

CHLOROPLAST SMALL HEAT-SHOCK PROTEINS PROTECT PHOTOSYNTHESIS DURING HEAVY METAL STRESS¹

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Plants can accumulate heavy metals when exposed to them at high levels. These metals can interfere with photosynthesis. Limited evidence suggests that increased synthesis of some heat-shock proteins (Hsps) may be a general plant response to metal stress, but the specific functions or structures protected by Hsps remain unidentified. Chloroplast small Hsps (smHsps) protect photosynthetic electron transport (Ph_e) during heat, oxidative, and photoinhibitory stress, but it is not known if chloroplast smHsps are synthesized during metal stress and protect photosynthesis. *Zea mays* (corn) plants were exposed to varying soil concentrations of Cu, Ni, Pb, and Zn to determine if chloroplast smHsps are induced by heavy metals, if smHsps protect Ph_e , and any effects on chloroplast smHsp and photosynthesis. Net photosynthesis (Ph_n) decreased with all metals—more so at higher levels and with longer exposures. Decreases in Ph_n resulted from damage to photosynthetic metabolism, including Ph_e . All metals increased chloroplast smHsp content, which increased with time of exposure. In vitro, Ph_e was protected from Pb (but not Ni) by purified chloroplast smHsp added to thylakoids. In vivo, Ph_n was protected from Ni and Pb by increases in smHsp in a heat-tolerant *Agrostis stolonifera* selection genotype expressing additional chloroplast smHsps compared to a near-isogenic heat-sensitive genotype. These results are evidence that Hsps protect photosynthesis from heavy metals and are among the first to demonstrate specific functions protected by Hsps during metal stress.

Key words: heat-shock proteins; heavy metals; photosynthesis; stress proteins.

Heavy metal contamination of the environment is currently a major global environmental problem, threatening the health of vegetation, wildlife, and humans (e.g., Singh et al., 1997; Salt et al., 1998; Taiz and Zeiger, 1998). Plants exposed to high levels of heavy metals typically accumulate the metals in their tissues (both roots and shoots), often to toxic levels that decrease growth or reproduction. The mechanisms utilized by plants to protect cells from excess heavy metals are not completely understood, but are known to include internal and external chelators, efflux pumping, vacuolar compartmentalization, and heat-shock proteins (Salt et al., 1998; Cobbett, 2000; Hall, 2002).

Photosynthesis is typically decreased by elevated levels of heavy metals, but the specific effects of a given heavy metal on photosynthesis vary among species, preventing broad generalizations about metal effects on photosynthesis. For example, copper has been shown to negatively affect components of both the light reactions (e.g., photosystem II [PSII], thylakoid membrane structure, chlorophyll content) (Gupta and Singhal, 1995; Ralph and Burchett, 1998; Szalontai et al., 1999; Pätsikkä et al., 2002; Vinit-Dunand et al., 2002) and CO₂-fixation reactions (Stiborova et al., 1986; Lidon and Henriques, 1991; Angelov et al., 1993; Moustakas et al., 1994). However, in studies that examined both light and CO₂-fixation components, the relative sensitivity of each to Cu varies among studies (Stiborova et al., 1986; Lidon and Henriques, 1991; Angelov et al., 1993; Moustakas et al., 1994). Similar

observations have been made for other metals, such as lead (Stiborova et al., 1986; Moustakas et al., 1994; Singh et al., 1997; Ralph and Burchett, 1998; Szalontai et al., 1999).

As mentioned, a general response of plants to elevated levels of heavy metals appears to be increased synthesis of various heat-shock proteins (Hsps) (e.g., Tseng et al., 1993; Neumann et al., 1994, 1995; Barque et al., 1996; Wollgiehn and Neumann, 1999; Hall, 2002). However, the specific cell components or processes targeted by Hsps during metal stress are not yet identified. Both cell membranes and proteins are primary sites of damage to cells during heavy metal stress, and limited evidence suggests that Hsps function to protect cell membranes from metal damage (Hall, 2002). In addition, given the well-characterized roles of Hsps in the protection and repair of soluble proteins during heat stress (e.g., Parsell and Lindquist, 1994) Hsps may function in this way during metal stress too. Recently, the chloroplast small (low molecular mass) Hsp has been shown to protect photosynthesis during heat, oxidative, and photoinhibitory stress, by protecting PSII or other aspects of thylakoids (Heckathorn et al., 1998; Lee et al., 1998, 2000a, b; Miyao-Tokutomi et al., 1998; Downs et al., 1999a, b; Nakamoto et al., 2000; Török et al., 2001). Also, chloroplast smHsps probably protect photosynthesis via more than one mechanism: by preventing irreversible protein aggregation (Török et al., 2001), by stabilizing chloroplast membranes (Török et al., 2001), and possibly as site-specific anti-oxidants (Hamilton and Heckathorn, 2001). Given this, and that heavy metals damage both membrane and soluble phases of chloroplasts through multiple mechanisms that include protein denaturation and oxidative damage (Hall, 2002), we hypothesized that the chloroplast smHsps are produced when heavy metals accumulate in the chloroplast and damage photosynthesis and that smHsps help protect photosynthesis from excess heavy metals. To test this, we exposed *Zea mays* plants

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to Cu, Ni, Pb, and Zn at levels sufficient to decrease in vivo net photosynthesis (Ph_n) (and chlorophyll content and PSII function, too), then determined if chloroplast smHsp levels were elevated with metal exposure. Having positive evidence of metal-induced decreases in Ph_n and increases in chloroplast smHsps, we directly determined if chloroplast smHsps could protect photosynthetic electron transport (Ph_{et}) when added to thylakoids exposed to high levels of heavy metals. In vivo protection of Ph_n by smHsp was confirmed in two, previously characterized, near-isogenic genotypes of *Agrostis stolonifera* differing in the number of chloroplast smHsp genes expressed and in the amount of smHsp protein produced during stress (Park et al., 1996; Luthe et al., 2000; Heckathorn et al., 2002).

MATERIALS AND METHODS

Plant material and growth conditions—Corn (*Zea mays* L. cv. 3475; Pioneer, Des Moines, Iowa, USA) was grown from seed in a topsoil/calclined-clay mix (1 : 1, v : v) under 22°–24°C days, 20°C nights, and a 12-h photoperiod at 400 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ photosynthetically active radiation (PAR). After approximately 2 wk, plants were transplanted to 8-cm pots (with no-drain bottoms) containing perlite and grown until plants had 3–4 fully expanded leaves (approximately one more week). At this time, plants were exposed to heavy metals by watering the soil surface of each pot once with 50 mL of nutrient solution (10 mmol/L NH_4NO_3 , 2 mmol/L KH_2PO_4 , 3 mmol/L CaCl_2 , 2 mmol/L MgCl_2 , 1 mmol/L MnSO_4 , 10 $\mu\text{mol/L}$ FeSO_4 , 1 $\mu\text{mol/L}$ H_3BO_3 , 1 $\mu\text{mol/L}$ Na_2MoO_4 , and 1 mmol/L ethylenediamine tetra-acetic acid [EDTA] to chelate the heavy metals) containing 500, 1000, or 2000 $\mu\text{mol/L}$ Cu-, Ni-, Pb-, or Zn-chloride. Plants were harvested after 96 and 168 h (4 or 7 d) and stored at -70°C ($n = 3$ –6 per treatment combination).

A heat-sensitive progenitor of *Agrostis stolonifera* Huds. var. *palustris* (creeping bentgrass) and a near-isogenic heat-tolerant selection mutant were propagated vegetatively. These two previously characterized genotypes differ in the number of chloroplast smHsp genes expressed (one and two, respectively). Consequently, the tolerant genotype produces approximately twice as much chloroplast smHsp protein as the sensitive genotype (Park et al., 1996; Luthe et al., 2000; Heckathorn et al., 2002). Increased smHsp accumulation in the tolerant genotype is genetically linked to increased plant thermotolerance (Park et al., 1996) and results in the increased tolerance of Ph_{et} and PSII to heat stress (Heckathorn et al., 2002). Vegetative plants were grown at 22°C days and 18°C nights for several weeks in pots as described, but at ca. 200 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR. Individual adult plants were then transferred to aerated flasks containing 2000 $\mu\text{mol/L}$ Ni or Pb and then monitored after 4 d.

Protein extraction and content—Proteins were extracted from tissues or chloroplasts in an extraction buffer containing 1% sodium dodecyl sulfate (SDS), 100 mmol/L Tris pH 8.0, 10 mmol/L EDTA, 1 mmol/L ϵ -amino caproic acid, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L benzamidine, 10 mmol/L ascorbate, 10 mmol/L dithiothreitol, 1 $\mu\text{mol/L}$ antipain and leupeptin, 10% glycerol (v/v), 10% sucrose (m/v), 2% polyvinylpyrrolidone (PVPP) and 2% polyvinylpyrrolidone (PVP) (m/v), and 0.05% bromophenol blue. Leaf proteins were extracted by grinding tissue (0.1 g) in liquid N_2 , then in extraction buffer (1 mL) with a mortar and pestle. Chloroplast proteins were then extracted by solubilizing isolated chloroplasts in extraction buffer. Protein extractions were heated for 2 min at 75°–85°C and then centrifuged at 21 000 $\times g$ for 2 min to remove insoluble debris. Protein concentration of each sample was determined in triplicate by the Coomassie dye-binding method of Ghosh et al. (1988), using bovine serum albumin as a standard and using a desktop scanner and National Institutes of Health imaging software (Scion) to perform densitometry.

Electrophoresis and immunoblotting—Protein extracts were fractionated by SDS-PAGE, using 16 \times 20 \times 0.15-cm 15% gels; equal total protein was loaded per lane (40 μg). Following SDS-PAGE, the proteins were transferred to nitrocellulose membranes by electroblotting for 90 min at 350 mA. The membranes were then probed with protein-specific antibodies and secondary

antibodies conjugated to alkaline phosphatase. Antibodies to the chloroplast small Hsps were polyclonal rabbit antibodies raised against oligopeptides of conserved sequences (described in Heckathorn et al., 2002). Secondary antibodies were detected with a colorimetric system consisting of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (densitometry performed as above).

Chloroplast isolation—Chloroplasts were isolated by a combination of differential and density gradient centrifugation, using a modification of the method described previously (Heckathorn et al., 1998). As before, leaves were ground, and chloroplasts were partially purified by differential centrifugation (5000 $\times g$ for 10 min), in a buffer containing 350 mmol/L sorbitol, 50 mmol/L Hepes (pH 7.75), 5 mmol/L EDTA, 5 mmol/L ascorbate, 2 mmol/L dithiothreitol, and 2% PVP (m/v). The resulting crude chloroplast pellet was resuspended in this same buffer, and the chloroplasts were subjected to density gradient centrifugation (7000 $\times g$ for 10 min) using a step-gradient containing 0.5, 1.0, 1.5, and 2.0 mol/L sucrose in 50 mmol/L Hepes (pH 7.75). Intact and broken chloroplasts were collected separately from the gradients with a pipet, repelleted by low-speed centrifugation (5000 $\times g$ for 10 min), resuspended in a small volume (10 : 1 volume of buffer : sample) of 1.0 mol/L sucrose in 50 mmol/L Hepes (pH 7.75), and then stored at -70°C . Chloroplast or thylakoid samples analyzed by SDS-PAGE were thawed and resuspended in protein extraction buffer (as earlier), while samples used in electron transport assays were diluted to 100 mmol/L sucrose and 25 μg chlorophyll/mL as described later.

Photosynthetic measurements—Photosystem II function of intact leaves was monitored by determining the ratio of variable-to-maximum fluorescence (F_v/F_m) of dark-adapted leaves with a pulse-amplitude-modulated fluorometer (model PAM 101/103; Walz, Effeltrich, Germany). Net photosynthesis of intact leaves was monitored by measuring net CO_2 exchange with an infrared gas analyzer-based photosynthesis system (model 6200; LI-COR, Lincoln, Nebraska, USA) (e.g., Heckathorn et al., 1996, 2002). Net photosynthesis and F_v/F_m were measured in the growth chamber at midday (1300–1500 h) on recently fully expanded leaves; net photosynthesis was measured at 400 or 200 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR (for corn and bentgrass, respectively) and 425 ± 20 $\mu\text{mol/mol}$ CO_2 , while F_v/F_m was measured after 30 min of dark adaptation.

Whole-chain photosynthetic electron transport was measured in isolated thylakoids by monitoring O_2 evolution from PSII in the presence of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), an artificial electron acceptor that receives electrons from PSI (thus intercepting flow from PSI to NADP^+ ; Allen and Holmes, 1986). O_2 evolution was monitored using a Clark-type O_2 electrode (Hansatech, Norfolk, England). Isolated thylakoids were resuspended to 25 μg chlorophyll/mL in 50 mmol/L HEPES (pH 7.75), 100 mmol/L sucrose, 4 mmol/L MgCl_2 , 4 mmol/L NaCl, 0.01% Triton X-100, and 4 mmol/L $\text{K}_3\text{Fe}(\text{CN})_6$ (added just prior to measurement) (Allen and Holmes, 1986). Electron transport was monitored for 15 min at 25°C, at either 150 or 1300 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR, with or without Pb or Ni (either 500 or 1000 $\mu\text{mol/L}$), and with or without purified chloroplast smHsp (in 50 μL aliquot) (as in Heckathorn et al., 1998; Downs et al., 1999b); rabbit whole serum was used as a negative protein addition control (50 μL). We examined only two of the four metals in these in vitro experiments, due to limited amount of purified smHsp, and randomly chose Ni and Pb as representative. Protection of electron transport by purified smHsp was titrated, and maximum protection was achieved with 1.125 mol of smHsp per mol of PSII, a stoichiometry of approximately 1 : 1 (assuming 250 chlorophyll molecules per PSII and PSI each and a PSII/PSI ratio of 1.8 [Buchanan et al., 2000]; tomato smHsp MW = 20 000 [Waters et al., 1996]).

Chlorophyll concentration of isolated thylakoids and leaves after extraction in dimethyl sulfoxide (DMSO) was determined spectrophotometrically using the equations of Barnes et al. (1992). For leaves, segments from the mid-length region of recently expanded leaves were removed using a cork borer (avoiding the mid vein), then leaf pieces were incubated in DMSO in the dark at 60°C for 1.5 h prior to measurement.

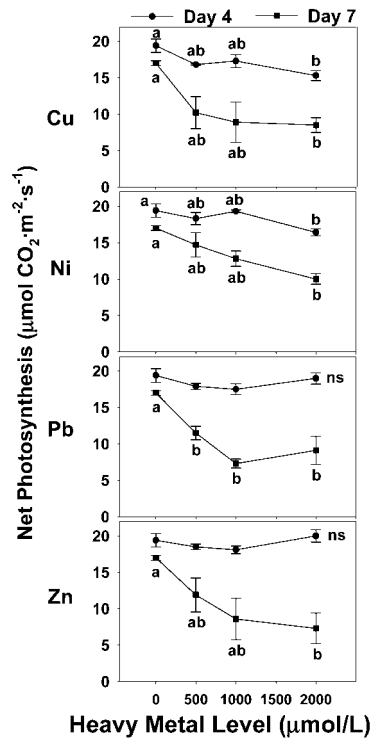


Fig. 1. In vivo net photosynthesis in leaves of *Zea mays* after 4 or 7 d exposure to heavy metals in soil. Plants were watered with a nutrient solution containing either 0, 500, 1000, or 2000 $\mu\text{mol/L}$ Cu, Ni, Pb, or Zn. Results are means ($n = 3-6$) \pm 1 SE. Within each metal \times day combination, significant differences among metal levels and controls are indicated by different superscripts ($P < 0.05$).

Purification of smHsp—Chloroplast smHsp was purified from heat-stressed tomato plants (*Lycopersicon esculentum* Mill. cv. Condine Red), using a method modified from Lee and Vierling (1998). Plants were heat stressed for 8 h during the day, and then chloroplasts were isolated from leaves as earlier, except that a two-step sucrose gradient was used (0.5 and 2.0 mol/L), resulting in a mixture of broken and intact chloroplasts. These chloroplasts were resuspended in a protein purification buffer (PP buffer) containing 20 mmol/L Tris (pH 8.0), 1% glycerol, 1 mmol/L EDTA, 0.1 mmol/L PMSF, 0.1 mmol/L benzamidine, 0.1 mmol/L ϵ -amino caproic acid, 0.1 $\mu\text{mol/L}$ leupeptin, 0.1 $\mu\text{mol/L}$ antipain, and 0.1% Triton X-100. Chloroplast proteins were then fractionated by ammonium sulfate precipitation [25, 50, 75, and 100% (NH_4)₂SO₄] and collected by centrifugation at 10000 $\times g$ for 10 min; chloroplast smHsps precipitated at 75% (determined by SDS-PAGE and immunoblotting). Following this, the smHsp-enriched fraction was resuspended in PP buffer and then diluted 100-fold with 20 mmol/L Tris (pH 8.0), to ensure that residual (NH_4)₂SO₄ was less than 30 mmol/L. The proteins in this fraction were separated by anion-exchange chromatography, using DEAE Sepharose (CL-6B; Sigma, St. Louis, Missouri, USA) in a 13 \times 2.5-cm gravity flow column. Proteins were eluted from the column using a step-gradient of NaCl in 20 mmol/L Tris (pH 8.0) (100, 200, 300, 400, and 500 mmol/L NaCl; 50 mL per step) and collected in 10-mL fractions. Anion-exchange fractions containing smHsp (five fractions between 400–500 mmol/L NaCl) were determined by SDS-PAGE and immunoblotting. These fractions were pooled, desalted by gel-filtration chromatography (1.5 \times 20-cm gravity flow column with Sephadex G-25; Amersham Biosciences, Uppsala, Sweden) using 10 mmol/L sodium phosphate (pH 7.3) for elution/buffer exchange (protein elution monitored spectrophotometrically). Proteins were then fractionated by hydroxyapatite chromatography (1.5 \times 15-cm gravity flow column with Bio-Gel HTP; Bio-Rad, Hercules, California, USA). Proteins were eluted from the column using 50 mL steps of 25, 50, 100, 200, and 400 mmol/L sodium phosphate (pH 7.3). Spectrophotometric analysis of each eluted fraction in-

TABLE 1. The effects of heavy metals in soil on leaf stomatal conductance (g_{st} , $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and internal CO₂ concentration (C_i , $\mu\text{mol/mol}$) in *Zea mays*.

Treatment	Day 4		Day 7	
	g_{st}	C_i	g_{st}	C_i
Control	157 (16) ^A	226 (22) ^A	64 (8) ^A	139 (32) ^A
Copper				
500	100 (6) ^B	187 (23) ^A	41 (6) ^A	104 (39) ^A
1000	93 (6) ^B	146 (34) ^A	44 (19) ^A	126 (30) ^A
2000	77 (7) ^B	133 (22) ^A	44 (8) ^A	238 (28) ^A
Nickel				
500	113 (13) ^A	180 (20) ^A	66 (12) ^A	119 (32) ^A
1000	109 (6) ^A	172 (19) ^A	63 (10) ^A	167 (40) ^A
2000	116 (11) ^A	206 (15) ^A	62 (3) ^A	260 (20) ^B
Lead				
500	99 (8) ^B	160 (5) ^B	51 (10) ^A	126 (14) ^A
1000	96 (8) ^B	147 (18) ^B	34 (4) ^A	128 (45) ^A
2000	100 (1) ^B	139 (11) ^B	40 (10) ^A	188 (43) ^A
Zinc				
500	113 (4) ^A	161 (7) ^A	51 (6) ^A	131 (28) ^A
1000	111 (16) ^A	159 (20) ^A	42 (13) ^A	226 (32) ^A
2000	118 (3) ^A	186 (15) ^A	37 (3) ^A	238 (56) ^A

Notes: Plants were treated for 4 or 7 d with nutrient solutions containing either 0, 500, 1000, or 2000 $\mu\text{mol/L}$ Cu, Ni, Pb, or Zn. Results are means ($n = 3-6$) and 1 SE. Within each metal \times day combination, significant differences among metal levels and controls are indicated by different superscripts ($P < 0.05$; one-way ANOVA for each metal, with levels of 0, 500, 1000, and 2000, followed by Tukey's).

TABLE 2. The effects of heavy metals in soil on total chlorophyll content (total chl; $\mu\text{g}/\text{cm}^2$) and the ratio of chlorophyll a to chlorophyll b (chl a : b) in leaves of *Zea mays*.

Treatment	Day 4		Day 7	
	Total chl	chl a : b	Total chl	chl a : b
Control	34.8 (0.7) ^A	5.4 (0.3)	20.8 (2.8) ^A	5.7 (0.2)
Copper				
500	30.0 (5.9) ^A	5.1 (0.5)	23.6 (2.6) ^A	4.2 (0.1)
1000	31.8 (3.7) ^A	5.2 (0.2)	19.2 (2.7) ^A	4.3 (0.1)
2000	25.7 (0.4) ^A	4.5 (0.2)	16.4 (1.0) ^A	3.8 (0.8)
Nickel				
500	35.9 (1.6) ^A	4.7 (0.4)	30.3 (2.8) ^A	4.6 (1.0)
1000	28.5 (3.0) ^{AB}	4.8 (0.3)	30.2 (2.6) ^A	4.3 (0.9)
2000	25.2 (1.8) ^B	4.3 (0.6)	23.5 (2.0) ^A	3.5 (0.5)
Lead				
500	30.8 (1.4) ^A	4.5 (0.6)	30.3 (0.4) ^B	4.3 (0.7)
1000	33.5 (2.7) ^A	4.7 (0.4)	32.4 (0.8) ^B	4.2 (0.7)
2000	29.8 (2.5) ^A	4.5 (0.4)	30.8 (2.4) ^B	4.3 (0.8)
Zinc				
500	35.9 (3.2) ^A	4.8 (0.5)	20.3 (1.8) ^A	3.7 (0.7)
1000	35.3 (1.1) ^A	4.5 (0.2)	22.7 (5.7) ^A	4.0 (0.3)
2000	36.9 (1.4) ^A	4.6 (0.3)	23.3 (0.7) ^A	3.8 (0.4)

Notes: Plants were treated for 4 or 7 d with nutrient solutions containing either 0, 500, 1000, or 2000 $\mu\text{mol/L}$ Cu, Ni, Pb, or Zn. Results are means ($n = 3-6$) and 1 SE. For total chl, within each metal \times day combination, significant differences among metal levels and controls are indicated by different superscripts ($P < 0.05$; as Table 1); sampling date effects were not significant for chl a : b.

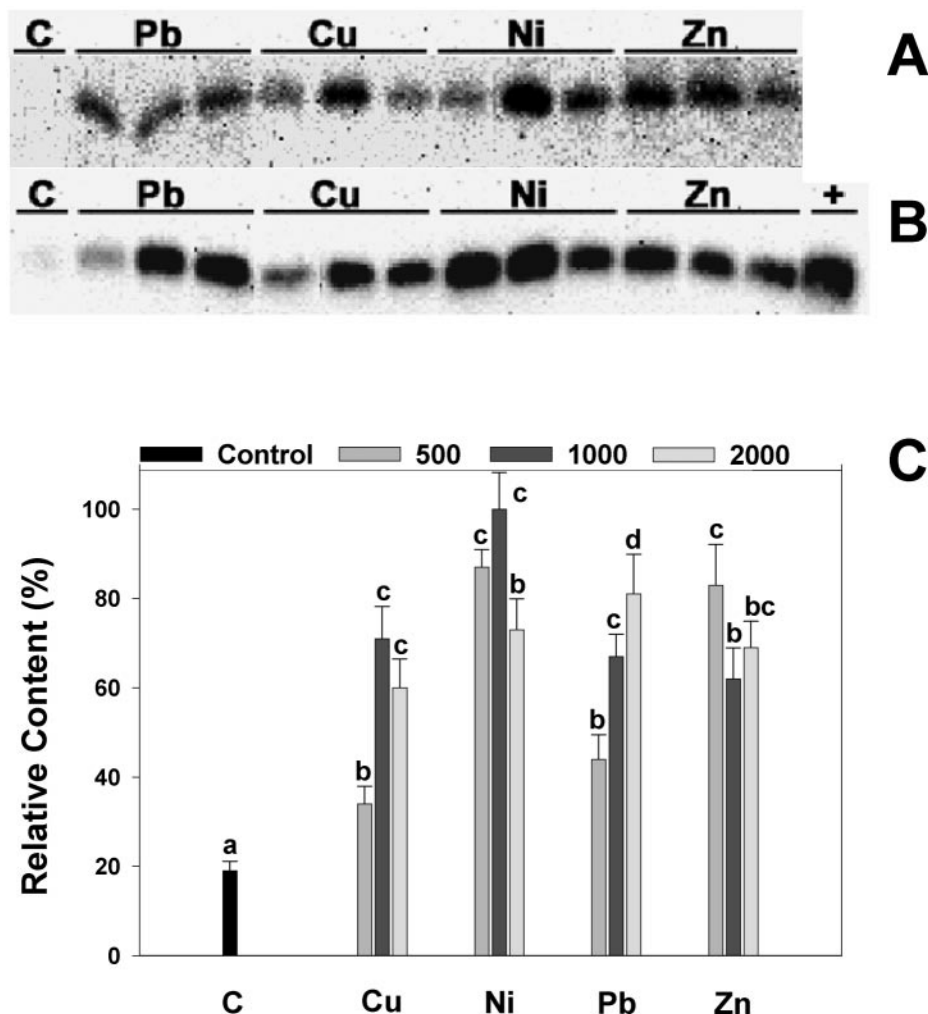


Fig. 2. Relative content of chloroplast small heat-shock protein (smHsp) in leaves of *Zea mays* after 4 or 7 d of exposure to heavy metals in soil. Plants were watered with a nutrient solution containing either 0 (control = C), 500, 1000, or 2000 $\mu\text{mol/L}$ Cu, Ni, Pb, or Zn. Representative immunoblots of samples collected on (A) day 4 and (B) day 7 of exposure. For each metal, the left lane = 500 $\mu\text{mol/L}$, the middle lane = 1000 $\mu\text{mol/L}$, and the right lane = 2000 $\mu\text{mol/L}$; "+" lane = standard sample from heat-stressed plants (leaf tissue from *Z. mays* treated at 42°C for 6 h). Equal total leaf protein (40 μg per lane; from a single leaf of an individual plant) was fractionated by SDS-PAGE, transferred to membranes, and probed with chloroplast smHsp-specific antibodies (visualized colorimetrically). (C) Densitometric analysis of all samples collected on day 7. Results are relativized to the highest level and are means ($n = 3$) + 1 SE. Within each metal \times day combination, significant differences among metal levels and controls are indicated by different superscripts ($P < 0.05$).

indicated three protein peaks, and immunoblotting indicated that the last of these three peaks (somewhere between 100 and 200 mmol/L sodium phosphate) contained only the chloroplast small Hsp (confirmed by silver staining). This fraction, containing small Hsp at a protein concentration of 0.0017 g/L, was stored at -70°C and used directly for electron transport assays (final concentration described above).

Statistical analyses—Results were analyzed by analysis of variance (ANOVA), first by including all main effects and their interactions in the models, and then, when appropriate (i.e., when significant main effects were observed at $P < 0.05$), only single levels of main effects (e.g., days) were included in ANOVA analyses. Differences among levels of main effects were analyzed by Tukey's test or by a *t* test, following significant ANOVA results.

RESULTS

All four metals examined (Cu, Ni, Pb, Zn) decreased Ph_n , and decreases were greater at higher metal levels and with longer exposure times (Fig. 1). After 4 d of exposure to metals,

significant decreases in Ph_n were observed only for Cu and Ni at the highest metal level (2000 $\mu\text{mol/L}$). However, after 7 d of exposure, all metals decreased Ph_n , with greater decreases at higher metal levels (except for Pb), and the decreases in Ph_n were greater than those observed after 4 d of treatment.

Decreases in Ph_n with Cu and Pb exposure were accompanied by significant decreases ($P < 0.05$) in stomatal conductance (g_{st}) after 4 d, but not after 7 d (Table 1) (decreases with Ni and Zn at day 4 were marginally significant at $P < 0.08$ and 0.09, respectively). On day 4, leaf internal CO_2 concentration (C_i) decreased significantly with exposure to Pb, with marginally significant decreases for Cu and Zn ($P < 0.09$ and 0.08, respectively), indicating potential increases in stomatal limitations to Ph_n for Cu-, Pb-, and Zn-treated plants. However, since Ph_n decreased on day 4 only in Cu- and Ni-treated plants, decreases in g_{st} contributed to decreases in Ph_n only in Cu-treated plants; decreases in Ni-treated plants had to result from decreases in photosynthetic metabolism. In contrast to day 4,

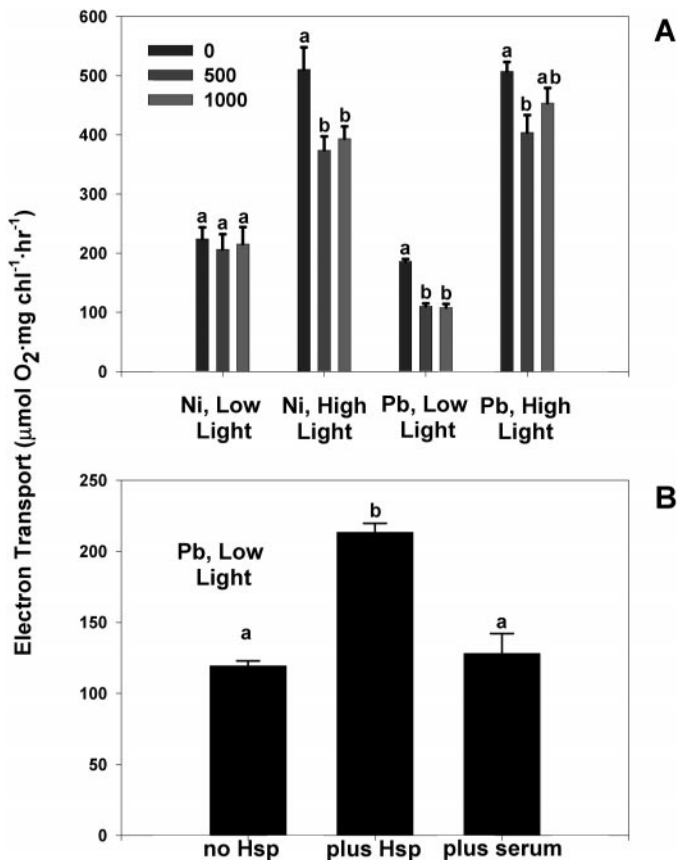


Fig. 3. The effects of heavy metals on in vitro photosynthetic electron transport and protection of electron transport from heavy metals by chloroplast small heat-shock protein (smHsp) in *Zea mays*. (A) Thylakoids were isolated from untreated plants and exposed to 0, 500, or 1000 $\mu\text{mol/L}$ Ni or Pb, under low- or high-light conditions (150 or $1300 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ photosynthetically active radiation, respectively). (B) Thylakoids were exposed to $500 \mu\text{mol/L}$ Pb at low light, either with added purified chloroplast smHsp (plus Hsp), with added pre-immune serum (plus serum), or without smHsp or serum (no Hsp). Results are means ($n = 3-4$) + 1 SE. Within each treatment combination, significant differences (A) among metal levels and controls or (B) among addition treatments are indicated by different superscripts ($P < 0.05$).

on day 7, no decreases in g_{st} occurred with metal treatment, but a trend of increases in C_i was observed (for Ni, $P < 0.05$; for Cu, $P < 0.08$), indicating that decreases in Ph_n on day 7 were due to metal effects on photosynthetic metabolism.

There was little indication that decreases in Ph_n with metal exposure in this study were due to decreases in total chlorophyll content (Table 2); from this, we conclude that decreases in Ph_n were not simply due to a decrease in the number of functional photosystem reaction centers. While sampling date (day 4 vs. 7) had a significant effect on total chlorophyll for all metals (two-way ANOVA; $P < 0.05$), metal exposure affected total chlorophyll only in Ni-treated plants on day 4 (decrease; $P < 0.05$) and in Pb-treated plants on day 7 (increase; $P < 0.05$). In contrast, for chlorophyll a : b, there were no significant effects of sampling date (or date by metal-level interactions), but Cu, Zn, and Ni decreased chlorophyll a : b ($P < 0.05$ for Cu, Zn; $P < 0.08$ for Ni). Decreases in chlorophyll a : b indicate proportionately greater effects on photosystem reaction centers, compared to light-harvesting complexes (LHC), since reaction centers are relatively rich in chlorophyll

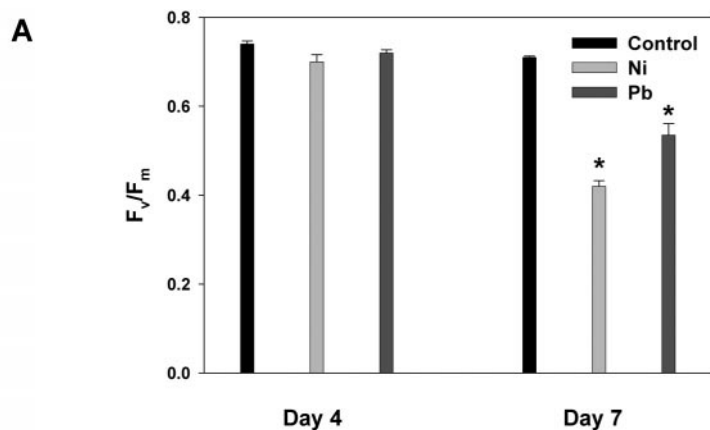


Fig. 4. The effects of heavy metals in soil on in vivo photosystem II (PSII) function in *Zea mays*. Plants were treated for 4 or 7 d with nutrient solutions containing 0 (Control) or $2000 \mu\text{mol/L}$ Ni or Pb. Photosystem II function was assayed by determining the ratio of variable-to-maximum fluorescence in dark-adapted leaves (F_v/F_m). Results are means ($n = 4$) + 1 SE. Within each metal \times day combination, significant differences between controls and metal treatments are indicated by asterisks ($P < 0.05$).

a, while the LHCs are rich in chlorophyll b (e.g., Taiz and Zeiger, 1998).

Exposure to all four metals induced the accumulation of chloroplast smHsp, with greater accumulations with longer exposures to the metals (Fig. 2A, B). By day 7, maximum levels of chloroplast smHsp were comparable among the four metal treatments (Fig. 2C), and the extent of smHsp accumulation was similar to that induced by severe acute heat stress (Fig. 2B, right lane marked “+” vs. lanes with metal-treated samples). Interestingly, in several instances, smHsp levels increased with increases in metal concentrations, especially with 500 vs. $1000 \mu\text{mol/L}$ metal (e.g., Cu on days 4 and 7, Pb on day 7, and Ni on day 4). In most cases, smHsp accumulation leveled off at intermediate metal concentrations and then often decreased at the highest metal concentration, relative to lower metal levels (e.g., Cu, Ni, and Zn on day 4 and 7).

As with in vivo Ph_n , Ni and Pb decreased in vitro photosynthetic electron transport (Ph_{et}) (Fig. 3A). With Ni, decreases in Ph_{et} were observed only at high light, while for Pb, decreases were evident at both low and high light levels. When purified chloroplast smHsp was added to thylakoids isolated from untreated control plants that had no detectable smHsp, smHsp increased Ph_{et} in Pb-treated thylakoids to levels comparable to untreated controls (Fig. 3B, plus smHsp vs. Fig. 3A, 0 mmol/L Pb at low light); pre-immune serum (negative control) had no effect on Ph_{et} . Interestingly, purified smHsp had no protective effect on Ph_{et} in Ni-treated thylakoids (not shown), suggesting that chloroplast smHsp protects other aspects of chloroplast function during Ni stress (confirmed below).

To determine if Ni and Pb decrease Ph_{et} in vivo, as observed in vitro, we treated plants with Ni and Pb and then monitored in vivo PSII function by determining the ratio of variable-to-maximum chlorophyll fluorescence in dark-adapted leaves (F_v/F_m). Exposure of plants to Ni and Pb did not affect F_v/F_m after 4 d of treatment, but by day 7, both Ni and Pb caused decreases in F_v/F_m (Fig. 4).

To confirm the protective effects of chloroplast smHsp on photosynthesis, we monitored Ph_n in heat-sensitive (wild-type progenitor) and near-isogenic, heat-tolerant selection mutants

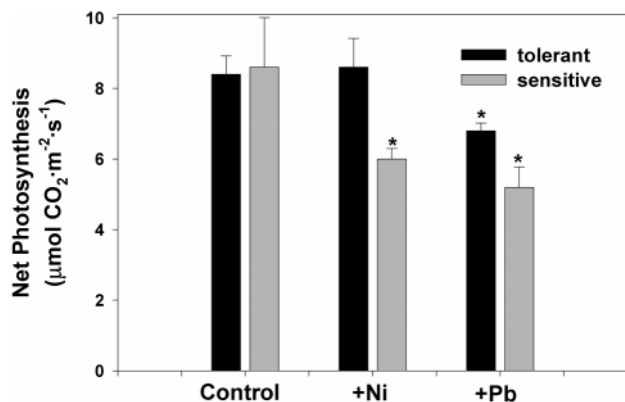


Fig. 5. The effects of heavy metals in soil on in vivo net photosynthesis in *Agrostis stolonifera* heat-tolerant selection mutants that express additional chloroplast small Hsp genes and accumulate more smHsp, relative to their heat-sensitive near-isogenic progenitor genotype. Plants were treated for 4 d with nutrient solutions containing 0 (Control) or 2000 µmol/L Ni or Pb. Results are means ($n = 3$) + 1 SE. Significant differences between controls and metal treatments are indicated by asterisks ($P < 0.05$).

of *A. stolonifera* that differ in the quantity of chloroplast smHsp that each produces (with the tolerant genotype accumulating approximately twice as much chloroplast smHsp than the sensitive genotype) (Fig. 5). Both Ni and Pb decreased Ph_n in the sensitive genotype, but Ph_n did not decrease in the tolerant genotype with Ni treatment, and Ph_n decreased less in the tolerant genotype than in the sensitive genotype with Pb treatment.

DISCUSSION

Heat-shock proteins are general stress proteins involved in the protection, repair, and degradation of damaged cell components, especially proteins, during most abiotic stresses (e.g., Parsell and Lindquist, 1994; Downs et al., 1999b; Hamilton and Heckathorn, 2001). Heavy metal accumulation damages cell components directly (e.g., by poisoning enzymes) and indirectly (e.g., by inducing oxidative damage to proteins and lipids) (Hall, 2002). Therefore, the production of certain Hsps might be predicted to increase during heavy metal stress in plants or other organisms. However, based on the few studies that have investigated the effects of heavy metals on production of heat-shock proteins in plants, the increased production of Hsps is indeed a general plant response to heavy metal accumulation (reviewed in Hall, 2002). These studies also indicate that members of the Hsp70 and small Hsp groups are especially responsive to heavy metals, and there is some evidence to suggest that one of the key functions of Hsps during heavy metal stress is to protect the cell membrane (a primary site of heavy metal damage) (Hall, 2002). However, with few exceptions, the specific Hsps induced by heavy metals and the cellular location of these proteins are unidentified, as are the specific roles or cell targets of Hsps during metal stress (Hall, 2002).

In the present study, the chloroplast small Hsp, which is typically not produced by plants in the absence of stress, was induced by exposure to all four heavy metals examined (Cu, Ni, Pb, Zn) to levels comparable to those induced by severe acute heat stress (Fig. 2). In corn, purified chloroplast small Hsp was able to protect photosynthetic electron transport in vitro from direct toxic effects of Pb (Fig. 3). In *A. stolonifera*,

the resistance of in vivo net photosynthesis to Ni and Pb was correlated with the increased production of chloroplast small Hsp in a heat-tolerant selection genotype, compared to a near-isogenic heat-sensitive progenitor genotype making less chloroplast small Hsp (Fig. 5). These results indicate that the chloroplast small Hsp can protect photosynthesis during heavy metal stress. Thus, this study provides some of the first evidence that Hsps of any kind are involved in protecting photosynthesis during heavy metal stress. Interestingly, in corn, purified small Hsp did not protect Ph_{et} from Ni in vitro, but in *A. stolonifera*, protection of Ph_n was indicated, supporting the prediction that small Hsp could protect other aspects of chloroplast function from heavy metals besides Ph_{et} , such as Calvin cycle enzymes (including rubisco), which are known to be readily damaged by excess heavy metals (Stiborova et al., 1986; Lidon and Henriques, 1991; Angelov et al., 1993; Moustakas et al., 1994).

Production of chloroplast small Hsp was induced (Fig. 2) even before damage to photosynthesis was evident (e.g., day 4 at 500 and 1000 µmol/L metal) (Fig. 1). In addition, the results suggest the possibility that at lower metal levels, chloroplast smHsp accumulation increases with metal accumulation in leaves, while at intermediate levels of metals, smHsp accumulation saturates, prior to declining at high levels of metals, that likely are so toxic that even smHsp production is inhibited. Together, the results indicate that production of chloroplast small Hsp is an early response to heavy metal accumulation in leaves and that the function of chloroplast small Hsp is to limit damage to photosynthesis, rather than involvement in repair or recovery from heavy metal damage (a similar role is suggested for heat stress; Waters et al., 1996; Downs et al., 1999a; Török et al., 2001). The ability of chloroplast small Hsp to protect photosynthesis from heavy metals was significant, both in vitro and in vivo, suggesting the potential utility of breeding, engineering, or selecting plants for increased production of chloroplast small Hsp (e.g., either constitutive production, more rapid induction, or increased accumulation during stress) in improving plant tolerance to heavy metals. Of course, increased production of the chloroplast small Hsp in response to heavy metal accumulation is only one of the many plant adaptive responses to heavy metals. Given that heavy metals damage a wide range of cell components and functions, significant increases in the tolerance of whole plants to heavy metals likely requires changes in multiple traits.

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