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Characterization of HSP-70 cognate proteins from wheat

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Summary. Animal and plant cells contain a family of constitutively expressed HSP-70 cognate proteins that are localized in different subcellular locations and are presumed to play a role in protein folding and transport. Utilizing antibodies raised against the yeast endoplasmic-reticulum-localized HSP-70 cognate termed BiP/GRP-78, as well as antibodies raised against the *Escherichia coli* HSP-70 protein DnaK, we have identified and characterized a large family of closely related proteins in wheat. One protein band of 78 kDa that is apparently closely related to yeast BiP was localized in the endoplasmic reticulum. This band cross-reacted with the yeast BiP but not with the DnaK-specific antibodies. The yeast BiP antibodies also recognized a cytoplasmic protein of 70 kDa that is probably related to the HSC-70 cognate proteins. These two proteins were further confirmed as HSP-70 cognates by their ability to bind to an ATP-agarose column. Probing of proteins from purified wheat mitochondrial preparations with the yeast BiP and DnaK-specific antibodies showed that this organelle contained a family of HSP-70-related proteins. The yeast BiP antibodies recognized two mitochondrial proteins of 60 and 58 kDa, but failed to detect any protein in the size rang of 70 to 80 kDa. However, the presence of immunologically distinct proteins of 90 and 78 kDa, as well as of lower molecular weight from this family in the mitochondria, was shown by probing with the DnaK-specific antibodies. A new protein of 30 kDa, cross-reacting with anti-yeast BiP antibodies, was detected only in developing seeds, close to their maturity. The evolution of HSP-70 cognate proteins in wheat as shown in this study is discussed.

Key words: HSP-70 – BiP/GRP-78 – Wheat – *Triticum aestivum* – Chaperone

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Introduction

Cells of all examined organisms possess a group of genes that are specifically induced by heat and other types of stress. These genes code for a family of proteins termed heat shock proteins (HSP) (Rothman 1989). The most prominent members of this multigene family are the genes coding for 70-kDa proteins (HSP-70). These proteins were shown to be among the highest evolutionary-conserved proteins between bacteria and eukaryotes, including animals and plants (Bardwell and Craig 1984; Rothman 1989; Marshall et al. 1990). The recent observation that these proteins bind strongly to ATP has been used for their affinity purification (Welch and Feramisco 1985). One function proposed for this class of proteins is an ATP-dependent binding to improperly folded or denatured proteins (Pelham 1986). Cloning and characterization of a variety of HSP-70-related genes have shown that HSP-70 represents in fact a family of proteins containing members that are localized in various subcellular organelles. Moreover, these proteins were found to be either constitutively expressed or induced by heat and stress treatments (for review, see Rothman 1989). Munro and Pelham (1986) were the first to clone a cDNA coding for an HSP-70-related protein that possessed a signal peptide for transport into the endoplasmic reticulum (ER). This protein was shown to be identical to a previously identified protein called the glucose-regulated protein (GRP-78), also known as the immunoglobulin's heavy chain binding protein (BiP). Haas and Wabl (1983) first identified BiP as being bound to immunoglobulin heavy chain in pre-B cells. It has subsequently been proposed that it associates transiently with normal, newly synthesized membrane and secretory proteins (Gething et al. 1986; Kassenbrock et al. 1988), and permanently with underglycosylated or mutant proteins that fail to

fold correctly (Bole et al. 1986; Gething et al. 1986; Dorner et al. 1987). Vogel et al. (1990) reported that yeast BiP appears as a catalyzer of both the import of secretory proteins into the ER as well as of their folding and assembly. Binding of BiP to its substrate was shown to be abolished upon addition of ATP (Munro and Pelham 1986; Flynn et al. 1989).

Another member of the HSP-70 family that is constitutively expressed was recently discovered in the cytoplasm of several eukaryotic organisms and was termed HSC-70. It was hypothesized that this protein is involved in the post-translational transport of proteins from the cytoplasm to different cellular organelles by binding to newly synthesized precursor proteins, thus preventing their misfolding prior to transport across the organelle membranes (Rothman and Kornberg 1986).

Recently, HSP-70-related proteins have also been discovered in mitochondria of various organisms as well as in plant chloroplasts, showing that proteins from this family are probably ubiquitous in each of the subcellular organelles. (Leustek et al. 1989; Marshall et al. 1990).

Despite extensive characterization of HSP-70 cognate proteins in animal and yeast cells, much less is known about their presence and function in higher plants. In the present report, we show that a large family of HSP-70-related proteins is present in various subcellular organelles of wheat cells. This family consists of at least two immunologically distinct groups of proteins, some of which have probably been more conserved than others.

Materials and methods

Plant material

Triticum aestivum cv Chinese Spring (CS) was grown under greenhouse conditions. Spikes at various days after pollination (DAP) were collected and stored at −80 °C. Grains were separated from the spikes under liquid N_2. Shoots and roots were collected at 5 days after germination from etiolated seedlings grown in a growth chamber at 22 °C.

Electrophoresis and western blotting

SDS-PAGE was performed according to the method of Laemmli (1970) using 12.5% gels. For western blotting, proteins were transferred to nitrocellulose filters as described by Towbin et al. (1979). Specific binding was visualized using an alkaline-phosphatase-conjugated goat antirabbit IgG preparation (Sigma Chemical Co., St. Louis/MO), according to the procedure of Burnette (1981).

Two-dimensional PAGE

Two-dimensional PAGE was performed according to the method of O'Farrell (1975). One-dimensional PAGE separation was performed on isoelectric focusing (IEF) gels containing ampholytes (LKB) at the pH range of 3 to 10 (0.3%) and 5–7 (1.2%). The IEF gels were then separated on a two-dimensional 12.5% SDS-PAGE. Visualization of the proteins was done by Coomassie Brilliant Blue R-250 staining.

Protein extraction

Developing grains, shoots, and roots were homogenized with a mortar and pestle in buffer A [0.1*M* TRIS-HCl (pH 8.0), 100 m*M* KCl, 50 m*M* MgAc, 1 m*M* DTT, 1 m*M* phenylmethylsulfonyl fluoride (PMSF)], and centrifuged at 4 °C, 14,000 × *g* for 10 min. The supernatant (termed S) was collected. The pellet was dissolved in buffer A containing 1% Triton-X-100 and centrifuged at 4 °C, 14,000 × *g* for 10 min. The second supernatant was collected and termed P. The S and P proteins were then acetone-precipitated and stored at −20 °C until used. Protein pellets were dissolved in SDS sample buffer [0.125 *M* TRIS-HCl (pH 6.8), 2.14 *M* β-mercaptoethanol, 4% (w/v) SDS, 20% (w/v) glycerol, and 0.002% (w/v) Bromophenol blue], boiled at 100 °C for 4 min, and loaded directly onto the gels.

Subcellular fractionation

Subcellular fractionation of developing wheat grains (5 g) was performed as described by Nigam and Blobel (1989). For the immunoblotting studies, an attempt was made to use approximately equivalent amounts of proteins.

Affinity binding to ATP-agarose columns

Developing wheat grains (25 g) were ground on ice with Polytron (five bursts of 30 s each) in buffer A at a tissue-to-buffer ratio of 1:3 and centrifuged at 4 °C, 260 × *g* for 10 min. The supernatant was filtered through four layers of cheesecloth. The filtered homogenate was then centrifuged at 4 °C, 27,000 × *g* for 40 min to yield a high-speed supernatant and a pellet. The pellet was then dissolved in 5 ml of buffer A (pH 9.0), hand-homogenized, and centrifuged at 4 °C, 27,000 × *g* for 40 min The resulting supernatant was adjusted to pH 7.8 using 1 *M* TRIS-HCl (pH 7.5). Both high-speed supernatant and the resulting pellet were applied directly to a 5-ml ATP-agarose column pre-equilibrated with buffer D containing 20 m*M* Tris acetate (pH 7.5), 20 m*M* NaCl, 0.1 m*M* EDTA, 15 m*M* β-mercaptoethanol, and 3 m*M* $MgCl_2$. After loading, the column was washed with buffer D containing 0.1 *M* NaCl and then with buffer D alone. The column was then developed with buffer D containing 3 m*M* ATP. Fractions were acetone-precipitated and analyzed by SDS-PAGE as described above.

Results and discussion

Identification of constitutively expressed HSP-70 cognate proteins in wheat tissues

Antibodies raised against the HSP-70-related protein BiP/GRP-78 from ER of yeast (anti-yeast BiP) were used to identify closely related proteins in wheat. Proteins from supernatant (S) and membrane pellets (P) of developing grains, shoots, and roots were fractionated on SDS-PAGE, and the gel was either stained or reacted in a western blot against anti-yeast BiP antibodies. Six major cross-reactive bands were detected, with apparent molecular masses of 78, 70, 62, 60, 58, and 30 kDa (Fig. 1). All of these proteins were related to the HSP-70 family, since they were not detected when the blot was reacted against a preimmune serum (data not shown). Most of these yeast-BiP-related proteins were detected in all of the tissues examined, but their relative intensities varied among different tissues. The 30 kDa band ap-

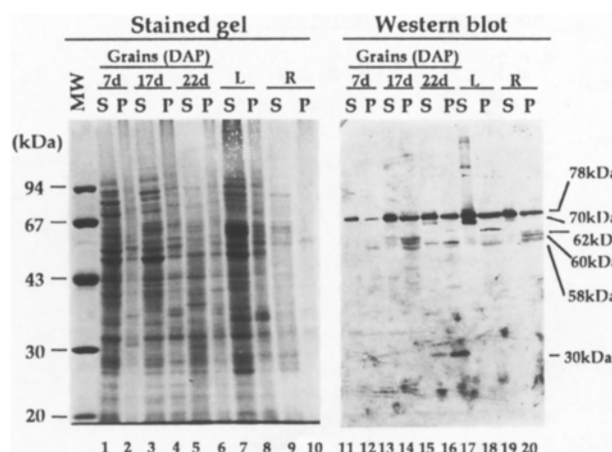

Fig. 1. Detection of HSP-70-related proteins in various wheat tissues. Plant material from developing grains at 7, 17, and 22 DAP; shoots (L) and roots (R) were homogenized in buffer A, centrifuged, and the pellet was further extracted with detergent to yield supernatant (S) and membrane (P) protein fractions. The proteins were subjected to SDS-PAGE and the gel was either stained with Coomassie blue (*lanes 1–10*) or reacted in a western blot against yeast-BiP-specific antibodies (*lane 11–20*). MW markers are indicated on the *left*, while the molecular masses of specific HSP-70-related proteins are indicated on the *right*

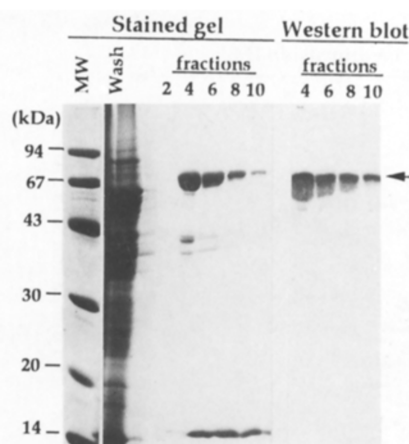

Fig. 2. ATP-agarose affinity chromatography of HSP-70-related proteins. Developing grains at 17 DAP were homogenized in buffer A. The supernatant proteins were applied to the column. After washing the column with buffer A containing 0.1 *M* NaCl (wash), the column was then eluted by 3 m*M* ATP and the fractions were analyzed by SDS-PAGE. The gels were either stained or reacted in a western blot against yeast-BiP-specific antibodies. MW markers are indicated on the *left*. *Arrow* on the *right* indicate the position of 78 kDa protein

peared solely in developing grains of 22 DAP. The 70 kDa band was detected only in the supernatant (Fig. 1; lanes 15, 17, 19) and therefore may reside in the cytoplasm. In contrast, a major part of the rest of the proteins appeared in the membrane fraction, indicating their localization within organelles.

It has been previously shown that HSP-70-related proteins can bind ATP (Welch and Feramisco 1985). We therefore utilized this property to further characterize the wheat HSP-70-related proteins. Developing wheat grains were homogenized in buffer A and the soluble fraction (S) was applied directly to an agarose column containing covalently linked ATP. After extensive washing of the column with high salt and low salt buffers, it was then developed with 3 m*M* ATP. A sample from every other fraction eluting off the column was fractionated on SDS-PAGE, and the proteins were either visualized by Coomassie blue staining of the gel or reacted in a western blot with anti-yeast BiP antibodies (Fig. 2). While most of the cellular proteins were washed off by the high salt, several proteins were bound to the column and were found to coelute off the column in the presence of ATP. The major stained protein eluted from the column was a broad band with an apparent molecular mass in the range of 70 to 78 kDa. In addition, several other proteins with apparent molecular masses of 42, 40, 17, and 16 kDa were also eluted (Fig. 2). The western blot described in Fig. 2 confirmed that the 70–78 kDa broad band belonged in fact to the HSP-70 family. The 42, 40, 17, and 16 kDa proteins eluted from the column with ATP did not cross-react with anti-yeast BiP antibodies, and therefore may belong to other families of ATP-binding proteins. We could not detect the 62, 60, and 58 kDa HSP-70-related proteins in the column eluants. Whether this is due to their low levels or their inability to bind ATP has not yet been determined. The 17 and 16 kDa proteins are probably GTP-binding proteins, since they were the only proteins that could also be eluted from the column with 1 m*M* GTP (data not shown). In order to test whether the broad 70–78 kDa band eluted from the ATP-agarose column consisted of more than one protein, proteins eluted from this column were separated on two-dimensional IEF SDS-PAGE (Fig. 3). Indeed, this broad band contained at least two ATP-binding proteins. In addition, the two-dimensional PAGE revealed that the 40 kDa family consisted of approximately six proteins and that the low molecular mass bands corresponding to the GTP-binding proteins were separated into three major spots.

We were next interested in studying whether the HSP-70-related proteins present within organelles could also bind ATP. In order to test this, the membrane pellet was washed at pH 9 and the resulting solubilized proteins were applied to the ATP-agarose column and subsequently eluted with ATP. The pH 9 treatment is known to solubilize proteins that are localized within subcellular organelles (Bulleid and Freedman 1988). Eluted proteins (every other fraction) were fractionated on SDS-PAGE and stained with Coomassie blue (Fig. 4). In this experiment, the 78 kDa HSP-70 related protein was the only

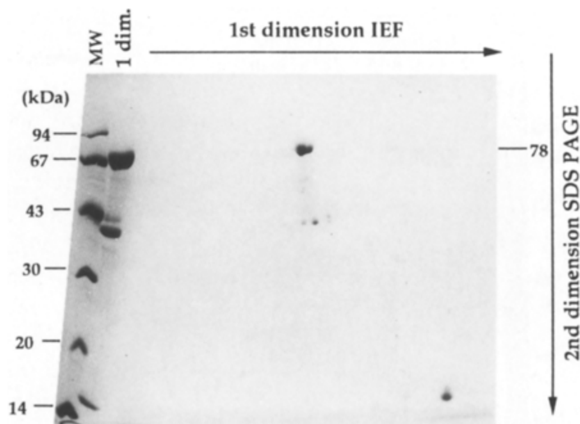

Fig. 3. Two-dimensional separation of the 78 kDa HSP-70-related proteins. Proteins purified by ATP-agarose column were analysed by a two-dimensional IEF SDS-PAGE. The gel was stained with Coomassie blue. MW markers are indicated on the *left*. The position of the 78 kDa proteins is indicated on the *right*. The "1 dim" lane represents a pattern of one-dimensional SDS-PAGE of the eluted proteins

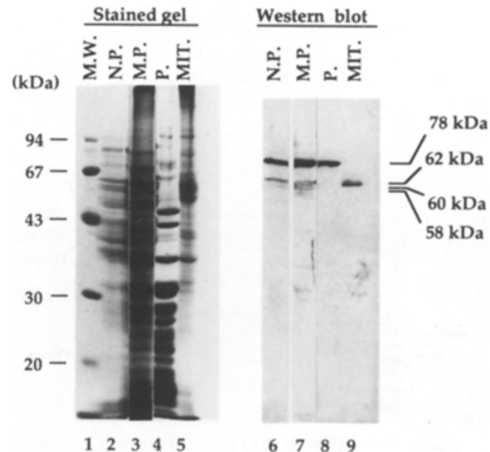

Fig. 5. Subcellular localization of HSP-70-related proteins from developing wheat grains at 17 DAP. Developing grains were homogenized and the homogenate was precipitated in two steps to yield nuclear (NP) and mitochondrial pellets (MP). The post-mitochondrial supernatant was then centrifuged over a sucrose cushion to yield an ER-rich sucrose pellet (P). Equivalent amounts of these proteins as well as from proteins extracted from purified mitochondria (MIT) were subsequently analyzed on SDS-PAGE or reacted in a western blot against anti-yeast BiP antibodies. MW markers are indicated on the *left*, molecular masses of specific HSP-70-related proteins are indicated on the *right*

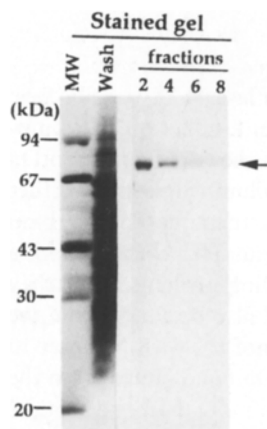

Fig. 4. ATP-agarose affinity chromatography of HSP-70 proteins present within organelles. Proteins from developing grains at 17 DAP were homogenized in buffer A. Proteins extracted from the pellet at pH 9.0 were applied to the column. The column was washed and eluted as described in Fig. 2. The fractions were analyzed by SDS-PAGE. The gel was stained with Coomassie blue. MW markers are indicated on the *left*. *Arrow* on the *right* indicates the position of 78 kDa protein

protein detected in the column eluate, indicating that the 70 kDa as well as the other ATP-binding proteins of lower molecular masses were localized in the cytoplasm.

Subcellular compartmentalization of HSP-70-related proteins in developing wheat grains

In order to further characterize the subcellular localization of the HSP-70-related proteins, developing grains at 17 DAP were subjected to subcellular fractionation. First, homogenates of those grains were fractionated by two steps of precipitation at $800 \times g$ and $10{,}000 \times g$, to yield nuclear and mitochondrial pellets. The postmitochondrial supernatant was loaded on a 1.3 *M* sucrose cushion and further fractionated by centrifugation to yield a sucrose pellet that is highly enriched in ER vesicles. Proteins from the nuclear and mitochondrial pellets, as well as from the ER-enriched sucrose pellet, were separated by SDS-PAGE and then either stained or reacted in western blots with yeast BiP antibodies (Fig. 5). The cytoplasmic 70 kDa protein was not detected in any of these membranous fractions, thus showing that these preparations were relatively free of cytoplasmic contamination. However, the rest of the HSP-70-related proteins were clearly detected (Fig. 5). The 60 and 58 kDa proteins appeared mainly in the mitochondrial pellet (Fig. 5, lane 7), indicating their localization within this organelle. The 62 kDa protein appeared with similar intensity in both the nuclear and mitochondrial pellets, but not in the ER-enriched sucrose pellet (Fig. 5, cf. lanes 6, 7, and 8). Thus, although it is apparently not localized within the ER, its exact organellar localization could not be identified.

The sucrose pellet showed a markedly different pattern of stained proteins than the rest of the fractions (Fig. 5, cf. lanes 2, 3, and 4), indicating that it was indeed highly enriched in ER microsomes. The fact that the 60 and 58 kDa mitochondrial proteins were not detected in

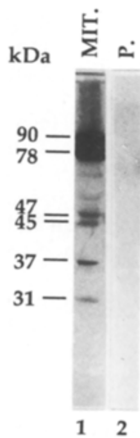

Fig. 6. Antigenic relationships of the wheat HSP-70-like proteins and *E. coli* DnaK. Protein from purified mitochondria (MIT) and proteins from the ER-enriched fraction (P) were separated by SDS-PAGE and reacted in western blots against DnaK-specific antibodies. Molecular masses of specific HSP-70-related proteins are indicated on the *left*

the sucrose pellet further confirmed the high purity of this ER fraction. The only yeast-BiP-related protein band that appeared in the sucrose pellet was that of the 78 kDa thus showing its localization within the ER. The localization of the 78 kDa band within the ER was also confirmed by its cosedimentation with the ER marker enzyme, NADH cytochrome C reductase, in a metrizamide density gradient analysis (data not shown). As expected, the 78 kDa protein was also detected in the nuclear and mitochondrial pellets (Fig. 5, lanes 6, 7) that are known to contain ER membranes. In order to confirm the mitochondrial localization of the 60 and 58 kDa proteins, we compared the western blot pattern of proteins extracted from purified wheat mitochondria to those of the mitochondrial pellet fraction. The 60 and 58 kDa BiP-related proteins that were enriched in the mitochondrial pellet of the subcellular fractions (Fig. 5, lane 7) were also the only detectable BiP-related bands in the western blot of purified mitochondria (Fig. 5, lane 9), indicating that no 78 kDa band reacting against yeast BiP antibodies was localized in this organelle.

HSP-70-related proteins in the range of 70 kDa were previously identified in the mitochondria of animal and plant cells (Bardwell and Craig 1984; Craig et al. 1989; Jindal et al. 1989; Leustek et al. 1989; Amir-Shapira et al. 1990). It was therefore possible that an approximate 70-kDa protein does exist in wheat mitochondria, but that it does not cross-react with the yeast-BiP-specific antibodies. To test this possibiltiy, proteins from purified wheat mitochondria and from the ER-enriched sucrose pellet were reacted in western blots against *E. coli* DnaK-specific antibodies. Interestingly, a major DnaK-related protein band with apparent molecular mass of ca. 78 kDa was detected in purified wheat mitochondria, together with several other proteins of 90, 47, 45, 37, and 31 kDa (Fig. 6, lane 1). In contrast, the 60 and 58 kDa yeast-BiP-related mitochondrial proteins were not reacted with the DnaK antibodies (Fig. 6, lane 1). Thus, our data show that at least two small families of immunologically distinct HSP-70-related proteins exist in the mitochondria of wheat cells. The 60 and 58 kDa mitochondrial proteins reacted with the yeast BiP antibodies are probably structurally distinct from the other six proteins reacted with the DnaK-specific antibodies. These two subfamilies were either diverged differently from the same ancestor or were evolved from another HSP-70-related gene. We could not detect any interaction of the ER-localized 78 kDa protein with the DnaK-specific antibodies (Fig. 6, lane 2). This result indicates that this 78 kDa protein is structurally distinct from the mitochondrial 78 kDa protein.

HSP-70-related proteins provide a powerful tool for evolutionary studies, since they represent a family of highly conserved proteins that are localized in different subcellular locations. A part of these proteins may still share some homology with HSP-70-related proteins derived from prokaryotic organisms. The fact that several immunologically distinct families are present in different organelles, as well as even within the same organelle such as the mitochondria, shows that the pattern of their evolution appears to be very complex. Recently, a family of HSP-70-related proteins was also detected in chloroplasts of higher plants (Amir-Shapira et al. 1990), but their immunological relationships have not yet been determined. Further cloning and molecular comparison of the various wheat HSP-70-related genes may provide more information concerning their structural and functional evolution.

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