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Influence of Plant Growth at High CO_2 Concentrations on Leaf Content of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase and Intracellular Distribution of Soluble Carbohydrates in Tobacco, Snapdragon, and Parsley[1]

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We have examined the possible role of leaf cytosolic hexoses and the expression of mannitol metabolism as mechanisms that may affect the repression of photosynthetic capacity when plants are grown at 1000 versus 380 μL L^{-1} CO_2. In plants grown at high CO_2, leaf ribulose-1,5-bisphosphate carboxylase/oxygenase content declined by ≥20% in tobacco (*Nicotiana sylvestris*) but was not affected in the mannitol-producing species snapdragon (*Antirrhinum majus*) and parsley (*Petroselinum hortense*). In the three species mesophyll glucose and fructose at midday occurred almost entirely in the vacuole (>99%), irrespective of growth CO_2 levels. The estimated cytosolic concentrations of glucose and fructose were ≤100 μM. In the three species grown at high CO_2, total leaf carbohydrates increased 60 to 100%, but mannitol metabolism did not function as an overflow mechanism for the increased accumulation of carbohydrate. In both snapdragon and parsley grown at ambient or high CO_2, mannitol occurred in the chloroplast and cytosol at estimated midday concentrations of 0.1 M or more each. The compartmentation of leaf hexoses and the metabolism of alternate carbohydrates are further considered in relation to photosynthetic acclimation to high levels of CO_2.

The photosynthetic response of plants grown at high concentrations of CO_2 involves adjustments at both the biochemical and molecular levels (Bowes, 1993). This response often results in a decreased photosynthetic capacity associated with increased carbohydrate levels (Long and Drake, 1992; van Oosten and Besford, 1995). This down-regulation of photosynthetic capacity occurs by reduced transcription of many photosynthetic genes (Krapp et al., 1993), perhaps as a consequence of increased Glc metabolism through cytosolic hexokinase (Jang and Sheen, 1994). However, we know relatively little about the suggested biochemical role of hexokinase as a sugar sensor. For example, one might hypothesize that mesophyll cytosolic Glc may increase in some species during growth at high levels of CO_2, since plants grown at ambient CO_2 normally have little, if any, leaf cytosolic Glc (e.g. tobacco < 1 mM; Heineke et al., 1994). If this were true, then differential control of mesophyll cytosolic Glc levels could in part account for some of the variation observed between species in their photosynthetic acclimation to high CO_2 concentrations (Sage et al., 1989; Sage, 1994).

Much of the information concerning the biochemistry of plant growth at high CO_2 is derived from studies of species such as tobacco, the photosynthetic carbohydrate metabolism of which mostly involves Glc, Fru, Suc, and starch. However, in many plant species the most prominent photosynthetic end products are sugar alcohols (e.g. mannitol or sorbitol) or sucrosyl-oligosaccharides (e.g. raffinose sugars or fructans). We were interested in whether some of the observed species variations in growth and performance at high CO_2 concentrations may partly be due to qualitative differences in species carbohydrate metabolism that may ultimately affect carbohydrate flux through leaf hexokinase.

Many plants are active in mannitol metabolism, but their performance has not been evaluated during plant growth at high CO_2 levels. Mannitol occurs in more than 70 families of angiosperms, including most species of the Oleaceae, Scrophulariaceae, Apiaceae, and Rubiaceae (Bieleski, 1982). Much of what is known about the leaf biochemistry of mannitol has come from studies of celery (*Apium graveolens*). In celery mannitol is synthesized in the cytosol (Rumpho et al., 1983) and is readily labeled from $^{14}CO_2$ by a pathway from Fru-6-P → Man-6-P → mannitol 1-P → mannitol (Loescher et al., 1992). Much of the photoassimilate in celery is translocated out of the leaf as mannitol (Davis and Loescher, 1992), and mannitol occurs in sieve-tube exudates of many species (Zimmerman and Ziegler, 1975). In sink tissues of celery mannitol is oxidized directly to Man (Stoop and Pharr, 1992).

Mannitol is thought to have several diverse functions, including being a storage form for reduced carbon and reductant, being a compatible solute and/or osmotically active compound, and being a sink for excess leaf carbohydrate (Loescher, 1987). In celery mannitol metabolism responds strongly to salt stress, and this is characterized by increased activities of Man 6-P reductase (up to 6-fold) and

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Abbreviations: Chl, chlorophyll; PAD, pulsed-amperometric detection.

increased whole-leaf mannitol levels (by up to 27 μmol g^{-1} fresh weight; Everard et al., 1994). Additionally, transformed tobacco (*Nicotiana tabacum*) that produce mannitol have shown an increased salt tolerance (Tarczynski et al., 1993). However, the significance of changing mannitol levels associated with environmental variation (including possibly CO_2 concentration) is not clear, which is primarily due to a lack of information about the leaf subcellular distribution of mannitol. To date, there is only one report of the intracellular localization of mannitol among higher plants. Keller and Matille (1989) showed that 81% of the mannitol in celery petiole protoplasts is located in the vacuole, but they still estimated the cytosolic concentration to be about 300 mM.

In this study we first examined the influence of plant growth at high CO_2 on leaf Rubisco content in relation to amounts and intracellular distribution of soluble sugars in tobacco (*Nicotiana sylvestris*), which does not make appreciable amounts of unusual leaf sugars. Mesophyll sugar compartmentation was examined using density gradient fractionation with nonaqueous solvents (Stitt et al., 1989; Moore et al., 1995). This technique is particularly well suited for carbohydrate localization, since no metabolism occurs during fractionation and analysis and since chloroplast, cytosol, and vacuole distributions can be precisely determined. We then compared the influence of growth at a high CO_2 level in tobacco with that of two species, snapdragon (*Antirrhinum majus*) and parsley (*Petroselinum hortense*), that are active in the metabolism of mannitol.

MATERIALS AND METHODS

Plants of tobacco (*Nicotiana sylvestris*), snapdragon (*Antirrhinum majus* var Liberty Scarlet), and parsley (*Petroselinum hortense* var Dark Moss) were grown in 20-L pots in greenhouses with natural irradiance, a 28/20°C day/night thermoperiod, and either 380 or 1000 μL L^{-1} CO_2. Plants were grown for 3 to 6 months, and leaves were used only from nonflowering stems. All plants were watered with half-strength Hoagland solution three times per week. Leaves were collected into liquid N_2 at midday, and visible leaf veins were removed from collected material prior to biochemical analyses.

Nonaqueous Density Gradient Fractionation of Leaves

Leaf samples were lyophilized, extracted in heptane, and processed, as described previously (Moore et al., 1995). Leaf extracts were fractionated on exponential gradients of heptane/tetrachloroethylene (16 mL of 1.35 g mL^{-1} to 8 mL of 1.55 g mL^{-1} for snapdragon or to 8 mL of 1.60 g mL^{-1} for parsley). Gradient fractions were assayed for Chl, PEP carboxylase activity, and α-mannosidase activity (Moore et al., 1995) as markers for chloroplasts, cytosol, and vacuoles, respectively.

Sugar distributions were calculated using a three-compartment, iterative method that uses the Marquardt-Levenberg algorithm (as in SigmaPlot, version 2.0 or newer, Jandel Scientific, San Rafael, CA). Marker and analyte distributions were input as percentage values for each fraction, and values from multiple gradients were analyzed together using the following equations:

$$f = cp^*A + cyt^*B + vac^*C \quad (1)$$

$$\text{fit } f \text{ to } D \text{ with weight } w \quad (2)$$

The independent variables (*A*, *B*, and *C*) are the marker distribution values and are input into respective columns (1, 2, and 3). The dependent variable (*D*) is the sugar distribution values, input into a separate column (4). The iterative calculation was set up to evaluate possible analyte distributions in steps of 0.1% (stepsize = 0.001). Two constraints were used in the calculation. First, the total amount of analyte in all three compartments was made to equal 100% (cp + cyt + vac = 1.0, where cp is chloroplast, cyt is cytosol, and vac is vacuole). Second, the amount of analyte in any one compartment was set to be ≥0.01% (cp > 0.0001, etc.). Additionally, the program was instructed to calculate compartment distribution values after weighting of the residuals by the respective inverse values of the dependent variable (w = 1/column 4). This step is necessary to obtain the best fit for predicted distribution values, because there is a considerable range in the input values of the analyzed markers and sugars (e.g. 1–50%) such that associated error terms are not uniform. For example, a relative variance of 5% for a gradient sugar/marker value of 1% will be very small in absolute value (1.00 ± 0.05), but the same variance for a fraction value of 50% will be relatively large (50.0 ± 2.5). Without weighting of the residual values, any fraction that contains a large percentage of the sugar or a given marker can skew the iterative calculation if there is much variance in the data. With minimal variances in measured values, weighting has little or no effect on predicted distribution values. For statistical analysis of the compartment distribution values, we determined the SD and 95% confidence interval (one-tailed Student's *t* test at $\alpha/2$) for each iterative calculation.

Carbohydrate Measurements

Vein-depleted leaf material (0.25 g fresh weight) or dried gradient fractions were extracted for 15 min in 4 mL of boiling 80% ethanol. Samples were centrifuged (4000*g*, 5 min), and the pellets were resuspended in hot 80% ethanol using sea sand and a glass rod and then sonicated briefly (pulsed at 35% duty cycle, 30% power output; sonifier model no. 200, Branson Ultrasonics, Danbury, CT). Samples were extracted a total of four times in 80% ethanol and then once in H_2O. Residual material from whole-leaf extracts was autoclaved, and the starch from replicate aliquots was hydrolyzed as described by Schulze et al. (1991). Released Glc was measured by high-performance anion-exchange chromatography-PAD as described below. Pooled extract supernatants were dried by rotary evaporation, resuspended in H_2O, and passed through a 5-mL Dowex 50 (H^+) column. The eluates were concentrated by rotary evaporation, brought to pH 5.0, and syringe-filtered through a C_{18} reverse-phase cartridge (600 mg, Alltech Associates, Deerfield, IL). Sugars from leaf extracts were then measured directly. Each gradient filtrate was concentrated and partially purified by passing the solution through a 3-mL column of QAE-Sephadex (formate form) and eluting the neutral sugars with H_2O (Redgwell, 1980). Each eluate was then dried by rotary evaporation and resuspended in about 1 mL of H_2O, and the solution was syringe-filtered (0.45 μm nylon, Alltech Associates). Recovery experiments using ^{14}C-labeled sugars indicated that >98% of the neutral sugars in the initial extract were present after their partial purification.

Soluble carbohydrates were measured by high-performance anion-exchange chromatography-PAD using a Dionex DX 300 system and a CarboPac PA1 or MA1 column (Dionex, Sunnyvale, CA). Sugars from snapdragon were measured with the PA1 column using a mobile phase of 150 mM NaOH isocratic with a 0 to 20 mM linear gradient of sodium acetate developed from 1 to 11 min after injection. Sugars from parsley were measured with the PA1 column, using an isocratic mobile phase of 200 mM NaOH. In both cases the flow rate was 0.85 mL min^{-1}. Different elution conditions were used for snapdragon sugars to resolve from Glc an unknown peak with an otherwise similar retention time. Sugar alcohols from both species were measured with the MA1 column, using a mobile phase of 0.6 M NaOH isocratic for 43 min. PAD was carried out with a gold working electrode using pulse potentials, durations, and integration periods as described by the manufacturer for carbohydrate detection. Sugar standards were measured daily, and plant samples were diluted sufficiently to provide signals within the linear range of the detector response (typically 0–20 nmol). Carbohydrate standards were mostly purchased commercially (Sigma). Galactinol was generously provided by Dr. Tsung Min Kuo (U.S. Department of Agriculture, Agricultural Research Service, Peoria, IL).

$^{14}CO_2$ Labeling Experiment

Stem sections from the 5th to the 10th nodes of snapdragon plants, or 10th-node leaves of parsley, were equilibrated in a 300-mL glass cuvette for 5 min with an irradiance of 800 μmol quanta m^{-2} s^{-1}. Leaves were pulse-labeled for 15 s with 20 μCi of $^{14}CO_2$ (initial radiospecific activity 54 Ci mol^{-1}) and chased for 30 min with $^{12}CO_2$ after transfer to an adjacent aerated cuvette. Metabolism was quenched by plunging the stem sections into liquid N_2. Leaves were separated from stems and leaf carbohydrates were partially purified as described above. The amount of ^{14}C-labeled sugars was determined by HPLC using a flow-through scintillation counter (Beta-One detector, Radiomatic Instrument and Chemical, Tampa, FL) connected after the electrochemical detector, with a postelectrochemical detector addition of 0.3 M acetic acid at 0.425 mL min^{-1}.

Rubisco and Chl Content Assays

Rubisco content was measured as described elsewhere (Evans and Seemann, 1984). Chl content of gradient fractions and whole-leaf material was determined after extraction in ethanol (Wintermans and De Mots, 1965).

RESULTS

Growth of tobacco, snapdragon, and parsley at high CO_2 resulted in about a 25% increase in individual leaf areas and in snapdragon resulted in the production of about 50% more flowers (data not shown). In tobacco leaves both the Chl content and Rubisco content on a Chl basis declined by 20% in mature leaves of plants grown at high CO_2 (Table I). In contrast, in leaves of both snapdragon and parsley grown at high CO_2, Chl content declined only slightly (parsley) or not at all (snapdragon), and relative Rubisco content was not affected. Labeling experiments with $^{14}CO_2$ established that mannitol is a photosynthetic product in both snapdragon and parsley but not tobacco (Table I). In snapdragon mannitol accumulated only a small amount of the ^{14}C-label and only during the chase period. In parsley mannitol was labeled during the pulse and after 30 min had accumulated about 17% of the label present in soluble sugars.

Since the metabolism of leaf carbohydrates in plants grown at high CO_2 is thought to be a factor that is associated with the decrease in leaf Chl and Rubisco content in species such as tobacco, we first examined leaf carbohydrate levels in tobacco, snapdragon, and parsley. Leaves of tobacco grown at high CO_2 had only modest increases in hexoses but a large increase in starch (Fig. 1). These carbohydrates totaled about 115 and 240 μmol hexose equivalents mg^{-1} Chl in plants grown in ambient and high CO_2,

Table I. *Leaf Chl and Rubisco contents, and labeling of mannitol from $^{14}CO_2$ in tobacco, snapdragon, and parsley grown at ambient and elevated levels of CO_2*

Chl and Rubisco values are means ± SD for three to six extractions of pooled leaf material. Mannitol labeling was after a 30-min chase period, at which time about 90% of the ^{14}C-labeled products were soluble sugars.

Species	Growth CO_2	Leaf Chl	Rubisco Content	[^{14}C]Mannitol
	μL L^{-1}	*mg g^{-1} fresh wt*	*nmol mg^{-1} Chl*	*% soluble sugars*
Tobacco	380	1.78 ± 0.08	53.1 ± 1.3	0
	1000	1.38 ± 0.03	42.4 ± 0.4	ND[a]
Snapdragon	380	1.31 ± 0.14	20.9 ± 0.6	3
	1000	1.35 ± 0.06	22.7 ± 1.2	3
Parsley	380	1.69 ± 0.05	35.0 ± 1.6	17
	1000	1.55 ± 0.03	36.4 ± 2.1	ND

[a] ND, Not determined.

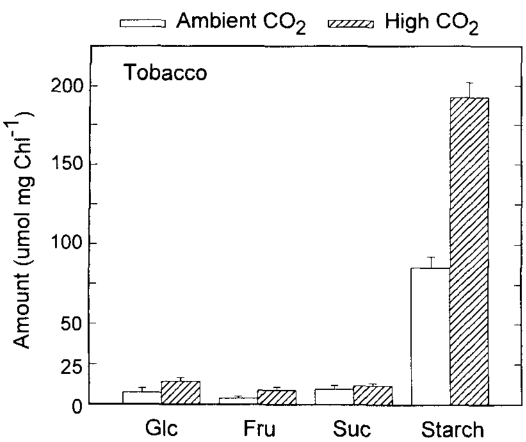

Figure 1. Influence of plant growth at high CO_2 on amounts of principle sugars in tobacco leaves. Plants were grown continuously at 380 or 1000 μL L^{-1} CO_2 and mature leaves were collected at midday. Values are means ± SD for four to seven extractions of several leaf collections. Starch is expressed as micromoles of Glc equivalents. Total amounts of these sugars in plants grown at ambient and at elevated CO_2 were 115 and 240 μmol Glc equivalents mg^{-1} Chl, respectively. *myo*-Inositol was also present but its levels were not quantified.

respectively. In snapdragon grown at high CO_2, levels of Glc, Fru, and starch increased severalfold, whereas Suc was not largely affected (Fig. 2). Mannitol was present at substantial levels under both growth conditions, and the levels were not affected by growth at high CO_2. Snapdragon leaves also contained a number of other prominent soluble sugars, of which the most abundant ones were either not affected by growth at high CO_2 (galactinol; O-α-D-galactopyranosyl-(1→1)-L-*myo*-inositol) or declined substantially (*myo*-inositol, xylitol, and sorbitol; Fig. 2). These carbohydrates totaled about 93 and 157 μmol hexose equivalents mg^{-1} Chl in plants grown in ambient and high CO_2, respectively.

Parsley leaves contained higher amounts of Suc, starch, mannitol, and *myo*-inositol than did snapdragon leaves but lower amounts of Glc and Fru, with no detectable levels of other soluble sugars (Fig. 3). In parsley grown at high CO_2, amounts of Suc, starch, mannitol, and *myo*-inositol increased 40 to 100%, whereas levels of Glc and Fru were constant. In parsley these carbohydrates totaled about 190 and 307 μmol hexose equivalents mg^{-1} Chl in plants grown in ambient and high CO_2, respectively.

To further examine the influence of growth at high CO_2 on leaf carbohydrate metabolism, we examined the intracellular distribution of leaf mannitol and other soluble carbohydrates after fractionation on density gradients of nonaqueous solvents (Fig. 4). In snapdragon and parsley about 40% of the leaf mannitol occurred in the cytosol, with generally somewhat smaller amounts in the chloroplast and vacuole (Table II). In snapdragon mannitol distribution was not effected by growth at high CO_2 but was effected by leaf age. In parsley, growth at high CO_2 did result in a somewhat lower percentage of mannitol within the mesophyll cytosol.

Growth at high CO_2 did not affect the midday intracellular distributions of leaf Glc, Fru, or Suc in tobacco, snapdragon, or parsley (Table III). Glc and Fru occurred almost exclusively in the vacuole of all three species (i.e. within resolution limits of 0.01% for the iterative calculation). Suc was predominantly cytosolic in tobacco (90%) and snapdragon (80%) but less so in parsley (55%). *myo*-Inositol in both snapdragon and parsley occurred largely in the chloroplast, but it also occurred in other compartments (Table IV). In snapdragon, galactinol, xylitol, and sorbitol occurred entirely in the vacuole.

DISCUSSION

If hexokinase does function as a flux-sensor (Jang and Sheen, 1994; Jang et al., 1997), then we had anticipated that decreased expression of Rubisco content at high CO_2 in species such as tobacco might be due to increased cytosolic Glc. However, we observed no detectable cytosolic hexoses in tobacco leaves collected at midday from plants grown at ambient or high CO_2 (Table III). Furthermore, leaf hexoses in snapdragon and parsley are also compartmentalized in the vacuole at midday, such that there were no species differences in the intracellular compartmentation of leaf Glc that might account for photosynthetic down-regulation

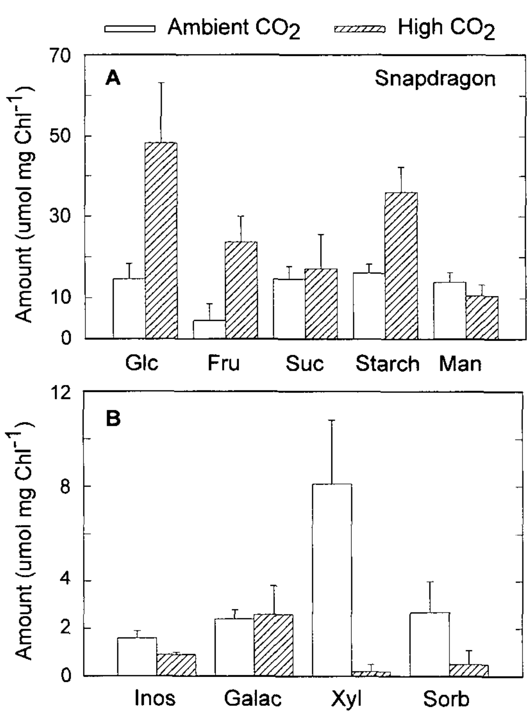

Figure 2. Influence of plant growth at high CO_2 on amounts of principal sugars in snapdragon leaves. Plants were grown continuously at 380 or 1000 μL L^{-1} CO_2 and mature leaves were collected at midday. A, Man, mannitol. B, Inos, *myo*-Inositol; Galac, galactinol; Xyl, xylitol; and Sorb, sorbitol. Values are means ± SD for four to seven extractions of several leaf collections. Starch is expressed as micromoles of Glc equivalents. Total amounts of these sugars in plants grown at ambient and at elevated CO_2 concentrations were 93 and 157 μmol Glc equivalents mg^{-1} Chl, respectively.

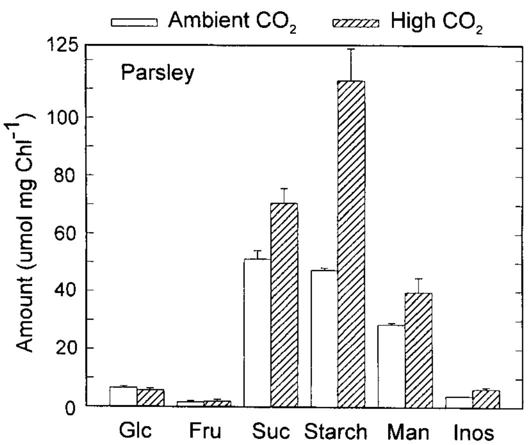

Figure 3. Influence of plant growth at high CO_2 on amounts of principal sugars in parsley leaves. Plants were grown continuously at 380 or 1000 $\mu L\ L^{-1}$ CO_2 and mature leaves were collected at midday. Man, Mannitol; Inos, *myo*-inositol. Values are means ± SD for four to seven extractions of several leaf collections. Starch is expressed as micromoles of Glc equivalents. Total amounts of these sugars in plants grown at ambient and at elevated CO_2 concentrations were 190 and 307 μmol Glc equivalents mg^{-1} Chl, respectively.

in tobacco grown at high CO_2. Using a previous estimate of mesophyll subcellular compartment volumes in tobacco (Heineke et al., 1994; Winter et al., 1994), we estimated that cytosolic hexose concentrations in all three species used in this study would be ≤100 μM based on the resolution limits for the iterative calculation. Heineke et al. (1994) also have estimated that daytime leaf cytosolic hexose concentrations in tobacco grown at ambient CO_2 are <1 mM (i.e. at their resolution limits). This result is not unreasonable, since leaf vacuolar hexose transporters (Rausch et al., 1987) and hexokinases (Schnarrenberger, 1990) typically have K_m values of about 100 μM. That we did not observe any enhanced cytosolic hexoses in tobacco grown at high CO_2 still is not inconsistent with the proposed function of cytosolic hexokinase as a sugar sensor (Jang and Sheen, 1994; Jang et al., 1997), since cytosolic hexose metabolism may be affected by futile cycling of Suc (Geigenberger and Stitt, 1991) or since levels of cytosolic hexoses possibly could be higher at other times during the day (e.g. during mobilization of vacuolar hexoses at the end of the day).

Mannitol metabolism likely does not function as an overflow mechanism for the accumulation of carbon under conditions of increased photosynthate production in snapdragon and parsley grown at high CO_2 concentrations, since leaf mannitol levels did not increase substantially and since there was not much difference in mannitol subcellular compartmentation (Figs. 1 and 2; Table II). Although we are uncertainof the intracellular compartment volumes, we estimate that mesophyll cytosolic and stromal mannitol concentrations range from about 75 to 105 mM in snapdragon and from about 190 to 270 mM in parsley (based on measured leaf levels, intracellular distribution values, respective leaf H_2O contents in snapdragon and parsley of 590 and 472 $\mu L\ mg^{-1}$ Chl, and assuming a relative cytosolic volume of 10% of leaf H_2O content and a relative stromal volume of 8%). The estimated cytosolic concentration in parsley is comparable to that previously estimated as occurring in celery petioles (Keller and Matille, 1989), but the estimated parsley value may be higher than what actually occurs, since cytosolic Suc levels calculated with the same assumptions would be rather high (600–700 mM). Since snapdragon and parsley did have at most only modest adjustments in photosynthetic components when grown at high CO_2, there still may be associated attributes of mannitol metabolism that provide some indirect benefit(s) for plant growth at high CO_2. For example, Tarczynski et al. (1993) have speculated that mannitol accumulation in transgenic tobacco may somehow stimulate cellular processes that result in the formation of new roots. Nonetheless, since both snapdragon and parsley did grow larger and more rapidly at high CO_2, perhaps their performance may be most simply attributed to a higher sink strength.

Mannitol metabolism appears to have different functions within snapdragon and parsley. Mannitol was labeled rather slowly from $^{14}CO_2$ in snapdragon but relatively more rapidly in parsley (Table I), as also occurs in celery (Loescher et al., 1992). In snapdragon only trace amounts of [^{14}C]mannitol were recovered in the stem tissue after a 1-h chase, indicating that little leaf mannitol is exported (data not shown). However, high rates of leaf mannitol export are well documented in celery (Davis and Loescher, 1992). Thus, in snapdragon mannitol metabolism may have a more restricted function such as being a compatible solute in the cytosol and stroma, whereas in parsley mannitol is likely also an active photosynthetic and long-distance transport metabolite. Since both species have substantial levels of cytosolic mannitol, the mechanism that controls mannitol export from a leaf must involve factors other than strictly the size of the mesophyll cytosolic mannitol pool. One possibility is that species such as parsley and celery may contain leaf cell-specific transporters for phloem loading of mannitol by an apoplastic pathway (Sauer et al., 1994). However, Flora and Madore (1996) provided evi-

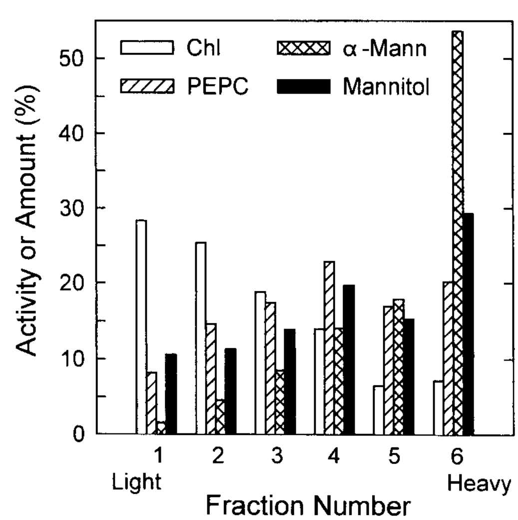

Figure 4. Nonaqueous density gradient fractions showing markers for cellular compartments and mannitol from mature parsley leaves. Chloroplast marker, Chl; cytosol marker, PEP carboxylase activity (PEPC); and vacuole marker, α-mannosidase activity (α-Mann).

Table II. *The intracellular distribution of mannitol in leaves of snapdragon and parsley grown at ambient and elevated levels of CO_2*

Species/Growth CO_2	Leaf Position	95% Confidence Interval	Distribution		
			Chloroplast	Cytoplasm	Vacuole
				%	
Snapdragon					
380 $\mu L\ L^{-1}$	5th Node	5.2	35.5	49.8	14.7
	10th Node	1.5	35.7	40.7	23.6
	20th Node	5.3	38.2	21.8	40.0
1000 $\mu L\ L^{-1}$	10th Node	6.4	33.9	45.3	20.8
Parsley					
380 $\mu L\ L^{-1}$	10th Node	1.9	19.0	42.9	38.1
1000 $\mu L\ L^{-1}$	10th Node	2.3	25.1	30.9	44.0

dence suggesting that species such as parsley may predominantly utilize a symplastic pathway for phloem loading of mannitol. In this case, snapdragon may lack the minor vein cellular specializations required for such phloem loading.

When grown at high CO_2, leaves of snapdragon, parsley, and tobacco maintained a similar relative proportion of Suc in the cytosol, as when grown at ambient CO_2 (Table III). A number of plants such as tobacco commonly maintain relatively constant leaf levels of Suc in the presence of either decreased (Barnes et al., 1994) or increased (Huber and Hanson, 1992) leaf Glc and Fru. The regulation of leaf Suc levels likely occurs independently of free Glc and Fru levels, being a consequence of the regulated activities of cytosolic Fru 1,6-bisphosphatase and Suc phosphate synthase in relation to Suc export and vacuolar Suc hydrolysis (Huber et al., 1985; Gerhardt et al., 1987; Huber, 1989). Whether small amounts of Suc may occur in the chloroplast stroma of snapdragon and parsley leaves is uncertain (Table III), but Suc notably is reported to occur at prominent levels in chloroplasts of transgenic tobacco that express cytosolic invertase activity (Heineke et al., 1994). That more Suc occurs in the mesophyll vacuole of parsley than in snapdragon presumably is due in part to a lower activity of vacuolar acid invertase in parsley. Spinach (*Spinacia oleracea* L.) also has a low activity of vacuolar invertase (Goldschmidt and Huber, 1992) and a high level of vacuolar Suc (Gerhardt et al., 1987).

Snapdragon leaves accumulated substantial levels of several other soluble carbohydrates, including *myo*-inositol, galactinol, xylitol, and sorbitol (Fig. 2). It is interesting that the levels of the latter three and of mannitol were moderately or strongly reduced in plants grown at high CO_2. Since growth of snapdragon plants was stimulated at high CO_2 and since we would certainly predict that photosynthetic rates were enhanced during growth at high CO_2, we conclude that leaf carbohydrate export was likely

Table III. *The influence of growth at high CO_2 on the intracellular distribution of Glc, Fru, and Suc in leaves of tobacco, snapdragon, and parsley*

Species/Growth CO_2	Sugar	95% Confidence Interval	Distribution		
			Chloroplast	Cytosol	Vacuole
				%	
Tobacco					
380 $\mu L\ L^{-1}$	Glc	4.1	<0.01	<0.01	100
	Fru	3.8	2.2	<0.01	97.8
	Suc	4.8	<0.01	91.5	8.5
1000 $\mu L\ L^{-1}$	Glc	4.7	<0.01	<0.01	100
	Fru	3.7	<0.01	<0.01	100
	Suc	3.4	<0.01	90.3	9.7
Snapdragon					
380 $\mu L\ L^{-1}$	Glc	7.6	<0.01	<0.01	100
	Fru	5.4	<0.01	<0.01	100
	Suc	3.1	1.5	83.8	14.7
1000 $\mu L\ L^{-1}$	Glc	4.0	<0.01	<0.01	100
	Fru	4.2	<0.01	<0.01	100
	Suc	3.4	4.0	77.9	18.1
Parsley					
380 $\mu L\ L^{-1}$	Glc	4.7	4.0	<0.01	96.0
	Fru	6.5	3.0	<0.01	97.0
	Suc	4.0	4.5	57.3	38.2
1000 $\mu L\ L^{-1}$	Glc	4.1	0.3	<0.01	97.0
	Fru	7.0	<0.01	<0.01	100
	Suc	5.0	2.0	50.6	47.4

Table IV. *Intracellular distribution of myo-inositol, galactinol, sorbitol, and xylitol in leaves of snapdragon and parsley grown at ambient and elevated levels of CO_2*

Species/Growth CO_2	Sugar	95% Confidence Interval	Distribution		
			Chloroplast	Cytosol	Vacuole
				%	
Snapdragon					
380 μL L^{-1}	*myo*-Inositol	3.2	54.9	27.3	17.8
	Galactinol	7.3	4.4	<0.01	95.6
	Xylitol	5.1	1.0	<0.01	99.0
	Sorbitol	3.0	<0.01	<0.01	100
1000 μL L^{-1}	*myo*-Inositol	3.5	53.1	33.7	13.2
Parsley					
380 μL L^{-1}	*myo*-Inositol	2.7	43.5	22.7	33.7
1000 μL L^{-1}	*myo*-Inositol	2.9	43.5	24.1	32.3

greater in plants grown at high CO_2. Thus, one speculative possibility is that the accumulation of these soluble sugars in snapdragon leaves grown at ambient CO_2 may have occurred in response to conditions of limiting rates of leaf carbohydrate export. Guy et al. (1992) suggested that Suc accumulation in spinach after a low-temperature treatment is primarily due to a reduction in the growth utilization of photosynthate. Also, fructans typically accumulate in leaves of certain grasses when there is a reduced growth utilization of Suc (Smart et al., 1994). Furthermore, in snapdragon the vacuolar location of leaf galactinol, xylitol, and sorbitol is consistent with the interpretation that they are accumulated as an overflow mechanism with minimal metabolic activity under the condition of relatively limiting leaf carbohydrate export.

There are several important aspects yet to be evaluated regarding the significance of qualitative differences in carbohydrate metabolism to plant growth and photosynthetic performance at high CO_2. First, the metabolism of mannitol, other sugar alcohols (e.g. sorbitol), or perhaps other sugars (e.g. raffinose or fructans) could be an important factor for improved plant performance under different environmental stress conditions that may be encountered during growth at high CO_2. For example, during salt stress in celery photosynthate is increasingly diverted from Suc to mannitol as an apparent mechanism to maintain plant growth (Everard et al., 1994). The chloroplastic location of a substantial portion of the leaf's mannitol (Table II) supports an additional suggested function of mannitol as a scavenger of hydroxyl radicals that can be produced in the chloroplast under conditions of drought or low-temperature stress (Smirnoff and Cumbes, 1989). Also, the accumulation of fructans in transgenic tobacco results in enhanced drought resistance (Pilon-Smits et al., 1995). Second, since plant growth at high CO_2 depends on source-sink relationships (Stitt, 1991), such performance potentially may be affected by the efficiency of leaf carbohydrate export. Species that transport galactosides (Van Bel et al., 1992; Turgeon, 1995) and possibly those that transport sugar-alcohols as well (Flora and Madore, 1996) likely utilize a symplastic pathway for phloem loading of such sugars. There are few comparative data available concerning the efficiency of carbohydrate export by symplastic versus apoplastic pathways, but there is some evidence that species utilizing symplastic loading actually may be less efficient in carbohydrate export (Van Bel, 1993). Third, some species may be able to avoid the phenomenon of Glc repression of photosynthetic gene expression that is thought to result in photosynthetic down-regulation during plant growth at high CO_2 (Van Oosten and Besford, 1996). Such species may minimize the rate of leaf Suc hydrolysis either by having relatively low invertase activities, as may occur in parsley, or by rapidly removing Suc by metabolism to alternate products such as raffinose sugars.

In summary, mesophyll cytosolic hexoses occurred at $\leq$100 μM at midday in tobacco, snapdragon, and parsley grown at ambient or elevated concentrations of CO_2. The vacuolar compartmentation of leaf hexoses may account for the lack of correlation between absolute levels of hexose accumulation and down-regulation of photosynthesis observed in this and previous studies (Goldschmidt and Huber, 1992; Nie et al., 1995). In addition, mannitol-producing plants may have associated, but as yet undefined, benefits related to vegetative sink strength that could be important for plant growth at high CO_2 levels.

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