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Expression of a Conserved Family of Cytoplasmic Low Molecular Weight Heat Shock Proteins during Heat Stress and Recovery[1]

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ABSTRACT

Plants synthesize several families of low molecular weight (LMW) heat shock proteins (HSPs) in response to elevated temperatures. We have characterized two cDNAs, HSP18.1 and HSP17.9, that encode members of the class I family of LMW HSPs from pea (*Pisum sativum*). In addition, we investigated the expression of these HSPs at the mRNA and protein levels during heat stress and recovery. HSP18.1 and HSP17.9 are 82.1% identical at the amino acid level and are 80.8 to 92.9% identical to class I LMW HSPs of other angiosperms. Heat stress experiments were performed using intact seedlings subjected to a gradual temperature increase and held at a maximum temperature of 30 to 42 degrees Celsius for 4 hours. HSP18.1 and HSP17.9 mRNA levels peaked at the beginning of the maximum temperature period and declined rapidly after the stress period. Antiserum against a HSP18.1 fusion protein recognized both HSP18.1 and HSP17.9 but not members of other families of LMW HSPs. The accumulation of HSP18.1-immunodetected protein was proportional to the severity of the heat stress, and the protein had a half-life of 37.7 ± 8 hours. The long half-life of these proteins supports the hypothesis that they are involved in establishing thermotolerance.

Plants respond to elevated temperatures by expressing several families of evolutionarily conserved HSPs[2] (12, 17, 23, 31). Unlike yeast and most animals, which produce only one to four LMW HSPs (17), plants synthesize many LMW HSPs, between 12 and 27 different polypeptides, depending on the plant species (E. Vierling, unpublished data) (9, 18, 20). There are at least four families of nuclear encoded LMW HSPs in higher plants (22, 26, 31), all of which are members of the eukaryotic LMW HSP gene superfamily (17, 23, 31). Three of these families have been described from more than one plant species; two families, class I and II, encode proteins that are primarily localized in the cytoplasm and the third encodes chloroplast-localized LMW HSPs. Class I HSPs have been cloned and sequenced from wheat (19), soybean (20), and *Arabidopsis thaliana* (10), and class II cytoplasmic LMW HSPs have been identified in pea (15), soybean (25), and maize (8). Chloroplast LMW HSPs have been sequenced in soybean (28), pea (29), maize (24), petunia (4), and *A. thaliana* (4). The relationship between these families has not been closely examined at the amino acid level.

It has long been known that plants can develop the ability to withstand otherwise lethal HS temperatures, a phenomenon referred to as acquired thermotolerance (12, 17, 23). Thermotolerance can be induced by several regimens: a previous moderate HS, a gradual temperature increase, a short, severe HS followed by a recovery period, and pretreatment with arsenite (1, 12, 16). All of these procedures induce accumulation of HSP mRNAs and synthesis of HSPs. These observations have led to the hypothesis that HSPs confer thermotolerance (16). Similar correlations between HSP synthesis and thermotolerance have been obtained in prokaryotes and other eukaryotes (17). However, the mechanism by which HSPs may effect thermotolerance has not been determined.

Although the expression of HSPs under laboratory conditions has been studied extensively, only a few studies have focused on the HS response in intact plants stressed under field conditions. Burke *et al.* (2) found that proteins with mol wts corresponding to HSP89, HSP75, and HSP21 are expressed in cotton grown in dry land fields but not in cotton grown in irrigated fields where canopy temperatures were 10°C lower. Likewise, cytoplasmic LMW HSP mRNAs were expressed in soybeans grown in nonirrigated fields when air temperature approached 40°C and to a lesser extent in irrigated fields where leaf temperatures were presumably cooler (13). Chen *et al.* (3) examined the expression of chloroplast HSP21 mRNA and protein in intact pea seedlings stressed in a growth chamber programmed to mimic the conditions on a hot day. Throughout the course of the day, temperature was increased gradually to a maximum midday temperature, ranging from 34 to 40°C, and then gradually decreased. HSP21 mRNA and protein both accumulated to levels proportional to the applied stress. Chloroplast HSP21 was expressed in both leaves and roots; the protein had a half-life of 52 ± 12.7 h in both tissues (3). Although these studies firmly establish that expression of HSPs occurs under natural conditions, they provide little information concerning the accumulation and stability of specific HSPs other than the chloroplast-localized proteins.

Our laboratory is interested in understanding the roles of

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[2] Abbreviations: HSP, heat shock protein; LMW, low molecular weight; HS, heat shock; poly(A) RNA, polyadenylated RNA; pI, isoelectric point.

LMW HSPs in the natural environment and ultimately in determining their function at the molecular level. As a first step toward these goals we have characterized two cDNAs and the corresponding proteins from a major family of LMW HSPs in *Pisum sativum*. The characterized HSPs are members of the class I family of LMW HSPs, and their comparison to previously sequenced LMW HSPs from pea reveals conserved features of plant LMW HSPs. To determine under what conditions these HSPs may function, their expression at both the mRNA and protein level was studied in intact pea seedlings stressed under growth chamber conditions designed to resemble hot days in a field environment. These are the first studies in which the abundance and stability of the class I LMW HSPs have been examined.

MATERIALS AND METHODS

cDNA Cloning, DNA and Protein Sequence Analysis

Pea HSP18.1 and 17.9 cDNA clones were isolated by previously described methods (29) from a cDNA library constructed in λgt10 with poly(A) RNA isolated from heat-stressed pea (*Pisum sativum*, cv "Little Marvel") leaves (29). The library was screened at reduced stringency with a soybean LMW HSP cDNA clone homologous to the soybean pCE53 cDNA described by Czarnecka *et al.* (5).

The cDNA clones were subcloned into either M13 or Bluescript (Stratagene, La Jolla, CA) vectors and both strands were completely sequenced (4, 29). Analysis of ORFs, as well as pairwise sequence comparisons between HSPs, was performed using the Gap program included with the Wisconsin GCG Sequence Analysis software package (6). Multiple amino acid sequence alignments were done with the CLUSTAL 3 alignment program (11). Both pairwise and multiple comparisons were performed with the default parameters specified by the software.

Hybridization Selection and *in Vitro* Transcription/Translation

The HSP cDNA plasmids were used to hybrid select mRNA according to the procedure described previously (29). The HSP18.1 cDNA insert was also subcloned into an SP6 transcription vector and transcribed using SP6 polymerase as recommended by the supplier (Boehringer Mannheim Biochemicals, Indianapolis, IN). Hybrid-selected mRNA was translated in a wheat germ cell-free system (28). Total poly(A) RNA and *in vitro* transcribed mRNA was translated in reticulocyte lysate extracts (Bethesda Research Laboratories, Bethesda, MD). Translations were performed in the presence of ^{35}S-Met (>1000 Ci mmol^{-1}; 40 TBq mmol^{-1}; New England Nuclear Inc., Boston, MA) using 0.25 to 0.75 mCi/mL of translation mix.

Plant Growth and HS

Peas were planted in vermiculite and grown in a growth chamber on a 22/18°C, 16-h day/8-h night cycle. Light intensity was 300 μE m^{-2} s^{-1}. Plants were watered with 0.25 strength Hoagland solution. Intact plants were heat stressed after the first leaves had expanded (8–9 d) as described by Chen *et al.* (3). The growth chamber temperature was increased 4°C/h until the desired stress temperature (30–40°C) was reached, maintained at that temperature for 4 h, and then decreased at 4°C/h until the temperature returned to 22°C. High humidity was maintained during HS to prevent transpirational cooling, and leaf temperature was measured using an IR thermometer (Everest Interscience, Fullerton, CA). Samples for protein and RNA analysis were obtained before the HS, at five time points spaced at 2-h intervals during the HS, and when the chamber temperature had returned to 22°C. To examine recovery after HS, plants were kept at normal growth temperatures for 7 d following the day of HS treatment. Samples for protein analysis were obtained each day of recovery at the time of day corresponding to the end of the maximum temperature treatment of the stress day.

RNA Isolation and Northern Analysis

RNA isolation and Northern analysis were performed as described previously (28). Total RNA was isolated from leaves of three plants and 10 μg of each RNA sample were separated on formaldehyde agarose gels and capillary blotted to Nytran membrane (Schleicher & Schuell, Keene, NH). The filters were hybridized and washed under high stringency conditions that are estimated to require 90% nucleotide identity for stable hybrid formation (29). HSP18.1 and HSP17.9 cDNA probes were labeled with [^{32}P]dATP (ICN Radiochemicals, Irvine, CA, 3000 Ci/mmol) by random priming. The hybridization signals were quantitated using a betascope (Betagen Corp., Waltham, MA).

Antibody Production

Antibodies against pea HSP18.1 were generated using antigen synthesized as a fusion protein in *Escherichia coli*. A portion of the HSP18.1 cDNA encoding the carboxyl-terminal 15.3 kD of the protein and 373 nucleotides of 3′-noncoding sequence was excised as a 775-base pair *Bam*HI-*Eco*RI fragment and cloned into the expression vector pATH22 (27). In pATH expression vectors, a protein or protein fragment is fused to the carboxyl-terminus of the amino-terminal 35 kD of TrpE and expressed under control of the Trp promoter. To prepare the fusion construct, the pATH22 and HSP18.1 cDNA plasmids were digested with *Xba*I and *Eco*RI, respectively, and the 5′ overhangs were filled in using Klenow polymerase. Both plasmids were then digested with *Bam*HI and ligated together. The size of the TrpE-HSP18.1 fusion protein was determined by SDS-PAGE to be 49 kD, which is in agreement with the predicted size. The TrpE-HSP18.1 fusion protein was overproduced in *E. coli* using the method of Spindler *et al.* (27) and purified by SDS-PAGE and electroelution (30). The purified protein was used to generate antibodies in New Zealand white female rabbits as described previously (30). Preimmune serum was taken from the rabbits before inoculation, and immune serum was taken 7 to 9 d after the third and subsequent injections.

Protein Electrophoresis

Protein was extracted from the first leaf pair of two plants as described by Chen *et al.* (3), and protein concentration was

determined using the BCA protein assay (Pierce Chemical Company, Rockford, IL). Samples were separated either on 12.5% acrylamide SDS gels (8–30 μg protein/lane) or on two-dimensional gels (250 μg protein/gel) (29).

Western Analysis and HSP Quantitation

Protein gels were electroblotted onto nitrocellulose (Schleicher & Schuell) and reacted with crude antiserum at a 1:500 dilution as described previously (30) except that no dried milk was added to the hybridization and wash solutions. Bound antibody was visualized with ^{125}I-protein A (ICN Radiochemicals, Irvine, CA; >30 mCi/mg) followed by autoradiography. For quantitative analyses, equal protein loading and integrity of protein samples was verified on Coomassie blue-stained gels in parallel to the immunoblots. HSP18.1 was quantified by cutting the ^{125}I-protein A-labeled bands from the nitrocellulose filters and determining their radioactivity with a scintillation counter (3). The amount of radioactivity in the bands was consistent with the intensity of the corresponding signal on the autoradiograms.

RESULTS

Isolation and Characterization of HSP18.1 and HSP17.9 cDNAs

When the pea HS leaf cDNA library was screened at reduced stringency with a class I soybean LMW HSP cDNA, >1% of the phage hybridized with the probe. The high percentage of hybridization is consistent with the estimated abundance of LMW HSP mRNAs (A. DeRocher, unpublished data) (29). Five phage were randomly selected and plaque purified, and their insert sizes were determined. The two longest cDNAs were further characterized by DNA sequence analysis.

Following sequencing the clones were designated HSP18.1 and HSP17.9, based on the calculated mol wts of the peptides encoded by their longest ORFs. The HSP18.1 and HSP17.9 nucleotide sequences have been deposited in the GenBank library with accession numbers M33899 and M33900, respectively. The HSP18.1 cDNA is 860 base pairs long, contains 13 nucleotides of 5′-noncoding sequence and 370 nucleotides of 3′-noncoding sequence, and has a 477-base ORF encoding a 159 amino acid protein (Fig. 1). A preliminary analysis of

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A. Multiple alignment of class I cytoplasmic LMW HSPs from different species.

                                          20                  40                  60                  80
P. sativum HSP18.1   MSLIPSFFS-GRRSNVFDPFSLDVWDPLKDFPFSNSSPSASFPRENPAFVSTRVDWKETPEAHVFKADLPGLKKEEVKVEVEDDR
P. sativum HSP17.9   --IIPRVFGTGRRTNAFDPFSLDLWDPFQNFQLARSATGTTN--ETAAFANAHIDWKETPEAHVFKADLPGVKKEEVKVEIEEDR
G. max HSP17.5-E     MSLIPGFFG-GRRSNVFDPFSLDMWDPFKDFHVPTSSVSA----ENSAFVSTRVDWKETPEAHVFKADIPGLKKEEVKVQIEDDR
A. thaliana HSP17.6  MSLIPSIFG-GRRTNVFDPFSLDVFDPFEGFLTP-SGLANAPAMDVAAFTNAKVDWRETPEAHVFKADLPGLRKEEVKVEVEDGN
T. aestivum C5-8     MSIV-------RRSNVFDPFADLWADPFDTFRSIVPAISGGSS-ETAAFANARVDWKETPEAHVFKVDLPGVKKEEVKVEVEDGN
                       ..       ** * ****    **.  *                 .  **    .**.********* *.**..******..*.
                                         100                 120                 140
P.s. HSP18.1          VLQISGERSVEKEDKNDEWHRVERSSGKFLRRFRLPENAKMDKVKASMENGVLTVTVPKEEIKKAEVKSIEISG
P.s. HSP17.9          VLKISGERKTEKEDKNDTWHRVERSQGSFLRRFRLPENAKVDQVKAAMENGVLTVTVPKEEVKKPEAKPIQITG
G.m. HSP17.5-E        VLQISGERNVEKEDKNDTWHRVERSSGKFTRRFRLPENAKVNEVKASMENGVLTVTVPKEEVKKPDVKAIEISG
A.t. HSP17.6          ILQISGERSNENEEKNDKWHRVERSSGKFTRRFRLPENAKMEEIKASMENGVLSVTVPKVPEKKPEVKSIDISG
T.a. C5-8             VLVVSGERSREKEDKNDKWHRVERSSGKFVRRFRLPEDAKVEEVKAGLENGVLTVTVPKAEVKKPEVKAIEISG
                      .* .****  * *.*** ******* * * *******.**.  .** .***** *****   ** . * *.* *

B. Multiple alignment of P. sativum HSPs from different LMW HSP classes.
                                                                                                        20
HSP18.1                                                                             MSLIPSFFS-GRRSNVFDPFS-LDV
HSP17.9                                                                               IIPRVFGTGRRTNAFDPFS-LDL
HSP17.7                                                                                 MDFRLMDLDSPLFNTLH-HIM
HSP21      MAQSVSLSTIASPILSQKPGSSVKSTPPCMASFPLRRQLPRLGLRNVRAQAGGDGDNKDNSVEVHRVNKDDQGTAVERKPRRSSIDISPFGLLDP
                                                                                                   .
                          40                  60                  80                  100
HSP18.1    WDPLKDFPFSNSSPSA------SFPRENPAFVSTRVDWKETPEAHVF--KADLPGLKKEEVKVEVEDDRVLQISGERSVEKEDKNDEWHRVERSS
HSP17.9    WDPFQNFQLARSATGT------TN--ETAAFANAHIDWKETPEAHVF--KADLPGVKKEEVKVEIEEDRVLKISGERKTEKEDKNDTWHRVERSQ
HSP17.7    DLTDDTTEKNLNAPTR------TYVRDAKAMAATPADVKEHPNSYVF--MVDMPGVKSGDIKVQVEDENVLLISGERKREEEKEGVKYLKMERRI
HSP21      WSPMRSMRQMLDTMDRIFEDAITIPGRNIGGGEIRVPWEIKDEEHEIRMRFDMPGVSKEDVKVSVEDDVLVIKSDHR--EENGGEDCWSRK--SY
                                                           *.**.   ..** .*.. ..  *  *  *        .
                                                           Consensus II
                120                 140
HSP18.1    GKFLRRFRLPENAKMDKVKASMENGVLTVTV---PKEEIKKAEVKSIEISG-
HSP17.9    GSFLRRFRLPENAKVDQVKAAMENGVLTVTV---PKEEVKKPEAKPIQITG-
HSP17.7    GKLMRKFVLPENANIEAISAISQDGVLTVTVNKLPPPEPKKPKTIQVKVA--
HSP21      SCYDTRLKLPDNCEKEKVKAELKDGVLYITI---PKT---KIERTVIDVQIQ
          .. **.*   . . *   .*** .*.    *     *      . .
                  Consensus I
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Figure 1. Comparison of HSP18.1 and HSP17.9 amino acid sequences to other plant LMW HSPs. A, Comparison of HSP18.1 and HSP17.9 to class I LMW HSPs from soybean (20), *A. thaliana* (10), and wheat (19). B, Comparison to class II (HSP17.7) (15) and chloroplast (HSP21) (29) LMW HSPs from pea. Sequences were aligned as described in "Materials and Methods." *, Identical amino acids; •, conservative replacements; –, gaps introduced to optimize alignment. Numbers reference the HSP18.1 sequence. The conserved carboxyl-terminal HS domain is shown in bold letters. Consensus regions I and II are described in the text.

the derived amino acid sequence of HSP18.1 was previously reported, via a communication from us, by Neumann *et al.* (23). The HSP17.9 cDNA has a 465-base ORF that encodes a 155-residue protein but does not contain a start Met codon. The HSP17.9 ORF is followed by 234 nucleotides of 3'-noncoding sequence. Comparison to related LMW HSP amino acid sequences (Fig. 1, discussed below) suggests that HSP17.9 is missing six nucleotides at the 5' end that would encode the start Met followed by a conserved Ser residue. Consequently, the mol wt of the HSP17.9 protein was calculated based on the assumption that Met and Ser are the first two residues of the complete protein.

The HSP18.1 and HSP17.9 nucleotide sequences are 71.8% identical throughout the coding sequence. Both the HSP18.1 and HSP17.9 cDNAs have two AATAAA polyadenylation consensus sequences. The HSP18.1 polyadenylation signals are 297 and 120 nucleotides from the 3' end of the cDNA, and those from HSP17.9 are 191 and 178 nucleotides from the 3' end.

Relationships of HSP18.1 and HSP17.9 to Other LMW HSPs

To determine the relationship of the HSP18.1 and HSP17.9 proteins to members of different LMW HSP gene families from plants, a series of pairwise and multiple comparisons to seven previously characterized LMW HSPs were performed. In addition to pea HSP18.1 and HSP17.9, the following HSPs were included in the analyses: pea chloroplast localized HSP21 (29), pea HSP17.7 (15), *A. thaliana* HSP17.6 (10), soybean (*Glycine max*) HSP17.5-E (20), soybean HSP17.9-D (25), wheat (*Triticum aestivum*) C5-8 (19), and maize (*Zea mays*) HSP18.3 (8). The derived amino acid sequences were used in all comparisons.

Results of pairwise comparisons between the nine LMW HSPs are shown in Table I. Pea HSP18.1 and HSP17.9 had the highest amino acid sequence similarity to the class I cytoplasmic HSPs (80.1–92.9%) which includes wheat C5-8, soybean HSP17.5-E, and *A. thaliana* HSP17.6. In contrast, HSP18.1 and HSP17.9 are only 54.5 to 62.2% similar to the class II LMW HSPs, although the class II sequences are 84.1 to 92.4% similar among different species. HSP18.1 and HSP17.9 are also <55% similar to pea chloroplast HSP21. We conclude that HSP18.1 and HSP17.9 belong to the class I family of LMW HSPs.

Multiple sequence alignments further demonstrate the relationship of HSP18.1 and HSP17.9 to other class I HSPs and to pea LMW HSPs belonging to class II or chloroplast HSP gene families. When class I HSPs from soybean, *A. thaliana*, and wheat are optimally aligned with HSP18.1 and HSP17.9, they share 64% similarity over the length of the sequence (Fig. 1A). In contrast, when HSP18.1 and HSP17.9 are compared with pea HSP17.7 and pea HSP21, the similarity along the shared sequence (amino acids 5–158 of HSP18.1) is only

Table I. *Percentage of Amino Acid Similarity/(Identity) among Plant LMW HSPs*

Amino acid sequence similarity was determined using the GCG program Gap. After finding optimal alignment between a pair of amino acid sequences, the total conserved and identical (parentheses) residues were divided by the length of the consensus sequence and the quotient was defined as percentage of identity or similarity.

Protein	Mol Wt	pI	Class I					Class II[a]		
			P. sativum HSP 18.1	*P. sativum* HSP 17.9	*A. thaliana* HSP 17.6	*G. max* HSP 17.5E	*T. aestivum* C5-8	*P. sativum* HSP 17.7	*G. max* HSP 17.9-D	*Z. mays* HSP 18.1
	kD									
Class I										
P. sativum HSP 18.1	18.1	5.96								
P. sativum HSP 17.9	17.9	7.78	82.1 (71.2)							
A. thaliana HSP 17.6	17.6	5.24	84.7 (71.9)	80.2 (66.0)						
G. max HSP 17.5-E	17.5	6.31	92.9 (85.1)	85.1 (73.4)	85.1 (71.4)					
T. aestivum C5-8	16.9	5.95	84.5 (71.6)	82.0 (70.0)	80.1 (68.2)	84.5 (72.3)				
Class II										
P. sativum HSP 17.7	17.6	6.82	59.7 (38.3)	57.7 (37.6)	59.6 (38.4)	59.2 (37.5)	62.2 (40.5)			
G. max HSP 17.9-D	17.9	6.29	59.6 (37.1)	54.5 (33.8)	56.4 (33.9)	56.3 (38.4)	60.0 (34.7)	92.4 (78.3)		
Z. mays HSP 18.3	17.8	5.19	59.0 (36.5)	61.2 (36.1)	58.8 (34.0)	60.7 (37.9)	61.7 (39.6)	84.1 (62.4)	84.9 (64.8)	
Chloroplast										
P. sativum HSP 21	26.2	6.67	52.0 (30.3)	54.3 (27.2)	57.1 (27.9)	56.8 (29.7)	58.6 (31.0)	53.1 (28.3)	57.2 (28.3)	58.4 (32.5)

[a] Class II LMW HSPs defined by Vierling (31), previously designated class VI (26).

25.4% (Fig. 1B). In the latter comparison, sequence homology is restricted to two regions: the carboxyl-terminal "HS domain" (4, 17, 31) found between amino acids 118 and 143 of HSP18.1 (consensus I) and a domain between amino acids 66 and 95 of HSP18.1 (consensus II). Consensus I is conserved among all eukaryotic LMW HSPs, whereas consensus II is not (4, 29, 31). However, the region encompassing consensuses I and II and the intervening sequence has a distinctive hydropathy profile found in all eukaryotic LMW HSPs, suggesting a greater conservation of structure than is indicated by the sequence homology. Together, the alignments show that sequence conservation among LMW HSPs within a gene family is maintained across divergent orders and far exceeds that of LMW HSPs from different gene families, even if they are gene families from the same plant species.

Characterization of HSP18.1 and HSP17.9 Gene Products

The proteins encoded by the HSP18.1 and HSP17.9 cDNAs clones were characterized by hybrid selection translation experiments. The HSP18.1 and HSP17.9 plasmids were used to hybrid select mRNAs from HS leaf poly(A) RNA. The hybrid-selected mRNAs and total poly(A) RNA from heat-stressed and unstressed leaves were translated *in vitro* in the presence of ^{35}S-Met and the translation products separated by SDS-PAGE (not shown). HSP18.1 and HSP17.9 hybrid-selected mRNAs produced translation products that migrated as polypeptides with estimated sizes of 20 and 19 kD, respectively. These polypeptides co-migrated with major translation products of total HS poly(A) RNA. Neither HSP18.1 nor HSP17.9 cDNAs selected messages from the unstressed leaf poly(A) RNA (not shown).

HSP18.1 and HSP17.9 hybrid-selected translation products were separated by two-dimensional gel electrophoresis to ascertain the pIs of the encoded proteins and to determine whether multiple isoelectric forms were present (Fig. 2). The HSP18.1 hybrid-selected translation products separated as two prominent polypeptides of approximately equal intensity with pIs of approximately 5.8 and 6.1 (Fig. 2C). These polypeptides co-migrated with prominent polypeptides seen in *in vitro* translation of total heat-stressed leaf RNA (Fig. 2A) but not in translations of unstressed leaf RNA (Fig. 2B). These two polypeptides could represent either the products of two distinct messages that were hybrid-selected by the HSP18.1 cDNA or a single polypeptide that was posttranslationally modified by the *in vitro* translation system. To distinguish

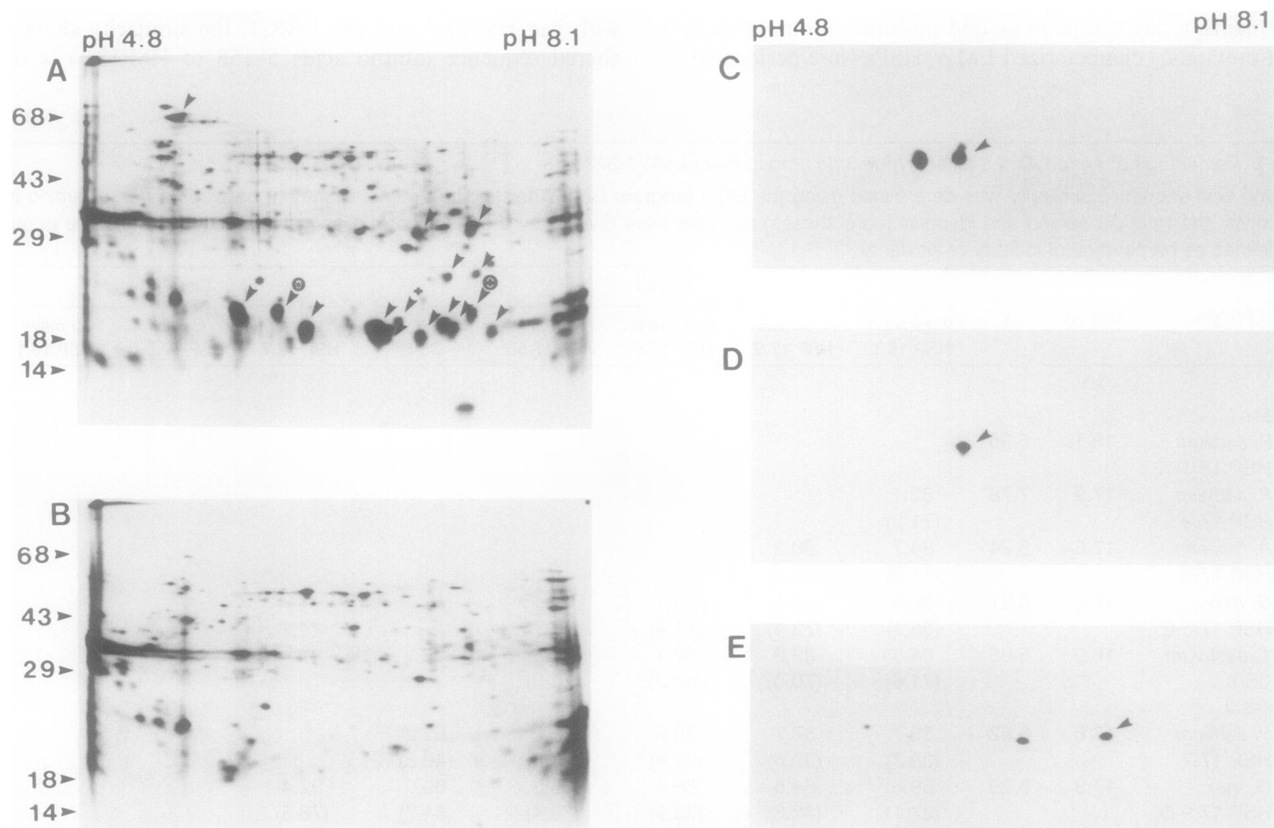

Figure 2. HSP18.1 and HSP17.9 encode distinct, heat-inducible mRNAs. *In vitro* translation products of the following mRNA samples are presented: HS leaf poly(A) RNA (A), unstressed leaf poly(A) RNA (B), HS leaf RNA hybrid-selected with the HSP18.1 cDNA (C), HSP18.1 mRNA that had been transcribed *in vitro* (D), and HS leaf RNA hybrid-selected with the HSP17.9 cDNA (E). The translation products were separated by two-dimensional gel electrophoresis and autoradiographed. Only the lower half of the gels are presented in C, D, and E. In A: arrowheads, proteins induced by HS; * above arrowhead, proteins corresponding to HSP18.1 hybrid-selected products; +, HSP17.9 hybrid-selected products; circles around + and *, the HSP18.1 and HSP17.9 gene products. In A and B: ordinates, molecular size in kD. In C and D: arrowheads, the HSP18.1 gene product. In E: arrowhead, the HSP17.9 gene product.

between these two possibilities, the HSP18.1 plasmid was transcribed *in vitro* and translated, and these products were separated on a two-dimensional gel (Fig. 2D). Only one polypeptide was produced, which co-migrated with the more alkaline, pI 6.1, peptide. The observed pI (6.1) is close to the predicted pI (5.96) calculated from the deduced amino acid sequence. This result indicates that this is the protein encoded by HSP18.1 and that the other protein is a closely related family member.

The translation of HSP17.9 hybrid-selected mRNAs revealed two major polypeptides, with pIs of 7.4 and 7.1, and three weaker spots, with pIs ranging from 5.9 to 6.7 (Fig. 2E). Because the HSP17.9 cDNA is not full length, it was not possible to perform an *in vitro* transcription/translation experiment, as was done for HSP18.1. However, we suggest that the major isoelectric forms most likely represent unique gene products, as seen for HSP18.1. The observed pI of the more alkaline of the two prominent signals (7.4) is close to the predicted pI of HSP17.9 (7.78) and is probably the corresponding protein.

Antibody Production and Characterization

To study the expression of class I LMW HSP gene products, antibodies that specifically recognize these proteins were generated. Because we lacked a convenient method to purify the HSPs, a TrpE-HSP18.1 fusion protein was constructed that included amino acid residues 25 to 159 of HSP18.1. The fusion protein was used as an antigen as described in "Materials and Methods." Antibody specificity was characterized by SDS-PAGE and Western blotting of protein extracted from heat-stressed and unstressed leaves. The HSP18.1 antiserum reacted strongly with a wide 19 to 20 kD band in heat-stressed leaf samples and showed no reactivity with samples from unstressed leaves (Fig. 3A). Preimmune serum did not react with protein from either heat-stressed or unstressed leaves (Fig. 3A).

To characterize further the proteins detected by the antiserum, a two-dimensional gel of HS leaf proteins was Western blotted and probed with HSP18.1 antiserum (Fig. 3B). The antiserum reacted at different intensities with five proteins of apparent sizes between 19 and 20 kD whose pIs ranged from 5.6 to 7.4. Two of the five prominently reacting polypeptides co-migrated with the polypeptides that had been identified as the products of the HSP18.1 and HSP17.9 cDNAs, indicating that this antiserum reacts with several members of the class I LMW HSP family. The reaction with HSP17.9 was confirmed by mixing ^{35}S-Met-labeled HSP17.9 hybrid-selected/translation products with HS leaf protein, separating the products on two-dimensional gels and transferring to nitrocellulose. The filters were autoradiographed, revealing the HSP17.9 hybrid-selected products. The filter was then reacted with HSP18.1 antiserum and ^{125}I-protein A and reexposed. The proteins synthesized in the HSP17.9 hybrid-selected translation co-migrated precisely with proteins detected by the antiserum (not shown). None of the proteins recognized by the antiserum co-migrated with LMW HSP gene products representing the pea HSP17.7 or chloroplast HSP21 gene families (not shown). Therefore, it is probable that the other proteins that react with the HSP18.1 antiserum are also members of the class I LMW HSP gene family.

Expression of HSP18.1 and HSP17.9 mRNA during and following HS

We were interested in how high temperatures affect LMW HSP expression at both the mRNA and protein levels in plants stressed under conditions they might encounter in the natural environment. Therefore, plants were heat stressed for 4 h using a temperature regimen designed to mimic conditions on a hot, humid day (Fig. 4A and "Materials and Methods"). Under this environmental regimen, leaf temperature closely parallels the growth chamber temperature (3). Plants were stressed to a maximum temperature of either 34 or 38°C, and RNA was isolated from leaves at the time points indicated (Fig. 4A). Figure 4, B and C, show Northern blots of total leaf RNA isolated from plants heat stressed at 38°C and probed at high stringency with HSP18.1 or HSP17.9 cDNAs. Both mRNAs show low but detectable expression at 10:00 AM, when the chamber had reached 30°C and the leaf temperature was approximately 31.5°C. The mRNA level was close to maximum by the time the chamber reached 38°C and the leaf

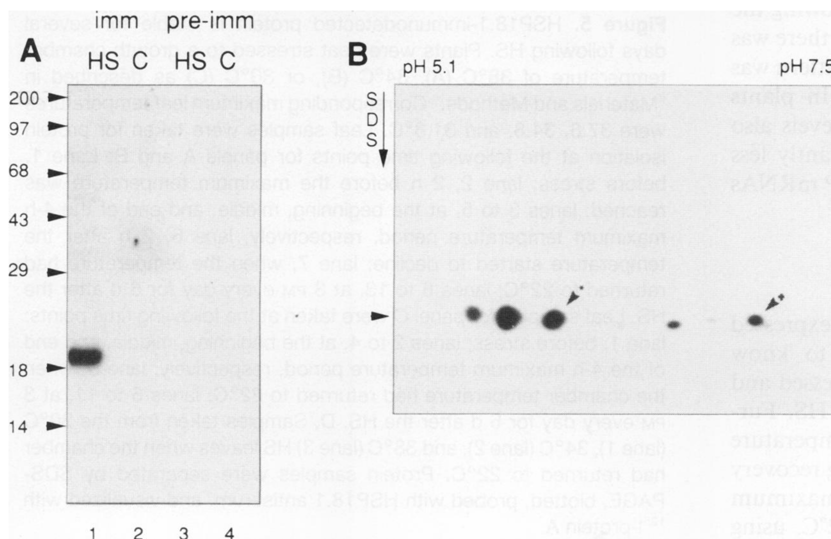

Figure 3. Antibodies against the TrpE-HSP18.1 fusion protein recognize HSP18.1 and closely related LMW HSPs. A, Proteins isolated from heat-stressed (lanes 1 and 3) and unstressed (lanes 2 and 4) leaves were separated by SDS-PAGE, Western blotted, and probed with HSP18.1 immune serum (lanes 1 and 2) or preimmune serum (lanes 3 and 4). Antibody binding was visualized with ^{125}I-protein A and autoradiography. Ordinate, molecular size in kD. B, protein isolated from heat-stressed leaves was separated by two-dimensional electrophoresis, Western blotted, probed with HSP18.1 antiserum, and visualized with ^{125}I-protein A and autoradiography. Arrowhead with an *, the HSP18.1 gene product; arrowhead with +, the HSP17.9 gene product.

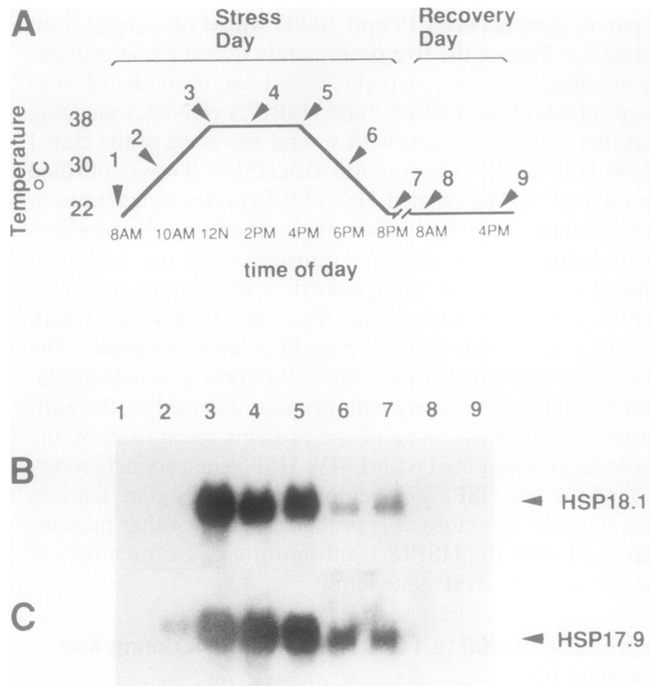

Figure 4. HSP18.1 and HSP17.9 mRNA expression during and following HS. A, HS regimen for a 38°C stress. Growth chamber temperature was raised 4°C/h until the desired stress temperature was reached, maintained at that temperature for 4 h, and then decreased 4°C/h until the chamber returned to 22°C. Numbered arrowheads, time points at which total RNA was isolated. B and C: Northern analysis of RNA samples probed with HSP18.1 (B) or HSP17.9 (C). Lane numbers correspond to sample numbers in panel A. RNA samples were separated on formaldehyde/agarose gels, transferred to nytran membrane, and probed with ^{32}P-labeled cDNA inserts.

temperature was approximately 36°C. The amount of HSP18.1 message peaked at the beginning of the HS and declined by 40% during the next 4 h. The mRNA levels declined to <10% of the maximum accumulation as the chamber returned to 22°C. By 8 AM on the day following the stress, only faint signals were detected, and by 2 PM, there was no detectable HSP18.1 or HSP17.9 message. This pattern was observed in two separate 38°C HS experiments. In plants stressed at 34°C, HSP18.1 and HSP17.9 mRNA levels also peaked at the beginning of the stress, but significantly less mRNA accumulated than at 38°C (not shown). HSP mRNAs were absent by the end of the 34°C stress day.

Expression of HSPs during and following HS

Because it was evident that HSP18.1 mRNA was expressed under physiological HS conditions, we wanted to know whether the corresponding proteins were also expressed and to what levels these proteins accumulated during HS. Furthermore, we wanted to determine the minimum temperature at which HSPs accumulate and their stability during recovery from HS. Intact pea plants were heat stressed to maximum temperatures of 28, 30, 32, 34, 36, 38, 40, and 42°C, using the gradual temperature increase regimen. At 28°C HSPs were not consistently expressed, and at 42°C the plants suffered extensive visible tissue damage. Therefore, plants treated at these temperatures were not further characterized. The maximum leaf temperatures were 31.8°C when the growth chamber temperature was programmed at 30°C; 33.0°C when it was set at 32°C, 34.3 to 34.8°C at 34°C, 35.4 to 35.8°C at 36°C, 37.5 to 37.7°C at 38°C, and 38.4 to 39.5°C at 40°C.

The relative amount of HSP18.1 and related proteins present in leaf tissues during HS and recovery was determined by SDS-PAGE and Western blotting. Analysis of typical HS and recovery experiments at 38, 34, and 30°C are shown in Figure 5. No HSP18.1-immunodetected protein is seen in leaves

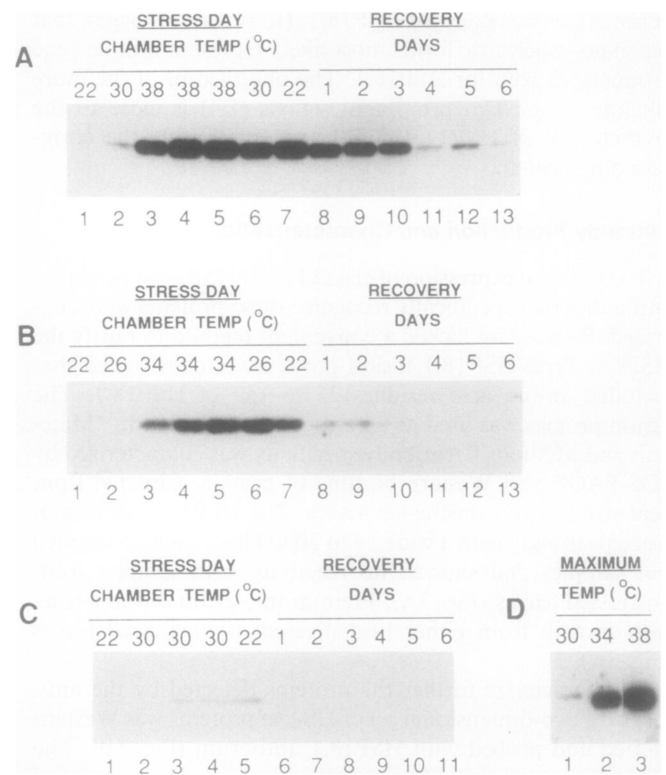

Figure 5. HSP18.1-immunodetected protein is stable for several days following HS. Plants were heat stressed to a growth chamber temperature of 38°C (A), 34°C (B), or 30°C (C) as described in "Materials and Methods." Corresponding maximum leaf temperatures were 37.3, 34.8, and 31.8°C. Leaf samples were taken for protein isolation at the following time points for panels A and B: Lane 1, before stress; lane 2, 2 h before the maximum temperature was reached; lanes 3 to 5, at the beginning, middle, and end of the 4-h maximum temperature period, respectively; lane 6, 2 h after the temperature started to decline; lane 7, when the temperature had returned to 22°C; lanes 8 to 13, at 3 PM every day for 6 d after the HS. Leaf samples for panel C were taken at the following time points: lane 1, before stress; lanes 2 to 4, at the beginning, middle, and end of the 4-h maximum temperature period, respectively; lane 5, when the chamber temperature had returned to 22°C; lanes 6 to 11, at 3 PM every day for 6 d after the HS. D, Samples taken from the 30°C (lane 1), 34°C (lane 2), and 38°C (lane 3) HS leaves when the chamber had returned to 22°C. Protein samples were separated by SDS-PAGE, blotted, probed with HSP18.1 antiserum, and visualized with ^{125}I-protein A.

before they are heat stressed. At all temperatures examined, the amount of HSP18.1-immunodetected protein peaked at the end of the stress day and remained high while the leaf temperature returned to 22°C. In the plants stressed at 38 and 34°C, the protein persisted for several days following the stress.

To determine the relative abundance of HSP18.1-immunodetected protein at different temperatures, samples from the 38, 34, and 30°C stresses were run on the same gel and Western blotted. Results showed that the abundance of HSP18.1-immunodetected protein was strongly correlated with the severity of the HS (Fig. 5D).

To determine more precisely the pattern of HSP18.1 accumulation and decline, HSP18.1-immunodetected protein was quantified for the 38°C stress treatment as described in "Materials and Methods" (Fig. 6). The values represent the means and SD from three independent HS experiments. Significant variation at some time points was apparently due to microheterogeneity in leaf temperatures and/or some variation in response between individuals. HSP18.1-immunodetected protein was approximately one-third maximum at the beginning of the 38°C temperature period and continued to increase throughout the stress period and during the temperature decline, with maximum accumulation after the temperature returned to 22°C. There was an approximately twofold decline in the amount of HSP18.1-immunodetected protein in the 20 h between the time the chamber returned to 22°C and the first recovery time point on the following day. There was a slower decline during the subsequent recovery period. This general pattern was observed at all stress temperatures except the 40°C HS experiments (not shown). In plants stressed to 40°C (38.4–39.5°C leaf temperature), the amount of HSP18.1-immunodetected protein did not decline significantly until after 1 to 3 d of recovery.

Because both HSP18.1 and HSP17.9 mRNAs are no longer detectable by the afternoon of the day following HS (Fig 6A), HSP18.1-immunodetected protein cannot be synthesized following this time point during recovery. Therefore, the half-lives of the proteins can be calculated by quantifying the amount of protein remaining at 24-h intervals during recovery and, from that, calculating the rate of decay. A half-life of 37.7 ± 8 h was estimated for the 38°C experiments shown in Figure 6B, assuming an exponential decay rate (half-life = −0.6931 × 24 h/ln [amount remaining]). The half-life of the protein was not significantly different at the other stress temperatures.

Because the HSP18.1 antiserum reacts with several class I LMW HSPs, it is possible that the persistent signal we observed represents a specific subset of these proteins that are more stable than other family members. Alternatively, the different family members may have similar half-lives. To distinguish between these possibilities, samples obtained during the HS and after 2 and 5 d of recovery were separated on two-dimensional gels and analyzed by Western blotting. There was no evidence of a substantial difference in the rates of decay between the proteins (not shown).

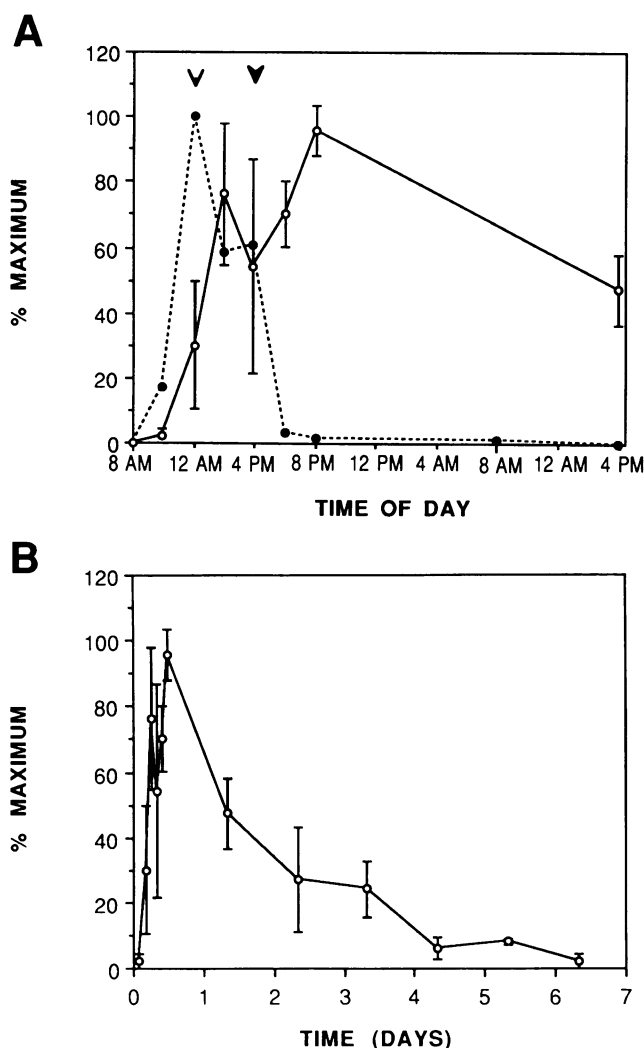

Figure 6. Quantitation of HSP18.1-immunodetected protein and mRNA during HS and recovery. Plants were heat stressed for 4 h at 38°C as described in "Materials and Methods." The growth chamber reached 38°C at noon (open arrowhead) when leaf temperatures were 35.0 to 36.6°C, and the plants reached their maximum temperatures (37.5–37.7°C) at 4 PM (solid arrowhead). ● (A), HSP18.1 mRNA abundance from a single experiment, quantitated as described in "Materials and Methods." ○ (A and B), the mean amount of protein (bar, SD) from three separate 38°C HS experiments.

DISCUSSION

We have characterized a major family of conserved LMW HSPs from pea and examined the expression of the corresponding mRNAs and proteins in intact seedlings. HSP18.1 and HSP17.9 are members of the class I LMW HSP family of higher plants (25, 31), indicated by their amino acid sequence homology with the known class I LMW HSPs. At the amino acid level, HSP18.1 and HSP17.9 are 83% identical to each other, which is comparable to the approximate 90% amino acid identity between individual soybean class I LMW HSPs (22). There is greater homology between the class I HSPs of pea and other angiosperms than between class I HSPs and either class II or chloroplast LMW HSPs of pea. However, all LMW HSPs contain a conserved carboxyl-terminal HS domain corresponding to residues 118 to 143 of HSP18.1 (4, 17, 31). This region contains the absolutely conserved Pro

121, Gly 135, Val 136, and Leu 137 residues found in all eukaryotic LMW HSPs. The homology relationships of the different families of plant LMW HSPs suggests that divergence of the different families occurred before the divergence of monocots and dicots.

Five proteins, including HSP18.1 and HSP17.9, reacted strongly with the HSP18.1 antiserum and four other proteins reacted weakly on two-dimensional Western blots. The antiserum did not detect pea HSP17.7 or HSP21, which are members of other classes of LMW HSPs. These results imply that the eight polypeptides detected by the HSP18.1 antiserum are all members of the class I family. Hybridization selection-translation experiments with HSP18.1 and HSP17.9 cDNAs yielded two and five proteins, respectively, and eight to ten bands are observed on Southern blots hybridized to HSP18.1 or HSP17.9 at low stringency (L. Lauzon, unpublished data). Taken together, these data support the conclusion that the class I LMW HSP gene family in pea contains on the order of eight members. This is substantially less than the 13 class I HSPs identified in soybean by hybridization selection (22). These eight class I proteins are a subset of the 16 to 18 pea LMW HSPs observed in our experiments (Fig. 2, A and B). A similar number of LMW HSPs has been identified in pea by others (18). The remaining eight to ten proteins are members of the other classes of LMW HSPs, some of which are also multigene families, and possibly class I LMW HSPs that are not detected by the antiserum.

The HSP18.1 and HSP17.9 mRNAs accumulated rapidly in response to HS, first appearing when the leaf temperature was approximately 31°C. In severely stressed tissue, the HSP18.1 mRNA is abundant; in plants that have been heat stressed at 38°C for 2 h, HSP18.1 mRNA accounts for approximately 0.5% of the poly(A) RNA in the leaves (A. DeRocher, unpublished data). The amount of mRNA peaked at the beginning of the 4-h HS period and had declined substantially by the end of the 4-h stress, although the leaf temperature was still high. A decrease in LMW HSP mRNA preceding a temperature decrease was also observed in field-grown soybeans (13) and in soybean seedlings stressed at 41°C for 4 h (12). These observations are consistent with those of Kimpel *et al.* (14) who reported maximum levels of LMW HSP transcription following 15 min of a moderate HS and a decline in transcription after 1 h at HS temperatures. This suggests that factors in addition to tissue temperature regulate HSP mRNA levels. It is interesting to note that HSP18.1 mRNA levels began to decline 6 to 8 h before the amount of HSP18.1-immunodetected protein reached maximum levels. HSP70 protein levels are believed to regulate HSP70 expression at both the transcriptional and posttranscriptional levels in *Drosophila* (7). Our results suggest that LMW HSP levels in plants may also be self-regulated or regulated by some other heat-inducible protein such as HSP70.

Although numerous studies in plants have documented that the rate of HSP mRNA synthesis increases with increasing temperature, this is the first study in which the accumulation and stability of class I LMW HSPs have been measured. The level to which HSP18.1-immunodetected protein accumulated was proportional to the HS temperature. There is 25 to 50-fold more HSP18.1-immunodetected protein produced during a 40°C HS than during a 30°C HS, and the maximum signal on the 30°C HS Western blot was 40-fold above the background in the unstressed leaf controls, as determined by quantitating the bands. Therefore, we estimate that there is a 1000 to 2000-fold increase in the amount of HSP18.1 during a 40°C HS compared with unstressed leaves. Estimates from Coomassie blue-stained gels indicate that class I LMW HSPs are a substantial component of the protein in heat-stressed leaves; approximately 0.5% of the protein seen in 38°C HS leaves (A. DeRocher, unpublished). This is much more abundant than chloroplast-localized HSP21 which accounts for only 0.01 to 0.02% of the total protein in 38°C HS leaves (3). Both HSP18.1 mRNA and protein were detected at tissue temperatures of approximately 31°C, a lower temperature than has been reported for soybean, in which HSP mRNAs are typically first detected at 34°C (1, 12). Because 31°C is well within the range of temperatures normally encountered by peas during their life cycle, this work emphasizes that HSP expression may occur frequently during the life of a plant, even in the absence of severe temperature stress.

Pulse chase experiments indicated that soybean LMW HSPs were still abundant 21 h following HS (12). Data showing that thermotolerance is retained up to 36 h after an inducing treatment have led to the hypothesis that HSPs are stable for at least this duration (21). Our data demonstrate that during recovery from HS the half-life of HSP18.1-immunodetected protein is 37.7 ± 8 h. This is similar to the 52 ± 12 h half-life estimated for the chloroplast-localized HSP21 (3). The proteins may persist for several days either because damage to the cells caused by the HS needs to be repaired by the LMW HSPs or because the LMW HSPs provide adaptive thermotolerance in the event of a future HS. More HSP18.1 accumulation at higher temperatures could be the result of either proportionally greater damage to the cell at increased temperatures or an increased requirement for thermoprotection in anticipation of a more severe stress. These alternatives are not mutually exclusive; the LMW HSPs could both repair and prevent cellular damage, the later resulting in adaptive thermotolerance. The stability of these proteins provides correlative evidence that HSPs provide thermotolerance.

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