

Plant Hsp90 family with special reference to rice

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Hsp90 family represents a group of highly conserved and strongly expressed proteins present in almost all biological species. Heat shock proteins in the range of 90 kDa have been detected in a range of plant species and *hsp90* genes have been cloned and characterized in selected instances. However, the expression characteristics of plant Hsp90 are poorly understood. Work on expression characteristics of rice Hsp90 is reviewed in this paper. Experimental evidence is provided for indicating that while the rice 87 kDa protein is transiently synthesized within initial 2 h of heat shock, high steady-state levels of this protein are retained even under prolonged high temperature stress conditions or recovery following 4 h heat shock. It is further shown that fifteen different wild rices accumulate differential levels of these proteins in response to heat shock treatment.

1. Introduction

Nearly all organisms ranging from bacteria to man respond to heat shock (HS) by synthesizing a set of novel proteins called heat shock proteins (Hsps). In plants, Hsps were reported for the first time in soybean suspension cells (Barnett *et al* 1980) and subsequently these proteins have been detected in a number of plant species (Singla *et al* 1997b). Detailed information on various classes of Hsps with respect to their molecular and biochemical characteristics has been covered in several reviews/chapters (Morimoto *et al* 1990; Schlesinger 1990; Vierling 1991; Howarth and Ougham 1993; Parsell and Lindquist 1993; Becker and Craig 1994; Schumann 1996; Csermely *et al* 1997; Singla *et al* 1997b). Hsp90 family represents a group of highly-conserved and strongly-expressed proteins present in almost all species (Lakhotia and Ray 1996; Nardai *et al* 1996; Pal *et al* 1996; Csermely *et al* 1997). *hsp90* genes have been cloned and sequenced from diverse organisms including *Drosophila melanogaster*, yeast, chicken, mammals and bacteria (Csermely *et al* 1998). Importantly, the nucleotide sequence of *hsp90* genes has been found to be highly conserved even amongst the members which are distantly related with respect to evolution. A striking feature of the Hsp90

proteins is that though they are induced by high temperature stress, levels of these proteins are reasonably high even at normal temperatures. The precise role(s) of Hsp90 family members are not known. In recent years, these proteins have been shown to function as molecular chaperones (Becker and Craig 1994; Csermely *et al* 1997). Detailed studies have shown that Hsp90, Hsp70 and Hsp56 form a complex which has been referred to as 'transportosome' or 'foldosome' (Hsp56 is also referred to as p55 or FKBP52 in this complex) (Pratt 1993; Stancato *et al* 1996). In this complex form, Hsp90 proteins have been shown to bring about the activation of steroid hormone receptors towards their binding to hormonal signal.

Hsps in the range of 90 kDa have been detected in a range of plant species (table 1). Further, *hsp90* genes have been cloned and sequenced in some plant species (table 1). High temperature stress adversely affects processes associated with seed germination and anthesis in rice crop (Yoshida 1977; Yoshida *et al* 1981; Pareek *et al* 1997a). While information on isolation and characterization of rice *hsp90* genes has progressed somewhat (see table 1), the expression characteristics of the rice Hsp90 proteins (OsHsp90) are poorly understood. We shall present information on the expression characteristics

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of OsHsp90 proteins in this paper. Little is known about the stability of Hsp90 proteins. We addressed this point by comparing the levels of rice Hsps in gels representing *de novo* synthesized and steady-state proteins. Further, we looked into the expression characteristics of OsHsp90 proteins in fifteen different wild rices by Western blotting.

2. Characteristics of the OsHsp90

2.1 OsHsp90 is a major stress protein

In response to high temperature stress, rice (*Oryza sativa* L. cv Pusa 169) seedlings (5 day old) accumulate 87

(OsHsp87) and 85 (OsHsp85) kDa proteins (collectively referred to as OsHsp90) to a significant extent (Pareek *et al* 1995). It was further shown that OsHsp90 make nearly 10% of the total soluble proteins after 8 h of HS (Pareek 1997).

2.2 OsHsp90 are members of the plant Hsp90 family

Hsp80 of *Neurospora crassa* has been shown to share a remarkable extent of homology with other eukaryotic Hsp90 family of proteins (Roychowdhury *et al* 1992). Anti *N. crassa* Hsp80 antibodies were checked for cross-reaction to rice OsHsp90 and it was found that OsHsp87

Table 1. Selective reports on Hsp90 proteins/genes in plants.

System	Gene/Protein	Characteristics	Mol. wt. (kDa)	Reference
<i>Arabidopsis thaliana</i>	<i>hsp81-1</i>	cDNA clone codes for protein having 66–69% identity with other members of Hsp90 family from microbial and animal systems; at least three genes in the family induced by heat shock and heavy metals	81.1	Takahashi <i>et al</i> (1992)
<i>A. thaliana</i>	pTT10	cDNA clone codes for a protein having 66–69% identity with other Hsp90 family members, a genomic clone <i>hsp81-2</i> isolated; induction by high temperature stress, salinity, IAA application as well as by heavy metals	80.6	Takahashi <i>et al</i> (1992)
<i>A. thaliana</i>	<i>hsp83</i>	cDNA clone codes for a protein having 63–68% homology with other Hsp90 members; at least three genes in the genome; induced by HS at 36°C	81.0	Conner <i>et al</i> (1990)
<i>Brassica napus</i>	<i>hsp90</i>	cDNA clone isolated using <i>hsp90</i> gene probe from <i>Pharbitis nil</i> ; multiple genes, induced by high and low temperatures; maximum transcripts detected in shoot apices and flower buds; high levels present at normal temperature in suspension cultures which decline upon heat shock	90.0	Krishna <i>et al</i> (1995)
<i>Catharanthus roseus</i>	<i>hsp90</i>	cDNA clone codes for a protein having 40% identity with a member of Hsp90 family from <i>A. thaliana</i> , single gene; induced by high temperature and by pathogens	93.4	Schroder <i>et al</i> (1993)
<i>Hordeum vulgare</i>	pBH6-60-1	cDNA clone codes for a protein having nearly 50% identity with members of Hsp90 family; induced by pathogen infection as well as by high temperature stress	92.8	Walther-Larsen <i>et al</i> (1993)
<i>Lycopersicon esculentum</i>	<i>hsc80</i>	cDNA clone codes for a protein having 65–88% identity with members of Hsp90 from other systems, single copy gene; a genomic clone A-4 has also been isolated	80.5	Koning <i>et al</i> (1992)
<i>Oryza sativa</i>	<i>hsp82</i>	cDNA clone codes for a protein having 61–62% identity with members of Hsp90 family from other systems; at least 4–5 copies in the genome; three genomic clones designated as <i>hsp82A</i> , <i>B</i> and <i>C</i> isolated; induced by high temperature stress	82.0	Breusegem <i>et al</i> (1994)
<i>Pharbitis nil</i>	<i>hsp83A</i>	cDNA clone codes for a protein having 70% amino acid identity with a member of Hsp90 family from <i>Drosophila</i> , at least six members in the genome; accumulates in response to photoperiods that induce flowering	80.8	Felsheim and Das (1992)

is an immunological homologue of the Hsp80 of *Neurospora*. It is, therefore, inferred that OsHsp87 belongs to eukaryotic Hsp90 family of proteins (Pareek 1997; Pareek *et al* 1998).

In support of this, sequence data of the four internal peptides of OsHsp87 (OsHsp87 protein purified from SDS-gels; Pareek *et al* 1995) revealed that OsHsp87 has nearly complete homology with the deduced amino acid sequence of *hsp82* gene previously cloned from rice (with only single amino acid difference of K in place of S in the sequence KPEEITK).

2.3 OsHsp90 are conserved stress associated proteins

OsHsp90 proteins have been found to accumulate in rice seedlings subjected to salt stress, water stress, low temperature stress and exogenous application of abscisic acid (ABA), apart from high temperature stress (Pareek *et al* 1995). These proteins also accumulated when rice seedlings grown in pots under natural conditions were subjected to water stress by withholding watering.

Further, seedlings of *Triticum aestivum*, *Sorghum bicolor*, *Pisum sativum*, *Zea mays*, *Brassica juncea*, and mycelia of *N. crassa* showed accumulation of the immunological homologues of the OsHsp90 in response to high temperature stress (Pareek *et al* 1995). Considering such general accumulation patterns, these proteins have also been referred to as stress-associated proteins 90 (SAP90).

2.4 Induced and constitutive levels of OsHsp90 differ in different growth stages of rice

OsHsp90 proteins have been detected in several growth stages of the rice plant (Pareek 1997; Pareek *et al* 1997b). Importantly, levels of OsHsp90 have been noted to be variable in different organs of the mature plant. High uninduced levels of OsHsp90 were specifically noted in lemma, palea and culm tissues. Dry seeds of rice, maize and sorghum were also found to contain high uninduced amounts of these proteins. When seed and callus tissues (containing significantly higher amounts of OsHsp90 in uninduced conditions) were subjected to HS, the level of these proteins declined (Pareek *et al* 1997b).

Tissue print-immunolocalization analysis revealed higher accumulation of OsHsp90 proteins in the vascular bundles/procambial cells and in outermost cell layers of various leaves as well as in the meristematic cells of the stem apex. In seeds, these proteins are most abundant in the seed coat and embryo (Pareek *et al* 1997b).

3. Materials and methods

3.1 Plant material and stress conditions

Seedlings of rice (*O. sativa* L. cv Pusa 169) were raised on moistened germinating paper at 28°C. Four day old

seedlings were transferred to 100 ml glass beakers containing supporting pad of absorbent cotton soaked in distilled water and were acclimatized overnight at 28°C. Following day, the 5 day old intact seedlings were subjected to heat (35, 40, 45°C), cold (5 ± 2°C), desiccation (air drying) or salinity (200 mM NaCl) stresses or ABA (10⁻⁶, 10⁻⁵, 10⁻⁴ M) for different durations. For salinity treatment, seedlings were carefully transferred on to double layers of autoclaved cotton pieces floating on NaCl solution. After completion of stress treatment, shoot and root tissues were analysed separately.

Seeds of 15 different wild rices were grown as described earlier (Singla *et al* 1997a). Details of the accession numbers of these rices and their origin are given elsewhere (Singla *et al* 1997a). Dehusked seeds of the wild rices were germinated on a moist filter paper and 5 to 8 day old seedlings were transferred to earthen pots under natural day/night conditions. Segments from the topmost leaf of vegetative (just prior to flowering) plants in each case were placed in a beaker containing distilled water and subjected to HS (45°C, 6 h). Following the HS, tissues were stored in liquid nitrogen till further use.

3.2 In vivo labelling of proteins

Shoot segments from 4 day old seedlings (cv Pusa 169) were employed for radiolabelling of proteins since rooted seedlings showed poor uptake of [³⁵S]methionine (Singla and Grover 1994). The excised shoot segments were floated on distilled water and were acclimatized for 12 h at the growth temperature (28°C) prior to HS. For each radiolabelling treatment, 185 mBq of [³⁵S]methionine (specific activity > 1000 Ci/mmol, Amersham, UK) was employed.

3.3 Protein extraction and gel electrophoresis

Extraction of total soluble proteins was carried out in a buffer consisting of 30 mM Tris-HCl (pH 8.5), 1 mM ascorbic acid, 1 mM EDTA-Na, 5 mM MgCl₂, 1 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride (Zivy *et al* 1983). Insoluble polyvinylpyrrolidone (50 mg/g fresh weight of the tissue) was also added to the extraction buffer. The crude homogenate was spun twice at 12,000 g at 4°C for 15 min each. The buffer soluble proteins were precipitated using 8 vols of chilled acetone (containing 10 mM 2-mercaptoethanol). Precipitated proteins were solubilized following Laemmli (1970).

Estimation of protein content was carried out following Bradford (1976). An aliquot of the Laemmli buffer solubilized proteins was precipitated using equal vol of 20% trichloroacetic acid, and the pellet was redissolved in 1 M NaOH to minimize the interference due to SDS and 2-mercaptoethanol in protein quantification. For

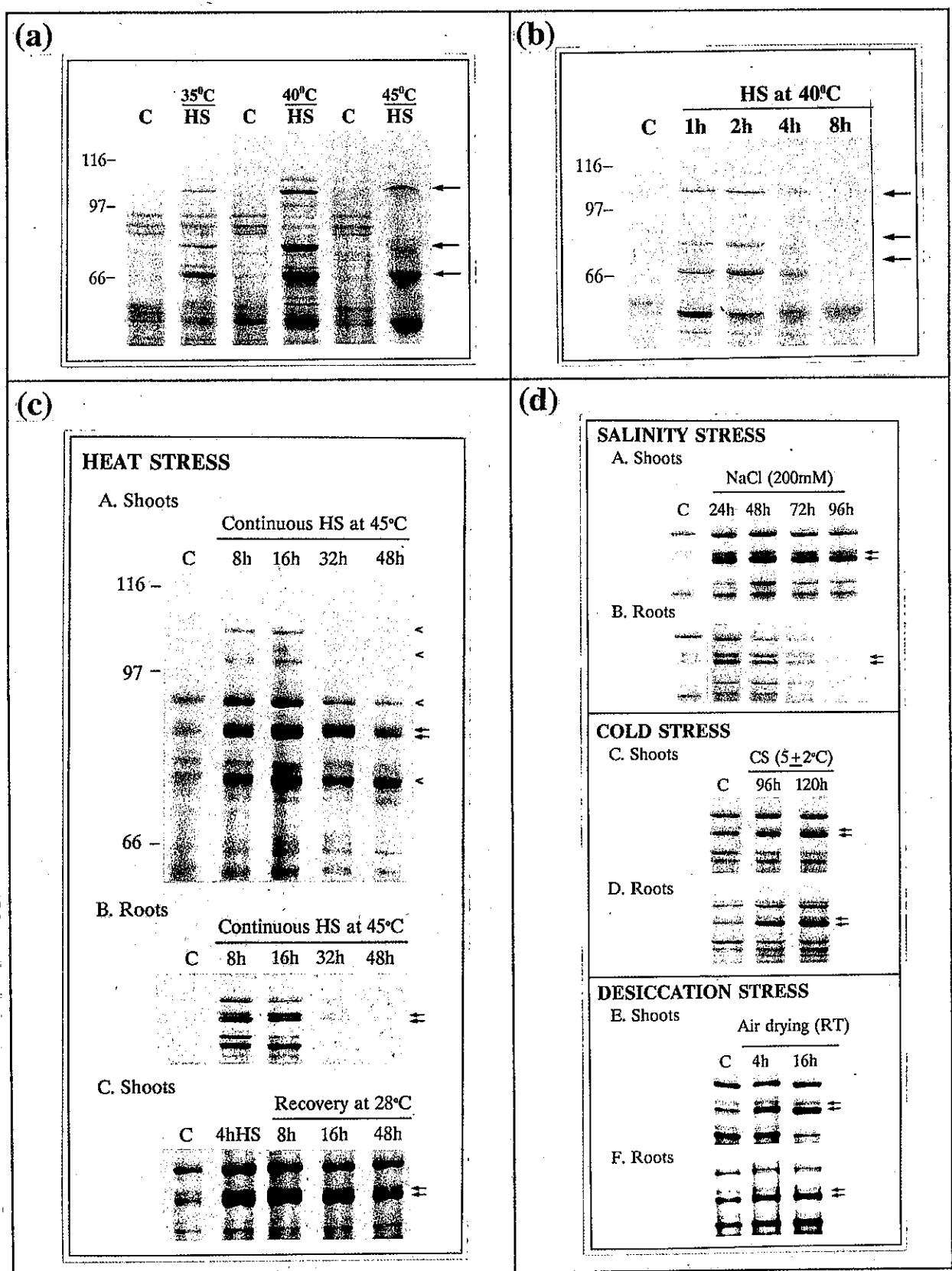


Figure 1.

radiolabelled protein samples, the degree of [³⁵S]methionine incorporation into proteins was determined by the procedure of Hames and Rickwood (1981).

The polypeptides were subjected to one-dimensional polyacrylamide gel electrophoresis, using either uniform or gradient gels (Singla and Grover 1994). Silver staining was performed according to the method of Damerval *et al* (1987).

4. Results and discussion

HS-responsive *de novo* synthesized proteins were analysed by feeding [³⁵S]methionine to shoot segments. [³⁵S]methionine was provided for the entire 6 h interval of HS at 35, 40 or 45°C. Autoradiograms of radiolabelled proteins (resolved on 7.5% polyacrylamide SDS-gel) revealed that increase in ambient temperature by a mere 7°C (from 28–35°C) resulted in an altered synthesis of a number of polypeptides. In the high molecular weight (HMW) range, prominent accumulation of 104, 87, and 70 kDa polypeptides was noted (figure 1a, shown by arrows). HS at 40°C resulted in a much higher accumulation of 87 kDa polypeptide (middle arrow, figure 1a). HS at 45°C appeared to damage the excised shoot segments as a loss of majority of the cellular proteins could be noticed excepting the prominent HMW-Hsps (including Hsp87) (figure 1a). For analysing the time-kinetics of OsHsp87 synthesis at 40°C, [³⁵S]methionine was provided to the shoot segments during the last 1 h of the HS. High amounts of 87 kDa polypeptide persisted till 2 h of HS only (middle arrow, figure 1b) reflecting that this protein is synthesized predominantly during the first 2 h of HS. Analysis of the cellular proteins after radiolabelling with [³⁵S]methionine showed that Hsp 87 was *de novo* synthesized in rice cells in response to high temperature stress. However, the point whether the 85 kDa protein is also *de novo* synthesized could not be resolved from the autoradiograms since the two pro-

teins were so close in position on gels that at times, it was difficult to separately analyse these proteins.

Steady-state proteins from shoot and root tissues of the seedlings from control (28°C) and those subjected to HS (45°C for various durations) were compared using 7.5% polyacrylamide SDS-gel electrophoresis followed by silver staining. Intact seedlings were subjected to HS in this study. The electrophoretograms of soluble proteins from control and heat shocked seedlings were found to be by and large similar excepting for a few polypeptides in the range of 70–110 kDa which prominently increased in both shoot and root tissues (figure 1c, shoots). Two closely-placed polypeptides of 87 and 85 kDa accumulated to high levels in response to HS (shown by arrows, figure 1c). The position of 87 kDa polypeptide in steady-state gel was identical to the same-sized polypeptide seen in autoradiogram showing *de novo* synthesized polypeptides (figure 1a). Both 87 and 85 kDa polypeptides in steady-state protein samples were detected with Coomassie blue staining as well. These polypeptides were seen under unstressed conditions (control) also but were lesser in amounts (figure 1c). Time-course analysis showed that the accumulation of 87 and 85 kDa polypeptides increased till 16 h of HS in shoots. High amounts of these polypeptides were maintained till 48 h of the continuous HS although most of the other HMW-Hsps disappeared after such a long period of stress (figure 1c). In case of roots too, these polypeptides accumulated in higher amounts till 16 h of HS (figure 1c). Extended HS treatment of 32 h proved to be detrimental for proteins of roots. When the seedlings were shifted back to normal temperature at 28°C after a 4 h heat shock at 45°C, no change in the level of either 87 or 85 kDa proteins could be detected till 8–16 h of recovery phase. High levels of both 87 and 85 kDa polypeptides persisted in shoots till 48 h at 28°C, time interval up to which analysis was carried out in this study (figure 1c).

Accumulation of 87 and 85 kDa polypeptides was

Figure 1. (a) Autoradiogram of [³⁵S]methionine labelled proteins from shoots of rice seedlings showing the effect of HS on accumulation of various heat inducible polypeptides. The polypeptides were resolved on 7.5% SDS-PAGE, and each lane was loaded with equal cpm. Details of treatment are given on top of each lane. C, Control; HS, heat shock at various specified temperatures for 6 h (radiolabelled methionine was provided for the entire 6 h of HS). Major heat shock inducible polypeptides are marked with arrows (middle arrow marks the position of 87 kDa polypeptide). The molecular mass (kDa) of standard proteins are shown on the left side. (b) Autoradiogram of [³⁵S]methionine labelled proteins from shoots of rice seedlings showing time-course of accumulation of various Hsps. The polypeptides were resolved on 7.5% SDS-PAGE and each lane was loaded with equal cpm. Details of each treatment are given on top of each lane. C, control; HS, heat shock at 40°C for various indicated durations (radiolabelled methionine was fed during the last 1 h of stress). Major heat shock-inducible polypeptides are marked with arrows (the second arrow from the top marks the position of 87 kDa polypeptide). The molecular mass (kDa) of standard proteins are shown on the left side. (c) Effect of HS on steady-state polypeptide alterations analysed by 7.5% SDS-PAGE stained with silver. Details on stress conditions are given on the top of each lane. Panels A and B show the profiles of soluble proteins from shoots and roots of rice seedlings, respectively. Panel C shows the protein profile of shoots during recovery from the heat shock. C, Control; HS, heat shock at 45°C, each lane was loaded with 20 µg of crude protein sample. Molecular masses (kDa) of protein standards are shown on the left side of panel A. Arrows indicate 87 and 85 kDa polypeptides. Arrowheads indicate a few other high molecular weight polypeptides which were found to be altered in response to HS (panel A). (d) Effect of salinity, cold and desiccation stress on the accumulation of 87 and 85 kDa polypeptides in shoots (A, C and E) and roots (B, D and F). Details of treatment are given on top of each lane. In each case 20 µg of crude protein sample was loaded. Arrows indicate the position of 87 and 85 kDa polypeptides. C, Control; CS, cold stress; RT, room temperature.

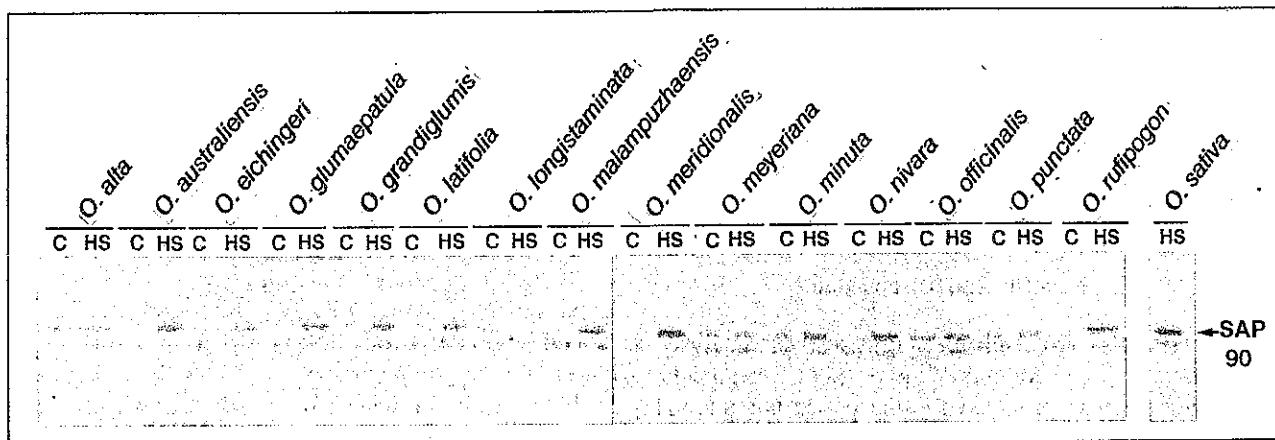


Figure 2. Western blot detection of Hsp90/SAP90 in leaf segments of various wild rices in response to HS. The last lane shows position of Hsp90 in heat shocked leaf segments of *O. sativa* as a reference. C, Control; HS, heat shock (45°C, 6 h). Each lane was loaded with 5 µg protein.

examined after the imposition of cold, salinity and air drying stress for different durations. These stresses were imposed on intact rice seedlings. Both 87 and 85 kDa polypeptides showed a high degree of accumulation in root and shoot tissues in response to all these stress conditions (figure 1d, arrows indicate the position of 87 and 85 kDa polypeptides). High level accumulation of these polypeptides was seen till 96 h in shoot tissues and 48 h in root tissues of the seedlings exposed to NaCl stress. Following cold stress, relatively higher levels of this protein were noted in roots as compared to shoots, till 120 h tested in this study. Similarly, accumulation of both 87 and 85 kDa polypeptides was noted till 16 h of desiccation stress (i.e., air-drying at room temperature) in both shoot and root tissues. Visual observation (figure 1d) as well as quantification of the 87 and 85 kDa polypeptides (pooled sample) by densitometry indicated that the heat shock, though given for a shorter interval as against other stresses (for instance 8 h of HS as against 48 or 96 h of salinity), caused maximum accumulation of these polypeptides in both shoot and root tissues (data not shown).

The above data from SDS-polyacrylamide gels revealed several important features of the expression of the OsHsp87 and OsHsp85 proteins. Both these proteins showed nearly similar kinetics with respect to their steady-state accumulation. These polypeptides appeared stable to a reasonable extent since their accumulation in seedlings could be noticed even after 48 h of the cessation of the stresses (figure 1c). We have earlier marked almost a similar pattern of accumulation and persistence of OsHsp104 (Singla and Grover 1994). Stable nature of Hsps have earlier been found in soybean, *Drosophila*, sorghum and maize and linked to their involvement in thermotolerance (Howarth and Ougham 1993). The levels

of accumulation of these proteins were highly pronounced and relatively speaking, maximum levels were seen in response to high temperature and minimum in response to low temperature stresses.

Further, fifteen different wild rices were tested for the extent of HS-induced accumulation of Hsp90 proteins. All the wild rices reacted positively with anti rice Hsp90 antibodies (figure 2). Importantly, however, the levels of this protein were variable. HS-induced levels of Hsp90 were found to be very high in case of *O. australiensis* as well as in *O. meridionalis*, but in the case of *O. alta*, the HS-induced levels of this protein were very low. Moreover, variations in constitutive levels of this protein were also seen among wild rices. In this case, high levels of Hsp90 were found in control tissues of *O. meyeriana* as well as *O. minuta*. On the other hand, OsHsp104 levels were more or less similar in these rice types as noted in a separate study (Singla 1996; Singla *et al* 1998) while the levels of OsHsp110 showed differential expression in these rices (Singla *et al* 1997a). It is noteworthy that these wild rices are endemic to diverse ecosystems. Detailed analysis of stress response of wild rices in light of OsHsp90 expression may provide useful information for testing the cellular roles of these proteins.

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