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Identification of stress-induced mitochondrial proteins in cultured tobacco cells

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Under stress conditions, small and large heat-shock proteins cooperatively fulfil molecular chaperone activities within many cellular compartments, including mitochondria. Here, we report the identification and characterization of 10 newly synthesized low-molecular-weight heat stress-induced proteins (SIPs) found solely in the mitochondrial fraction of suspension-cultured cells of tobacco (*Nicotiana tabacum* L., strain Virginia Bright Italia-0). These SIPs were shown to be encoded by the nuclear genome and none was detected in non-stressed cells. Mass spectroscopy analysis revealed that eight SIPs belonged to the group of small heat-shock proteins, whilst one protein shared significant homology with transcription factors. De novo synthesis of all SIPs was detected within 10 min of the commencement of heat stress and continued for the whole 12 h stress interval. Moreover, nine of the SIPs were stable for 24 h following stress termination. SIP1, which showed the most intense synthesis profile, was found to be phosphorylated. The most intense stress response was observed mainly during the exponential growth phase of the cell culture. Native electrophoresis of mitochondrial protein complexes revealed an association of all the SIPs in high-molecular-weight complexes with no free SIPs left in mitochondria. These complexes were localized to the mitochondrial membrane fraction.

Introduction

Eukaryotic cells respond to stress treatments by the expression of heat-shock proteins (HSPs). The function of these molecular chaperones is to prevent irreversible protein aggregation and to promote correct folding of proteins after the onset of stress (Vierling 1991, Parsell and Lindquist 1993, Morimoto et al. 1994). According to their molecular weight, HSPs are classified into several families: HSP120, HSP100, HSP90, HSP70, HSP60 and the family of small HSPs (sHSPs). While the amino acid sequence of large HSPs (HSP60 to HSP120) is highly conserved from yeast to animals and plants, the homology within the large group of sHSPs is less apparent and is limited only to the short amino acid sequence at the C-terminal region (De Jong 1993, Caspers et al. 1995, Rassow et al. 1997).

High-molecular-weight HSPs are also present in cells under normal physiological conditions and their chaperone activities have been demonstrated in a variety of cell processes, in protein translocation across membranes, in the formation of multiprotein complexes and in the targeting of proteins for degradation (Zhang

Abbreviations – EST, expressed sequence tag; HSP, heat-shock protein; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; sHSP, small heat-shock protein; SIP, stress-induced protein; VBI-0, Virginia Bright Italia-0.

and Glaser 2002). Increased demand for chaperone activities under stress conditions turns the constitutive HSP expression level into overexpression and induces the transient activation of sHSPs. The expression of sHSPs positively correlates with strong resistance to stress treatment in both plant and animal cells (Landry et al. 1989, Downs and Heckathorn 1998). However, the main difference between the two eukaryotic kingdoms lies in the number of sHSPs induced by one agent. In human cells, the expression of sHSP27 has a protective activity against various kinds of treatment, including heat stress, oxidative stress, chemotherapeutic drugs and staurosporine-induced apoptosis, and phosphorylation has been shown to play an important role in regulating sHSP27 activity (Landry et al. 1989, Mehlen et al. 1996, Garrido et al. 1997). Plants, on the other hand, have evolved a different strategy. One stress impulse triggers the expression of a diverse family of sHSPs with molecular weights ranging from 12 to 38 kDa (Neumann et al. 1989, Vierling 1991, Banzet et al. 1998). These molecules form homopolymeric complexes from 200 to 800 kDa (Helm et al. 1993, Lenne and Douce 1994) that selectively bind proteins in their non-native state to prevent their aggregation, and to maintain them in a conformation capable of ATP-dependent refolding by other chaperones (Sun et al. 2002). Under stress conditions, the synthesis and accumulation of sHSP molecules are very high and their abundance can reach 1% of total cellular proteins (Hsieh et al. 1992).

The presence of small and large HSPs in the cytoplasm, endoplasmic reticulum, chloroplasts and mitochondria under stress conditions demonstrates the essential cooperation of both types of HSP molecule in their molecular chaperone activities within many cellular compartments in plants (Waters et al. 1996). The positive correlation between the stress-induced expression of a broad set of sHSPs and the maintenance of cell homeostasis raises questions about the specific function of these particular sHSP genes. The first indications of a possible functional hierarchy of sHSP species, sHSP15, sHSP17 and sHSP22, were suggested by three different types of stress in tomato stem calli cells (Banzet et al. 1998). Heat stress granules formed in tobacco protoplasts comprise both class I and class II sHSPs arranged into class-specific dodecamers. However, class I oligomers are formed only in the presence of class II sHSPs (Kirschner et al. 2000). Phosphorylation, an important tool of functional variability of sHSPs in animal cells, has been identified for the first time in maize mitochondrial sHSP22 (Lund et al. 1998). In animals, mitochondria are commonly regarded as the sensor of stress (Lai et al. 1996, Arrigo 1998). However, such a role in plants is still under discussion (Banzet et al. 1998, Jones 2000).

In this work, we report the contribution of mitochondria to the heat stress response of suspension-cultured cells of tobacco [*Nicotiana tabacum* L., strain Virginia Bright Italia-0 (VBI-0)]. A proteomic study led to the identification of eight low-molecular-weight proteins as stress-induced sHSPs. The kinetics of their expression during stress treatment, their stability and the demonstrated serine phosphorylation of one sHSP suggest the possibility of the above-mentioned specific function or functional hierarchy of particular sHSP species in plant mitochondria. The presented results will be exploited as input data for the further characterization of sHSP complexes.

Materials and methods

Plant material and cultivation conditions

Tobacco cell strain VBI-0, derived from the stem pith of *Nicotiana tabacum* L. cv. 'Virginia Bright Italia' (Opatrný and Opatrná 1976), was cultivated in standard Heller liquid medium (Heller 1953) supplemented with the auxins, alpha-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid (each 5×10^{-6} *M*). Cells were subcultured every 2 weeks (inoculation density of approximately 5×10^4 cells ml^{-1}) and cultivated in darkness on an orbital shaker (IKA KS501) at 180 rpm (diameter, 30 mm). For the heat stress treatment, the temperature was set to 40°C. To identify stress-induced proteins (SIPs) and to follow the kinetics of their expression and degradation, cell cultures were pulse or pulse-chase labelled with an L-[^{14}C]-amino acid mixture (1.85 GBq mg^{-1} atom carbon, Amersham Biosciences, Little Chalfont, UK; 10 kBq ml^{-1} medium). The duration of the stress treatments and labelling periods are given in the figure legends of the relevant experiments.

Isolation and subfractionation of mitochondria

Suspension cultures were separated from the cultivation medium by filtration and immediately used for mitochondrial isolation. Intact mitochondria were isolated as described previously (Hájek et al. 2004). Plant material was homogenized between two nylon meshes and centrifuged stepwise in three steps (2000 *g*/5 min, 6000 *g*/10 min and 40 000 *g*/80 min) with PERCOLL solution in the last step. Mitochondria forming a yellowish ring in PERCOLL solution were collected by filtration and immobilized on the surface of a 0.22 μm nylon filter.

Isolated mitochondria were further analysed by subfractionation as follows: filters were covered with 1 ml of hypotonic buffer (150 m*M* Bis-Tris/HCl, pH 7.0, 1.5 *M* aminohexanoic acid, 0.4 m*M* phenylmethylsulphonyl fluoride (PMSF), 5 μg ml^{-1} leupeptin, pepstatin and aprotinin, according to Schagger et al. 1994), and protein complexes were released by freezing and thawing. Matrix and membrane-bound mitochondrial subfractions were separated according to Hájek et al. (2004).

Protein extraction and electrophoretic separation

Proteins from whole mitochondria and from individual subfractions were quantitatively isolated by the phenol/chloroform protocol as described previously (Hájek et al. 2004).

Extracted mitochondrial proteins were separated by one-dimensional (1-D) and two-dimensional (2-D) SDS-PAGE. The first 2-D SDS-PAGE run was performed on a Protean IEF Cell (Bio-Rad, Hercules, CA), with a strip size of 11 cm and an ampholyte range of pH 3–10 (Bio-Rad). The second dimension (15% SDS-PAGE) was performed on a Biometra (Whatman Biometra, Goettingen, Germany) apparatus according to Laemmli (1970). Separated proteins were visualized by silver staining of polyacrylamide gels according to Blum et al. (1987). Radioactively labelled proteins were visualized by fluorography. For mass spectroscopy, proteins were stained with Coomassie Brilliant Blue-R (86%, Serva, Heidelberg, Germany).

For immunodetection, separated proteins were transferred onto nitrocellulose membranes (NC 45; Serva) by semidry blotting (Towbin and Gordon 1984). Protein phosphorylation was detected by commercially available monoclonal antibodies developed against phosphoserine, phosphothreonine and phosphotyrosine, according to the manufacturer's instructions (Sigma Aldrich Corp., St. Louis, MO). [^{14}C]-Labelled proteins were visualized by fluorography after 14 days of X-ray film (Kodak, Rochester, NY) exposure.

2-D patterns were analysed by Lucia G image analysis software, vs. 4.7 (Laboratory Imaging, Prague, Czech Republic, www.lim.cz).

Native electrophoresis of mitochondrial protein complexes

Isolated mitochondria were disrupted in hypotonic native electrophoresis loading buffer (150 m*M* Bis-Tris/HCl pH 7.0, 1.5 *M* aminohexanoic acid, 0.4 m*M* PMSF, 5 μg ml^{-1} leupeptin, pepstatin and aprotinin mixture) and the mixture was layered on the top of preparative 3–15% polyacrylamide gradient gel (Schagger et al. 1994). Protein complexes separated on preparative native gels were cut from the gel and small pieces were placed into wells for 1-D SDS-PAGE. Radioactively labelled monomers of disassembled protein complexes were visualized by fluorography.

Determination of amino acid uptake and incorporation

VBI-0 cells were heat stressed for 1 h in the presence of [^{14}C]-labelled L-amino acid mixture (Amersham Pharmacia Biotech). Mitochondrial and cytoplasmic fractions were isolated according to Hájek et al. (2004).

For amino acid uptake determination, 200 μl of sample was mixed with 800 μl H_2O and 7.5 ml of scintillation solution (mixture of two volumes of 0.7% 2,5-diphenyloxazol in toluene with one volume of Triton X-100). Radioactivity was measured by scintillation counting (Packard Tri Carb 2900TR; GMI Inc., Ramsey, MN).

Amino acid incorporation was determined by mixing 500 μl of sample with 100 μl of 60% TCA. The mixture was boiled for 20 min and the resulting protein precipitate was quantitatively collected by filtration on GF/C glass filters (Whatman Biometra). Filters were washed with acetone, dried and overlaid with 7.5 ml of scintillation solution (0.7% 2,5-diphenyloxazol in toluene). Radioactivity was measured by scintillation counting.

Partial peptide mapping

Proteins from isolated heat-stressed mitochondria were separated by 2-D SDS-PAGE and SIPs were cut from the gel. Gel fragments containing analysed proteins were inserted into wells of the gradient SDS polyacrylamide gel (3% stacking gel; 10–20% polyacrylamide resolving gel). Gel slices were overlaid with the sample buffer containing 100 ng of endoproteinase V8 per well. Samples were focused in the stacking gel at a constant 80 V and then digested without electric current at 20°C for 45 min. Partial protein fragments were separated in the resolving gradient gel at 180 V at 12°C. Separated peptides were silver stained according to Blum et al. (1987), but with 4 mg ml^{-1} of $AgNO_3$ in the staining solution.

Mass spectrometry

Proteins were cut from 2-D gels and subjected to in-gel tryptic digestion (Shevchenko et al. 1996). The resulting partially digested peptide mixture was analysed by nano-electrospray quadripole time of flight (ESI-QUAD-TOF) mass spectrometry (QTOF II,

Micromass, Manchester, UK) according to Wilm et al. (1996). The data obtained were interpreted using Masslynx software. The resulting data were analysed by Matrix software (www.matrixscience.com/cgi/) and fragments were sequence identified by database search against several publicly available databases (SwissProt, www.ebi.ac.uk/swissprot/; NCBI nr, www.ncbi.nlm.nih.gov; and dbEST, www.ncbi.nlm.nih.gov/dbEST/) using the MASCOT search engine (Perkins et al. 1999). Unannotated expressed sequence tag (EST) hits were further analysed by BLAST searches (www.ncbi.nlm.nih.gov/BLAST/; http://www.arabidopsis.org/Blast/) using the peptide fragments obtained (Altschul et al. 1990).

Results

Heat stress-induced proteins of low molecular weight

To follow the changes in the pattern of SIPs, a VBI-0 cell suspension was treated at 40°C for 2 h in the presence of [^{14}C]-labelled amino acids. The application of heat stress treatment had no substantial effect on the total or cytoplasmic uptake of the radioactive label, which fell within the range of 300 000–350 000 dpm. In contrast, the translation efficiency under stress conditions, expressed as the level of total and cytoplasmic incorporation of [^{14}C], decreased by more than 50% compared with the non-stressed control. In mitochondria, the situation was quite different. During heat-shock treatment, a similar decrease in the level of de novo synthesized proteins accompanied a substantial decrease in amino acid uptake. This demonstrates impaired free amino acid import from the cytosol to the mitochondria by stress.

The application of stress conditions led to the emergence of a set of newly synthesized small proteins in the mitochondrial fraction with a molecular weight of the order of 21 kDa, as visualized by 1-D SDS-PAGE fluorography (Fig. 1). A more detailed examination by 2-D SDS-PAGE of mitochondrial protein spectra resolved the few protein bands visible by 1-D electrophoresis into a set of more than 30 low-molecular-mass heat SIPs (Fig. 2A). Under identical labelling and fluorography conditions, almost no de novo synthesized proteins were detected in a non-stressed control (Fig. 2B). The broad spectrum of total mitochondrial proteins in the control variant on a 2-D gel silver stained prior to fluorography (Fig. 2C) verified the good quality of protein separation, and demonstrated the expression of SIPs only after the onset of stress, accompanied by the weak expression of other mitochondrial proteins during 30 min of labelling.

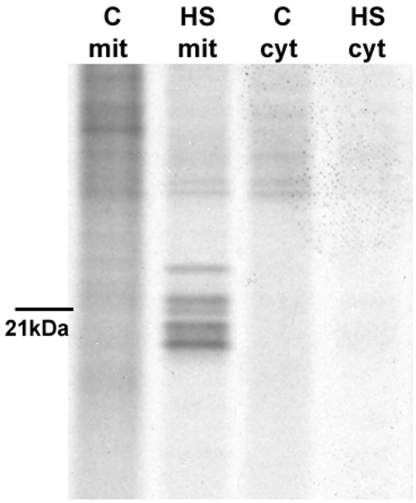

Fig. 1. One-dimensional SDS-PAGE of [^{14}C]-labelled proteins in two subcellular fractions of control (C) and 40°C heat-stressed (HS) tobacco (Virginia Bright Italia-0, VBI-0) cell suspension culture. Cells were heat stressed for 2 h and were labelled for the last 30 min. cyt, cytosol; mit, mitochondrial fraction.

According to the intensity of SIP expression, 10 proteins were selected for further analysis. Using a combination of 2-D and 1-D markers (Bio-Rad), the molecular weight and pI values for selected proteins were determined (see legend to Fig. 2A). The molecular weights of all selected SIPs ranged from 18 to 23 kDa. Eight of the 10 SIPs exhibited acidic characteristics with pI values ranging from 5.1 to 6.0. Only two proteins (SIP5 and SIP10) possessed neutral pI values (7.4 and 7.5), documenting that these two proteins were not consistent with the acidic character of the rest of the SIP group.

In order to determine whether SIPs were encoded by the nuclear or mitochondrial genome, tobacco VBI-0 cells were cultivated under stress conditions in the presence of cycloheximide and chloramphenicol. Both drugs are potent inhibitors of translation elongation. Cycloheximide inhibits the translation of nuclear-encoded mRNAs in the cytosol of eukaryotic cells, and chloramphenicol, acting in bacteria, inhibits the translation of only those eukaryotic mRNAs which are encoded by mitochondrial and chloroplast genomes (see Alberts et al. 2002). In the presence of 100 mg ml^{-1} cycloheximide, the synthesis of all 10 SIPs was completely suppressed and the fluorogram was clear, even without low expression, as shown in Fig. 2B. On the other hand, 200 mg ml^{-1} chloramphenicol had no effect on SIP synthesis and the fluorogram corresponded to Fig. 2A. This demonstrates that all SIPs are encoded by the nuclear genome.

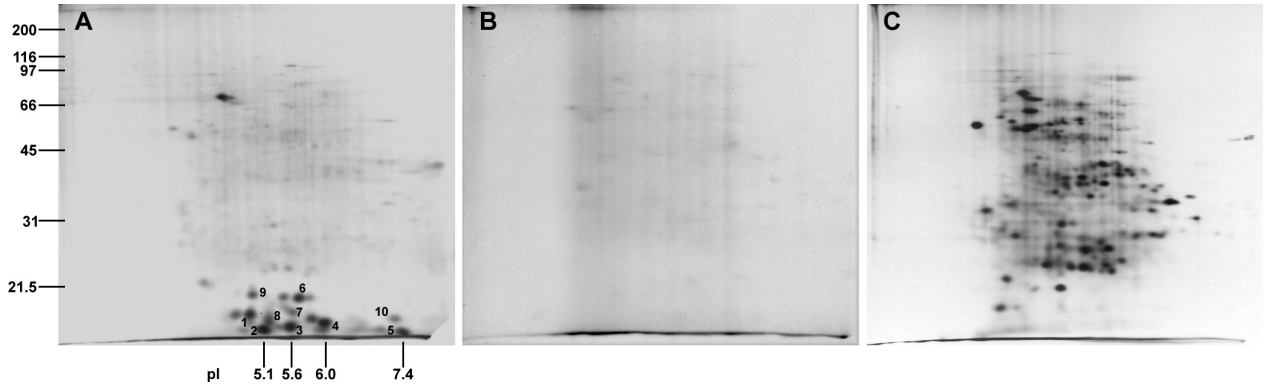

Fig. 2. Two-dimensional SDS-PAGE separation of Virginia Bright Italia-0 (VBI-0) mitochondrial proteins. (A) Fluorogram of de novo synthesized proteins after 2 h of heat stress and [^{14}C]-labelling for the last 30 min. The ten most intense stress-induced proteins (SIPs) were characterized: SIP1 (20 kDa, pI = 5.1), SIP2 (18 kDa, pI = 5.3), SIP3 (18.5 kDa, pI = 5.6), SIP4 (19 kDa, pI = 6.0), SIP5 (18.5 kDa, pI = 7.5), SIP6 (22.5 kDa, pI = 5.8), SIP7 (20 kDa, pI = 5.7), SIP8 (20 kDa, pI = 5.4), SIP9 (23 kDa, pI = 5.2), SIP10 (21 kDa, pI = 7.4). (B) Fluorogram of de novo synthesized proteins after 30 min of [^{14}C]-labelling of the control variant. (C) Silver-stained total proteins visualized on the gel used for fluorography of the control variant (B).

Identification of heat stress-induced proteins

Mitochondrial SIPs separated by 2-D SDS-PAGE and immobilized on polyvinylidene difluoride (PVDF) membranes were N-terminally sequenced using Edman's degradation. The N-terminal microsequence of nine of the 10 proteins was unsuccessful because of the modification of their amino termini. SIP6 was the only successfully N-terminally sequenced protein. The resulting sequence, LMPYTRP, exhibited limited homology with three proteins, all of which are putative transcription factors. These are *Caenorhabditis elegans* homeobox protein YRM6_CAEEL Q09602, *Euglena gracilis* mitochondrial inner membrane complex III zinc finger protein CR9_EUGGR P43266 and *Candida albicans* putative transcriptional activator 1_CANAL P33181.

More precise analysis of mitochondrial heat SIPs was performed using mass spectrometry. Individual SIPs were separated by 2-D SDS-PAGE, excised from the gels and analysed by ESI-QUAD-TOF mass spectrometry. The peptide fragments resulting from mass spectrometry analyses are shown in Table 1. The analysis of peptide fragments revealed that SIP1, 2, 3, 4, 5 and 7 fell into the family of sHSPs. Their scores in MASCOT software analysis (www.matrixscience.com/cgi; Perkins et al. 1999) were 119, 29, 104, 35, 38 and 72, respectively. The lower scores obtained for proteins SIP8 and SIP9 (27 and 31) led to their classification into the sHSP family with a lesser degree of probability. According to their shared peptides, SIPs were divided into two subgroups. The first subgroup was formed by proteins SIP3,

Table 1. Peptide fragments of stress-induced proteins (SIPs) identified by mass spectrometry. HSP, heat-shock protein; sHSP, small HSP.

SIP	Fragment	GenBank	Species	Annotation
SIP1	AAMENGVLTVTVPKEEVK	T06449	*Pisum sativum*	Putative HSP
SIP2	VQVEDDNVLLISGER	AAP33012	*Citrus x paradisi*	HSP19 class II
	EYPNSYVFIDDMPGLK	AAP33012	*Citrus x paradisi*	HSP19 class II
SIP3	AMAATPADVK	CAA12390	*Lycopersicon peruvianum*	HSP20.2
	VQVEDDNVLLISGEK	CAA12390	*Lycopersicon peruvianum*	HSP20.2
	DGVLTVIVQK	CAA12390	*Lycopersicon peruvianum*	HSP20.2
SIP4	VQVEDDNVLLISGER	AAP33012	*Citrus x paradisi*	HSP19 class II
	EYPDSYVFVVDMPGLK	AAC36312	*Lycopersicon esculentum*	sHSP class II HCT2
SIP5	VQVEDDNVLLISGER	AAP33012	*Citrus x paradisi*	HSP19 class II
	EYPDSYVFVVDMPGLK	AAP33012	*Citrus x paradisi*	HSP19 class II
SIP6	VQASIAANTWVVSGSPQTK	NP 173230	*Arabidopsis thaliana*	Transcript. factor At1g17880
SIP7	AAMENGVLTVTVPKEDVK	AAM28293	*Ananas comosus*	sHSP class I
SIP8	VLQISGER	CYPZ77	*Daucus carota*	sHSP17.7
SIP9	ASMENGVLTVTVPK	CAA53286	*Oryza sativa*	HSP17.8
SIP10	No homology to known protein			

SIP4 and SIP5. The second subgroup comprised SIP1, SIP2 and SIP7. Two proteins, SIP6 and SIP10, did not belong to the sHSP family. SIP6 protein shared homology with transcription factors, as also suggested by N-terminal sequencing results (see above), and SIP10 exhibited no homology to any protein present in the available databases.

The database homology search revealed *Arabidopsis thaliana* homologues of SIPs. For SIPs 1, 2, 3, 4, 5, 7 and 9, homology was found only to sHSPs. Their alignment with SIP fragments is shown in Fig. 3. Peptide fragments of SIP8 shared no homology to any *Arabidopsis* protein. Fragments of SIP6 were homologous to transcription factor (At1g17880; pI = 7.5; 17.9 kDa) and to putative transcription factor BTF3 (At1g73230; pI = 5.9; 18 kDa).

Partial peptide mapping represented the last method used for SIP characterization. Mass spectrometry data based on short conservative peptide fragments (Table 1) were extended to non-conserved protein regions. SIPs separated by 2-D SDS-PAGE were excised from the gel and subjected to partial peptide mapping using the V8 endoproteinase. Maps of 1-D SDS-PAGE-separated peptides were visualized by silver staining. Eight peptide maps were analysed; there was insufficient material recovered for SIP9 and SIP10 analyses (Fig. 4). According to their partial peptide maps, several groups of SIPs were distinguished. The first group comprised SIP3, SIP4 and SIP5, which exhibited a high degree of similarity, as already suggested by mass spectrometry. The partial peptide maps of the other proteins differed from one another and proteins SIP1, SIP2, SIP6, SIP7 and SIP8 formed individual groups.

Kinetics of synthesis and stability of heat stress-induced proteins

The kinetics of SIP expression were studied during heat stress treatment in the presence of [^{14}C]-labelled amino acids, and were quantified using Lucia software (Fig. 5). The first faint signal of newly synthesized SIPs in mitochondria was detectable on the fluorogram after 10 min of stress treatment. When cell cultures were labelled for the last 30 min of stress treatment, a distinct pattern of proteins appeared after 30 min of stress (data not shown) and remained identical for 90 and 120 min of stress. After 2 h of stress treatment, an increase of over 90% in the quantity of all SIPs was observed (Fig. 5, 2 h). Nine of the 10 selected SIPs (i.e. all except SIP5) were synthesized throughout the 12 h heat stress period. These proteins exhibited very similar expression patterns (Fig. 5, 12 h). The peak synthetic activity of all SIPs was observed after 6 h of stress treatment, when their expression levels were 1.5–15 times higher than

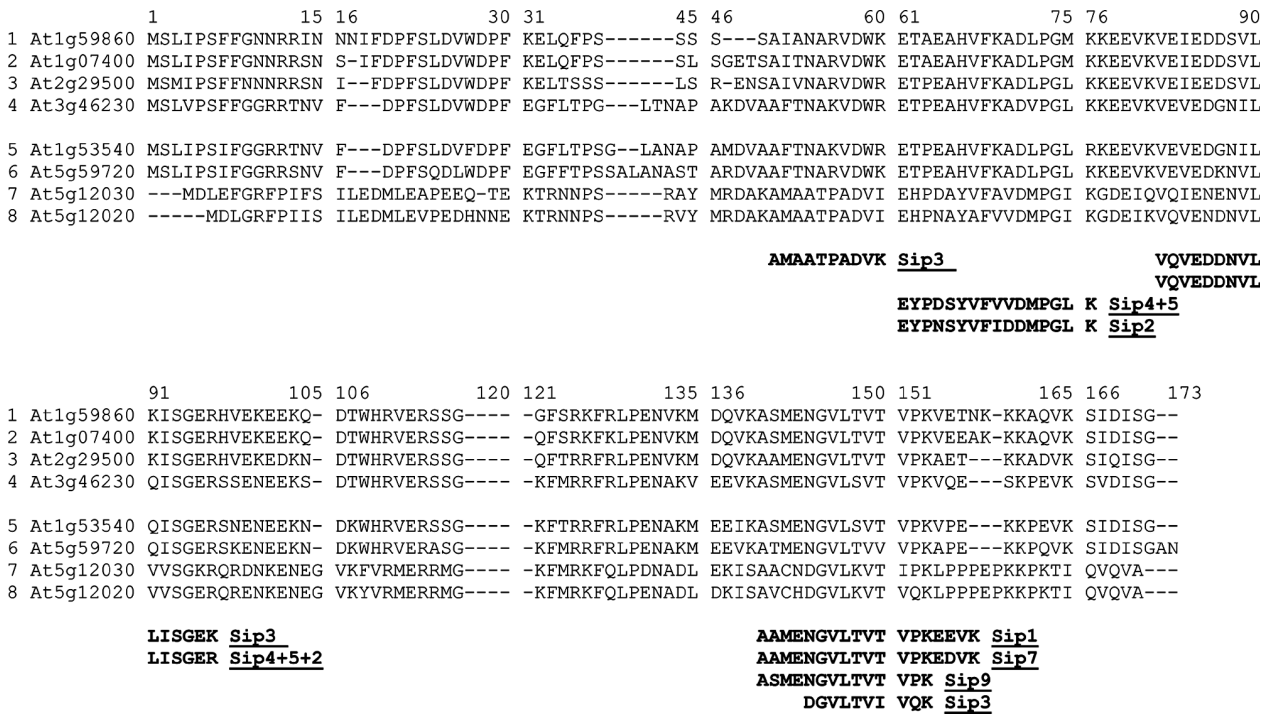

Fig. 3. Sequence alignment of stress-induced protein (SIP) peptide fragments identified by mass spectrometry with *Arabidopsis thaliana* small heat-shock proteins.

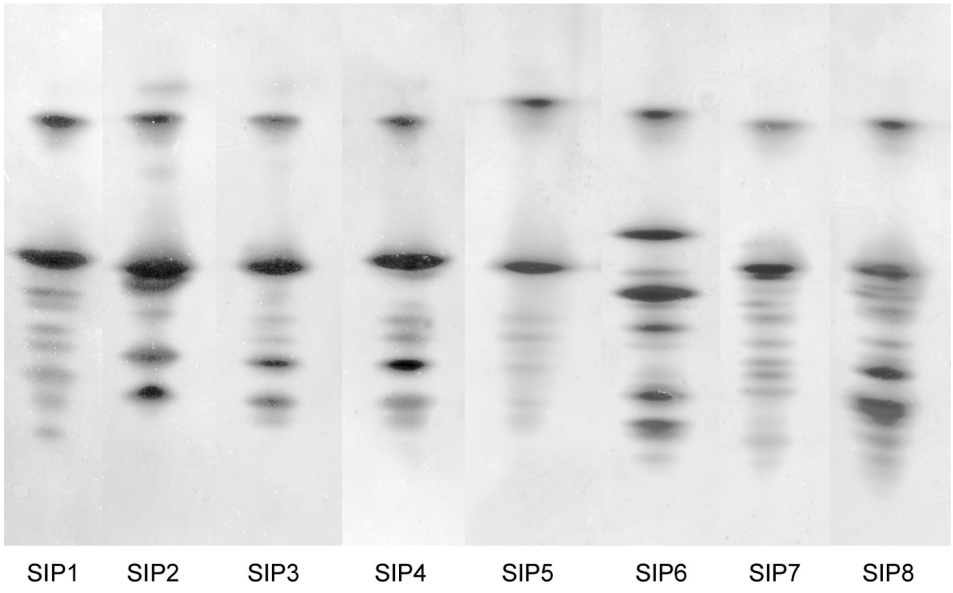

Fig. 4. Partial peptide maps of eight stress-induced mitochondrial proteins from Virginia Bright Italia-0 (VBI-0) cell suspension culture. Three to four spots were cut from corresponding two-dimensional SDS-PAGE. Spots were placed into one-dimensional SDS-PAGE wells and cleaved by V8 endoproteinase for 90 min at 20°C.

those after 2 h of stress. The maximum increase in synthetic activity was detected in proteins SIP1 and SIP6 (6 h signals 7.8 times and 15.5 times higher, respectively, than those at 2 h). Significant changes in individual expression profiles were observed after 12 h of stress treatment. Expression levels of SIP3, 4 and 10 decreased below the level at 2 h, the expression level of SIP2 decreased to the 2 h level and the synthesis of SIP1, 6, 7, 8 and 9 remained higher than that at the 2 h level throughout.

The stability of de novo synthesized SIPs was examined by pulse-chase experiments. VBI-0 cell suspension cultures were labelled with a [^{14}C]-amino acid mixture for 1 h at 40°C. The remaining free radioactive amino acids were washed out immediately after the 1 h labelling period, cells were cultivated under standard conditions in medium without label and two chase intervals were followed. The pulse-chase experiment revealed that, 6 h after the onset of stress, the quantity of all SIPs, except SIP6, decreased to 30–15% of the quantity observed immediately after stress (Fig. 6). In contrast, SIP6 was extremely stable and its amount remained the same throughout the 12 h of the pulse-chase experiment. After 24 h, all induced proteins were still detectable but, with the exception of SIP6, only at negligible

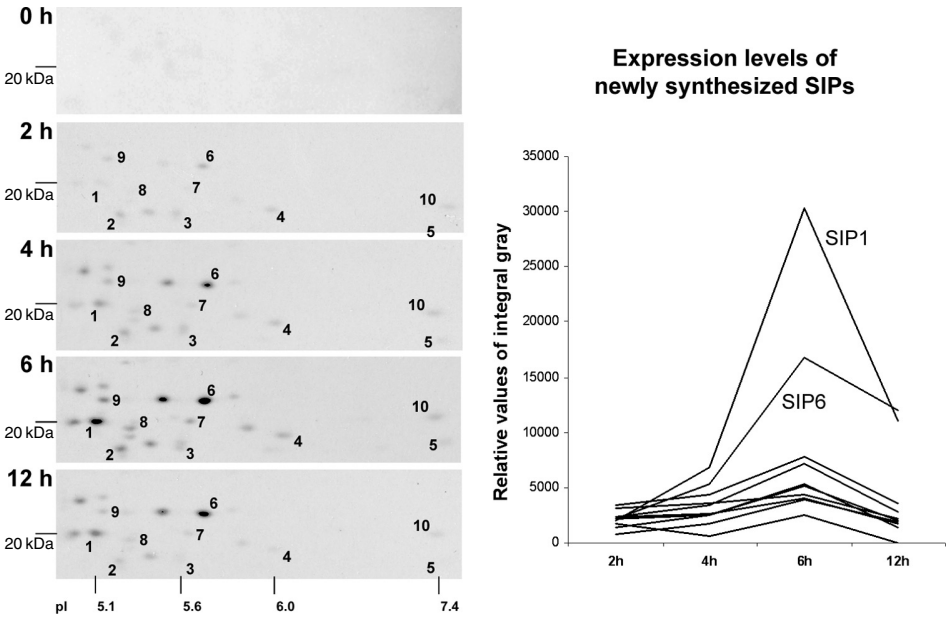

Fig. 5. Kinetics of expression of heat stress-induced proteins (SIPs) in the mitochondrial fraction of tobacco (Virginia Bright Italia-0, VBI-0) cells. Cells were heat stressed for 2, 4, 6 and 12 h, and [^{14}C]-labelled mitochondrial proteins synthesized during the last 30 min of each stress treatment period were identified by two-dimensional SDS-PAGE fluorography. Images were enhanced digitally to increase contrast.

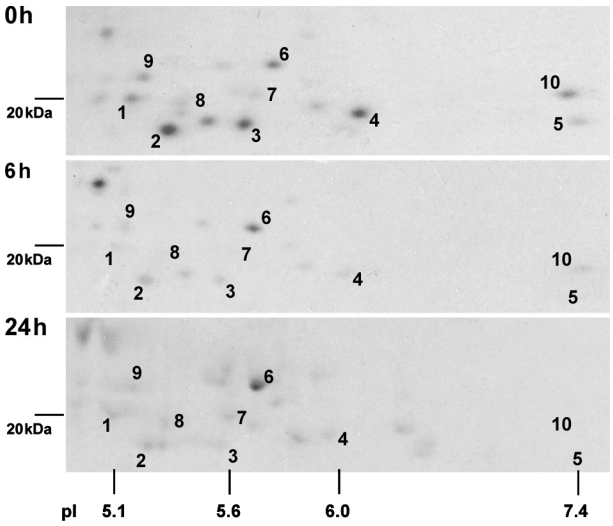

Fig. 6. Stability of heat stress-induced proteins in the mitochondrial fraction of tobacco (Virginia Bright Italia-0, VBI-0) cells was analysed by pulse-chase experiments. A heat stress period of 12 h was followed by [^{14}C]-pulse labelling and subsequent 6 or 24 h chase. Images were enhanced digitally to increase contrast.

levels. SIP6 was very stable and its signal did not decrease significantly after a 24 h chase.

The sensitivity of VBI-0 cells to heat stress during a standard cultivation cycle, as indicated by SIP synthesis, was followed. Both qualitatively and quantitatively, the pattern of expression of SIPs remained stable during the whole culture cycle. However, the most intense synthesis of the 10 selected SIPs was observed on the seventh day of subculture, during the cell elongation phase (Fig. 7).

Post-translational modifications of heat stress-induced proteins

The phosphorylation of stress-induced mitochondrial proteins was tested immunologically using monoclonal antibodies developed against phosphoserine, phosphothreonine and phosphotyrosine. The antiphosphoserine antibody treatment highlighted SIP1 especially and SIP10 faintly (Fig. 8). In contrast, treatment with antiphosphothreonine and antiphosphotyrosine antibodies gave no positive signal for any protein studied.

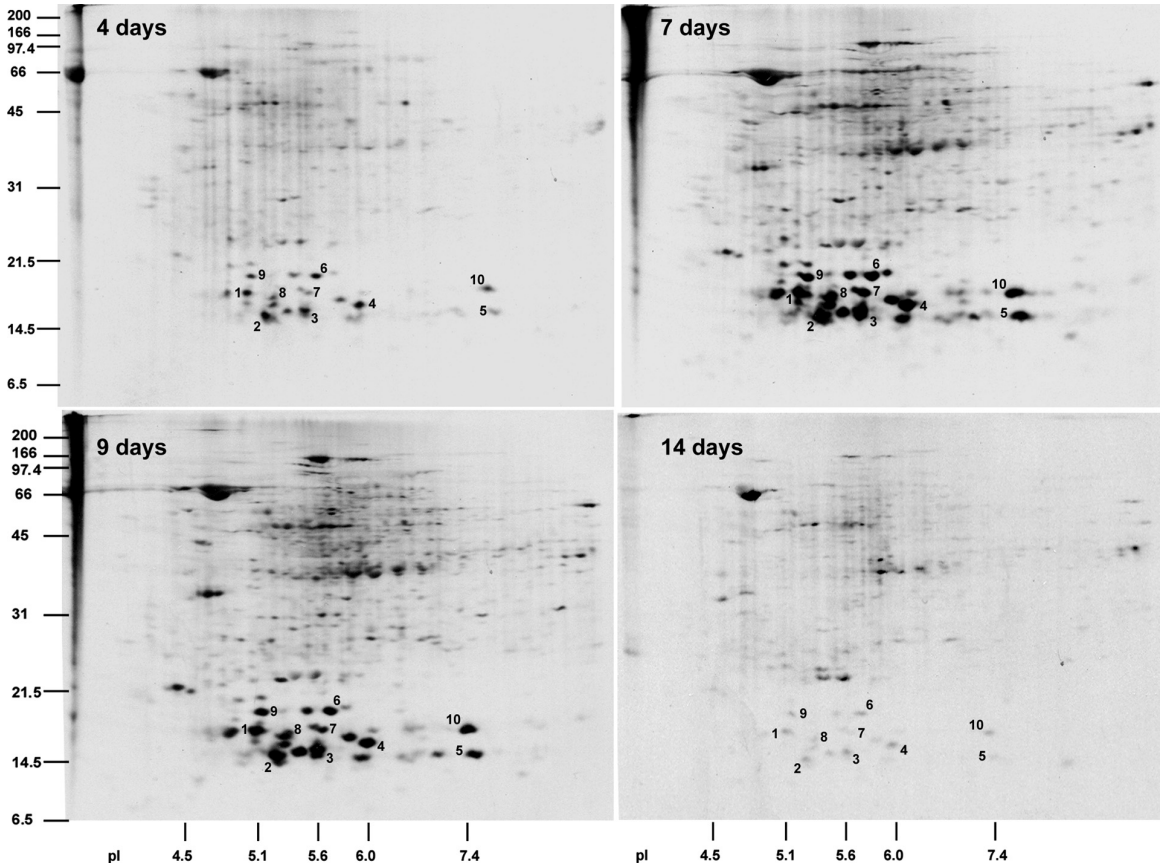

Fig. 7. Sensitivity to heat stress treatment in individual phases of the Virginia Bright Italia-0 (VBI-0) cell cultivation interval. A stress period of 1 h in the presence of [^{14}C]-label was applied to cells cultivated for 4, 7, 9 and 14 days, and stress-induced proteins in the mitochondrial fraction were visualized by two-dimensional SDS-PAGE fluorography.

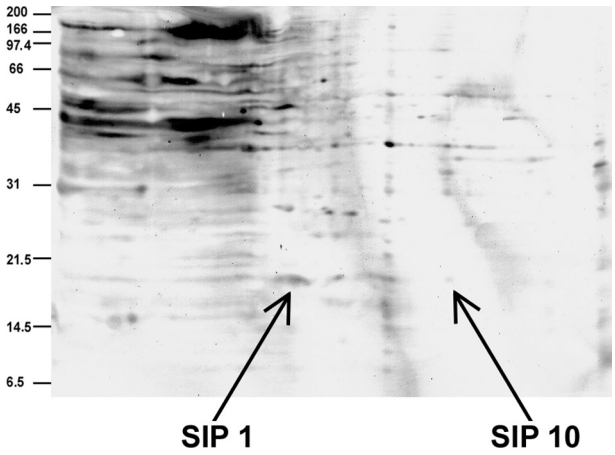

Fig. 8. Immunodetection of the phosphorylated form of stress-induced proteins (SIPs) after 2 h of heat stress in the mitochondrial fraction of tobacco (Virginia Bright Italia-0, VBI-0) cells. Mitochondrial proteins were separated by two-dimensional SDS-PAGE, blotted and probed with antiphosphoserine monoclonal antibody. Phosphorylated SIP1 and SIP10 are marked.

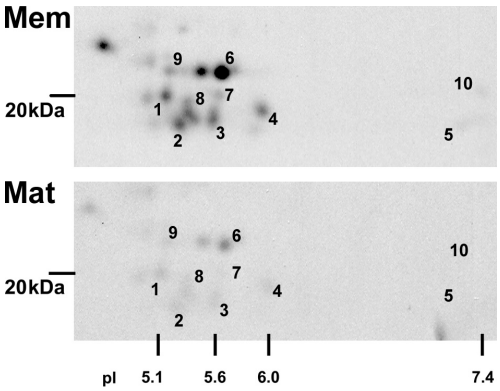

Fig. 9. Localization of heat stress-induced proteins in the mitochondria of tobacco (Virginia Bright Italia-0, VBI-0) cells. The two-dimensional SDS-PAGE fluorography patterns of [^{14}C]-labelled mitochondrial proteins of two mitochondrial subfractions were compared: membrane (Mem) and matrix (Mat).

Localization of heat stress-induced protein complexes in mitochondria

Cell suspension cultures of VBI-0 were heat stressed in the presence of a [^{14}C]-labelled amino acid mixture for 4 h. Mitochondrial membrane and matrix fractions were separated, and proteins from both fractions were analysed by 2-D SDS-PAGE. Newly synthesized SIPs were found preferentially in the mitochondrial membrane fraction (Fig. 9).

Protein complexes from heat-stressed mitochondria were separated by native gel electrophoresis on a 3–15% gradient gel. The gel was cut longitudinally into 25 pieces and the proteins in each piece were separated by SDS-PAGE. This process disrupted protein complexes into monomers, which were detected by fluorography (Fig. 10). SIPs were predominantly localized together in fractions 9–14 within the zone of the gradient gel corresponding to approximately 3–10% polyacrylamide, indicating the existence of functional high-molecular-weight complexes of heat SIPs in mitochondria. Free SIPs were not detected in heat-stressed mitochondria.

Discussion

The induced expression of a cluster of low-molecular-weight proteins as a specific reaction of plant cells to stress treatment has been repeatedly described by many authors (Neumann et al. 1989, Vierling 1991, Hsieh et al. 1992, Waters et al. 1996, Banzet et al. 1998). However, an explanation of the role of these particular protein species in stress response is missing. The use of tobacco cell suspension cultures (strain VBI-0) enabled the exploration of the kinetics of the synthesis and successive degradation of these proteins during the whole period of stress treatment and after its termination, exploiting the pulse-chase technique. No quantitative changes in [^{14}C]-amino acid uptake by tobacco cells under stress conditions were detected, and a considerable decrease in [^{14}C]-label incorporation under stress represented the commonly accepted effect of stress on the total translation efficiency. The broad spectra of the constitutively synthesized proteins under physiological conditions were exchanged in favour of fewer strongly expressed SIPs. Cellular levels of [^{14}C]-free amino acids were sufficient to analyse de novo protein synthesis in the control as well as under stress conditions.

Heat stress treatment of VBI-0 cells triggered a strong reaction in mitochondria (Fig. 1), in comparison with an incomparably weaker reaction in the cytoplasm. The isolation of mitochondria, exploiting a rapid and reproducible method (Hájek et al. 2004), offered a good chance to analyse the mitochondrial stress response. From the large group of de novo synthesized low-molecular-weight heat SIPs, the 10 most intensively expressed proteins were selected for further study (Fig. 2). This cluster of 10 SIPs was sufficiently abundant to compare them at the level of the biochemical characteristics and expression kinetics of particular species.

Proteomic analyses revealed that SIP1, 2, 3, 4, 5 and 7 belonged to the family of sHSPs, while SIP8 and 9 could also be classified as sHSPs with a high degree of probability (Table 1). Two remaining SIPs analysed in this work did not belong to the sHSP group. SIP10, with a neutral pI value of 7.4, showed no homology to any

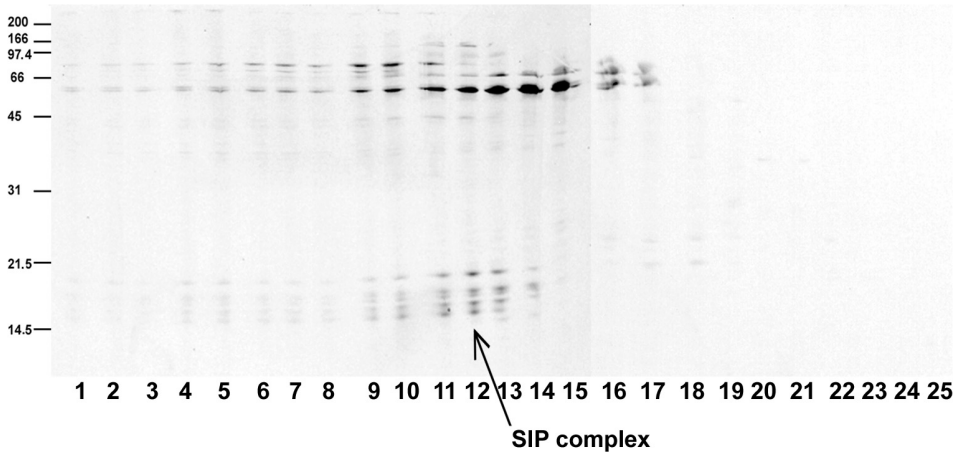

Fig. 10. Fluorogram of proteins comprising heat stress-induced macromolecular complexes synthesized in tobacco (Virginia Bright Italia-0, VBI-0) cells. Mitochondrial protein complexes were separated by native 3–15% gradient gel electrophoresis. Native gel was cut into 25 fragments which were run on one-dimensional SDS-PAGE to release individual proteins. Position of the stress-induced protein (SIP) complex is marked.

known protein sequence. SIP6 shared a significant degree of homology with transcription factors from several different organisms. However, both of these proteins were always detected together with the sHSP group. Moreover, SIP6 was localized strictly to mitochondria. Consequently, one possible function of this protein may be the repression of mitochondrial transcription under stress conditions, perhaps through binding of the protein to mitochondrial DNA. These suggestions were based on findings that plant mitochondria lose their DNA during cell differentiation (Fujie et al. 1994).

Basic biochemical data from the analyses of the sHSPs with molecular masses ranging from 18 to 23.5 kDa (Fig. 3) corresponded with the data previously published for sHSP22 from *Pisum sativum* (Lenne et al. 1995), sHSP22 from *Zea mays* (Lund et al. 1998), sHSP23 from *Chenopodium rubrum* (Debel et al. 1997) and sHSP23.5 from *Arabidopsis thaliana* (Visioli et al. 1997). Seven of the eight characterized stress-induced sHSPs exhibited acidic characteristics in agreement with previously published data on other sHSPs (Morrow et al. 2000). SIP5, with a neutral pI value of 7.5, was the only exception. However, SIP5 shared the same peptide fragment identity with SIP3 and SIP4. Together, these proteins formed a distinct subgroup within the set of eight characterized SIPs. The close sequence relationship of these proteins was confirmed by partial peptide mapping (Fig. 4). Sequence fragments obtained for these proteins were then compared with 28 sequences of *Arabidopsis thaliana* sHSPs downloaded from the TAIR server (www.arabidopsis.org). When compared with the eight most similar sHSPs, it became clear that the amino acid sequences of all peptide mass digest fragments suitable for database searches lay strictly within two of the most conserved amino acid sequence domains of sHSPs (Fig. 4).

The 2-D pattern of sHSPs detectable 30 min after stress (Fig. 2A) remained qualitatively stable throughout the 12 h stress treatment and was maintained for up to 12 h after its termination (data not shown). An identical pattern was observed during the course of the whole subculture interval. However, higher levels of all SIPs during the cell elongation phase suggested higher sensitivity to stress at this stage (Fig. 7). Quantitative analyses utilizing pulse-chase experiments confirmed the frequently observed intense expression of sHSPs induced by stress and the high stability of these molecules after the termination of stress conditions (Lenne et al. 1995, Waters et al. 1996). A 30 min pulse-label during the course of stress demonstrated two phases of synthetic activity for all SIPs. Synthesis increased during the first 6 h of the stress treatment, when it reached a maximum, and then decreased continuously for a further 6 h. However, levels of synthetic activity for individual sHSP species were found to be different. The synthesis of sHSP22 (SIP1) was significantly high throughout the whole stress period. In contrast, the synthesis of sHSP18.5 (SIP5) was so low that, by the end of the stress period, the protein was undetectable (Fig. 5). The stability of sHSP molecules was verified by 1 h pulses followed by a 6 h and 24 h chase. After 6 h, the qualitative pattern of protein synthesis was unchanged, with only a slight quantitative decrease of 15–30%. A substantial decrease in synthesis occurred 18 h later. Only SIP6, a transcription factor homologue, which showed the highest synthetic activity during the 12 h stress interval, exhibited an extremely stable and high quantitative level even after 24 h of the pulse-chase experiment (Fig. 6)

Inspired by the previously published data on maize mitochondrial HSP22 phosphorylation (Lund et al. 1998), we performed phospho-immunoassays of the

analysed SIPs. Despite the preliminary character of the phospho-immunotests performed, the observed serine phosphorylation of SIP1 represents one more key result of this study. Moreover, the mass spectrometry sequence fragment of the 20 kDa SIP1 shared significant homology with the C-terminal part of maize HSP22. Within their 14-amino acid aligned region, these sequences were 71% identical with only three mismatches (data not shown). The combination of the above results strongly suggests that SIP1 represents a tobacco homologue of the previously described maize HSP22 (Lund et al. 1998). In animal cells, the relationship between the serine phosphorylation status of sHSP27 and the oligomerization/dissociation of sHSP complexes has been documented (Ehrnsperger et al. 1997, Haslbeck et al. 1999). The sHSP27 phosphorylation is also known to influence actin cytoskeleton dynamics and to modulate actin filament stability (Guay et al. 1997). Taken together, the observed strong phosphorylation of the mitochondrial HSP within SIPs introduces heterogeneity into this coordinately induced protein cluster, and is likely to reveal a new perspective on organized sHSP function in plant mitochondria in connection with other structures.

The exclusive presence of newly synthesized small heat SIPs in the mitochondria of stressed VBI-0 cells suggests an important role for this organelle in the exponentially growing plant cell. The majority of SIPs colocalized with the mitochondrial membrane fractions, whereas, in contrast, matrix fractions contained only traces of their presence (Fig. 9). Moreover, no free sHSPs were detected in mitochondria and the cluster of SIPs was shown to be stable and to form high-molecular-weight complexes. Newly synthesized SIPs were localized only in high-molecular weight complexes (Fig. 10). We conclude that SIPs, in agreement with the literature, form homopolymeric complexes assembled from a broad spectrum of subunits (Nover et al. 1989, Helm et al. 1993), and these complexes are likely to participate in the formation of high-molecular-weight HSP chaperones described previously (Lee et al. 1997, Smýkal et al. 2000).

Conclusions

The data presented here clearly demonstrate the complexity of the heat stress response in tobacco VBI-0 cell suspension culture. In non-differentiated, proliferating cells, heat stress preferentially induced the synthesis of a significantly stable group of SIPs in mitochondria. Within the SIP group, eight sHSPs were accompanied by one transcription factor and one protein with unknown function. sHSPs represented a heterogeneous cluster of proteins synthesized in variable quantities. Moreover, the preferentially synthesized protein SIP1 (sHSP22) was found to be serine phosphorylated. SIPs formed multimolecular protein complexes localized in the mitochondrial membranes.

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