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Thermal Regulation of Phosphoenolpyruvate Carboxylase and Ribulose-1,5-Bisphosphate Carboxylase in C_3 and C_4 Plants Native to Hot and Temperate Climates[1]

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ABSTRACT

Exposure of leaf sections from 2-week-old seedlings of sorghum (*Sorghum bicolor* L.) (C_4 plant), corn (*Zea mays* L.) (C_4), peanut (*Arachis hypogaea* L.) (C_3 plant), and soybean (*Glycine max* L.) (C_3) to 40 or 45°C for up to 4 hours resulted in significant increases in the levels of 102 kilodalton (C_4), 52 kilodalton (C_3 and C_4), and 15 kilodalton (C_3 and C_4) polypeptides. These proteins comigrated, respectively, with authentic phosphoenolpyruvate carboxylase (PEPC) and the large (RLSU) and small (RSSU) subunits of ribulose-1,5-bisphosphate carboxylase (Rubisco) during both one- and two-dimensional SDS-PAGE and reacted with antisera raised against these enzymes. After 4 hours at 50°C, levels of the polypeptides either remained relatively stable (PEPC, RLSU) or increased (RSSU) in sorghum and peanut (plants native to hot climates). In corn and soybean (plants native to temperate climates), levels of the proteins either fell sharply (corn) or showed strong evidence of incomplete processing and/or aggregation (soybean). In addition to changes in levels of the proteins, the activities of PEPC and Rubisco in extracts of leaves exposed to 50°C fell by 84% and 11% of their respective control values in sorghum and by 54% each in peanut. In corn and soybean, the activities of both enzymes were depressed at 40°C, with measured values at 50°C not exceeding 5% of those from the nonstressed controls.

Of all ecological variables, temperature is perhaps the most important factor affecting the distribution of flowering plants (2). Like most organisms, flowering plants respond to supraoptimal temperatures, in part, by synthesizing a set of unique polypeptides commonly known as HSPs[2] and also by altering the activity and synthesis of constitutive proteins which *in toto* may contribute to thermal tolerance (9–13, 18, 21). In the case of the photosynthetic apparatus, the effect of thermal stress on protein synthesis has not been well documented, but previous studies have established that changes in light intensity, temperature, salinity, pH, soil-water content, and mineral nutrients from their respective optimal levels can alter the efficiency of CO_2 fixation by affecting both PEPC (EC 4.1.1.31) and Rubisco (EC 4.1.1.39) (3, 16, 20, 23). Thus, physiological limitations imposed by environmental stress may derive, in part, from damage to specific proteins that control photochemical events and/or carbon fixation. Differences in the stability and/or synthesis of these proteins during stress suggest a possible basis for the adaptation of plant species to different thermal regimes. We have examined that possibility during the present study by following the activities and turnover of PEPC and Rubisco in peanut and sorghum (C_3 and C_4 plants native to hot climates [22]) and in soybean and corn (C_3 and C_4 plants native to temperate climates [22]) during exposure of their leaf tissue to a time-course of elevated temperatures.

MATERIALS AND METHODS

Plant Materials

Seeds of sorghum (*Sorghum bicolor* L. cv HO-K 5291), corn (*Zea mays* L. cv Seneca 60-11), peanut (*Arachis hypogaea* L. cv Valencia), and soybean (*Glycine max* L. cv Jackson) were germinated in flats (54 × 27 × 6 cm) of Pro-mix BX (Premier Brands, New Rochelle, NY) and placed in a growth chamber at 25°C. Seedlings were watered daily and grown under a 16-h photoperiod with 450 $\mu E \cdot m^{-2} \cdot s^{-1}$ of mixed fluorescent and incandescent light for 2 weeks before sampling.

Temperature Stress

Excised leaves were cut into 40 × 5 mm (sorghum and corn) or 20 × 5 mm pieces (peanut and soybean) and washed in 0.01% Tween-20. One g of leaf segments from a given species was placed in a closed Petri dish (100 × 15 mm) containing 10 mL of incubation mixture (50 mM Hepes-KOH [pH 7.0], one-quarter strength Murashige and Skoog salt mixture [GIBCO], and 0.01% Tween-20) and held at 25°C for 4.0 h to overcome wound shock (5) before incubation at 25, 40, 45, or 50°C for 0.5, 1.0, 2.0, or 4.0 h under fluorescent

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[2] Abbreviations: HSP, heat shock protein; PEPC, phosphoenolpyruvate carboxylase; Rubisco, ribulose-1,5-bisphosphate carboxylase; AHA, 6-amino-*n*-hexanoic acid; BH, benzamidine hydrochloride; CBBR-250, Coomassie brilliant blue R-250; PVPP, polyvinylpolypyrrolidone; Chaps, 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulphonate; RLSU, large subunit of Rubisco; RSSU, small subunit of Rubisco; BCIP, 5-bromo-4-chloro-3-indolylphosphate-toluidine salt; DMF, *n,n*-dimethylformamide; NBT, *p*-nitro blue tetrazolium chloride; PEP, phospho*enol*pyruvate; Rubp, ribulose-1,5-bisphosphate; ACS, aqueous counting scintillant; IEF, isoelectric focusing.

lights (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) on a laboratory bench. At the end of each treatment, leaf segments were rinsed with distilled water, frozen in liquid N_2, and either stored at −70°C or lyophilized and then stored in sealed containers at −70°C for subsequent processing.

Chl and Protein Extractions

Ten-mg samples of lyophilized leaves were placed in 30-mL Corex tubes with 20 mL of ice-cold acetone made to 5 mM AHA, 1 mM BH, and 1 mM PMSF, and then homogenized for 1 min at full speed in a Polytron (PT-A-10-S, Brinkmann Instruments). The homogenates were stored overnight at −20°C and then centrifuged at 10,000*g* for 15 min at 4°C. The Chl content in the acetone fraction was determined by measuring absorbance at 645 and 663 nm (1). The pellets were vacuum-dried, resuspended in 2 mL of sample buffer (62.5 mM Hepes-KOH [pH 6.8], 1 mM Na_2EDTA, 10% [v/v] glycerol, 5% [v/v]) 2-mercaptoethanol, 5 mM AHA, 1 mM BH, 1 mM PMSF, and 3% SDS), incubated for 3 min in a boiling water bath, cooled, and then centrifuged for 1 min at 14,000 rpm in an Eppendorf centrifuge (model 5415, Brinkmann Instruments). The supernatants were analyzed for total protein using the Coomassie blue method previously described (7) with BSA (Fraction V, Sigma) as a standard.

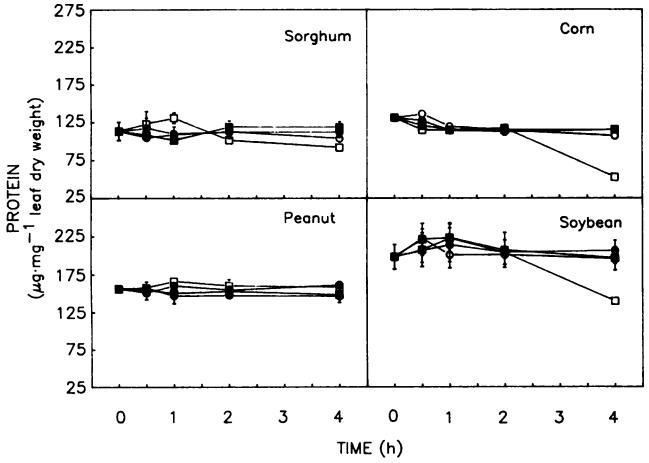

Figure 1. Effect of temperature on leaf protein in sorghum, corn, peanut, and soybean. Excised leaf segments from 2-week-old seedlings were preincubated at 25°C for 4 h prior to exposure to 25 (●), 40 (○), 45 (■), or 50°C (□) for up to 4.0 h. Each data point represents a mean of four replicates ± 1 SE.

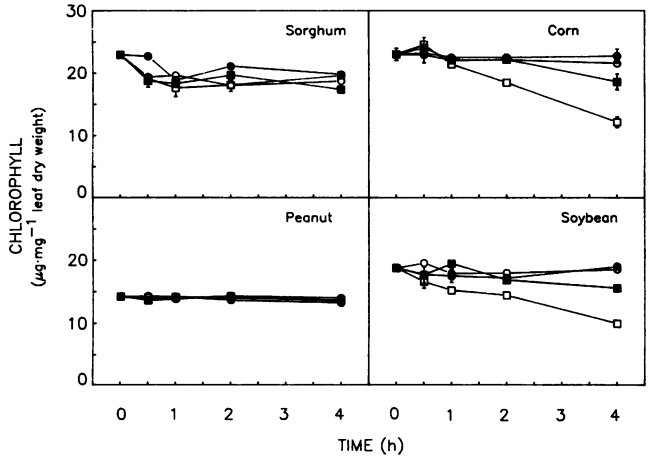

Figure 2. Effect of temperature on leaf Chl in sorghum, corn, peanut, and soybean. Excised leaf segments from 2-week-old seedlings were preincubated at 25°C for 4 h prior to exposure to 25 (●), 40 (○), 45 (■), or 50°C (□) for up to 4.0 h. Each data point represents a mean of four replicates ± 1 SE.

PAGE

Aliquots of the total protein extract were analyzed by SDS-PAGE (0.75 mm × 7 cm) using 12% separating and 5% stacking gels. After completion of a run, the gels were stained for 30 min with 0.1% (w/v) CBBR-250, destained for 2 h (7), and photographed.

Two-dimensional PAGE was performed as described by O'Farrell (14). Samples were prepared from 20 fresh-frozen leaf segments ground with a mortar and pestle using 2 mL of ice-cold solubilization buffer containing 50 mM Hepes-KOH (pH 7.4), 1 mM $MgCl_2$, 50 mM DTT, 5 mM AHA, 1 mM BH, 1 mM PMSF, 1% (w/v) PVPP, and 50 μg each of pancreatic RNAse and DNAse per mL. The mixture was allowed to stand on ice for 30 min, followed by the addition of SDS to a final concentration of 1% (w/v), and then boiled for 1 min. After removal of insoluble material by centrifugation, a 100-μL aliquot of the supernatant was removed for protein estimation before the protein was precipitated from the solubilization buffer by the addition of five volumes of ice-cold acetone. After 1 h at −20°C, the protein was pelleted by centrifugation, dried under vacuum, and resuspended in 9.5 M urea, 2% (w/v) Chaps, 1 mM PMSF, and a total of 2% Ampholines (Pharmacia) with 1.6% covering a pH range of 5 to 8 and 0.4% covering the range of 3 to 10.

Antisera

Spinach Rubisco (Sigma) was purified by SDS-PAGE on a 12% gel. The RLSU, corresponding to a molecular mass of about 53 kD, was excised and approximately 500 μg were homogenized in 1 mL of 50% Freund's complete adjuvant (Difco) and injected subcutaneously into the back of a 2.5 kg male New Zealand White rabbit. After 4 weeks a booster injection was given in 50% Freund's incomplete adjuvant, and 2 weeks later blood was collected from a lateral ear vein. Antisera against sorghum PEPC and the RSSU from spinach Rubisco (Sigma) were prepared as described previously (15, 19). The specificity of the antibodies was confirmed by two-dimensional PAGE followed by immunoblots.

Electrotransfer and Immunodetection of Proteins

Western Blot Analysis

Proteins were transferred from polyacrylamide gels to nitrocellulose membranes (0.2 μm) in buffer A (16 mM glycine, 25 mM Tris-HCl [pH 8.7], 0.02% SDS, and 20% [v/v] methanol) using a Bio-Rad transblot apparatus set at 0.4 A for 4 h (19). After transfer and shaking the nitrocellulose membrane for 1

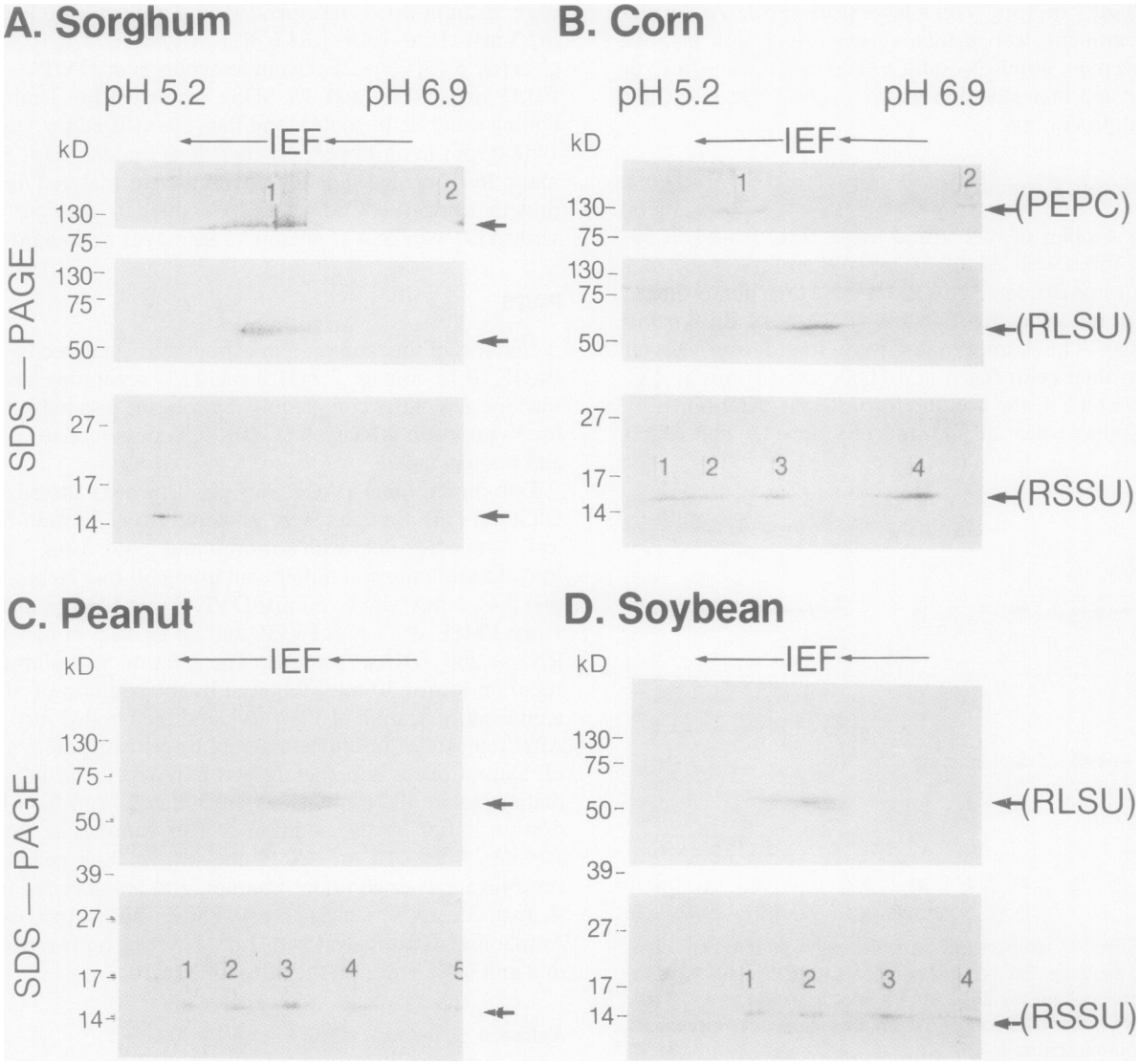

Figure 3. Immunological identification of PEPC, RLSU, and RSSU in crude extracts from excised leaves of 2-week-old seedlings of sorghum (A), corn (B), peanut (C), and soybean (D). Ten-μg (sorghum or corn) or 5-μg (peanut or soybean) of each plant extract were separated by two-dimensional SDS-PAGE and then transferred to a nitrocellulose membrane. The upper half of each membrane was immunoblotted against rabbit antisera specific for PEPC or RLSU and the lower half against antisera for RSSU. Protein spots labeled 1–5 are discussed in the text. Mol wt markers are indicated in kD to the left of each blot.

h in 30 mL of buffer B (25 mM Tris-HCl [pH 7.6], 140 mM NaCl) containing 1% BSA, the appropriate antibody was added and shaking was continued at room temperature for 12 h followed by three 10-min washes, each in 30 mL of buffer B. The membrane was then incubated at room temperature for 1 h in 30 mL of buffer B containing 10 μL of secondary antibody (goat anti-rabbit IgG, H + L) conjugated to alkaline phosphatase, and then washed for 10 min in buffer B, containing 1% Triton X-100 plus 5 mM Na_2EDTA with two additional washes in buffer B before transferring to 25 mL of alkaline phosphate buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 5 mM $MgCl_2$). The bound antibody was visualized by addition of 83 μL of 50 mg BCIP · ml^{-1} of DMF and 166 μL of 50 mg NBT · ml^{-1} of 70% DMF. The reaction was terminated within 3 to 5 min by addition of stop buffer (20 mM Tris-HCl [pH 8.0] and 5 mM Na_2EDTA) and the membrane was dried and photographed. A Bio-Rad scanning densitometer (model 1650) in the reflectance mode provided quantitative estimates of protein levels in each band.

Enzyme Assays

Fresh-frozen leaf segments (500 mg), previously infused with 0.37 mM $MgSO_4$, were ground in 1 mL of ice-cold extraction buffer (50 mM Hepes-KOH [pH 7.0], 1 mM Na_2EDTA, 10 mM DTT, 5 mM AHA, 1 mM BH, 1 mM PMSF, 1% [w/v] PVPP) with a mortar and pestle and clarified by centrifugation for 1 min at 14,000 rpm in an Eppendorf centrifuge. The supernatant was recentrifuged for 30 min at 14,000 rpm and diluted to approximately 25 μg of protein per 50 μL and assayed for enzyme activity. Activities were not affected by preincubation with additional Mg^{2+} (5 mM) or HCO_3^- (10 mM) for 10 min at 30°C. The assay buffer used

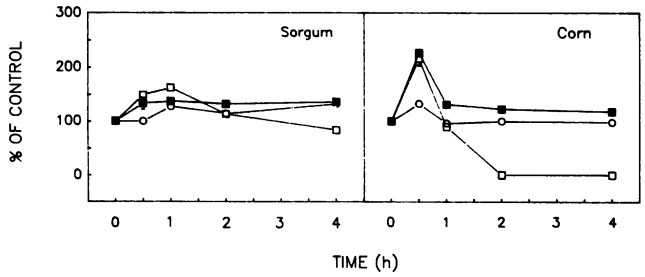

Figure 4. Quantitation of PEPC from densitometric scans of Western blots of leaf extracts from 2-week-old sorghum and corn. Freshly cut leaf segments were preincubated at 25°C for 4 h prior to heat stress at 40 (○), 45 (■), or 50°C (□). Protein concentrations are plotted as percentages of controls (25°C). Each value is a mean of four replicates ± 1 SE.

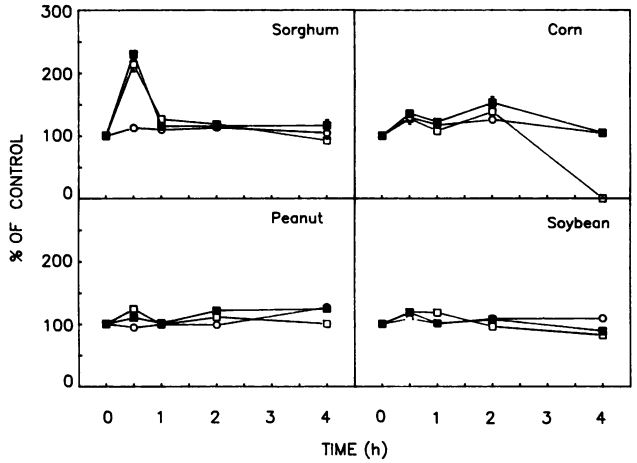

Figure 5. Quantitation of RLSU from densitometric scans of Western blots of leaf extracts from 2-week-old sorghum, corn, peanut, and soybean. Freshly cut leaf segments were preincubated at 25°C for 4 h prior to heat stress at 40 (○), 45 (■), or 50°C (□). Protein concentrations are plotted as percentages of controls (25°C). Each value is a mean of four replicates ± 1 SE.

to measure PEPC and Rubisco contained 100 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 10 mM L-glutamic acid, 0.1 mM Na_2EDTA, 20 mM $NaHCO_3$, 0.5 μCi $NaH^{14}CO_3$ (57 mCi · $mmol^{-1}$ CO_2), and either 32 μL of 20 mM PEP or 25 μL of 6 mM Rubp in a final volume of 250 μL (6, 15, 24). To initiate the reaction, 50 μL of enzyme extract and 200 μL of assay mixture were warmed to room temperature and mixed in a screw-capped vial (15 × 45 mm) for 10 min at 30°C. Activities remained linear for over 20 min. The reaction was terminated by addition of 250 μL of 6 M acetic acid, and the vial was left open in a fume hood for 12 h before adding 250 μL of ACS (Amersham) prior to scintillation counting in a Beckman LS7000. The levels of endogenous substrate present in leaf extracts were estimated by the omission of Rubp and PEP from assay reaction mixtures. Boiled enzyme extracts were used as controls in all tests.

RESULTS

Effects of Temperature on Leaf Protein and Chl

Exposure of intact, 2-week-old seedlings of sorghum and peanut (C_4 and C_3 hot climate plants) to a temperature of

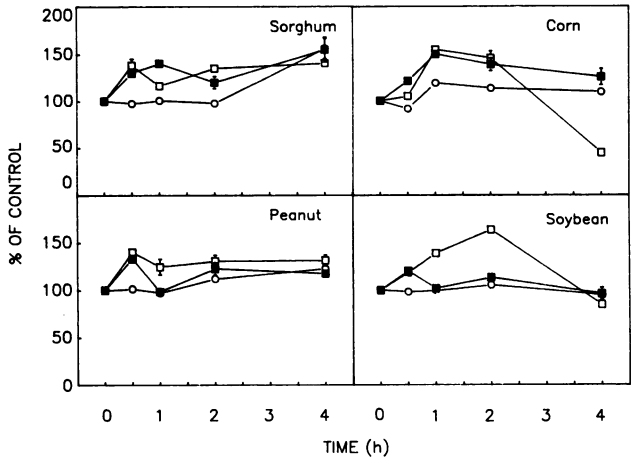

Figure 6. Quantitation of RSSU from densitometric scans of Western blots of leaf extracts from 2-week-old sorghum, corn, peanut, and soybean. Freshly cut leaf segments were preincubated at 25°C for 4 h prior to heat stress at 40 (○), 45 (■), or 50°C (□). Protein concentrations are plotted as percentages of controls (25°C). Each value is a mean of four replicates ± 1 SE.

45°C for 24 h did not induce any detectable morphological changes (not shown), nor did exposure to temperatures of up to 50°C for 4 h affect the amount of protein or Chl per unit dry weight of excised leaf segments (Figs. 1 and 2). In contrast, 24 h at 45°C caused severe wilting (not shown) in 2-week-old corn and soybean (C_4 and C_3 temperate climate plants), and the levels of protein and Chl in excised leaf segments showed large decrements after 4 h at 50°C (Figs. 1 and 2).

Effects of Temperature on Levels of the Carboxylating Proteins

An examination of crude proteins isolated from heat-shocked leaf sections from each of the four species by one-dimensional SDS-PAGE revealed marked changes in proteins in the 102, 52, and 14 to 15 kD bands. The three polypeptides comigrated, respectively, with purified PEPC previously isolated from sorghum (15) and with the large and small subunits of Rubisco from spinach (Sigma). In sorghum, PEPC usually appeared as a doublet at the 102 and 98 kD positions, and the RSSU was always detected at an M_r of 14 kD in soybean and at 15 kD in the other species.

The identities of the three proteins were confirmed by two-dimensional Western hybridization (Fig. 3). One major spot (Fig. 3-1) at the acidic end of the IEF gradient was identified as PEPC in both sorghum and corn, but in a few gels a spot that tested positive, and represents aggregated protein, is also seen at the basic (loading) end of the gradient (Fig. 3-2). PEPC was not detected in either peanut or soybean whereas the RLSU was detected as a single elongated spot in all four species (Fig. 3). A single, comparatively tight spot of the RSSU was identified in sorghum, but multiple spots, which may represent different products of a multigene family (4), were detected in corn, peanut, and soybean (Fig. 3).

One-dimensional Western blot analysis was used to estimate the levels of PEPC, RLSU, and RSSU in extracts prepared from leaves exposed to high temperatures (Figs. 4–6). During the first 0.5 h at 45 or 50°C, the three proteins rose to

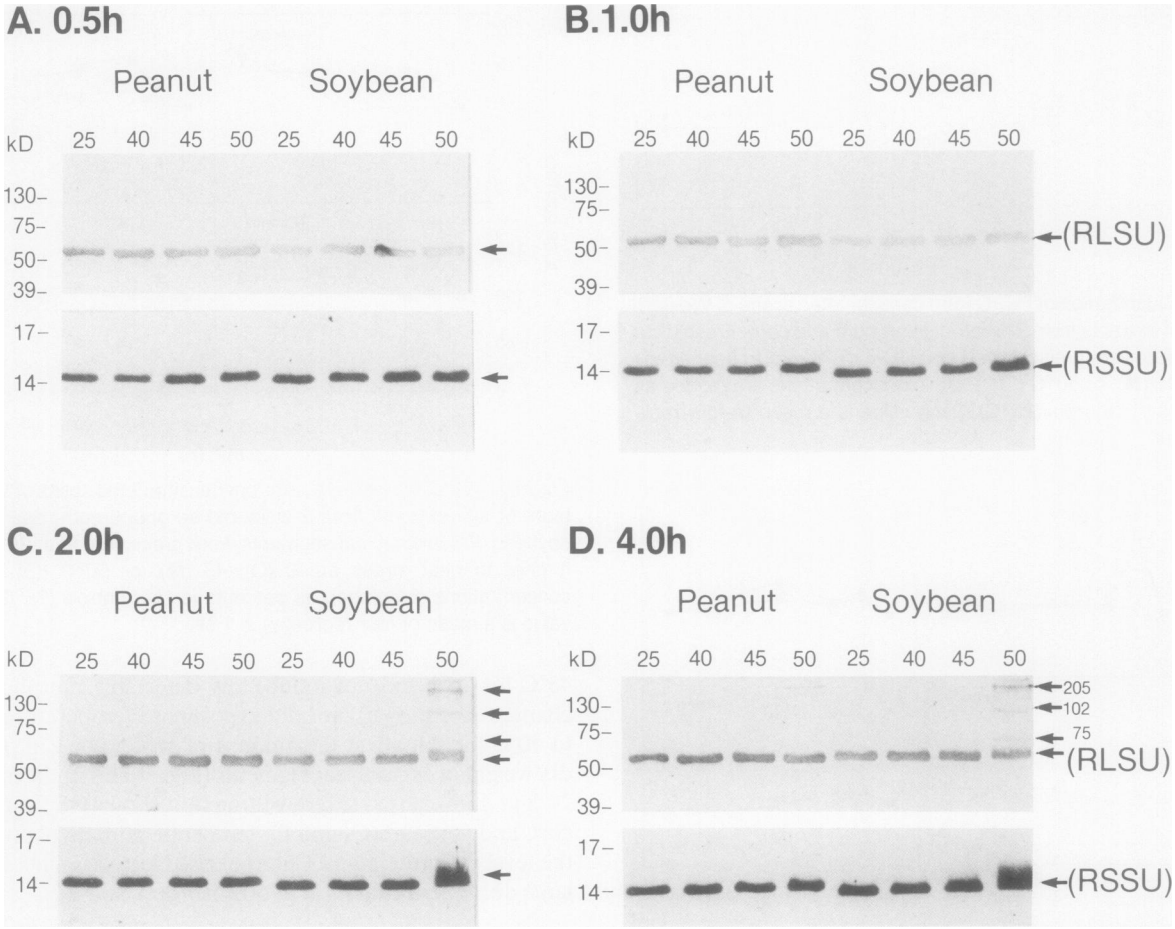

Figure 7. Immunodetermination of RLSU and RSSU in one-dimensional Western blots prepared from leaf extracts of 2-week-old seedlings of peanut and soybean. Freshly cut leaf segments were preincubated at 25°C for 4 h prior to exposure to treatment temperatures for periods of 0.5 (A), 1.0 (B), 2.0 (C), or 4.0 h (D). After one-dimensional SDS-PAGE of protein extracts, the samples (2.5-μg) were transferred to nitrocellulose membranes. The upper half of each membrane was immunoblotted against antisera for RLSU and the lower half against antisera for RSSU. Mol wt markers are indicated in kD on the left of each blot.

levels well above the controls in both sorghum and corn, although the effect on the RSSU in corn at 50°C was marginal (Figs. 4–6). Exposure to 50°C for 2 to 4 h sharply reduced the accumulation of the proteins in corn, and after 4 h at that temperature, the PEPC and RLSU bands were not detectable. In sorghum, the three proteins were more stable, and their levels remained at or above control levels with the exception of a small decline in PEPC after extended exposure to the highest temperature. In both C_4 species, all three proteins remained at or above control values at 40 and 45°C for the entire 4 h period.

The effects of high temperatures on the RLSU and RSSU in peanut and soybean were moderately (RLSU) to strongly (RSSU) stimulatory during the first 2 h of heat treatment (Figs. 5–7). After 4 h at 50°C, both subunits remained at 100% and 134% of respective control values in peanut, but in the less heat tolerant soybean, their levels had declined by 20 to 25% of the control values. Moreover, the M_r value of the RLSU in soybean increased by 3 to 5 kD after 2 h or more at 50°C, whereas the RSSU band appeared more diffuse (Fig. 7). Evidence suggesting irreversible aggregation of the RLSU during heat stress was seen with the immunodetection of three additional polypeptides at 205, 102, and 75 kD in soybean after 2 h at 50°C (Fig. 7C).

Effects of Temperature on Carboxylating Activity

The activities of PEPC and Rubisco were monitored in fresh extracts from leaf sections previously exposed to elevated temperatures (Figs. 8 and 9). Although PEPC bands were not detected during Western blot analysis of peanut and soybean, activity assays confirmed the presence of the enzyme in both C_3 plants, with measured activities in peanut about twice those detected in soybean. Activities of Rubisco at 25°C were similar in corn, peanut, and soybean but were approximately four times higher than those recorded for sorghum (Fig. 9). At high temperatures, the activities of both enzymes were more stable in heat-tolerant sorghum and peanut than in corn or soybean. At 50°C, specific activities of PEPC and Rubisco fell by 84% and 11% of respective control values in sorghum and by 54% each in peanut. In the less stress-resistant seedlings of corn and soybean, activities decreased significantly after 4 h at 45°C, with measured values at 50°C not exceeding

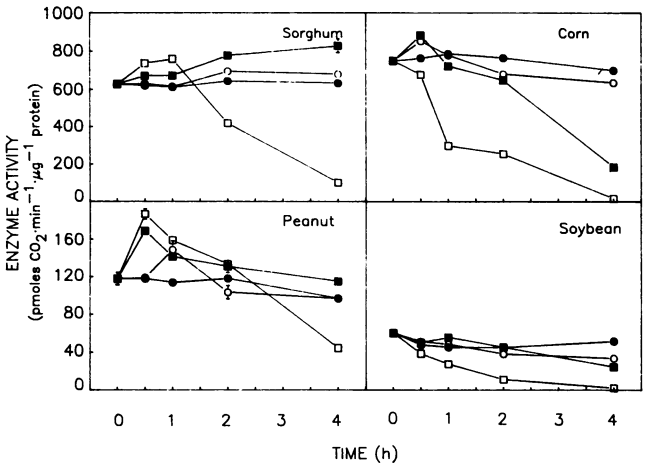

Figure 8. Activity of phosphoenolpyruvate carboxylase in leaf extracts from 2-week-old seedlings of sorghum, corn, peanut, and soybean. Freshly cut leaf segments were preincubated at 25°C for 4 h prior to exposure to 25 (●), 40 (○), 45 (■), or 50°C (□) for up to 4 h. Activities were monitored in fresh extracts at 30°C for 10 min. Each data point is the mean of four replicates ± 1 SE.

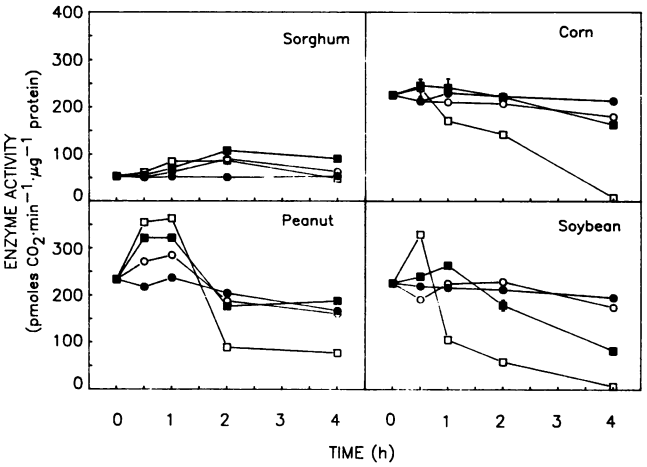

Figure 9. Activity of ribulose-1,5-bisphosphate carboxylase in leaf extracts from 2-week-old seedlings of sorghum, corn, peanut, and soybean. Freshly cut leaf segments were preincubated at 25°C for 4 h prior to exposure to 25 (●), 40 (○), 45 (■), or 50°C (□) for up to 4 h. Activities were monitored in fresh extracts at 30°C for 10 min. Each data point is the mean of four replicates ± 1 SE.

5% of those obtained from the controls (Figs. 8 and 9).

DISCUSSION

High-temperature stress in the natural environment can severely reduce photosynthetic efficiency and thereby limit the yield of crop plants. Conventional wisdom would suggest that at high temperatures C_4 plants are genetically programmed to have a better photosynthetic performance than C_3 plants. However, the presence or absence of the C_4 pathway per se may not be causally linked to factors that confer tolerance to high temperatures. For example, results from the present work and previous agronomic studies (22) indicate that sorghum, a C_4 plant, and peanut, a C_3 plant, show greater tolerance to high temperatures than either corn (C_4) or soybean (C_3), suggesting that more than one mechanism may be used to ameliorate the effects of heat stress. In this regard, the present study indicates that the thermostability of the carboxylating enzymes and/or the synthetic machinery which produces them may be important components of plant resistance to heat stress, an hypothesis supported by the maintenance of higher levels and activities of PEPC and Rubisco in sorghum and peanut than in corn or soybean.

Although the large subunit of Rubisco was more thermolabile than the small subunit in the four species under study, both subunits and the activity of functional Rubisco showed greater resistance to high temperature stress than PEPC by all measurements used in our tests. The resistance of Rubisco to heat stress has also been observed in several other species (3). For example, its stability at high temperatures exceeded whole-leaf photosynthesis in *Atriplex sabulosa, Tidestromia oblongifolia, Nerium oleander*, and *Hedera helix*, whereas the activities of PEPC, adenylate kinase, and NAD glyceraldehyde-3-phosphate dehydrogenase were markedly reduced at temperatures similar to those that irreversibly inhibited net photosynthesis.

The nature of the protective mechanism that affords heat resistance remains unclear, but *in vitro* studies suggest that enzyme stability at high temperatures may be achieved via physical effects of specific free amino acids including glutamine and aspartate or by other low mol wt metabolites such as malate (8, 17). Rathnam (17) reported that malate was more effective than aspartate in *Digitaria sanguinalis*, a preferentially malate forming C_4 species, whereas both aspartate and malate were equally effective in *Panicum maximum*, a C_4 species in which both acids are major carboxylation products.

In previous studies of plants adapted to contrasting temperature regimes, no differences were observed in the thermal stability of PEPC or Rubisco (16); however, the observed tolerance to high temperatures might be explained by increased rates of protein synthesis sufficient to offset decreased enzyme activity and/or loss by heat denaturation. Indeed, in the present study, the titer of the carboxylating enzymes as a fraction of total leaf protein either increased (sorghum, corn, peanut) or remained steady (soybean) as the temperature was raised from 25 to 45°C (Figs. 1, 4, 5, and 6). The levels remained steady in sorghum and peanut when the temperature was raised to 50°C but fell sharply in the less heat-tolerant corn and soybean. Although the detailed mechanism of PEPC and Rubisco synthesis at high temperatures remains to be elaborated, the present investigation does provide at least part of the rationale for understanding why sorghum and peanut are adapted to areas too hot for corn and soybean. In addition, the data in Figures 1 and 2 suggest that the thermal stability of total protein and/or total Chl at high temperatures may serve as useful indicators of plant resistance to high temperature stress.

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