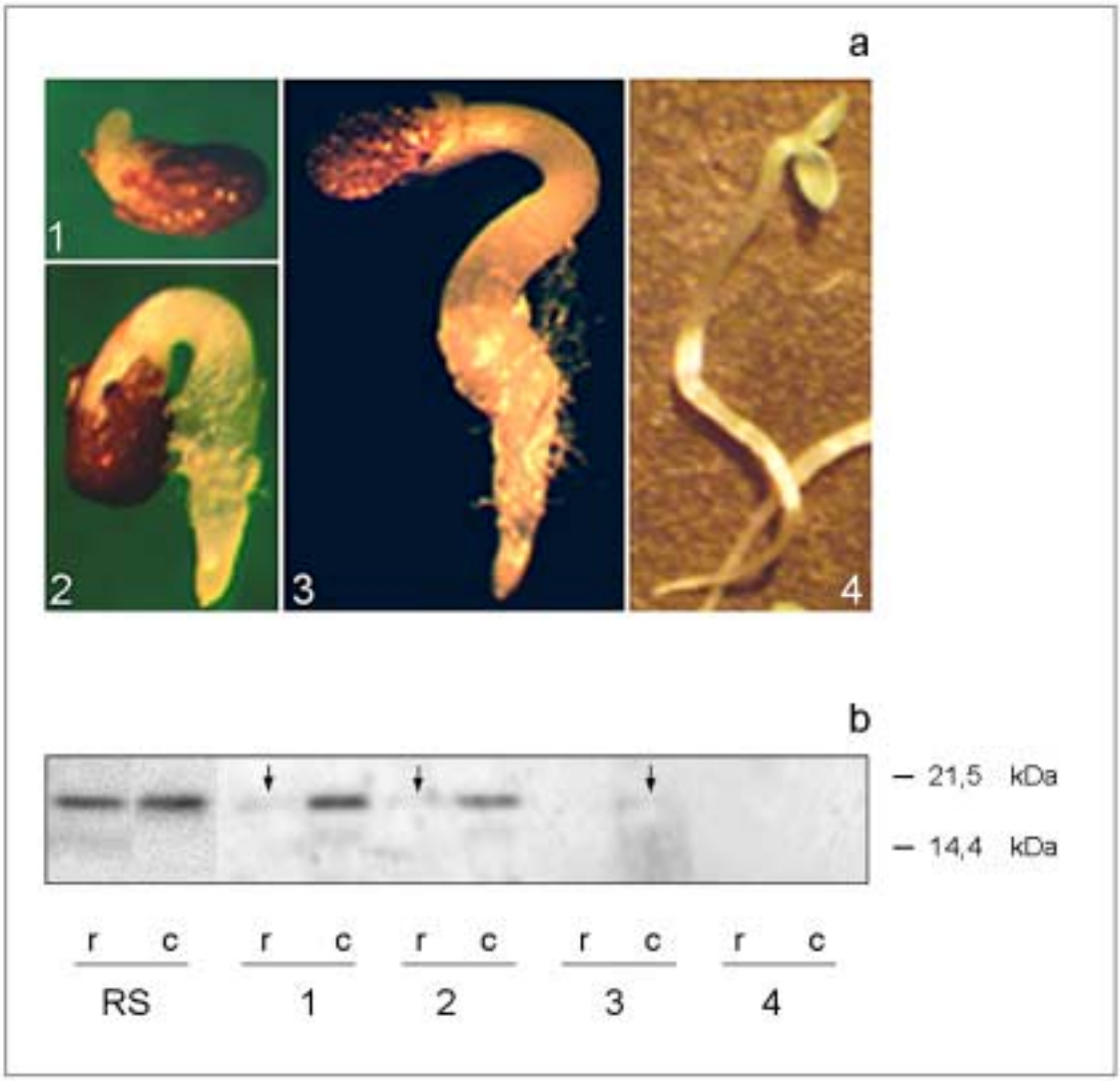

Figure 23. Cytosolic sHSP's during *Nicotiana tabacum* seed germination.
a - stages of tobacco plants during germination; **b -** Western blot analysis of the HSP17's during germination. 1, 2, 3, 4 correspond to the stages marked in **a**; RS-ripe seeds; r-root tips; c-cotyledons. The protein amount of each sample corresponds to the equal number of the seedlings.

when sHSP's disappeared. According to their morphological state, all seedlings were divided into four groups (Fig. 23, a). The first group consisted of seeds with 1-2 mm emerged root tips, the second - of young seedlings with elongated hypocotyl and the primary radicle without root hairs. Seedlings of the third group had a more elongated hypocotyle, their cotyledons were light green and remained within the seed coat. The primary roots were several millimetres in length and copious root hairs were developed. The fourth group consisted of seedlings with a length of about 1 cm. In this group the cotyledons had a bright green colour. The seed coat was completely withdrawn or adhered sometimes to the cotyledons. A reduced level of cytosolic sHSP's was observed in the cotyledons of group 1, 2 and 3 and the root tips of group 1 and 2 (Fig. 23, b). In seedlings of group 4 no sHSP's were found.

3.4.3 sHSP 2-D pattern during tobacco germination

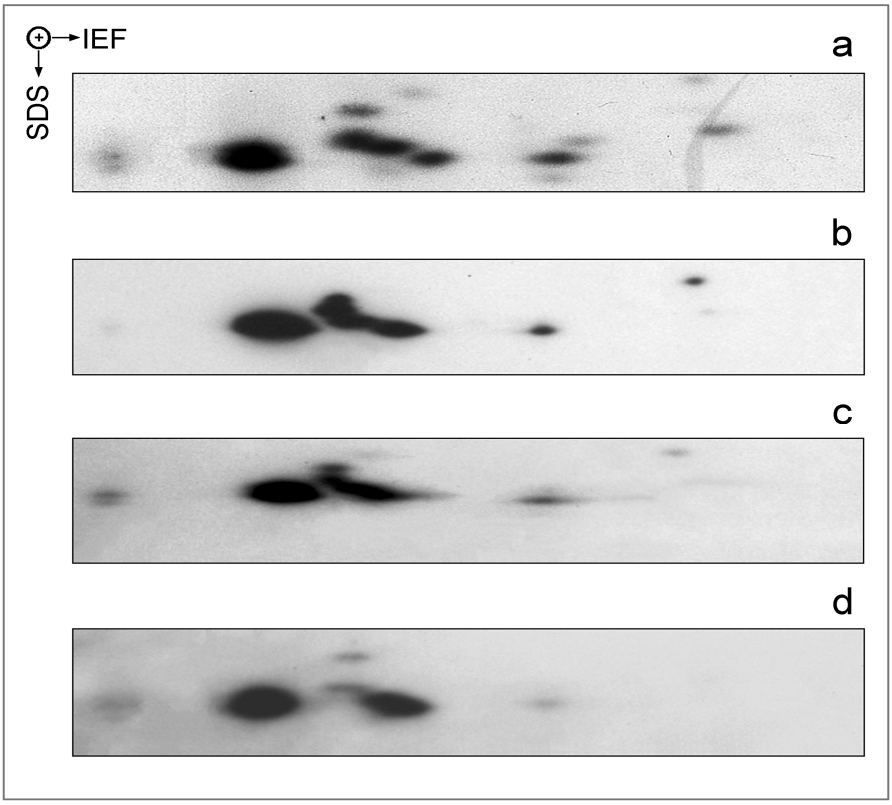

Figure 24. Two-dimensional Western blot analysis of cytosolic sHSP's during tobacco seed germination.
a - mature seeds; **b, c, d -** 2, 4, 6 day imbibed seeds. Proteins of each sample (150 μg) were separated by isoelectric focusing followed by SDS-PAGE and Western blot analysis using tomato HSP17 antiserum.

To determine the isoform pattern of developmentally induced sHSP's during the course of seed germination, protein samples, prepared from 2, 4, 6 days imbibed seeds, were analysed by two-dimensional Western blotting (Fig. 24). The sHSP isoform pattern during germination was the same as in ripe seeds (Fig. 24, a). No preferential degradation of a single isoform was observed.

3.5. sHSPś in plant vegetative organs

3.5.1 sHSP's in resting vegetative organs

The accumulation of developmentally induced cytosolic small heat shock proteins in seeds at normal growth temperatures was reported for several species (Hernandez and Vierling, 1993; Coca et al., 1994; DeRocher and Vierling, 1994; zur Nieden et al., 1995; Wehmeyer et al., 1996; Collada et al., 1997). Cell division cessation, accumulation of reserve materials and the presence of the quiescent stage are characteristic for these plant organs. Thus, the synthesis of non-stress induced small heat shock proteins may be characteristic for plant organs and/or tissues which are in the same physiological state as seeds. To verify this assumption different plant quiescent storage organs were analysed for the presence of small heat shock proteins.

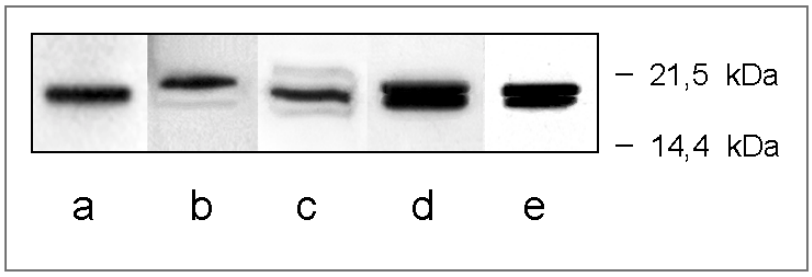

Figure 25. Cytosolic sHSP's in quiescent plant organs.
Proteins (10 µg/lane) were isolated from twigs of *Acer platanoides* (**a**) and *Sambucus nigra* (**b**), *Aristolochia macrophylla* tendrils (**c**), potato tubers (**d**) and *Narcissus* bulbs (**e**) and separated by SDS-PAGE followed by Western blot analysis.

Protein samples were prepared from underground resting vegetative storage organs of potato, *Narcissus*, one-year-old twigs of *Acer platanoides* and *Sambucus nigra*, and *Aristolochia macrophylla* tendrils, all collected during resting time. The Western blot analysis revealed that sHSP's were present in potato tubers, *Narcissus* bulbs and in investigated tendrils and twigs (Fig. 25).

3.5.2 Potato tuber sHSP's

3.5.2.1 sHSP mRNAs of potato tubers

The presence of sHSP mRNAs in mature seeds was demonstrated for sunflower and pea (Almoguera and Jordano, 1992; DeRocher and Vierling, 1994). However, the amount of sHSP mRNAs in seeds was much lower of that found in heat stressed

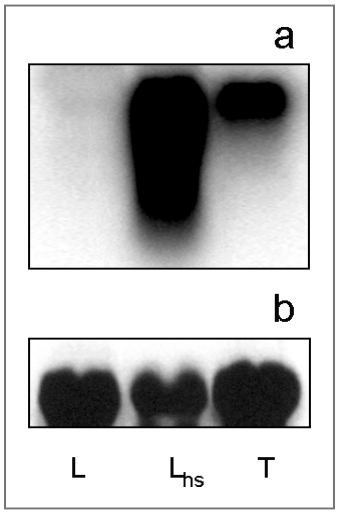

Figure 26. sHSP mRNAs in potato tubers and heat stressed leaves.
a - Northern blot analysis of total RNA (60 μg/lane). Tomato cDNA of class I HSP17 was used as hybridisation probe. **b -** ethidium bromide stained 25S rRNA. L-leaves; L_{hs}-heat stressed leaves; T-tubers.

tissues. To determine whether the same is true for sHSP mRNAs accumulated in vegetative storage organs, RNA was isolated from potato tubers and heat stressed leaves and analysed on Northern blots, probed with tomato class I HSP17 cDNA. Figure 26 shows that sHSP mRNAs are present in mature potato tubers at a much lower level in comparison with heat stressed tissues.

3.5.2.2 2-D pattern of potato tuber sHSP's

To determine and compare the stress and non-stress induced isoforms in potato, protein samples prepared from tubers and heat stressed leaves were separated by 2-D gel electrophoresis, followed by Western blot analysis with antibody against tomato HSP17. Figure 27 shows that the pattern of non-stress and heat stress induced sHSP's is complex and very similar. However, three tuber specific, and one heat stress specific, polypeptides were observed (Fig. 27, arrows). The relative

amount of some polypetidies which where expressed both under developmental control and upon heat stress was different in both samples.

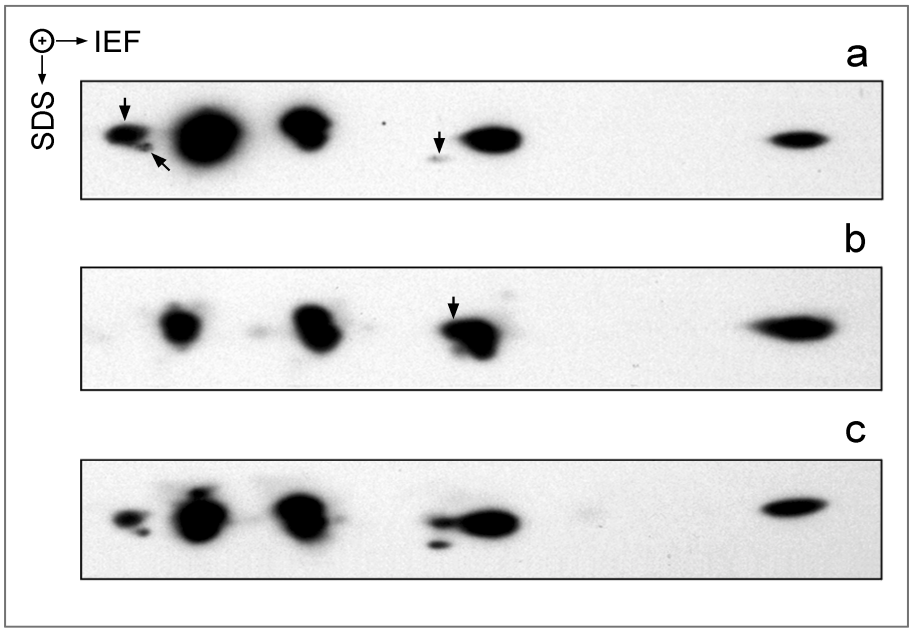

Figure 27. 2-D analysis of cytosolic sHSP's of potato tubers and heat-stressed leaves.
a - potato tuber proteins, **b -** heat stressed leaf proteins, **c -** potato tuber proteins mixed with heat stressed leaf proteins. Proteins of each sample (50 µg) were separated by isoelectric focusing followed by SDS-PAGE and Western blot analysis using tomato HSP17 antiserum. Arrows indicate tuber (**a**) and heat stressed leaf (**b**) specific isoforms.

3.5.3 sHSP's in vegetative organs after resting stage

In *Nicotiana* seeds no sHSP's were observed after new plant formation. To investigate if sHSP disappearance in vegetative plant organs also coincides with

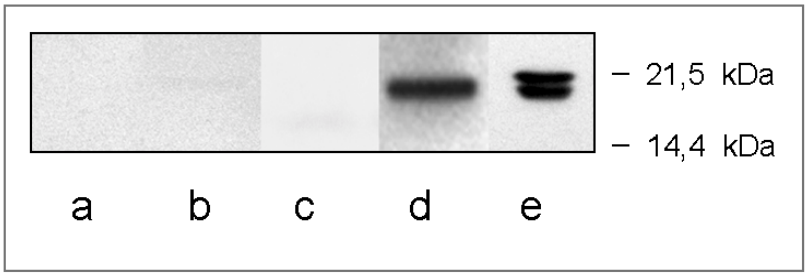

Figure 28. Cytosolic sHSP's in vegetative plant organs after resting stage.
Protein (10 µg/lane) isolated from twigs of *Acer* (**a**) and *Sambucus* (**b**), *Arystolochia* tendrils (**c**), potato tubers (**d)** and *Narcissus* bulbs (**e**) were separated by SDS-PAGE followed Western analysis.

activation of plant growth, protein samples were prepared from potato tubers and *Narcissus* bulbs after shooting, and from twigs and tendrils after budding. The Western blot analysis (Fig. 28) revealed that developmentally expressed cytosolic sHSP's were not present in twigs of *Acer* and *Sambucus* or tendrils of *Aristolochia* (Fig. 28, a, b, c, respectively). In potato and *Narcissus* underground organs, sHSP's were present after new plant formation and the levels of these proteins were not much below that of the resting stage (Fig. 28, d, e).

3.6 sHSP localisation in vegetative organs

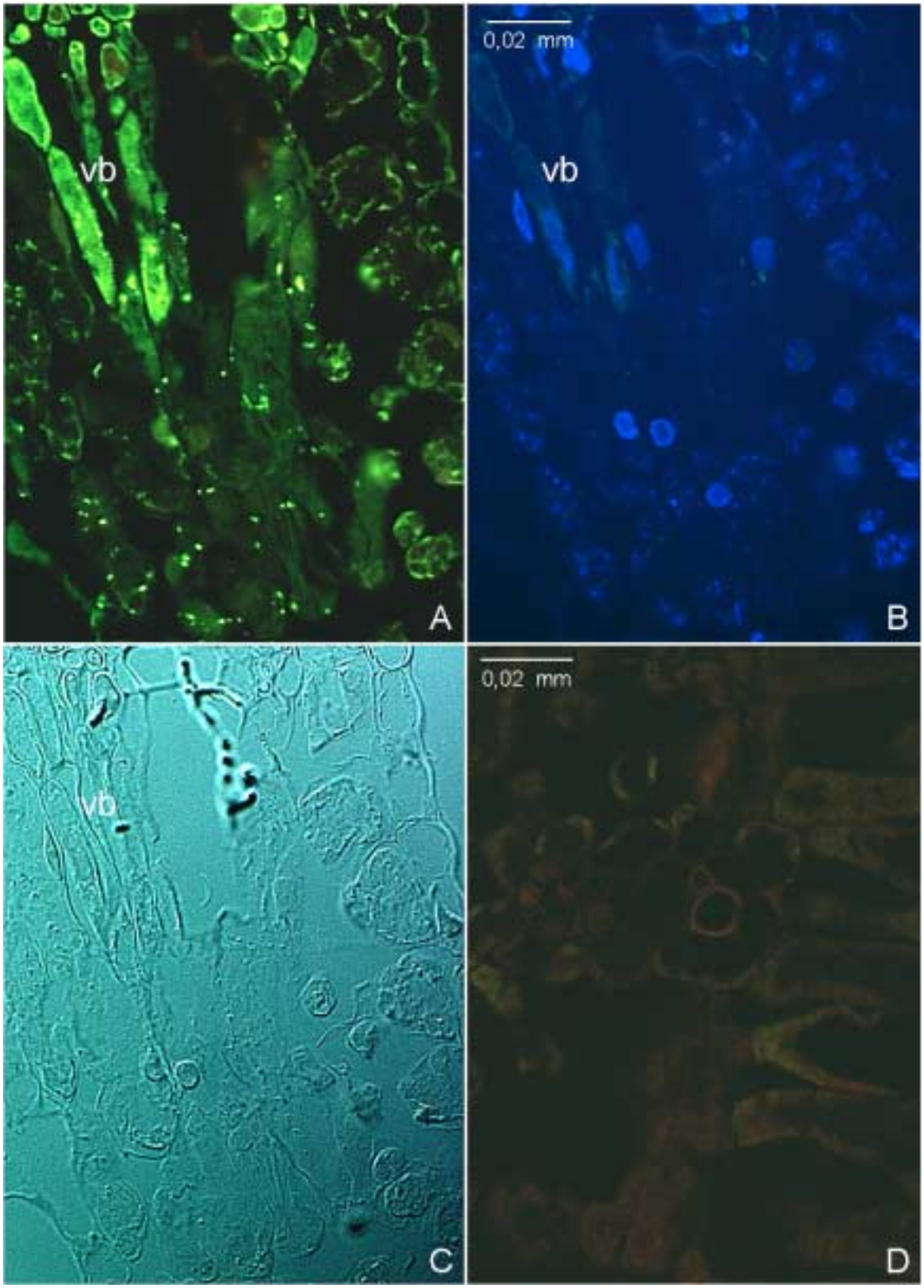

Figure 29. Localisation of sHSP's in heat stressed potato leaves.
A - localisation of sHSP's in heat stressed young leaves; **B** - DAPI staining of section shown in **A**; **C** - phase contrast photograph of a section shown in **A**; **D** - preimmune serum control with non-stressed leaf. **vb** - vascular bundle.

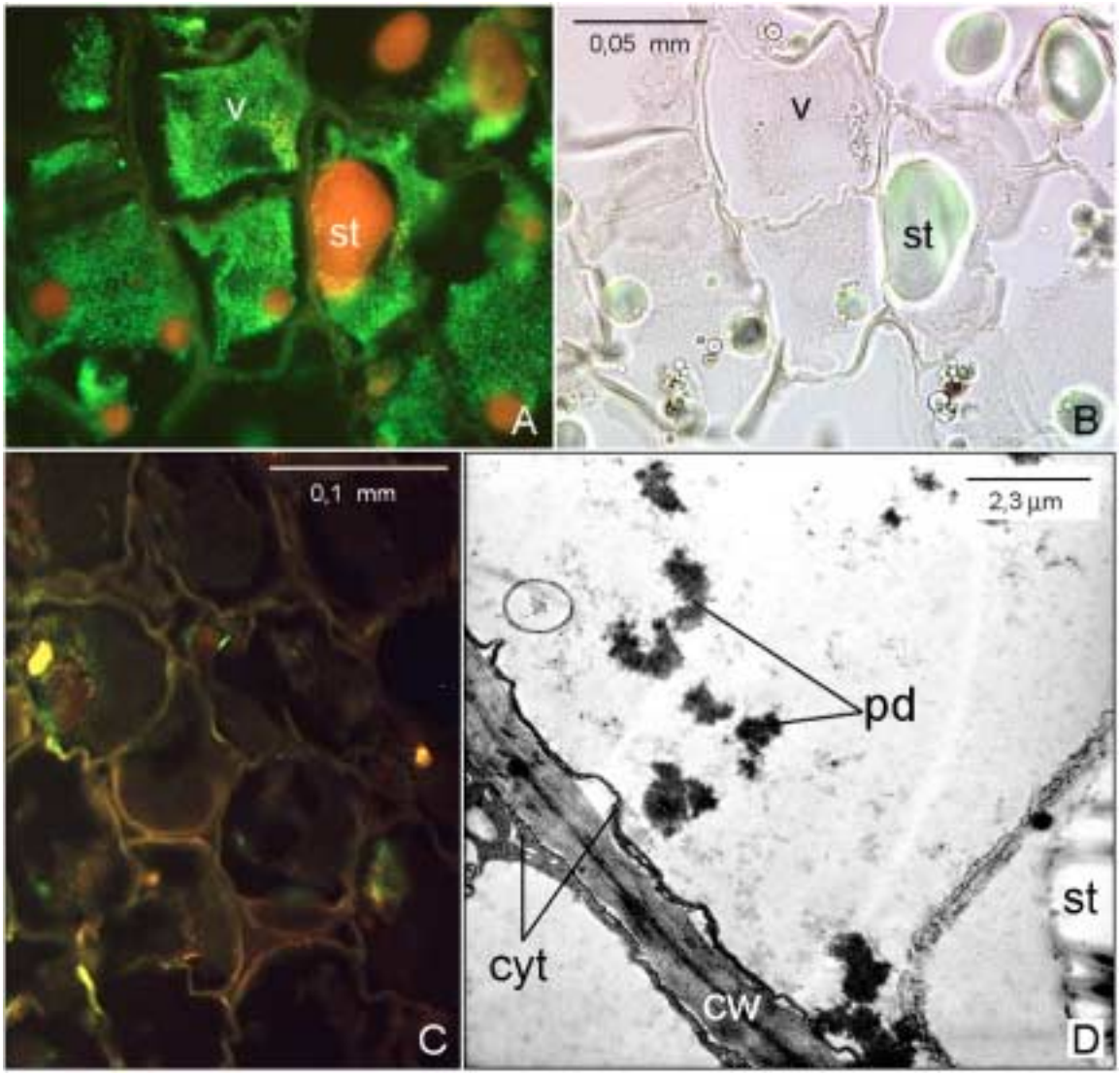

Figure 30. sHSP localisation and ultrastructure of potato tuber parenchyma cells. **A** - immunohistochemical staining of sHSP's in parenchyma cells; **B** - differential interference contrast photograph of a section shown in **A**; **C** - preimmune serum control for unspecific binding in tuber parenchyma cells; **D** - electron micrograph of parenchyma cell. **cw** - cell wall; **cyt** - cytosol; **pd** - protein deposits; **st** - starch; **v** - vacuole.

Figure 30 shows the typical localisation of sHSP's in the cytoplasm and in heat stress granules of heat stress potato leaves. The strongest immunolabelling for sHSP's was observed in the vascular bundle. In mature potato tubers cytosolic sHSP's were found in parenchyma cells of the pith and cortex. But, in contrast to heat stressed leaves, in these cells sHSP's localised homogeneously in the central vacuoles (Fig. 30, A). Electron micrograph of tuber parenchyma cells demonstrated the presence of protein deposits in the vacuoles (Fig. 30, D).

Figure 31 (A) shows the histological structure of *Narcissus* bulb scales, consisting of a non-storage parenchyma and an outer storage parenchyma. Both are bound by epidermal layers. *Narcissus* bulb scales show a labelling for sHSP's especially in epidermal cells, in the storage parenchyma and in the bundles (Fig. 32, A). In epidermal and storage parenchyma cells of *Narcissus*, sHSP's were detected in the vacuoles (Fig. 32, B).

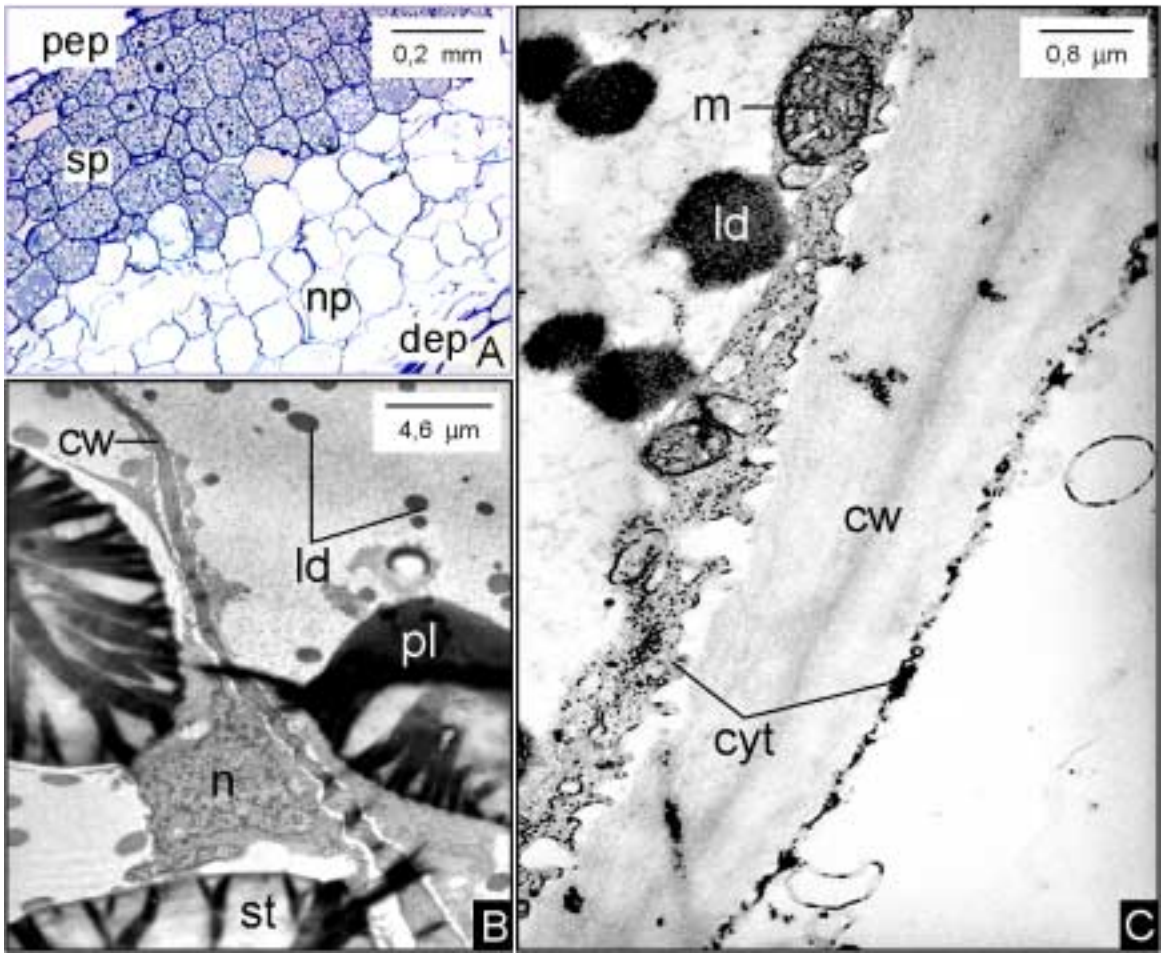

Figure 31. Structure of *Narcissus* bulb scale cells.
A - azure/methylene blue staining of a scale cross section; **B** - electron micrograph of storage parenchyma cell; **C** - electron micrograph of storage (left side) and non-storage (right side) parenchyma cells. **cw** - cell wall; **cyt** - cytoplasm; **dep** - distal epidermis; **ld** - lipid droplets; **m** - mitochondria; **n** - nucleus; **np** - non-storage parenchyma; **pep** - proximal epidermis; **pl** - plastids; **sp** - storage parenchyma; **st** - starch.

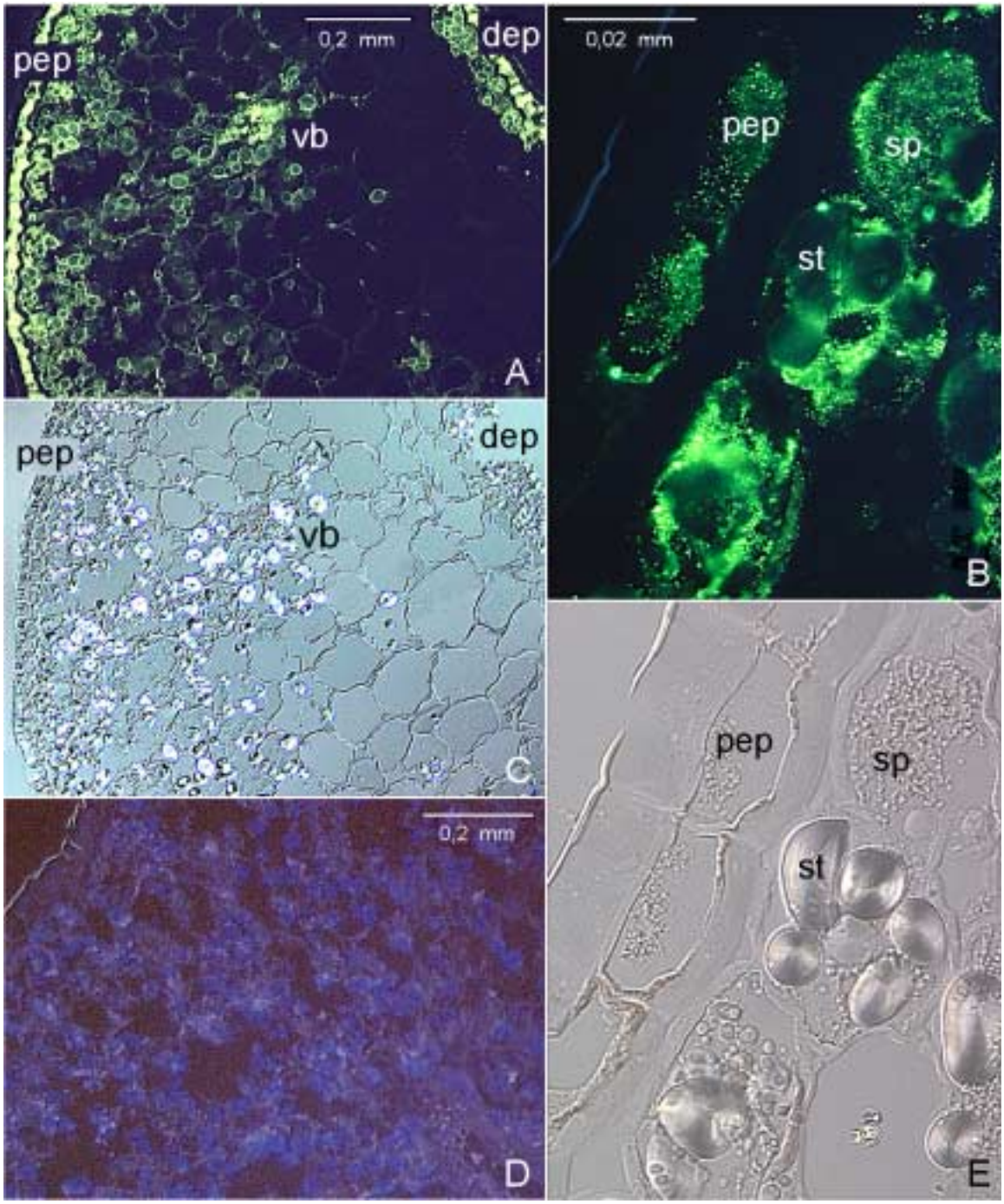

Figure 32. Localisation of sHSP's in *Narcissus* bulb scales.
A - immunohistochemical staining of sHSP's in a bulb scale; **B** - sHSP's localisation in proximal epidermis and storage parenchyma cells; **C, E** - phase-contrast photographs of sections shown in **A** and **B,** respectively; **D** - preimmune serum control for unspecific binding. **dep** - distal epidermis; **pep** - proximal epidermis; **sp** - storage parenchyma cells; **st** - starch; **vb** - vascular bundle.

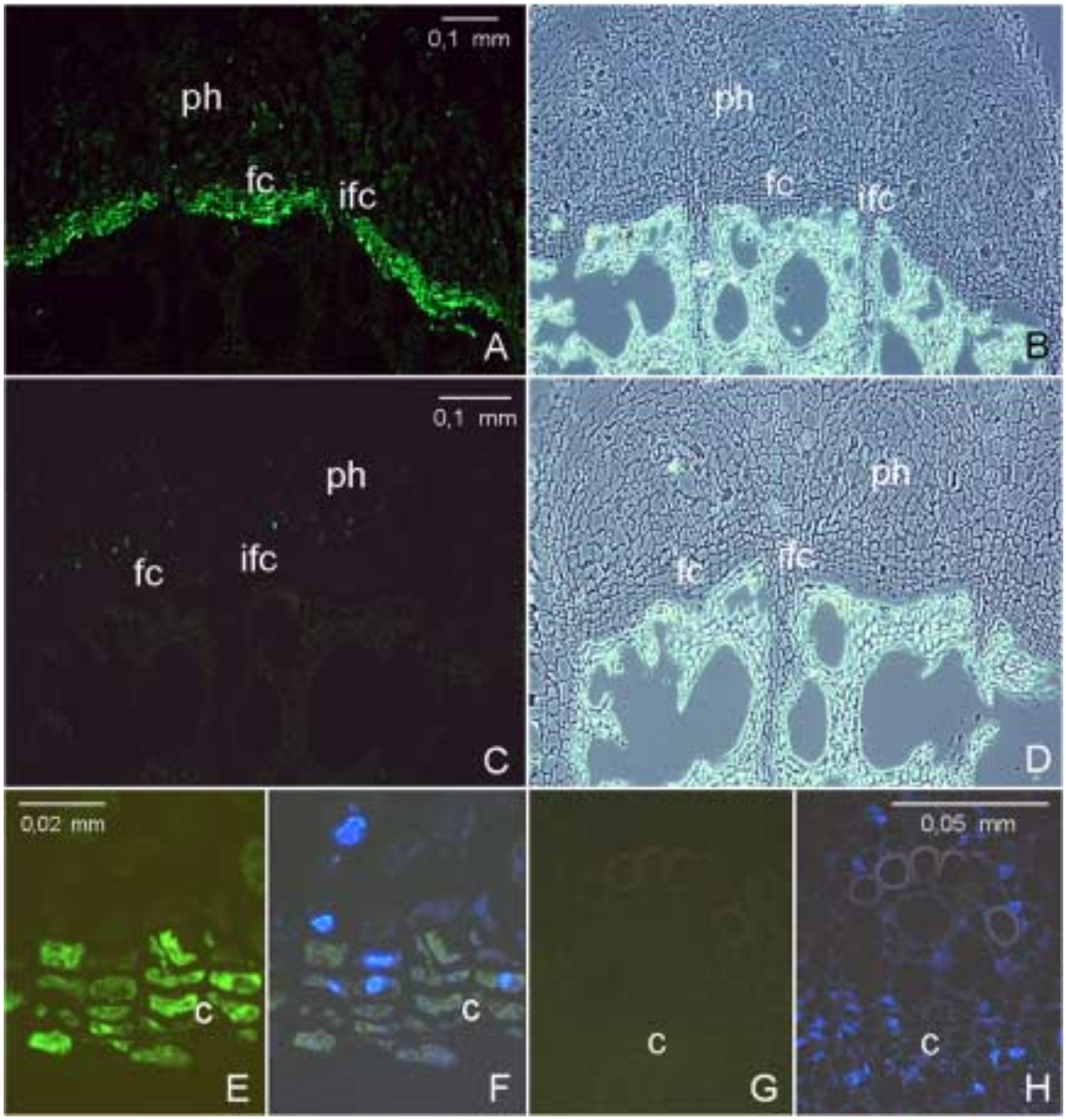

Figure 33. Localisation of sHSP's in *Aristolochia* tendrils.
A - immunohistochemical staining of developmentally induced sHSP's in cross section through one-year old dormant *Aristolochia* tendril; **C** - preimmune serum control for unspecific binding; **B, D -** phase-contrast photographs of sections shown in **A** and **C**, respectively; **E** - immunohistochemical staining of developmentally induced sHSP's in cells of dormant fascicular cambium; **G** - immunohistochemical staining of developmentally induced sHSP's in cells of fascicular cambium after resumption of the growth activity; **F, H** - DAPI staining of sections shown in **E** and **G**, respectively. **c** - cambium; **fc -** fascicular cambium; **ifc** - interfascicular cambium; **ph** - phloem.

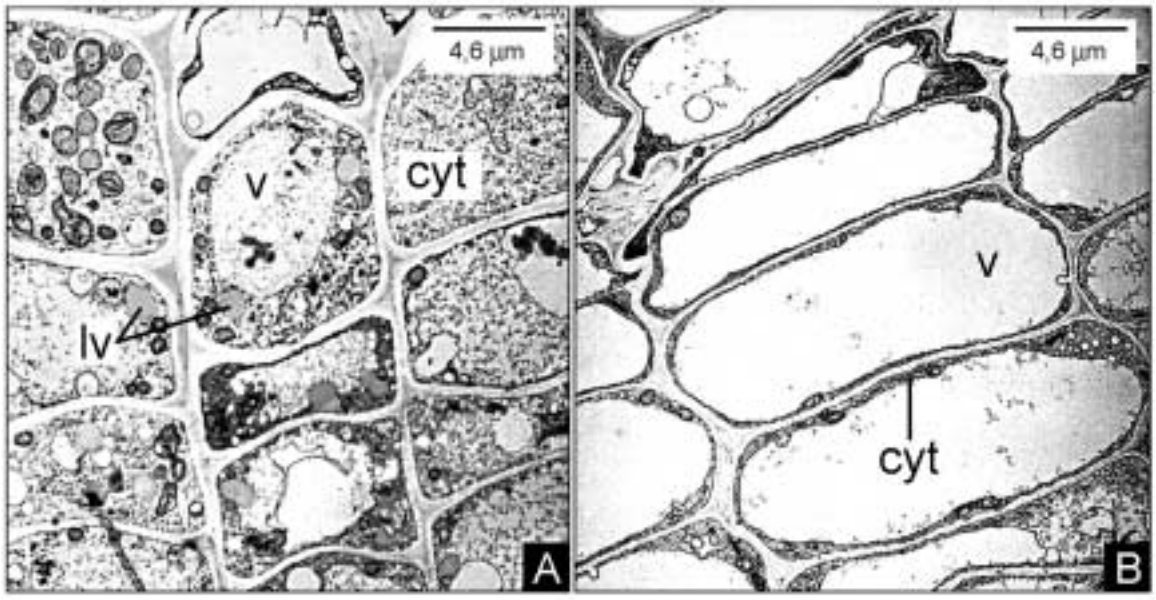

Figure 34. Ultrastructure of *Aristolochia* tendril cambium cells.
A - electron micrograph of dormant cambium cells; **B -** electron micrograph of the *Aristolochia* cambium cells after resumption of the growth activity. **cyt** - cytoplasm; **lv** - lipid vacuoles; **v** - vacuole.

In cross-section of yearling dormant *Aristolochia* tendrils sHSP's were detected exclusively in the fascicular cambium. In spring time, after budding, sHSP's could not be found any longer. An ultrastructural comparison of dormant and non-dormant tendril cross sections showed remarkable differences (Fig. 34). During the dormancy, cambium cells were densely cytoplasmic with numerous organelles, large lipid bodies, and several vacuoles filled with conspicuous flocculent material, obviously proteinaceous deposits. After budding and reactivation of cambial activity, a huge central vacuole without proteinaceous material was observed instead of numerous small vacuoles. Likewise, the lipid bodies were missing and the abundant cytoplasmic material occupied only the small region on the cell boder.

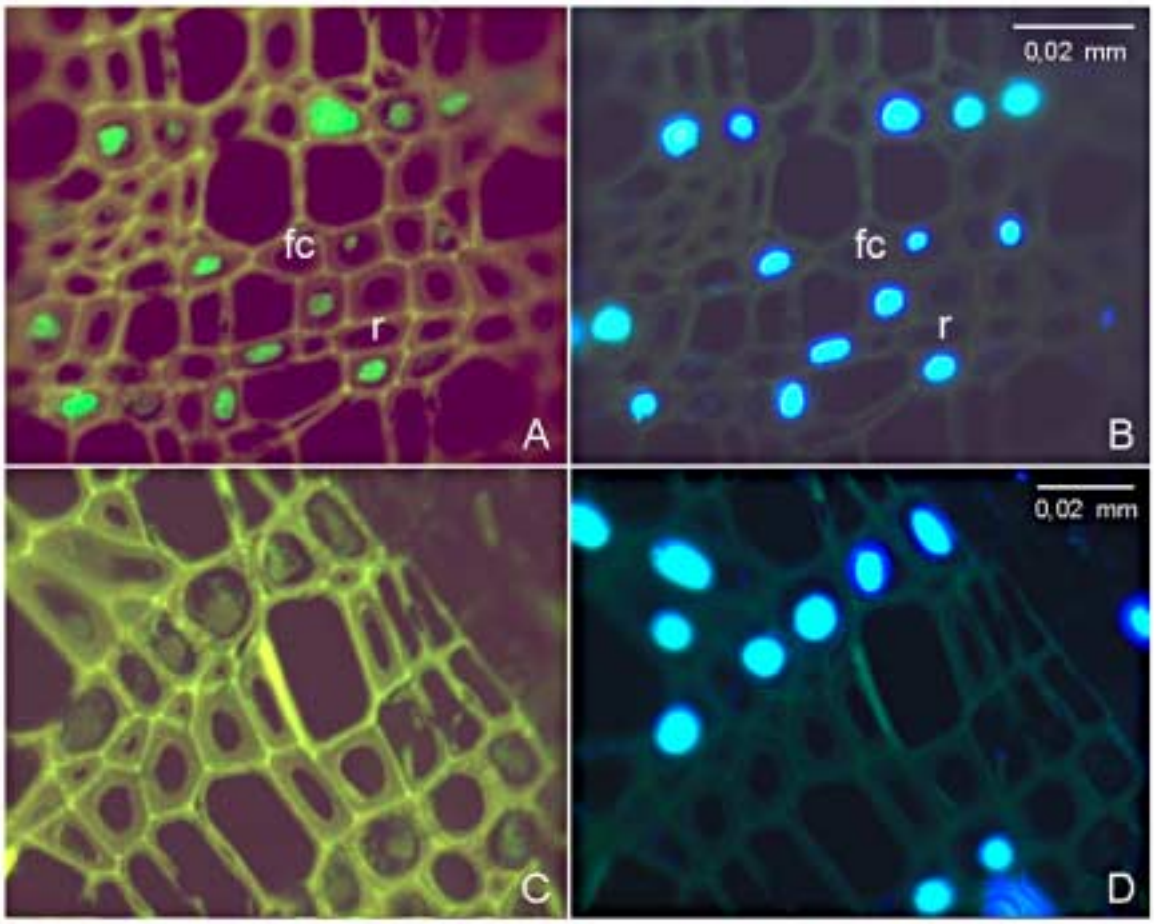

Figure 35. Localisation of sHSP's in *Sambucus* twigs.
A - immunohistochemical staining of developmentally induced sHSP's in cross section through the xylem of one-year old dormant *Sambucus* twig; **C** - preimmune serum control for unspecific binding in dormant *Sambucus* twig; **B, D** - DAPI staining of sections shown in **A** and **C,** respectively; **fc -** fiber cells; **r** - ray cells.

In transverse section of dormant *Sambucus* twigs sHSP's were observed predominantly in the nuclei of ray and fiber cells (Fig. 35, A).

4. Discussion

4.1 sHSP in *Nicotiana tabacum* seeds

In *Nicotiana tabacum* seeds cytoplasmic sHSP's (class I and class II) are expressed under developmental control during the late stages of seed development. The expression of mRNA and /or proteins of this gene family has been shown in ripe seeds of pea (Vierling and Sun, 1989; DeRosher and Vierling, 1994), wheat (Helm and Abernathy, 1990), sunflower (Almoguera and Jordano, 1992; Coca et al., 1994), alfalfa (Howarth, 1990), a variety of legumes (Hernandez and Vierling, 1993) and more recently in other species (zur Nieden et al., 1995; Collada et al., 1997). In seeds sHSP's are usually detected starting at the mid-maturation stage of development (Almoguera et al., 1993; DeRosher and Vierling, 1994; Wehmeyer et al., 1996), approximately at a period of reserve accumulation (Wehmeyer et al., 1996). In tobacco seeds this time point is referred to as the 17th day after pollination (DAP) (Fig. 7). The induction of sHSP synthesis in plants, with the exception of their expression in seeds, usually occurs in response to different stresses. It could be that the physiological state of the seed tissue at the time of sHSP accumulation is similar to that observed under stress. The cytosolic CuZnSOD was used as marker to determine the state of seed tissues during embryogenesis. It is known that SOD activity is increased by a number of environmental stresses (Bowley and Oliver, 1992). It was also reported that in mature *Nicotiana plumbagenifolia* seeds the amount of cytosolic CuZnSODs mRNAs are the highest in comparison to other parts of the plant (Herouart et al., 1994). In tobacco seeds, during embryogenesis, the level of cytosolic CuZnSOD did not change significantly (Fig. 5). These observations suggest the absence of stress in the developing seeds and the non stressed nature of sHSP induction in seeds.

At the beginning of sHSP's synthesis the tobacco embryo is in cotyledonary stage. The water content in seeds at this stage is about 28 % (Fig. 1). This value is much lower the critical moisture level necessary for cell division and growth (Levitt, 1980; Kigel and Galili, 1995). Thus, at 17 DAP tobacco seed tissues no longer grow. The beginning of sHSP expression coincides to the maximal ABA level in seed tissues (Fig. 1). ABA alone have been shown not to induce expression of sHSP's (Czarnecka et al., 1984). However, the ABI3 seed-specific transcription factor, which regulates various seed-specific genes (Giraudat et al., 1992; Parcy et al., 1995) and activates the heat shock promoter through

the heat shock factor (Rojas et al., 1999), is expressed in developing seeds prior to the increase in levels of endogenous ABA (Shiota et al, 1998). Since the increase in ABA content in tobacco seeds coincided with sHSP expression, it could be that ABI3 is a *trans*-regulating element that triggers the synthesis of some developmentally induced sHSP's. Additionally, non-HSE dependent activation of the sHSP promoter was observed in the early stages of seed maturation (Almoguera et al., 1998).

In tobacco seeds the messenger RNA transcripts for sHSP's were first observed on 17 DAP and synthesised concurrently with the proteins which they encode (Fig. 6, 7). The mRNA accumulation which paralleled the production of sHSP's, suggested the transcriptional regulation of sHSP synthesis in tobacco seeds. A correlation between the synthesis of sHSP mRNA and accumulation of the corresponding proteins was observed during embryogenesis of sunflower (Coca et al., 1994), pea (DeRocher and Vierling, 1994) and also under stress conditions (Jin et al., 1997). There are only a few reports which demonstrate the presence of sHSP mRNAs in unstressed tissues. For example, an *Arabidopsis thaliana* cDNA clone encoding a 17,6 kDa protein, with 64 - 68 % amino acid homology to class II sHSP's, was cloned from leaf mRNA of plants grown at 21° C (Bartling et al., 1992). Another sHSP cDNA was cloned from mRNA of unstressed, seven-day-old, *Papaver somniferum* seedlings (Facchini and De Luca, 1994). The accumulation of one sHSP transcript in Duglas-fir during germination has also been reported (Tranbarger and Misra, 1995). However, this transcript was not observed in ripe seeds. In *Nicotiana* seeds the amount of sHSP mRNA at 17 DAP was very low and significantly increased during seed ripening (Fig. 6). This could be due to the constant (or increased) gene transcription occurring throughout seed development (assuming that the degradation rate is slower then the rate of mRNA synthesis) and/or due to the degradation of most other mRNAs during late stage of seed maturation (Chandler et al., 1984; Walling et al., 1986; Nielsen et al., 1989; Ingle et al., 1965). Increased amounts of sHSP mRNA in *Nicotiana* during seed development is in contrast to sHSP mRNA accumulation in pea developing seeds, where a relatively constant level of their synthesis was observed (DeRocher and Vierling, 1994). In tobacco the highest level of sHSP mRNAs was detected in ripe seeds, however it was much lower than in heat stressed vegetative tissues. The synthesis of lower levels of developmentally induced sHSP mRNAs in comparison to that induced under heat stress were observed in potato tubers and were also reported for pea seeds (DeRocher and Vierling, 1994). Assuming that the rate of mRNA translation is

the same in seeds and stressed tissues, DeRocher and Vierling, (1994) suggested that sHSP mRNAs in seeds are more stable then in stressed tissues, thus much less mRNA would be required to synthesise a given amount of protein in seeds than in heat stressed tissues (DeRocher and Vierling, 1994). In this context it is interesting that in carrot transgenic cell lines containing a carrot HSP17.7 gene driven by the 35S promoter, the level of HSP17.7 mRNA at 23° C was relatively low (Malik et al., 1999). Malik et al. (1999) assumed that instability of HSP17.7 mRNA is due to high rate of turnover and that self-recognition may play an important role in this process. In contrast the soybean HSP mRNA in transgenic tobacco cells at room temperatures was stable (Schöffl et al., 1987).

During tobacco seed imbibition a reduced level of sHSP mRNAs (in comparison to ripe seeds) was detected at 2 DAI (Fig. 21). Sunflower developmentally induced sHSP mRNAs were also detected at 2-3 DAI (Cocca et al., 1994). These mRNAs could be residual embryogenesis-specific, stored in dry seeds and/or newly synthesised. This is probable since most of the messages including the HSP mRNAs in imbibed embryos of some seeds, have a short life-time (Caers et al., 1979; Howarth, 1990). Using inhibitors of mRNA synthesis it was shown that *Sorgum bicolour* HSP mRNAs stored in dry seeds disappeared about 10 h after imbibition (Howarth, 1990). However, it could be that the turnover of tobacco sHSP mRNAs occurs at a lower rate than in other species. In this case sHSP transcripts detected after imbibition are those accumulated during zygotic embryogenesis.

Western blot analysis of 2D gels of tobacco seeds and heat stressed leaves showed a complex pattern of polypeptides reacting with tomato anti-HSP17 antibodies (Fig. 10). These polypeptides are different not only in their pI but also in their molecular weight. In heat stressed leaves it appears that sixteen isoforms are expressed (Fig. 10, c). Thirteen small heat shock polypeptides were observed in tobacco seeds (Fig. 10, a). The comparison of both sHSP patterns revealed differences not only in isoform number and abundance of HSP's expressed in seeds and heat stressed leaves, but also demonstrated specific isoforms: one - in seeds (Fig. 10, a, arrow) and three - in heat stressed leaves (Fig. 10, c, arrows). The difference in pattern of developmentally and stress expressed sHSP's were also reported for pea, maize, tomato, *Nicotiana rustica* and pea (DeRocher and Vierling, 1994; zur Nieden et al., 1995; Wehmeyer et al., 1996). In all investigated plants, some isoforms expressed in heat stressed leaves were absent in seeds. On the other hand seeds possessed polypeptides which were not heat inducible (DeRocher and

Vierling, 1994; zur Nieden et al., 1995). During tobacco seed maturation, and upon imbibition and germination, no changes in the isoform pattern were observed (Fig. 11, 24).

The isoform pattern of sHSP's expressed in tobacco embryo and endosperm was similar with the exception of two proteins (Fig. 12). Additionally, differences in the amount of some sHSP's expressed in both embryo and endosperm were observed.

The presence of specific stress and developmentally induced sHSP's argues for the differential synthesis of these proteins during stress and embryogenesis. The specific polypeptides could be the products of different genes (Nover and Scharf, 1984) and/or the products of different mRNA produced by the same gene due to use of alternative transcription initiation sites or alternative splicing, and/or the products of a single transcript due to use the alternative translation. Furthermore, it could be that some sHSP isoforms are the products of posttranslation modification (Nover and Scharf, 1984).

During tobacco seed imbibition and early germination, sHSP's persisted for several days. sHSP's were not detected in protein extract prepared from ten day old seedlings (Fig. 22). At the stage of imbibition and early germination, sHSP's were detected in both the cotyledons and axes (Fig. 23). The decline of sHSP's during these processes was firstly observed in the axes of 2 DAG seedlings. In germinating pea seeds sHSP's started to disappear also in roots (DeRosher and Vierling, 1994). DeRosher and Vierling (1994) suggested that the observed decrease in sHSP amount could be caused either by their active degradation or by their dilution by newly synthesised proteins. The tobacco seedlings which have no more sHSP's have well developed hypocotyl, root with root hairs and bright green cotyledonary leaves. Since in tobacco seeds sHSP disappearance was observed in growing tissues, it could be that the presence of developmentally induced small heat shock proteins are characteristic for tissues which are in a quiescent state.

4.2 sHSP's in vegetative quiescent organs

The expression of small heat stress proteins was detected in vegetative plant organs of potato, *Narcissus*, *Acer platanoides, Sambucus nigra* and *Aristolochia macrophylla*. In potato and *Narcissus* we found sHSP's in tubers and bulbs (Fig. 25). In *Acer* and *Sambucus* - in one-year-old twigs, and in the liane *Aristolochia* - in tendrils (Fig. 25). It seems that the presence of sHSP's in storage quiescent organs is a general phenomenon.

The cell division cessation and the accumulation of reserve materials are characteristic for all investigated quiescent vegetative organs and also for seeds where developmentally induced sHSP's were also found. The stage of plant development when sHSP's disappear differs among investigated vegetative organs. In the twigs of *Acer* and *Sambucus* and tendrils of *Aristolochia* the sHSP's were observed only during dormant stage in winter time (Fig. 25). Following the start of budding, in spring time, the heat stress proteins were no longer detectable (Fig. 28). On the other hand, in potato tubers and *Narcissus* bulbs, sHSP's were found both in subterraneous organs of dormant plants whose overground organs were dead (Fig. 25), and in storage organs of plants with very well developed new overground shoots (Fig. 28).

4.3 Immunohystological localisation of sHSP's

The immunohistological localisation of cytosolic sHSP's in *Nicotiana* seeds and investigated vegetative organs differs among members of investigated plant group and in comparison to that of stressed tissues.

In young and completely developed tobacco leaves following heat stress treatment small heat shock proteins were found in all tissues, with a higher concentration in cells of vascular bundles (Fig. 14, 15). A similar picture of heat stress induced sHSP localisation was observed in young potato leaves (Fig. 29). Previously it was shown that in soybean seedlings during heat stress sHSP's of class I were not limited to specific cell types. However, the highest levels of cytosolic small heat shock protein synthesis were detected in tissues with high metabolic and mitotic activities, such as meristematic regions of roots, shoots, and the cambium cells in the vascular tissues (Jinn et al., 1997). Triphenyltetrazolium chloride (TTC) staining, which is a test for plant cell viability, has demonstrated that these tissues were more sensitive to heat stress (Jinn et al., 1997). The lowest amount of sHSP's were observed in the mature regions of hypocotyls. In sunflower plants under water stress, sHSP's were induced in lateral meristems of stems and roots, but not in leaves (Almoguera et al., 1993). Moreover, in these plants a differential tissue specificity was observed in water-stressed and heat-shocked cytosolic sHSP's. The latter are localised mostly around the xylem vessels in the stem. The water-stress induced sHSP's accumulate in the fascicular and interfascicular cambium. Thus, it seems that the

tissue specificity of stress induced sHSP accumulation depends on the physiological state of the plant, which in turn depends on a variety of external and internal factors.

In *Nicotiana* seeds the immuno detection reveals the expression of developmentally induced cytosolic sHSP's in all cell types of embryo and endosperm (Fig. 18). In embryo the strongest hybridisation signal was observed in provascular tissues and in shoot meristem (Fig. 18, E, G). This pattern of sHSP expression is similar to the pattern of GUS expression in seeds of transgenic tobacco plants containing beta-glucoronidase reporter gene driven by a soybean heat shock promoter and to the pattern observed in *in situ* hybridisation experiments (Prändel et al., 1995). In endosperm the distribution of sHSP's was uniform throughout (Fig. 18, C).

In one-year-old dormant *Aristolochia* tendrils cytosolic sHSP's localised only in fascicular cambium (Fig. 33). In mature potato tubers cytosolic sHSP's are localised in parenchyma cells of pith and cortex (Fig. 32). The immunostaining for sHSP's in *Narcissus* bulb scales shows labelling predominantly in epidermal cells, in the outer cell layers of storage parenchyma and in the bundles (Fig. 33). In *Sambucus nigra* twigs cytosolic sHSP's were found to be localised in secondary xylem: in rays and in fiber cells (Fig. 35). Thus, the tissue specificity of sHSP accumulation is also characteristic for developmentally induced small heat shock proteins.

The intracellular localisation of developmentally and stress induced cytosolic sHSP's varies within the investigated plant group and between the tissues. In heat stressed tobacco leaves sHSP's localisation does not simply depend upon the presence or absence of heat stress, but also on physiological state - in young leaves sHSP's were localised mainly in the cytosol, in heat stressed fully differentiated leaves sHSP's were found in the cytosol and nuclei (Fig. 15). A differential localisation of sHSP's depending on the physiological state of the cell was also demonstrated for yeast HSP26 (Rossi and Lindquist, 1989). When log-phase cells growing in glucose were heat shocked, HSP26 concentrated in the nuclei and continued to concentrate in the nuclei when these cells were returned to normal temperatures for recovery. In wild-type cells grown in galactose or acetate, HSP26 failed to concentrate in nuclei following a heat shock. In tomato cell cultures under stress treatment sHSP's were found predominantly localised in the cytosol distributed homogeneously or in granules observed after a long heat treatment (Neumann et al., 1987; Nover et al., 1989). In addition to the cytoplasma, sHSP's were also found in nuclei under stress conditions (Lin et al., 1984; Wollgiehn et al., 1994; Wollgiehn and

Neumann, 1995; Jinn et al., 1997). It was also reported that the 18.3 kDa sHSP from heat stressed *Chenopodium rubrum* has a unique DNA-binding domain (Knack et al., 1992). Knack et al. (1992) assumed that it might represent a sHSP which is translocated into the nucleus. The presence of cytosolic sHSP's in this organelle under stress treatment is not a peculiarity of plant sHSP's. A nuclear localisation under heat stress was also demonstrated for HSP23 in salivary glands of *Drosophila* and for mammalian HSP28 in HeLa cells (Arrigo et al., 1981; 1988). In tobacco seeds, a nuclear localisation of sHSP's was detected in embryonic cells, with highest amounts in cells of bundle and apical meristematic tissues (Fig. 18). Previously an association of developmentally expressed sHSP's with nuclei was also shown for some seeds (zur Nieden et al., 1995). A predominantly nuclear localisation of sHSP's was observed in *Sambucus* ray and fiber cells (Fig. 35). The presence of stress and developmentally induced cytosolic sHSP's in the nucleus could be caused by their diffusion through the nuclear pore and/or active transport due to the presence of nuclear localisation sequence or in a protein complex with other nuclear proteins. Until quite recently, no nuclear localisation sequence for sHSP's was known. The first and at present the only one report demostrating the presence of nuclear targeting signals in a heat stress induced cDNA clone coding for the cytosolic class II sHSP of tomato, was published in 1998 (Kadyzhanova et al., 1998). This clone has a sequence which has homology to a Xenopus type nuclear localisation signal and a second sequence which contains a putative SV40 large T-antigen nuclear targeting signals. Both sequences could be responsible for the translocation of cytosolic sHSP's from cytosol to nucleus during stress or during definite stages of plant development. Some members of stress and developmentally induced sHSP's found in the nuclei could be polypeptides which have nuclear localisation sequences. However, it was shown that in heat stress tomato cell cultures, where sHSP's are predominantly localised in the cytosol, the sHSP patterns of nuclear and total cell proteins are very similar (Wollgiehn and Neumann, 1995). Thus, the transport of sHSP's in complexes with other nuclear proteins is most probable, at least under stress conditions. The presence of plant sHSP's in the nucleus under stress treatment or in particular developmental stages could be due to their actual localisation in this organelle or due to their translocation from the cytosol as was demonstrated for αB-crystalline (Klemenz et al., 1991; Inaguma et al., 1992; Voorter et al., 1992). In these cases the translocation of sHSP's seems to be dynamic and depend upon the physiologic state of the cell.

The accumulation of developmentally expressed sHSP's have been shown in protein bodies of *Lycopersicon esculentum* and *Nicotiana rustica* seeds and for the cotyledon cells of *Vicia faba* (zur Nieden et al., 1995), although such localisation does not apply to all investigated seeds and tissues. In *Zea mays* embryo and *Vicia faba* hypocotyl cells sHSP's were observed in the cytoplasm and the nuclei and no small stress proteins were found in protein bodies (zur Nieden et al., 1995). A cytoplasmic localisation of sHSP's was also reported for cotyledonary cells of chestnut seed (Soto et al., 1999). In the storage organs of potato and *Narcissus*, cytosolic sHSP's localised homogeneously in the central vacuoles of the epidermis and storage parenchyma cells (Fig. 30, 32). This cell organelle is the main compartment for storage proteins in potato. It is interesting that in seeds of many plant species protein bodies originate from the subdivision of the central vacuole (Craig et al., 1979, 1980; Hoh et al., 1995). Thus, there are four cell compartments where developmentally induced cytosolic sHSP's were observed - cytosol, nucleus, protein bodies and vacuole. It is not clear how cytosolic sHSP's are transported into the vacuole. Since sequence analysis demonstrated that none of the known cytosolic sHSP's contain an N-terminal or C-terminal vacuolar targeting signal, it could be that these proteins or at least some members of sHSP family have internal targeting signals as was shown for phytohaemagglutinin and legumin (Tague et al., 1990; von Schaewen and Chrispeels, 1993; Saalbach et al., 1991). It could be that sHSP's are transported into the vacuole via autophagy or an alternative mechanism.

Since, on one hand, the isoform patterns of heat stress and developmentally induced proteins in tobacco and potato were very similar, but, on the other hand, the localisation is so different, and that most heat stress induced sHSP cDNAs do not reveal targeting sequences specific for cellular compartments, it could be that developmentally induced proteins possess these sequences as the products of different transcripts encoded by alternative transcription initiation or alternative splicing of the same gene.

4.4 sHSP's and storage proteins

The presence of sHSP's in the investigated seeds and vegetative organs seems to coincide to the presence of storage proteins. Such correlation was observed during the course of tobacco seed development (Fig. 7). Moreover, it was found that the embryos of transgenic tobacco seeds, which do not accumulate storage proteins have no sHSP's (Fig. 9).

Previously it was reported the correlation in the synthesis of sHSP's and 12S seed storage proteins in developing *Arabidopsis* seeds (Wehmeyer et al., 1996). We have found that during the course of tobacco germination the disappearance of sHSP's also correlates with the disappearance of storage proteins (Fig. 22). The electronmicrographs of tobacco endosperm and embryo cells demonstrated the presence of numerous protein bodies at the stage of sHSP's accumulation (Fig. 19). In the seeds of anti-ABA-scFv transgenic plants, protein bodies were observed only in endosperm cells where developmentally sHSP's were detected (Fig. 20). Vacuoles filled with dense proteinaceous material were observed in dormant fascicular cambium cells of *Aristolochia* tendrils (Fig. 34, A). After budding and reactivation of cambial activity the storage vacuoles have been replaced by a huge vegetative vacuoles and the vacuolar proteinaceous material has disappeared (Fig. 34, B). In mature potato tubers cytosolic sHSP's are localised in the vacuole of parenchyma cells of pith and cortex (Fig. 30, A). The electronmicrographs of these cells demonstrate the presence of numerous proteinaceous deposits in vacuoles (Fig. 30, D). The immunostaining for sHSP's in *Narcissus* bulb scales shows the labelling especially in epidermal cells, in the outer cell layers of storage parenchyma (Fig. 32). The cells of storage parenchyma were also intensively stained for proteins (Fig. 31, A (dark blue colour)). In *Sambucus nigra* twigs cytosolic sHSP's were found to be localised in secondary xylem: in rays and in fiber cells. The ray cells, generally remain parenchymatic and are particularly noted for storage of food reserves (Esau, 1965). Fiber cells may also function as storage cells (Esau, 1965).

4.5 Possible sHSP's function

The function of cytosolic sHSP's is still largely unknown. Stress induced sHSP's seem to play a role in cell protection during particular stress situations, i.g. heat stress (Vierling, 1991; Arrigo and Landry, 1994), water stress (Almoguera et al., 1993; Wehmeyer et al., 1996), heavy metal stress (Neumann et al., 1994), cold stress (Sabehat et al., 1996; Soto et al., 1999; Ukaji et al., 1999) etc.. Some *in vitro* and *in vivo* experiments indicate that sHSP's may function in the acquisition of thermotolerance. It was shown that cells that have not experienced heat stress and have not accumulated sHSP's do not survive a lethal heat shock. Enhanced high temperature resistance was observed in *E.coli* cells expressing plants sHSP's (Yeh et al., 1997; Soto et al., 1999). In plants, transgenic cell lines in which

a gene coding for one sHSP (HSP17.7) is constitutively expressed, were more thermotolerant than control lines (Malik et al., 1999). Moreover, cell lines expressing antisence sHSP mRNA which demonstrate reduced synthesis of many sHSP's under stress treatment, were less thermotolerant (Malik et al., 1999). Direct genetic evidence of a role for sHSP's in acquisition of thermotolerance was demonstrated for cyanobacterium *Synechocystis* (Lee et al., 1998b) and *Neurospora crassa* (Plesofsky and Brambl, 1999). In *Synechocystis* a reduced thermotolerance was found when its single sHSP was deleted and in *Neurospora crassa* when the HSP30 gene was disrupted. Recently it was demonstrated the correlation between the accumulation of sHSP's and the acquisition of chilling tolerance (Sabehat et al., 1996, 1998; Kadyrzhanova et al., 1998). An effect of sHSP's in maintaining cell viability at low temperatures (4° C) have been found in *E.coli* cells overexpressing recombinant chestnut sHSP's (Soto et al., 1999).

The chaperone function of sHSP's demonstrated in a few *in vitro* and *in vivo* studies (Jinn et al., 1989, 1995; Horwitz, 1992; Jakob et al., 1993; Lee et al., 1995, 1997; Chang et al., 1996; Collada et al., 1997; Forreiter et al., 1997; Veinger et al., 1998) could explain a possible mechanism of sHSP's function. Similar to results obtained for bacterial and mammalian sHSP's plant small heat shock proteins could bind denatured protein in an ATP-independent manner. From such complex substrate proteins can be refolded in an ATP-dependent fashion by HSP70 system (Lee and Vierling, 2000). Based on these experiments a working model of heat stress induced sHSP's function in cell protection was recently developed (Waters et al., 1996). At moderate temperatures sHSP's are thought to bind to target proteins, preventing their missfolding and aggregation. At higher temperatures irreversible binding of sHSP's to heat-sensitive proteins prevents the accumulation of insoluble protein aggregates. In the following steps the target proteins can be refolded by means of other chaperones or degraded by the protelytic machinery. Additionally it was assumed that sHSP's contribute to the protection of house-keeping mRNA during heat stress by the formation of heat shock granules including mRNAs, sHSP's and some other proteins (e.g. HSP70) (Nover et al., 1989). Such heat stress granules are not found in tissues containing non stress induced sHSP's (zur Nieden et al., 1995).

The information of organ, tissue and the cellular localisation of cytosolic sHSP's both in heat stressed plants and in reproductive and vegetative organs, could aid in the

understanding of their function. It seems that at elevated temperatures sHSP's function within all kind of tissues. In contrast, non stress induced sHSP's are found only in particular tissues and cells. Additionally, the intracellular localisation of heat stress and non stress sHSP's is different. The role of heat stress induced cytosolic sHSP's seems to be confined to the cytoplasm and nuclei, while the developmentally induced sHSP's - to the cytosol, vacuole, nuclei and protein bodies. The physiological state of the tissue is probably important for stress induced sHSP's distribution within cells - in young tobacco leaves these proteins accumulated predominantly in the cytosol, whereas in completely developed leaves they are localised in the cytoplasm and at a high level in the nuclei. The finding of non stress induced sHSP's in multiple plant cell organelles could be explained by their function in these organs. However, it could be that diversity of sHSP localisation reflects not only the functional sites of these proteins in the cell, but also the possible compartments for protein storage. Moreover, the variety of subcellular sites of sHSP's distribution could be due to the intracellular relocalisation of sHSP's which could depend on the physiological state of the cell. This was shown for a few eukaryotic sHSP's and αB-crystallin under different stress treatment (Arrigo et al., 1981, 1988; Voorter et al., 1992; Klemenz et al., 1991).

Plants organs which accumulated non stress induced sHSP's are quiescent. No cell division takes place at this stage. It could be that there is a correlation between the accumulation of sHSP's and the arrest of cell division. A disruption of the cell cycle at the G1/S and G2/M phases of cell cycle and the protein synthesis inhibitor function of HSP27 during heat stress were described for mammalian cells (Kühl and Rensing, 2000; Cuesta et al., 2000). In this regards it is interesting that the embryo cells of most seeds are also arrested at G1 and G2 phases of the cell cycle (Bowley and Black, 1985). The high level of sHSP's in meristematic cells of the tobacco seeds could be explained by high potential mitotic activity of these cells. Besides this possible function, non stress induced sHSP's accumulating in the investigated plant organs could act to protect proteins against possible unfavourable conditions during a quiescence stage and/or rest, or during the resumption of growth activity. In orthodox seed cells (seeds that are able to withstand a complete loss of cellular water) it was proposed that sHSP's contribute to the desiccation tolerance (Almoguera and Jordano, 1992; Coca et al., 1994; DeRosher and Vierling, 1994) since the water loss in the tissues during maturation can cause the destabilisation of protein conformation. In underground storage organs, the water stress could be during the

quiescent stage of plant development since different processes tacking place in cells utilise water. The differential localisation of sHSP's in plant organs could reflect the sites subjected to the strongest dehydration and/or be more sensitive to water loss. Besides desiccation as the result of physiological changes taking place in plants there are different environmental factors which can lead to protein denaturation. Among them are chilling and extracellular freezing. The main effect of extracellular freezing is also desiccation due to water moving from cells to the extracellular space where ice crystals are formed.

The finding that recalcitrant chestnut (*Castanea sativa*) seeds, which usually have a very high water content and are highly sensitive to desiccation (Jaynes, 1974; Payne et al., 1983), contain a highly abundant of sHSP-Ch sHSP (Collada et al., 1997) is in contrast to the previous hypothesis. Collada et al. (1997) suggested that the accumulation of sHSP's in this case was necassary for cell protection since high-moisture tissues are more sensitive to environmental damage. This could be the explanation for accumulation of sHSP's in quiescent underground storage organs.

All the hypothesises mentioned above are based on the chaperone function shown for some sHSP's. However, our investigation and previous studies have demonstrated that the isoform patterns of both developmentally and stress induced sHSP's are complex. It could be that different stress and developmentally induced members of the cytosolic sHSP gene family have the same and/or different functions. It is also possible that the isoforms induced both under heat stress and at non stress temperatures are multifunctional proteins and could have different functions in stress and unstressed tissues. This double use of a distinct protein has been called gene sharing and was demonstrated for pro- and eukyryotic proteins (Blumenthal and Carmichael, 1979; Holmgren, 1985; Huber et al., 1986; Russel and Model, 1986; Meyer-Siegler et al., 1991; Jimenez-Asensio et al., 1995). In some cases gene sharing is facilitated by the different features of the protein or it oligomeric state (Piatigorsky, 1998). It was also shown that the same protein in different cellular compartments could play distinct roles, suggesting the regulation of protein function by environmental factors (Smalheiser, 1996). In this regard it is interesting that mammalian the HSP27, which has chaperone function, is also a heat shock induced inhibitor of cellular protein synthesis (Cuesta et al., 2000). It was shown that HSP27 specifically bound eIF4G-an adapter protein of a cap-binding initiation complex which is required for translation of most mRNAs and trapped it in insoluble heat shock granules.

SUMMARY

The aim of the current work was to investigate developmentally induced cytosolic (class I and II) sHSP's to get information on the function of these proteins in plants. *Nicotiana tabacum* seeds served as model system in our experiments. Northern and Western blot analysis were used to determine the presence of sHSP's and their mRNAs in seeds at different stages of development and germination. We found that in tobacco seeds the accumulation of sHSP's and their mRNAs started at mid-maturation stage of embryogenesis at the 17 DAP. At this stage a maximal level of ABA in seeds was observed. sHSP's persisted in tobacco ripe seeds and in seeds of about ten days after imbibition and disappeared afterwards complietely. The presence of sHSP's in tobacco seeds correlated to the presence of globulins (storage protein of tobacco seeds) as during seed development as during germination. A additional evidence for the correlation between the expression of small heat shock proteins and globulins we get from our expirements with transgenic tobacco plants (anti-ABA-scFv), expressing a single-chain Fv (scFv) antibody against the abscisic acid. Embryos of transgenic plants have reduced level of storage protein in comparison to wild type plants. We have not found any sHSP's in anti-ABA-scFv embryo cells, which also do not accumulate any globulins. However, both proteins are present in endosperm. Additionally, the formation of protein bodies in transgenic seeds was observed only in endosperm. In contrast, in wild type seeds protein bodies were found as in embryo as in endosperm cells.

Since ripe seeds are resting organs we have inv estigated state other plant quiescence organs with respect to their sHSP's content. We have demonstrated the presence of sHSP's in potato tubers and narcissus bulbs. Additionally we have found these proteins in resting tendrils of the liane *Aristolochia* and twigs of *Sambucus* and *Acer*. It seems that the accumulation of small heat shock proteins in resting storage organs is a general phenomenon.

Northern blot analysis of potato RNAs showed the presence of sHSP transcripts in mature tubers. However, their amount was significantly less than that observed in heat stressed tissues. It seems that a lower sHSP mRNA expression level is characteristic for non stress induced transcripts, since in mature tobacco seeds sHSP mRNAs were also present in reduced amount in comparison to that under HS treatment.

2D Western blot analysis of potato and tobacco demonstrated a complex pattern of developmentally and heat stress induced sHSP's. One seed and four heat stress specific sHSP's were detected in tobacco. The relative amount of some polypeptides observed in both patterns was different. During tobacco embryogenesis and seed germination the isoforms pattern of sHSP's did not change. A differential expression of small heat shock proteins were observed in embryo and endosperm of mature seeds. Thus, the regulation of sHSP synthesis is different under stress and non stress condition. Additionally the synthesis of one non-stress induced sHSP's in tobacco seed is organ specific.

The investigation of heat stress induced sHSP localisation showed that young leaves of tobacco and potato these proteins are accumulated in all tissues with the highest amount in the sieve cell/companion complex of vascular bundles, presumably caused by the high metabolic activity of these cells. The immunodetection of sHSP's in heat stressed leaf parenchyma cells demonstrated the presence of intensively labelled sports, likely due to the heat shock granules, located in the weakly labelled cytoplasma. In young tobacco leaves we have found sHSP's only in the cytosol. However, in mature leaves an association of sHSP's with nuclei was observed. Therefore, the localisation of heat stressed induced sHSP's could depends of the physiological state of tissues.

Developmentally induced sHSP's were found in different tissues and different cellular compartments. In tobacco seeds these proteins are localised in the endosperm and embryo tissues. In the embryo the strongest immunolabeling of sHSP's was observed in vascular bundls and in the cells of the shoot meristeme. Embryo cells revealed the presence of sHSP's in cytosol and nuclei. A labelling of protein bodies was found in storage parenchyma cells of embryo and endosperm. No heat shock granules were observed in seed cells. In storage parenchyma cells of potato tubers and narcissus bulb scales sHSP's were detected in vacuoles. In dormant *Aristolochia* tendrils they were localised in the fascicular cambium. In resting *Sambucus* twigs sHSP's were found predominantly in the nuclei of ray and fiber cells. The diversification of the cellular compartmentation of non-stressed induced sHSP could reflects the sites of their function and/or the place for their storage.

The function of developmentally induced sHSP's still remains unknown. Since a disruption of cell cycle at G1 and G2 phases and a protein synthesis nhibitor function of sHSP's were demonstrated for heat stressed mammalian cells and the embryonic cells of most ripe seeds are arrested at G1 and G2 stages of cell cycle, we assumed that in seeds

sHSP’s participate in cell cycle arrest. The second possible function of sHSPs could be based on their chaperone properties. We have found non-stress induced sHSP’s in surviving plant organs which are important for further plant development. These organs have low metabolic activities and presumably a cessation of the protein synthesis and repair mechanisms. Different internal and external factors (water loss, extracellular freezing, free radicals, etc.) could cause protein degradation in these plant organs what could couse the reduction of the plant viability. We assume that non-stress induced sHSP’s acting as chaperones, protect proteins against unfavourable influences during resting stage of the plants.

Zusammenfassung

Im Rahmen der vorliegenden Arbeit sollten die entwicklungsbedingt exprimierten cytosolischen kleinen Hitzestressproteinen (sHSP) der Klasse I und II untersucht werden, um Anhaltspunkte für ihre Funktion in der Pflanze zu erhalten. Die Entwicklung von Tabaksamen *(Nicotiana tabacum)* diente dabei als Modellsystem. Die Anwesenheit von sHSP und ihrer m-RNA's in verschieden Stadien der Samenreife und -keimung wurde durch Northern und Western blot Analysen verfolgt. Die Synthese der sHSP's und ihrer m-RNA's beginnt gleichzeitig am 17. Tag nach der Anthese. Zu diesem Zeitpunkt befinden sich die Samen im mittleren Reifezustand, und enthielten die höchste Menge an Abszisinsäure. Im Verlaufe der Keimung konnten die sHSP's noch bis zum 10.Tage nach der Imbibition nachgewiesen werden. Es bestand eine enge Korrelation zwischen Speicherglobulinen und sHSP's, sowohl während der Samenreife als auch während der Keimung. sHSP's konnten nur nachgewiesen werden, wenn gleichzeitig Speicherproteine vorhanden waren. Das wurde auch durch unsere Untersuchungen an Samen von transgenen Tabakpflanzen bestätigt, die einen single chain Antikörper gegen Abszisinsäure exprimieren. In den Embryonen dieser Pflanzen sind weder Proteinbodies noch Speicherglobuline zu finden; ebenso konnten wir keine sHSP's nachweisen. Das Endosperm dagegen ist normal ausgebildet und enthält neben Proteinbodies und Speicherglobulinen auch gut nachweisbare Mengen an SHSP's.

Da eines der speziellen Merkmale von Samen höherer Pflanzen der ruhende Stoffwechsel ist, wurden Pflanzenteile, die sich ebenfalls in einem ruhenden Zustand befinden, auf ihren Gehalt an sHSP's untersucht. So konnte die Anwesenheit von sHSP's in Narzissenzwiebeln und Kartoffelknollen nachgewiesen werden. Außerdem fanden wir sHSP's in Ranken von *Aristolochia* und Zweigen von *Sambucus* und *Acer*, die alle während der Winterruhe untersucht wurden. Mit dem Austreiben der Knospen im April/Mai verschwanden die sHSP. Im Gegensatz dazu waren sHSP's in den unterirdischen Speicherorganen von Kartoffel und Narzisse nicht nur im Ruhezustand nachweisbar, sondern auch nachdem die Pflanzen gut ausgebildete oberirdische Sprosse entwickelt hatten.

Die Menge der sHSP's Transkripte in reifen Kartoffelknollen und in sich entwickelnden Tabaksamen war jeweils niedriger, als die in den entsprechenden hitzegestressten Blättern. Da der Gehalt an sHSP's in Samen und den untersuchten vegetativen

Speicherorganen jedoch signifikant höher als in den hitzegestressten Blättern ist, scheint eine im Vergleich mit hitzegestresstem Gewebe verminderte Transkriptmenge für nicht-stress induzierte sHSP's charakteristisch zu sein.

2D Western blot Analysen zeigten ein komplexes Polypeptidmuster sowohl für hitze-induzierte als auch für nicht stressinduzierte cytosolische sHSP. Dabei unterschied sich beim Tabak das Muster der hitzestress-spezifischen sHSP durch vier zusätzliche Polypeptide vom Muster der samenspezifischen sHSP, während das Muster der samenspezifischen ein zusätzliches Polypeptid im Vergleich mit hitzestress-induzierten sHSP's enthielt. Außerdem zeigte der Vergleich der Polypeptide von nicht-stress- und stressinduzierten sHSP's Unterschiede im Gehalt einzelner vergleichbarer Polypeptide. Jeweils spezifische Polypeptide wurden auch in Kartoffelknollengewebe und hitzegestressten Kartoffelblättern festgestellt.

Im Verlauf der Samenreife und -keimung änderte sich das Polypeptidmuster nicht qualitativ. Dagegen wurde im Endosperm und im Embryo von reifen Tabaksamen jeweils ein unterschiedliches Polypeptid gefunden. So scheint die Regulation der sHSP-Synthese nicht nur nach Stress und unter bestimmten Entwicklungsbedingungen unterschiedlich zu sein, sondern im Falle der nicht entwicklungbedingt exprimierten sHSP auch unterschiedlich für verschiedene Gewebe des entsprechenen Organs.

Die immunhistologische Lokalisierung der sHSP's in jungen Tabak- und Kartoffelblättern nach Hitzestress zeigte, daß diese Proteine in allen Blattgeweben zu finden sind, wobei der Siebröhren-Geleitzellen-Komplex des Phloems wegen der hohen metabolischen Aktivität eine besonders intensive Immunmarkierung aufweist. Innerhalb der Zellen sind die sHSP's nach Hitzestress wie zu erwarten im Cytoplasma und dort bevorzugt in den Hitzestressgranula lokalisiert. In reifen Tabakblättern wurden die sHSP's zusätzlich in den Kernen gefunden, so daß eine vom physiologischen Zustand des Gewebes abhängige hitzestressinduzierte intrazelluläre -Verteilung von sHSP's vermutet werden kann.

In Geweben, die sHSPs entwicklungsbedingt exprimieren, wurden Strukturen, ähnlich denen der Hitzestressgranula, nie gefunden. In Tabaksamen wurden die sHSP's im Embryo und im Endosperm lokalisiert. Dabei zeigten wiederum die provaskulären Bündel eine intensive Immunmarkierung. Sowohl im Embryo als auch im Endosperm waren die Proteinbodies markiert. Zusätzlich wiesen die meristematischen Kerne im Sproßapex des Embryo eine auffällige Immunfärbung auf. In Kartoffelknollen und

Narzissenzwiebeln wurden die sHSP's in den Vakuolen des Speicherparenchyms nachgewiesen. In ruhenden Ranken von *Aristolochia* waren die sHSP's nur im faszikulären Cambium zu finden und in ruhenden Zweigen von *Sambucus* in den Kernen der Markstrahlen und der Faserzellen des Xylems. Diese unterschiedliche Verteilung der nicht stressinduzierten sHSP's sowohl zwischen verschiedenen Gewebeteilen als auch auf intrazellulärer Ebene deutet auf unterschiedliche Orte ihrer Funktion und/oder ihrer Speicherung hin.

Die Funktion der entwicklungsbedingt exprimierten sHSP's ist im Gegensatz zu der von hitzestressinduzierten sHSP's noch weitgehend unbekannt. Da nach Hitzestress in Säugerzellen eine Unterbrechung des Zellcyklus zu Beginn der G1 und G2 Phase gefunden wurden und andererseits die embryonalen Zellen der meisten Samen zwischen der G1 und G2 Phase des Zellcyklus festgelegt sind, nehmen wir an, daß die sHSP's an der Arretierung des Zellcyklus in ruhenden Pflanzenorganen beteiligt sind.

Eine zweite Funktion der nicht-stress induzierten sHSP's basiert wahrscheinlich auf ihrer Chaperoneigenschaft. Wir haben nicht-stress induzierte sHSP's in Überdauerungsorganen gefunden. Diese Überdauerungsorgane sind wichtig für die weitere Entwicklung der betreffenden Pflanzen und zeigen verminderte metabolische Aktivität und vermutlich einen Stillstand von Proteinsynthese und Reparaturmechanismen. Unter diesen Umständen sind sie aber den verschiedensten inneren und äußeren Belastungen wie Wasserverlust durch Austrocknung oder durch extrazelluläres Ausfrieren im Winter, freie Radikale usw. ausgesetzt. Dabei können Proteinschäden auftreten und in deren Folge Vitalitätsminderungen.

Wir nehmen an, daß die sHSP's in ruhenden ausdauernden Pflanzenorganen als Chaperone fungieren, indem sie lebenswichtige Proteine vor schädigenden Einflüssen während der verminderten Stoffwechseltätigkeit schützen.

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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 benutzen Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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