Molecular and Physiological Analysis of a Heat-Shock Response in Wheat

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ABSTRACT

We have isolated two cDNA clones from wheat (Triticum aestivum L. var Stephens), designated WHSP16.8 and WHSP16.9, that are highly similar in sequence to the low molecular weight heat-shock protein genes previously isolated from soybean. RNA blot analysis confirms that these sequences are present in heat-shocked wheat seedlings, but not in control tissues. The WHSP16.8 and WHSP16.9 cDNAs were isolated by screening a lambda gt11 expression library with antibodies to HMGC (a chromosomal protein of wheat). Immunoblot analysis has demonstrated that the antibodies raised against HMGC also recognize a group of proteins that are induced by heat shock and have molecular weights estimated by sodium dodecyl sulfate electrophoresis consistent with the molecular weights of the proteins deduced from the sequences of the cDNAs.

Heat-shock proteins are a set of proteins whose synthesis is induced upon heat stress. The induction of heat-shock proteins is a highly conserved response that has been observed in every species studied thus far. The heat-shock proteins are also induced by other environmental stresses, and in some organisms, certain heat-shock proteins are induced during the course of normal development. The function of the heat-shock proteins is as yet unknown. However, evidence suggests that these proteins may provide organisms with a mechanism for thermotolerance (10).

The low mol wt heat-shock proteins are the predominant heat-shock-induced proteins in plants. These proteins are a complex group, with as many as 30 members present in soybean (12). Characterization of the low mol wt heat-shock protein genes of soybean has revealed that there are several classes of genes within this multigene family (17). The functions of these different classes of heat-shock genes are not yet clear.

Although recent reports have appeared documenting monocot cDNAs homologous to dicot low mol wt heat-shock genes (13, 28, 29), most previous studies on the low mol wt heat-shock proteins of monocots have been limited to the analysis of protein profiles of heat-stressed tissues. The induction of low mol wt heat-shock proteins in monocots has been documented (1, 2, 6, 8, 12, 30), and the number of low mol wt heat-shock proteins induced in several monocot species has been determined (12). These studies reveal that in wheat, 11 low mol wt proteins are induced and 1 enhanced by heat shock.

We have isolated two wheat (Triticum aestivum L. var Stephens) cDNA clones that are highly similar to the soybean 17-kD heat-shock protein genes described by Nagao et al. (17). The work presented in this article shows that these cDNA clones represent heat-shock-inducible transcripts in wheat. Because the wheat heat-shock cDNA clones were isolated through screening an expression library with the antibodies to the HMGC chromosomal protein of wheat (25), it seemed likely that the HMGC antibodies would cross-react with low mol wt heat-shock proteins of wheat. Immunoblot analysis of protein extracts from heat-stressed tissues has confirmed this conjecture. Use of the HMGC antibodies has revealed a set of immunologically related proteins that are induced upon heat shock in wheat and have mol wts consistent with those deduced from the cDNA sequences.

MATERIALS AND METHODS

Plant Material

Wheat (Triticum aestivum L. var. Stephens) seeds were surface sterilized with 0.5% sodium hypochlorite and allowed to imbibe for 3 h. Seedlings were grown in the dark at 20°C for 5 d and were then transferred to 40°C for heat-shock treatments. The etiolated coleoptiles and first leaves were harvested into liquid nitrogen and stored frozen at −70°C.

Preparation and Screening of a Wheat cDNA Library

Total RNA was isolated from the coleoptiles and first leaves of 4-d-old wheat seedlings that had been grown in the dark at room temperature. Phenol/chloroform extraction and lithium chloride precipitation were carried out as described by Thompson et al. (26). The total RNA was passed through a poly (U) Sepharose (BRL) column, and mRNA was eluted at 60°C in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.1% SDS (16). Synthesis of cDNA was carried out by the method of Gubler and Hoffman (5) using RNaseH and Pol I for second strand synthesis. The cDNA clones were treated with

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3 Abbreviations: HMGC, high mobility group; IEF, isoelectric focusing; BCIP, 5-bromo-4-chloro-3-indolyolphosphate; NBT, nitro blue tetrazolium.
Klenow and EcoRI methylase prior to the addition of EcoRI linkers. The cDNA was ligated into the EcoRI site of the lambda gt11 expression vector (Promega) and plated out with Y1090 cells. The library was screened with polyclonal antibodies prepared against the HMGc chromosomal protein of wheat (25) using the methods described by Huynh et al. (7). Alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies were used together with the BCIP and NBT substrates, which form a colored precipitate for visualization of positive clones (Protoblot Immunoscreening System, Promega).

**DNA Sequencing and Sequence Analysis**

Positive cDNA clones were subcloned into M13 (15) for sequencing by the Sanger dideoxy method (20). Sequencing reactions were carried out using the modified T7 polymerase (Sequenase, U.S. Biochemicals) and [γ-32P]ATP (Amersham). Dried gels were exposed to film for 1 to 2 d.

DNA sequences were aligned using the Cornell DNA Sequence Analysis Programs (4). The University of Wisconsin Sequence Analysis Programs were used to search the Genbank, European Molecular Biology Laboratory, and National Biomedical Research Foundation sequence data bases and to calculate percent similarities (3).

**Northern Blot Analysis**

An RNA miniprep procedure (27) was used to extract RNA from heat-shocked and control seedlings using the guanidinium isothiocyanate extraction buffer. This procedure yielded about 50 μg of RNA from 200 mg of tissue, as determined by absorbance at 260 nm. For analysis of each time point of heat treatment, 10 μg of total RNA was subjected to electrophoresis on formamide-formaldehyde denaturing gels (11) and blotted onto Nytran filters (Schleicher and Schuell). Radioactive probes were synthesized using the Random Primed DNA Labeling Kit (Boehringer Mannheim) and [α-32P]dCTP (Amersham). Blots were prehybridized and hybridized in 6 × SSPE (1.08 mM sodium chloride, 60 mM sodium phosphate, pH 7.7, 6 mM EDTA), 10 × Denhardt’s solution, 0.2% (w/v) SDS, 0.02% (w/v) sodium pyrophosphate, 10 μg/mL poly(A), 20 μg/mL salmon sperm DNA, and 50 μg/mL heparin (22). Radioactive probe was added to 2 × 10⁶ cpm/mL and hybridized at 65°C. Blots were washed in 0.1 × SSPE at 65°C for 30 min and exposed to film for 2 to 5 d.

**Immunoblot Analysis**

Total cellular proteins were extracted from the etiolated coleoptiles and first leaves of 4-d-old wheat seedlings, as described by Lin et al. (9). Tissue was ground in liquid nitrogen with a mortar and pestle and was then suspended in a buffer of 50 mM Tris-HCl, pH 8.5, 2% (w/v) SDS, 2% 2-mercaptoethanol, and 0.1 mM PMSF. Insoluble material was pelleted by centrifugation at 10,000g for 15 min. Protein extracts were quantitated by the dye binding method of Schaffner and Weissman (21). Proteins were precipitated with 4 volumes of acetone and resuspended in SDS sample buffer for electrophoresis on 18% SDS-polyacrylamide gels (23). Prestained low mol wt protein markers (Diversified Biotech) and wheat HMGc proteins (24) were run as standards. Protein banding patterns were visualized by Coomassie staining. The protein extracts were subjected to two-dimensional gel electrophoresis, with IEF in the first dimension and 18% SDS-PAGE in the second dimension. The IEF was performed as described by O’Farrell (18) using ampholytes from LKB. Total proteins (100 μg) were resuspended in 25 μL of O’Farrell sample buffer and loaded onto tube gels. The IEF tube gels were run at 3500 V for 15 h followed by 1 h at 800 V. The gels were then incubated in equilibration buffer (18) for 1 h and stored frozen at −70°C. Proteins were separated in the second dimension on 1.5-mm thick 18% SDS running gels with a 6% stacking gel (23). The second dimension gels were run for 15 h at 10 mA of constant current. The separation of proteins on two-dimensional gels was visualized by silver staining (14).

Proteins were blotted onto 0.1 micron nitrocellulose (Schleicher and Schuell) using the Novablot semidyed blotter from LKB and a single buffer of 39 mM glycyne, 48 mM Tris (base), 0.0375% (w/v) SDS, and 20% (v/v) methanol. Electrophoretic transfer was performed at 0.8 mA/cm² for 1 to 2 h. Transfer of proteins was monitored by staining the gels with Coomassie blue after the blotting procedure. All of the low mol wt proteins transferred out of the gels and were bound to the nitrocellulose, but there was incomplete transfer of the high mol wt proteins. Protein blots were blocked in 3% (w/v) BSA in Tris-buffered saline (1 × Tris-buffered saline: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl) and incubated with a 1:1000 dilution of the HMGc antibody (25). Antibody binding was visualized using alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G secondary antibodies with BCIP and NBT (Promega).

**RESULTS**

**Sequence Analysis**

Three homologous clones (C2–1, C3–1, and C5–8) were isolated through the screening of a wheat cDNA expression library with antibodies to the wheat HMGc chromosomal protein. Sequence analysis of the C5–8 wheat cDNA identified homology to the 17-kD heat-shock protein gene family of soybean (12). The C2–1 and C3–1 cDNA clones are identical to one another, but differ in sequence from the C5–8 clone. Hence, the three cDNA clones isolated represent two distinct heat-shock-induced transcripts, which will be identified hereafter as WHSP16.8 (C2–1/C3–1) and WHSP16.9 (C5–8).

The nucleotide sequences of WHSP16.8 and WHSP16.9 are presented in Figure 1. These two cDNA sequences are identical at 86% of the nucleotide residues. The highest degree of sequence similarity between WHSP16.8 and WHSP16.9 is in the coding region. However, the 5’ and 3’ untranslated regions of these two wheat cDNA clones also have regions of sequence similarity. The deduced amino acid sequences of WHSP16.8 and WHSP16.9 are aligned in Figure 2. The amino acid sequences of six of the soybean low mol
wt heat-shock proteins and the consensus sequence of the soybean heat-shock proteins (19) are also presented in Figure 2. Five of the six soybean heat-shock protein sequences are highly similar and are designated class I heat-shock proteins (19). However, gmhsp17.9-D has a markedly different amino acid sequence and is designated a class VI heat-shock protein (19). The deduced wheat heat-shock protein sequences are much more similar to the soybean consensus sequence than to the gmhsp17.9-D amino acid sequence. This suggests that the WHSP16.8 and WHSP16.9 cDNA clones encode class I heat-shock proteins. The deduced amino acid sequence for WHSP16.9 is identical to the soybean heat-shock protein consensus sequence at 73% of the amino acid residues, with the C-terminal two-thirds of the proteins having the most sequence similarity. The comparison of amino acid sequences of the wheat and soybean heat-shock proteins indicates that the two wheat heat-shock proteins are more similar to one another (95%) than they are to the soybean heat-shock proteins.

Heat-Shock Induction of mRNA

RNA from heat-shocked and control tissues was analyzed by northern blotting to determine whether the wheat cDNA clones that have sequence similarity to the soybean heat-shock protein genes represent heat-shock-induced transcripts in wheat. RNA isolated from a time course of heat-shocked tissues was hybridized with the WHSP16.9 cDNA clone (Fig. 3). There was no evidence for the presence of WHSP16.9 message in control tissues; however, within 1 h of exposure to 40°C temperature, the message begins to appear, and after

Figure 1. Alignment of the nucleotide sequences of WHSP16.8 and WHSP16.9. The start and stop codons are underlined.

Figure 2. Alignment of wheat and soybean low mol wt heat-shock protein amino acid sequences. The deduced amino acid sequences of the WHSP16.8 and WHSP16.9 wheat cDNA clones are aligned with the amino acid sequences of six soybean low mol wt heat-shock proteins and the soybean heat-shock protein consensus sequence (19). Dashes represent gaps in the sequence introduced for alignment. Dots represent identical amino acid residues.

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Because of the high proportion of basic amino acid residues in the HMG proteins, they do not migrate into the IEF gels (data not shown). Therefore, the HMG proteins are not present in these two-dimensional gels. The separations were carried out with control protein extracts and extracts of seedlings heat-shocked at 40°C for 5 h (Fig. 5). One gel of each extract was silver stained to visualize the protein spots, and for each, a duplicate gel was transferred to nitrocellulose for immunoblot analysis. The stained gels reveal several low mol wt proteins that appear in the heat-shock tissue extract, but not in the control.

The antibodies to HMGc were used to probe the two-dimensional protein blots. The results of these immunoblots

Figure 3. Induction of wheat heat-shock protein mRNAs. RNA gel blot analysis of total RNA that was isolated from a time course of heat-shock treatments and probed with the WHSP16.9 cDNA clone. Total RNA was extracted from wheat seedlings that were grown in the dark for 5 d at 20°C and transferred to 40°C for 0-, 1-, 2-, 3-, 4-, or 5-h heat treatment. Total RNA (10 μg) from each time point was subjected to electrophoresis on a formaldehyde-formamide denaturing gel and blotted onto Nytran for hybridization to the radioactively labeled WHSP16.9 cDNA clone.

2 h, the message is fully induced. These results indicate that WHSP16.9 does, in fact, represent a heat-shock-induced message. RNA mol wt markers show that the mRNA that hybridizes to WHSP16.9 is approximately 700 to 800 bases long, which is consistent with the length of the cDNA clone (740 base pairs).

Heat-Shock Induction of Proteins

The fact that several wheat cDNA clones that encode heat-shock proteins were isolated by screening an expression library with polyclonal antibodies to the wheat HMGc chromosomal protein suggested that these antibodies cross-react with low mol wt heat-shock proteins. Immunoblot analysis was performed on total protein extracts from a time course of heat-shocked seedlings in order to determine if the HMGc antibodies recognize heat-shock-induced proteins of wheat. Figure 4 shows that, indeed, proteins recognized by the HMGc antibody preparation are synthesized upon heat-shock. As shown in the upper panel of Figure 4, the cell extract from control tissues contains two proteins (arrowed) that are recognized by the HMGc antibodies. The more rapidly migrating protein is most likely HMGc because it is present in control tissues and comigrates with HMGc (darkest staining band in the HMG marker lane). The slower migrating arrowed protein is probably HMGb, which cross-reacts with HMGc antibodies (25). This band comigrates with HMGb and does not enter IEF gels, a characteristic of the wheat HMG proteins. The HMGc antibodies also react with a group of proteins that migrate between the two arrowed bands and gradually accumulate over the time course of heat shock. This places the heat-shock-induced proteins into a mol wt range consistent with that predicted by the cDNA sequences. On these gels, HMGc comigrates with myoglobin with a molecular mass of 17.2 kD (24).

The results mentioned above show that the antibodies cross-react with several heat-shock-induced proteins. To better resolve these proteins, we have separated them on a two-dimensional system with IEF in the first dimension (pH range of 4–7) and SDS polyacrylamide in the second dimension.

Figure 4. Immunoblot analysis of protein extracts from a time course of heat-shocked tissue. Total cellular protein was extracted from wheat seedling shoots that were grown at 20°C for 5 d in the dark and were then transferred to 40°C for 0, 1, 2, 3, 4, or 5 h. The total cellular proteins were then separated by SDS-PAGE, along with wheat HMG proteins as a control. Top panel, the proteins were transferred to a nitrocellulose filter and detected by antibody binding. Bottom panel, the proteins were stained with Coomassie blue. Two bands are arrowed in the top panel of proteins visualized by reaction with HMGc antiserum. The faster migrating band is presumably HMGc because, in addition to being recognized by the HMGc antiserum, it comigrates with HMGc. This band comigrates with 17.2-kD myoglobin (24). The slower migrating arrowed band is probably HMGb. It comigrates with HMGb, and HMGb cross-reacts with HMGc antiserum (25). Over the time course of heat shock, several bands of heat-shock-induced proteins appear in the region between the two arrowed bands.
indicate that the HMGc antibodies react with at least six heat-shock-induced proteins of similar molecular masses (approximately 17 kD), but different isoelectric points. The control blot indicates that there is one protein of low abundance present in control tissues that is also recognized by the HMGc antibodies.

**DISCUSSION**

Although the low mol wt heat-shock proteins are the predominant class of stress-induced proteins in higher plants, the characterization of genes encoding these proteins has primarily focused on the heat-shock protein genes of dicots. In this study, we have presented the sequences of two cDNAs that by homology to soybean cDNAs encode two low mol wt heat-shock proteins of wheat. Furthermore, we have shown that the messages represented by these cDNAs are, indeed, induced by heat shock. The cDNAs were originally obtained by screening an expression library with polyclonal antibodies generated with a preparation of HMGc (a chromosomal protein) as antigen. In addition to reacting with HMGc, the antiserum cross-reacts with a group of low mol wt proteins that are induced by heat shock in etiolated wheat seedlings. Thus, the cDNAs we have isolated appear to represent heat-shock genes by the manner in which their protein and mRNA are induced, as well as by sequence homology.

We have, as yet, no definitive explanations of why the antiserum to HMGc cross-reacts with the low mol wt heat-shock proteins. The two most obvious explanations are that either these proteins share a common epitope or the heat-shock proteins are present in wheat germ (the source of the HMG preparations) and contaminate the HMG proteins in the antigen preparations. Significant contamination of our antigens with heat-shock proteins seems unlikely because heavily overloaded gels of the HMG preparations show no sign of the heat-shock proteins (24, 25). We have no compelling reason to think that the two groups of proteins share a common epitope; however, we note that if they do, the sharing of the epitope may provide us with a mechanism to study the biological functions of both of these groups of proteins.

A question also arises concerning how we obtained cDNAs for the heat-shock proteins when northern blots consistently show no detectable hybridization of the heat-shock cDNA probes with RNA isolated from control wheat seedlings. We suspect that the explanation for this is that the wheat seedlings from which RNA had been isolated to make the cDNA library were, in fact, heat stressed. Bolstering this speculation is the fact that the seedlings were grown at room temperature in the laboratory during the summer months in North Carolina. Despite air conditioning, elevated room temperatures at this time are common.

**LITERATURE CITED**