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Establishment of Thermotolerance in Maize by Exposure to Stresses Other than a Heat Shock Does Not Require Heat Shock Protein Synthesis[1]

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ABSTRACT

Maize (*Zea mays*) seedlings were pretreated prior to heat shock with either a progressive water stress of −0.25 megapascal PEG/hour from 0 to −1.25 megapascal over a 6-hour time period, or various concentrations of copper, cadmium, or zinc for 4 days. When the subsequent heat shock of 40 or 45°C was administered for 3 hours, the seedlings showed an induced thermotolerance to these temperatures, which were otherwise lethal to control (water grown) seedlings. Thermotolerance was exhibited by both the root and the shoot of pretreated seedlings, even though the water and heavy metal stresses were applied only to the roots. Neither of these pretreatments had induced the synthesis of detectable levels of heat shock proteins (Hsps) at the time of heat shock. Pretreatment of seedlings with a progressive heat shock of 2°C/hour from 26 to 36°C, which did induce Hsps 18, 70, and 84, resulted in tolerance of a severe water stress of −1.5, −1.75, or −2.0 megapascal for 24 hours. But these seedlings producing Hsps were no better protected against water stress than those pretreated with a progressive water stress which did not produce Hsps. Hsps appear not to act as general stress proteins and their presence is not always required for the establishment of thermotolerance.

Elevated temperatures (heat shock) induce a set of new proteins, Hsps,[3] in all plant (excluding the pollen tubes of *Tradescantia* [1, 27]), microbial and animal species so far studied (6, 7). In several cases the application of heavy metals (8, 13), water stress (8, 14), plant hormones (8, 14), arsenite (16), and ethanol (23) will also induce the synthesis of Hsps (21). The maize Hsp profile consists of two groups in the range of 70 to 90 kD and 18 to 27 kD (4). The function(s) of the Hsps is not understood although it is thought that they are important in relation to the induction of thermotolerance, *i.e.* in the ability to survive a normally lethal temperature as a result of a prior exposure to a sublethal, but nonetheless stressful, temperature (10, 18). Thermotolerance has been demonstrated where Hsps are induced by a stress other than a heat shock (8, 23); however, the pollen tubes of *Tradescantia* (27) and rat fibroblasts (26) are capable of mounting a thermotolerance response in the absence of newly synthesized Hsps. Additionally, with the isolation of disruption and deletion mutants, it has been shown that one of the major Hsps of eukaryotic cells, HSP 26, is not required for thermotolerance in yeast cells (22) and in *Tetrahymena thermophila* cells it has been established that thermostabilization of the translational machinery, during a heat shock, does not require prior synthesis of Hsps (12).

Here we demonstrate that thermotolerance can be acquired by maize seedlings, subjected to other stresses prior to heat shock, in the absence of Hsp synthesis or Hsp70 mRNA. This phenomenon, described as cross-adaptation (11), was induced by pretreating seedlings through the roots with heavy metals or water stress (PEG).

MATERIALS AND METHODS

Plant Material and Stress Conditions. Maize kernels (*Zea mays*, DK246, Dekalb, Chatham, Ont., Canada) were sown on Whatman No. 3MM filter paper, soaked in deionized water or appropriate heavy metal solutions ($CuSO_4$, $CdCl_2$, or $ZnCl_2$, pH 6.5 ± 0.2) for 4 d at 26°C. Water stress was achieved by placing seedling roots in a shallow solution of increasing water potential, −0.25 MPa/h, from 0 MPa to −1.25 MPa PEG 8000 (Sigma Chemical Co.) (19), on Whatman No. 3MM filter paper. The mesocotyls were never in direct contact with the stressing solution. Heat shock at 40 or 45°C was applied to intact seedlings for 3 h prior to RNA extraction or 2 h prior to *in vivo* protein labeling. Three tissue regions were examined in this study: (a) growing—a 5 mm segment of the shoot directly below the mesocotyl node; (b) nongrowing—a 5 mm segment of the shoot taken 15 mm below the mesocotyl node; (c) root—the distal 5 mm of the primary root.

Seedling growth measurements were made by determining the length (in mm) of the shoot and root of 10 seedlings, from the kernel to the distal tip. Using the mean of these values, growth was determined as a percentage of control, water grown seedlings.

***In vivo* Protein Labeling and Gel Electrophoresis.** Following a 4-d heavy metal treatment, a progressive water stress, or a 2-h heat shock at 40 or 45°C to intact seedlings, 5 × 5 mm segments of the growing, nongrowing, and root regions were excised and incubated for 1 h, at the appropriate temperature, in 400 μl of treatment solution containing 50 μCi ^{35}S methionine (1250 Ci/mmol, Amersham, Dorval, Quebec). The segments were rinsed in deionized water and ground in 400 μl SDS-PAGE sample buffer (15) with acid-washed sea sand. The samples were boiled for 2 min, followed by centrifugation in an Eppendorf benchtop centrifuge (model 5414) at 15,600*g* for 4 min. Proteins in the supernatant were separated on 7 to 15% SDS-PAGE gradient gels according to the procedure of Laemmli (15). Equal amounts of TCA-precipitable radioactivity were loaded per treatment and,

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[3] Abbreviation: Hsps, heat shock proteins.

following electrophoresis, fluorography of Coomassie blue-stained gels was carried out using EnHance (NEN, Boston, MA) and Kodak XAR film.

Total RNA Isolation and Northern Hybridization. Fifty segments from each of the three regions studied were ground in liquid N_2 before extraction in an emulsion of 1 ml (50:50, v/v) phenol (saturated with 10 mM NaCl, 2 mM EDTA, and 1% [w/v] SDS). After centrifugation, the aqueous phase was added to an equal volume of saturated chloroform:phenol:isoamyl alcohol (50:50:1, v/v/v), and reextracted repeatedly until a clear interface resulted. The final aqueous phase was made to 300 μM ammonium acetate and 2 vol of 95% (v/v) ethanol added. The RNA precipitated overnight at −20°C, was collected by centrifugation, redissolved in 100 ul of water and stored at −80°C.

Total RNA (10 μg) was electrophoresed on a 1.5% agarose gel containing 10 mM methylmercuryhydroxide (Alfa Products, Danvers, MA) and then transferred to diazobenzyloxymethyl (DBM) paper (Transa-bind; Schleichter and Schuell Inc., Keene, NH) according to Alwine *et al.* (2). Northern hybridization was carried out using the maize HSP70 genomic fragment pMON 9502 (donated by Dr. J. Winter, Monsanto, St. Louis, MO: see Rochester *et al.* [24]), at 50°C in 50% formamide. Posthybridization washes were carried out at 70°C with buffer ranging from 4 × SET, 0.2% SDS to 0.1 × SET, 0.2% SDS (1 × SET = 0.15 M NaCl, 0.03 M Tris HCl [pH 8], 2 mM NaEDTA). The blot was then exposed to preflashed Kodak XAR-5 film using a Cronex intensifying screen (Dupont, Lightening Plus).

Atomic Absorption Spectroscopy. Tissue was extracted into phosphate-buffered saline (pH 6) and the supernatant analyzed using atomic absorption spectroscopy (3) (Perkin Elmer 5000, Perkin Elmer Corp., Norwalk, CT).

RESULTS AND DISCUSSION

Effect of a Preheat Shock Treatment on Seedling Growth. After 4 d in the presence of 200 μM $CuSO_4$, $CdCl_2$, or $ZnCl_2$ (pH 6.2 ± 0.2) similar levels of uptake of each heavy metal into the buffer soluble fraction of the primary root of seedlings were observed. However, no Cu, Cd, or Zn (other than embryonic Zn) was found in the shoots of these seedlings (Table I). It was thought that the relatively high metal content of the root might exert an inhibitory effect upon translocation of metal ions to the shoot. Seedlings grown in 50 μM metal solutions, a noninhibitory concentration, demonstrated higher levels of metal uptake into the roots, and yet did not show metal translocation to the shoot. Incubation of seedlings in the presence of any of the three metals had no effect on the internal distribution of Ca, Mg, or Fe.

Although the extent of accumulation of Cu, Cd, or Zn into the roots of treated seedlings was similar, their effect on seedling growth differed considerably (Fig. 1). Seedlings grown for 4 d in Cu showed a marked reduction in both root and shoot growth—approximately 50% at 200 μM—whereas Cd or Zn were much less inhibitory; at 200 μM, Cd caused a 30% reduction of growth but Zn had an insignificant effect. At lower concentrations, Cu again caused a reduced growth of both root and shoot after 4 d. The presence of Cd and Zn at the lower concentrations, as expected, had a minimal effect on root or shoot growth.

In contrast to seedlings treated with heavy metals, 4-d-old seedlings, subjected to a 6-h progressive water stress in increments of −0.25 MPa PEG/h from 0 to −1.25 MPa, showed no decrease in root or shoot length but a 10% decrease in their total fresh weight, from 0.53 ± 0.06 g per 10 seedlings to 0.48 ± 0.05 g, was recorded.

Effect of Preheat Shock Treatment on *in Vivo* Protein Synthesis. Analysis of the pattern of *in vivo* protein synthesis after pretreatment, but prior to heat shock, required the uptake and incorporation of labeled amino acid (^{35}S methionine). Sufficient uptake was not possible with intact seedlings and so after each pretreatment the specified regions were excised and incubated in the appropriate pretreatment solution, plus labeled amino acid, for 1 h. This protocol is not ideal because of the possible induction of a wound response (9). However, by minimizing the incubation time (1 h) we have attempted to limit this response.

Minor differences were observed in the patterns of *de novo* protein synthesis between the growing and nongrowing shoot regions, whether seedlings were incubated in heavy metals or water (Fig. 2, A and B). The protein profiles of the growing tissue were very similar between treatments, a notable exception being a very low M_r protein induced by Cd treatment (Fig. 2A). The profiles from nongrowing tissue showed one protein (94 kd), which is not present in control tissue, to be induced by all three

Table I. *Metal Ion Concentrations in Seedlings grown for 4 d in the Presence on Metal Salts*

Atomic absorption measurements were made on the soluble root and shoot extract of seedlings grown in 50 μM or 200 μM $CuSO_2$, $CdCl_2$, $ZnCl_2$, or H_2O for 4 d prior to extraction or seedlings treated with a progressive water stress (pws) of −0.25 MPa/h from 0 MPa to −1.25 MPa PEG. Embryo tissue was dissected from 24-h water-imbibed seeds.

Root Medium	Organ	Mg	Ca	Fe	Cu	Cd	Zn
				μg/g fresh wt ± SE			
Copper	Root	6.7 ± 0.8[a] (9.3)[b]	1.1 ± 0.14 (1.4)	0.3 ± 0.14	0.8 ± 0.16 (1.4)	0[c]	0.5 ± 0.26 (0.7)
	Shoot	8.7 ± 0.2 (8.9)	0.9 ± 0.17 (0.6)	0.3 ± 0.14	0	0	0.3 ± 0.14 (0.4)
Cadmium	Root	4.6 ± 0.5 (11.7)	0.7 ± 0.11 (1.3)	0	0	0.7 ± 0.26	0.2 ± 0.06 (0.7)
	Shoot	7.1 ± 0.5 (10.6)	0.4 ± 0.10 (0.6)	0.3 ± 0.07	0	0	0.3 ± 0.08 (0.4)
Zinc	Root	4.3 ± 0.46 (10.5)	1.2 ± 0.16 (1.6)	0	0	0	0.7 ± 0.15 (1.9)
	Shoot	8.0 ± 0.35 (9.8)	0.7 ± 0.18 (0.6)	0	0	0	0.4 ± 0.14 (0.5)
Water	Root	4.6 ± 0.16	1.0 ± 0.16	0	0	0	0.3 ± 0.14
	Shoot	8.1 ± 0.27	0.6 ± 0.14	0.3 ± 0.14	0	0	0.5 ± 0.24
Embryo		65.2	0.9	0.4	0	0	0.5
Pws	Root	11.8	1.3	0	0	0	0.6
	Shoot	7.0	0.7	0	0	0	1.5

[a] $n = 3$ for 200 μM. [b] $n = 1$ for 50 μM (values in parentheses). [c] 0 means less than 1 μg metal/g fresh weight was detected.

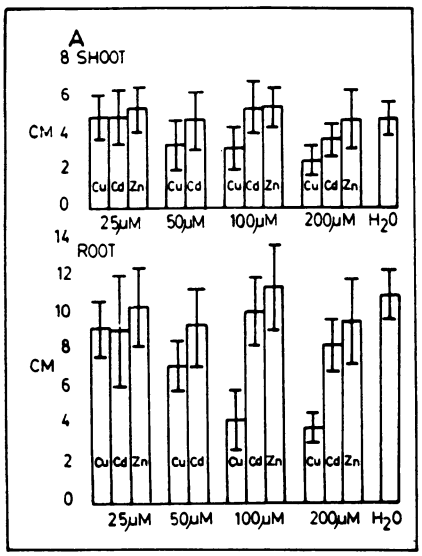

FIG. 1. Effects of various concentrations of Cu, Cd, and Zn on root and shoot growth of maize seedlings. Measurements were taken after 4 d continuous application of metals in darkness at 26°C (n=10, P=0.05). Using Duncan's multiple range test, LSD of shoots was 1.08 cm, and of roots 2.21 cm.

metals, and one protein (80 kd) present in control and Zn-treated tissue, to be absent from Cd- and Cu-treated tissue (Fig. 2B). However, the protein profiles from the root tissue varied with the metal treatment. Zinc-treated seedlings displayed few, if any differences in the root protein synthesis profile from control roots grown in water, whereas several proteins, of various M_r, synthesized in both control and Zn-treated roots, were not synthesized in Cu- and Cd-treated roots (Fig. 2C). Four proteins were induced by both Cu and Cd treatment, but not by Zn. None of these newly synthesized proteins corresponded to any of the maize Hsps (Fig. 2D). Growth in lower concentrations (25–100 μM) of Cu, Cd, or Zn also did not induce Hsps (data not shown). Similarly, application of a progressive water stress, −0.25 MPa/h from 0 to −1.25 MPa PEG, to 4-d-old seedlings did not appear to induce any specific water stress proteins or Hsps (Fig. 2E is the root protein profile from control [water grown] and water-stressed seedlings). The growing and nongrowing profiles also showed no protein differences between control and water-stressed seedlings (data not shown).

Although, by themselves, neither metals nor water stress elicited Hsp synthesis at the conclusion of the pretreatment period, such pretreated seedlings exposed to 3 h of heat shock (40°C) showed no reduction in the ability of their growing, nongrowing, and root regions to synthesize Hsps (data not shown).

Northern Blot Analysis of the Induction of Thermotolerance using the Hsp70 Genomic Probe. The mRNA for Hsp70, which is synthesized in response to heat shock in all organisms examined to date (7), was not induced in seedlings after 4 d in 50 μM (not presented) or 200 μM Cu, Cd, or Zn (Fig. 3). As expected, when the pretreated seedlings were subjected to a 3-h heat shock of 40°C the Hsp70 mRNA was synthesized normally (Fig. 3). Thus, the absence of induced Hsp mRNA at the end of a heavy metal or water stress treatment was not due to a permanent suppression of the heat shock genes but rather to a failure to induce Hsp gene transcription.

Thermotolerance in Seedlings Pretreated with Heavy Metals or Water Stress. A function of Hsps in thermotolerance had been presumed from experiments on yeast (18, 20) and *Dictyostelium* (17) where pretreatment with a sublethal temperature in the presence of cycloheximide, which prevented Hsp synthesis, also prevented the establishment of thermotolerance. However, the possibility that cycloheximide was affecting the synthesis of other important proteins cannot be overlooked. Here, by applying pretreatments of various heavy metals or a progressive water stress to the roots of maize seedlings, we were able to induce thermotolerance within these seedlings without concomitant synthesis or Hsps or, in the case of Hsp70, its mRNA.

Seedlings exposed to a 6-h progressive water stress, or to various concentrations of Cu, Cd, or Zn over a 4-d period, were now better able to withstand a subsequent 3-h heat shock at 40 or 45°C (Figs. 4, A and B, and 5). Untreated seedlings (*i.e.* incubated in water) exposed to a 40°C heat shock (a nonlethal treatment) showed an 80% reduction in root growth and a 50 to 60% reduction in shoot growth during a subsequent recovery period of 3 d. Seedlings preexposed to 25 μM Zn showed only a 10% reduction in root growth, while Cd and Cu treatment resulted in a 40 and 50% reduction, respectively, in recovery root growth following a 3-h, 40°C heat shock (Fig. 4A). This protection decreased with increasing metal concentration to the point where pretreatment with 100 to 200 μM Cu provided no protection of root growth after a 40°C heat shock. Growth in the

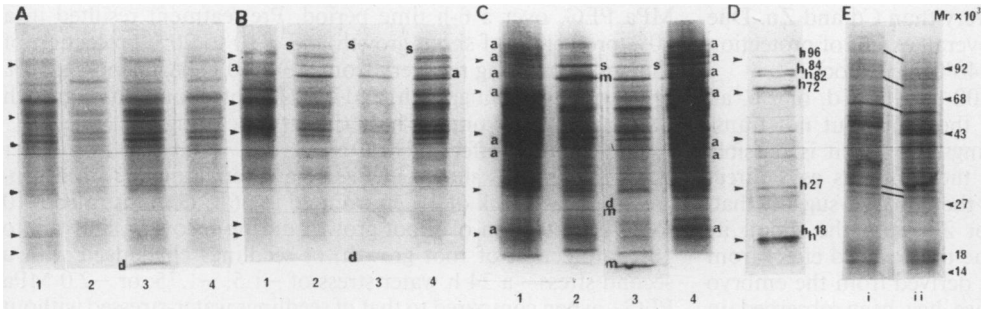

FIG. 2. Fluorographs of *in vivo* labeled ^{35}S methionine proteins of: A, growing; B, nongrowing; and C, root tissue after pretreatment with water (1), 200 μM Cu (2), 200 μM Cd (3), or 200 μM Zn (4) for 4 d at 26°C. D, Hsp profile induced by a heat shock of 40°C for 3 h. E, (i) a progressive water stress of −0.25 MPa PEG/h from 0 to −1.25 MPa over 6 h, or (ii) water. Hsps are indicated by h. a, Absence of a protein in Cu- and Cd-treated tissue, which is present in control and Zn-treated tissue; d, Cd-induced proteins; m, Cd- and Cu-induced proteins; s, proteins induced by all three metals. Note that in lane 2 of (C) the lowest M_r protein m was present, but was too faint to appear with the photographic exposure used. More prolonged exposure resulted in loss of definition of the higher M_r protein bands. M_r = mol wt markers. Arrows indicate position of M_r markers in relation to other lanes.

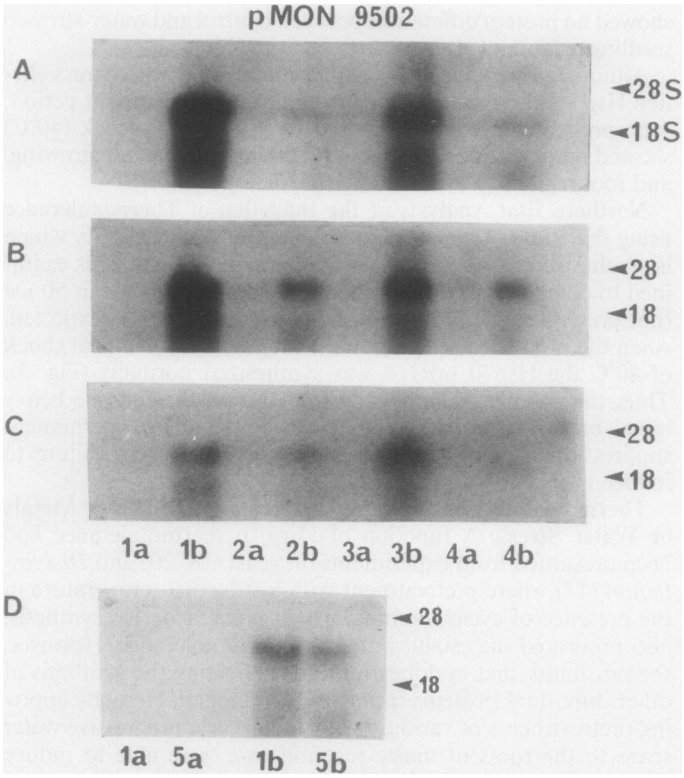

FIG. 3. Lack of Hsp70 mRNA induction by pretreatment of seedlings with (1a) water, (2a) 200 μM $CuSO_4$, (3a) 200 μM $CdCl_2$, or (4a) 200 μM $Zn Cl_2$, for 4 d at 26°C. A subsequent heat shock at 40°C for 3 h (1b–4b) resulted in the induction of Hsp70 mRNA in pretreated seedlings. A, growing shoot; B, nongrowing shoot; C, root; D, indicates similar results when seedlings are subjected to a progressive water stress at 26°C (5a) and subsequently 40°C for 3 h (5b).

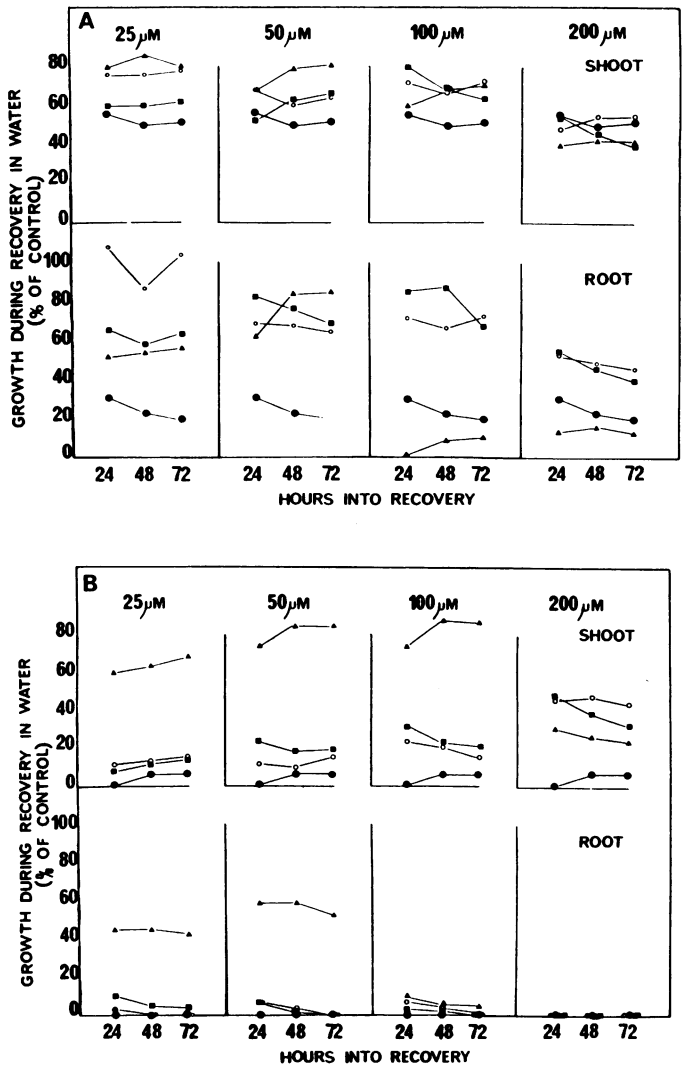

FIG. 4. Effect of a 4-d heavy metal pretreatment on the thermotolerance of seedlings (as measured by the recovery growth [in mm] of both root and shoot, over a 3-d postheat shock period, denoted as hours into recovery) subjected to (A) a 3-h 40°C heat shock and (B) a 3-h 45°C heat shock. Control seedlings were maintained at 26°C on water in the dark. Each point is the mean of 10 measurements. (■), Cd; (▲), Cu; (○), Zn; (●), water.

presence of 25, 50, or 100 μM metal provided protection for recovery shoot growth: Zn provided 25% protection, Cd 10%, and Cu 30% at 25 μM, but again the level of protection diminished with increasing metal concentration.

Heat shock of 45°C for 3 h was lethal when applied to maize seedlings without a pretreatment, *i.e.* seedlings grown in water only (Fig. 4B). However, a pretreatment with 25 to 50 μM Cu provided a 40 to 50% protection to root growth during recovery, whereas a pretreatment with 25 to 200 μM Zn or Cd, or 100 to 200 μM Cu was unable to provide any protection for recovery root growth. Pretreatment with 25 to 200 μM Cu, Cd, or Zn provided protection to shoot growth during recovery from a 3 h 45°C heat shock. In this case, Cu (25, 50, and 100 μM) appeared to be approximately 40 to 50% more effective in the protection of root and shoot growth during recovery than Cd and Zn. Due to the severity of the heat shock, the overall extent of protection was not as pronounced as that after a 40°C heat shock.

After a 4-d treatment with 50 or 200 μM Cu, Cd, or Zn, all three metals had been absorbed into the roots, but not transported to the shoots of treated seedlings. Hence, it is possible that the thermotolerance induced in the root was as a direct result of the accumulation of metals therein. We suggest that, because of the absence of Cu, Cd, or Zn from the shoot, its increased thermotolerance must be due to a relayed effect from the root (NB, the Zn in the shoot was derived from the embryo [Table 1]). A similar type of response has been observed in wounded pea (9), tomato, and potato plants (25) where tissue away from the site of the wound, both basipetally and acropetally, showed wound-induced changes in cell properties, especially in the cell membrane, mediated by a putative wound signal called proteinase inhibitor inducing factor (PIIF). PIIF activity is associated with cell wall fragments, released during injury (5).

A similar thermotolerance effect was demonstrated in seedlings pretreated with a −0.25 MPa/h water stress from 0 to −1.25 MPa PEG, over a 6-h time period. Pretreatment resulted in a 20% protection of shoot growth and a 10 to 20% protection of root growth during recovery from a 3-h 40°C heat shock, a 30% protection of shoot growth, and a 20% protection of root growth during recovery from a 3-h 45°C heat shock (Fig. 5).

The converse effect of heat shock and water stress on water stress tolerance is shown in Figure 6. Pretreatment with a progressive heat shock of 2°C/h from 26 to 36°C demonstrated a 10 to 20% protection of shoot growth during recovery, and a 5 to 10% protection of root growth, of seedlings challenged with a second stress—a 24 h water stress of −1.5, −1.75, or −2.0 MPa PEG—when compared to that of seedlings water-stressed without pretreatment (Fig. 6). In this case the progressive heat shock pretreatment induced low levels of the 18, 72, and 84 kd Hsps in both the root and the shoot prior to water stress (Fig. 7). However, the level of protection afforded these seedlings was not

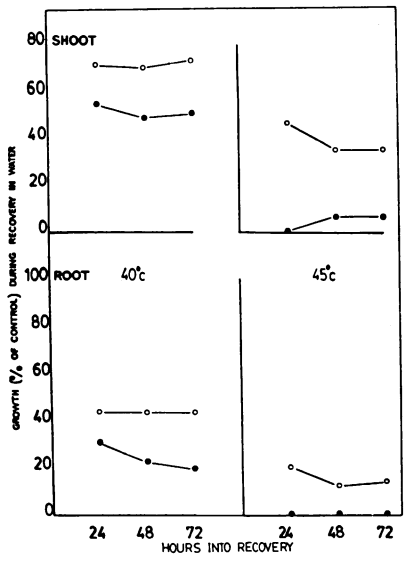

FIG. 5. Effect of a 6-h progressive water stress (−0.25 MPa PEG/h) on the recovery growth of seedlings subsequently subjected to a 3-h 40°C or 3-h 45°C heat shock. (○), Water stress; (●), unstressed control.

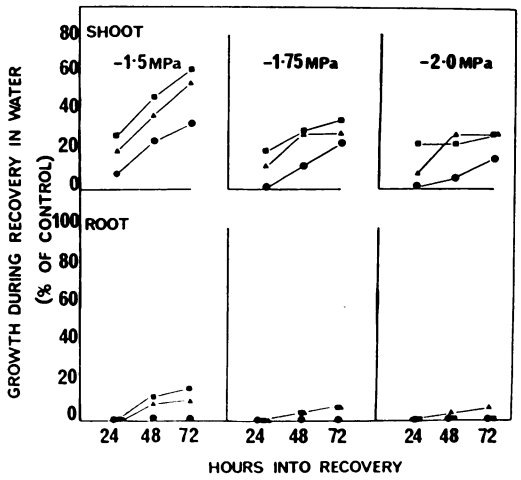

FIG. 6. Tolerance of seedlings to a 24-h water stress of −1.5, −1.75, −2.0 MPa PEG, by pretreatment with a progressive heat shock (2°C/h from 26–36°C), or a progressive water stress of −0.25 MPa PEG/h from 0 to −1.25 MPa, prior to water stress. (▲), Progressive heat shock; (■), progressive water stress; (●), control—given water stress without pretreatment.

substantially different from seedlings treated with a progressive water stress prior to the 24-h water stress (Fig. 6).

In relation to the seedlings imbibed in solutions of heavy metal, we cannot rule out the possibility of an early transient period of Hsp production prior to the completion of this pretreatment. While it would seem unlikely that any early Hsps remain stable for the 4-d pretreatment, their presence at undetectable levels, or the secondary results of their transient presence, could contribute to thermotolerance. It is noteworthy, however, that subjecting corn seedlings to heat shock for 3 h, which resulted in Hsp synthesis, followed by 2 d at 26°C, did not lead to increased thermotolerance of a second heat shock of 40°C, as measured by root or shoot growth (data not presented). Hence transient Hsp synthesis is unlikely to lead to subsequent thermotolerance. Also, it is evident that the presence of Hsps, induced by progressive heat shock (Fig. 7) did not substantially improve stress tolerance of seedlings subsequently subjected to severe water stress (Fig. 6) above that of water-stress pretreated seedlings, where Hsps are

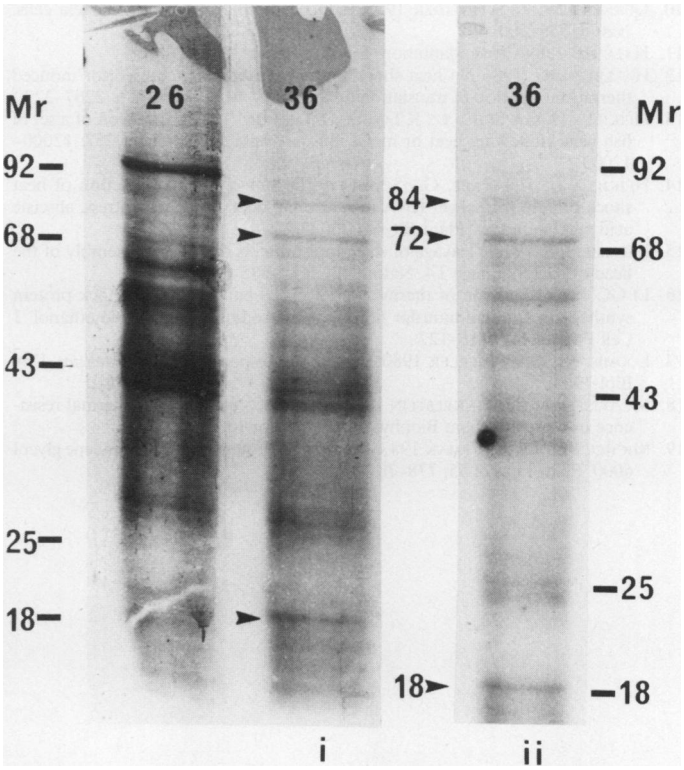

FIG. 7. Fluorograph of *in vivo* ^{35}S methionine-labeled proteins showing the induction of low levels of Hsps in both the (i) growing and (ii) root regions of seedlings subjected to a progressive heat shock of 2°C/h from 26 to 36°C prior to the seedlings being given a 24-h water stress. Arrows indicate Hsps. The lane marked 26 is the unstressed growing region of the water control at 26°C.

not detectable. The ability of tissues to have synthesized or accumulated Hsps at the time of application of heat stress is not universally essential for the establishment of thermotolerance, and Hsps may not be required to act as general protectants against other common physiological stresses.

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